Hepatitis C Virus (HCV)

OVERVIEW OF THE HCV INFECTION AND DIAGNOSTIC TESTING GUIDELINE

The Hepatitis C virus (HCV) is a small, enveloped virus that belongs to the *Flaviviridae* family. It is a blood-borne virus, which means the screening of blood and organ donors for HCV is of the highest importance. Intravenous drug use and unsafe medical practices are the main transmission routes.

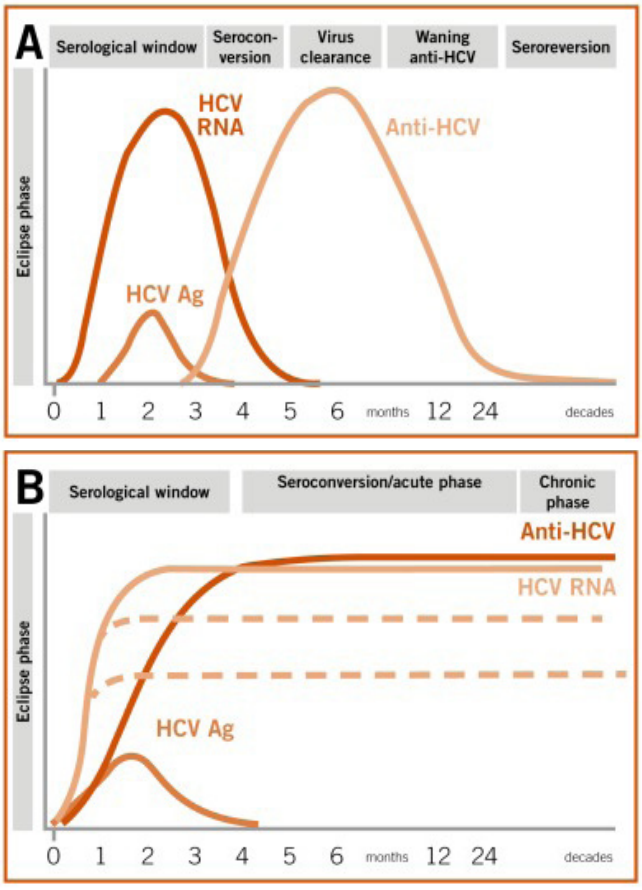

Figure 1. Serological window for HCV detection. (A) Self-resolving infection, (B) Chronic HCV infection.

Blood transfusion is also a way of transmission, which puts any patients requiring transfusion at risk, and this risk intensifies for those patients who require repeated or regular transfusions. Approximately 15–45% of people who are infected with HCV will clear the virus spontaneously, which will usually happen within 6 months of exposure, remaining HCV seropositive. Those people who do not go on to develop chronic HCV infection can often stay asymptomatic for a long time. There is a 20–30% risk of liver fibrosis and subsequently a risk of cirrhosis, liver failure, and hepatocellular carcinoma.

HCV infection is asymptomatic in the majority of patients. Therefore, it remains difficult to diagnose clinically until more advanced stages of fibrosis are present. HCV infection diagnosis relies heavily on clinical laboratory tests, which include anti-HCV antibody detection, detection of HCV core antigen (HCVcAg), and nucleic acid testing (NAT) for HCV RNA. In clinical practice, HCV infection diagnosis is a two-step process that starts with an anti-HCV assay, which is typically used to screen for virus exposure and is followed by the more complex and expensive NAT to confirm viremia.

Active HCV infection is characterized by HCV RNA (the first appearance is in an initial phase of 1 - 2 weeks) and HCV Core antigen (the second appearance is around 7~9 weeks). The approximate time course of virological and immunological markers of self-resolving and chronic HCV infection are marked in Figure 1.

CLINICAL UTILITY

- **Screening and diagnosis of HCV infections to support early detection**
- **Early identification to help prevent progression, reduce transmission, and improve long-term outcomes**

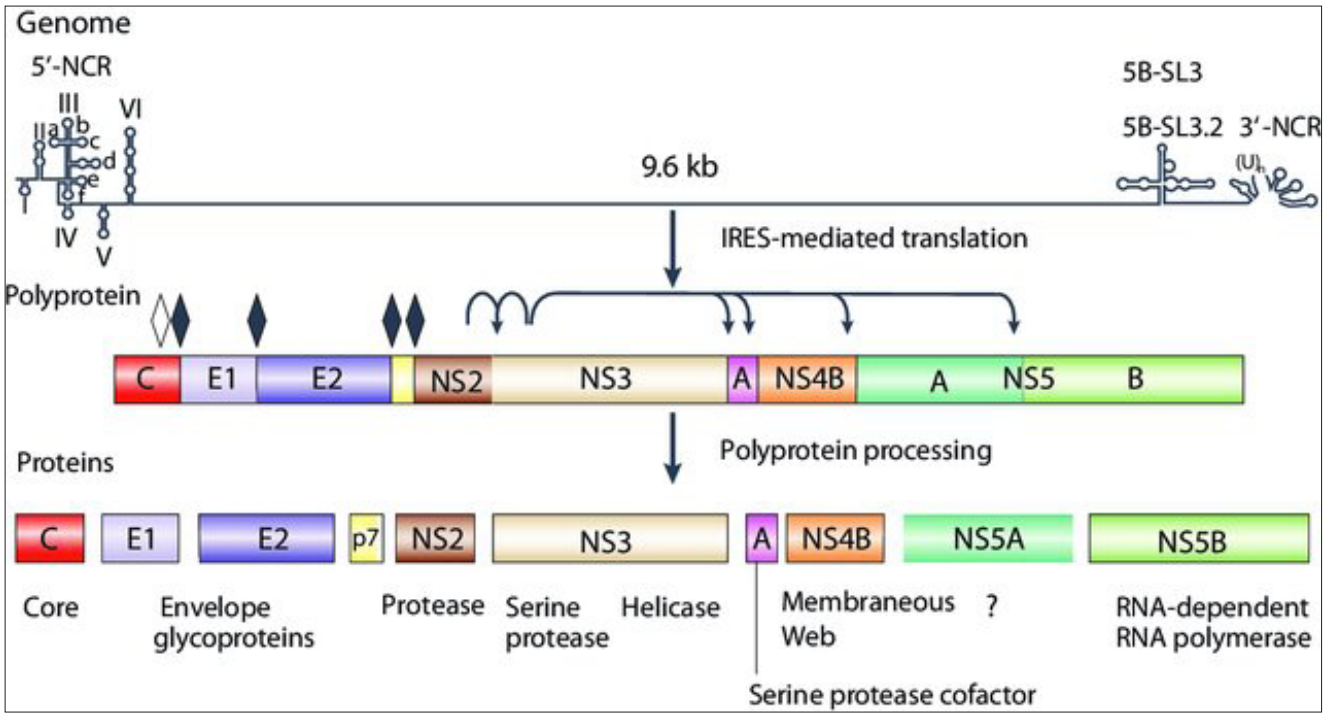

Figure 2. Genetic organization and polyprotein processing of HCV.

According to the 2017 WHO Guidelines on Hepatitis B and C Testing (ISBN978-92-4-154998-1), HCV screening begins with anti-HCV testing and it is followed by a nucleic acid test for HCV RNA to confirm chronic infection.

The positive-sense RNA genome of HCV is approximately 9.6 kb in length and the single large open reading frame encodes one polyprotein of 3008-3037 aa. After co- and post-translational cleavage by both viral and host proteases, at least ten discrete viral proteins are produced (see Figure 2).

- Structural Proteins:
  - Core Protein: Forms the viral capsid
  - E1 and E2: Envelope glycoproteins that decorate the outer surface.
- Nonstructural Proteins regulate viral replication and assembly:
  - p7: Functions as a viroporin (ion channel) aiding particle release.
  - NS2: A protease that cleaves itself from the polyprotein.
  - NS3 & NS4A: Form a complex with protease and RNA helicase activity.
  - NS4B: Induces rearrangements of host cell membranes to create the viral replication complex.
  - NS5A: A phosphoprotein that is essential for RNA replication and virus assembly.
  - NS5B: The RNA-dependent RNA polymerase responsible for replicating the viral genome.

Out of the ten HCV antigens, four are routinely used in commercial assays (Core, NS3, NS4, and NS5).

HCV CLASSIFICATION AND GEOGRAPHIC DISTRIBUTION

The classification of HCV is based on the topology of phylogenetic trees that are derived from viral variant relationships. Genotypes are further divided into subtypes. Based on the consensus criteria, for a virus to be confirmed as a new subtype, it must have a unique genetic sequence that is different from other known subtypes by at least 15% and sequence information from at least two other isolates in the core/E1 (>90% of the sequence corresponding to positions 869 to 1292 of the H77 reference sequence) and the NS5B region (>90% of positions 8276 to 8615) of the virus's genome.

Phylogenetic analysis permitted the classification of HCV into 8 major genotypes and 86 subtypes. The global distribution of HCV genotypes and subtypes also varies by geographic region, ethnicity, and risk groups. Genotypes 1 and 3 are the most prevalent and they account for more than 30% of all infections. Conversely, Genotypes 2, 4, 5, and 6 each account for less than 10% of infections. Genotype 7 has been found in a few people from Central Africa.

The HCV genotype distribution varies geographically. Genotype 1 predominates in Europe and North America, while genotypes 2 and 3 are also commonly reported in these regions. Genotype 2 is most prevalent in Central Africa, while Genotype 3 is common in India, Pakistan, Thailand, other Asian countries, and Northern Europe. Genotypes 4 and 5, which are likely disseminated through migration and intravenous drug use, are increasing in prevalence. Genotype 4 is primarily found in Central Africa and the Middle East, whereas Genotype 5 is more frequently found in Southern Africa. The HCV Genotype 6 has a high variation and is classified as more than five subtypes. These subtypes are highly prevalent in East and Southeast Asia,

and are mostly found in Laos and Vietnam, as well as Northern and Northeast Thailand.

Recombinant proteins for anti-HCV detection

Hytest offers a panel of recombinant proteins corresponding to the HCV core, NS3, and NS4 proteins for the qualitative detection of anti-HCV antibodies in human serum or plasma.

These proteins were selected based on their high immunogenicity and antibody response. To cover the maximal genetic diversity for HCV proteins, the most common fragments were used for protein engineering.

Cat. #8HC51 HCV, NS4 antigen, recombinant is a chimeric protein that is expressed in a prokaryotic cell line (*E. coli*). This protein consists of three NS4 fragments from two different isolates. The first immunodominant region (aa 1689-1767, serotype 1a) contains the C-terminal domain of NS4A and a small fragment of the N-terminal domain of NS4B. From there, through the GSGS linker, there is a mosaic of two immunodominant regions [aa 1689-1738]+[aa 1921-1940] from another serotype of the virus (1b). The secondary structure of the corresponding domains of the full-length protein is predominantly alpha-helical. At the N-terminus, there is an artificially introduced methionine residue; at the C-terminus, a 10His tag and cysteine residue are attached via a GSGS linker.

Cat. #8HC52 HCV, NS4 antigen, recombinant is a chimeric protein that is expressed in a mammalian cell line (Expi293F). This protein consists of three NS4 fragments from two different isolates. The first immunodominant region (aa 1689-1767, serotype 1a) contains the C-terminal domain of NS4A and a small fragment of the N-terminal domain of NS4B. From there, through the GSGS linker, there is a mosaic of two immunodominant regions [aa 1689-1738] + [aa 1921-1940] from another serotype of the virus (1b). The secondary structure of the corresponding domains of the full-length protein is predominantly alpha-helical. The N-terminus contains an HSA fusion partner, human serum albumin, attached via a GSGS linker. At the C-terminus, a 10His tag and a cysteine residue are also attached via a GSGS linker.

Cat. #8HC53 HCV, NS3 antigen, recombinant is expressed in a prokaryotic cell line (*E. coli*). This is an immunodominant fragment 1192-1457 aa of the NS3 protein, which is known in the literature as "c33c." The sequence of the H77 serotype 1a isolate (https://www.uniprot.org/uniprot/P27958) was used as a basis, with three substitutions (S1358L, S1327P, and K1247Q). At the N-terminus, there is an artificially introduced methionine residue; and at the C-terminus, a 10His tag and another cysteine residue are attached via a GSGS linker.

Cat. #8HC55 HCV, core antigen, C-terminal Cys, recombinant is expressed in a prokaryotic cell line (*E. coli*). Core protein is a dimeric alpha-helical protein that consists of two domains: an N-terminal two-thirds hydrophilic domain (D1) and a C-terminal one-third hydrophobic domain (D2). The D1 domain contains many positively charged amino acids and has characteristics that are similar to the capsid proteins of related pestiviruses and flaviviruses. The D2 domain is required for the correct folding of the D1 domain, and it is critical for the membrane properties of the core. Our protein is a core fragment (10-150 aa, H77 serotype, 1a isolate), which consists of the entire D1 domain and one-half of the D2 domain. At the N-terminus, there is an artificially introduced methionine residue; and at the C-terminus, a 10His tag and cysteine residue are attached via a GSGS linker.

Cat. #8HC56 HCV, core antigen, recombinant is a chimeric protein that is expressed in a mammalian cell line (Expi293F). It is a core protein fragment (10-150 aa, H77 serotype, 1a isolate) that consists of the entire D1 domain and one-half of the D2 domain. At the N-terminus, the fusion partner TnC (human Troponin C) attached via the GSGS linker; while at the C-terminus, a 10His tag and cysteine residue are also attached via a GSGS linker.

Cat. #8HC59 HCV, core antigen, recombinant is expressed in a prokaryotic cell line (*E. coli*). It is a core protein fragment (10-150 aa, H77 serotype, 1a isolate) that consists of the entire D1 domain and one-half of the D2 domain. At the N-terminus, there is an artificially introduced methionine residue; at the C-terminus, and a 10His tag is attached via a GSGS linker for protein affinity purification.

Recombinant HCV-specific proteins, short descriptions of them, and some of their physicochemical properties (molecular weight, theoretical pI, etc.) are summarized in the Ordering Information table (see page 8).

PERFORMANCE EVALUATION USING TWO ASSAY FORMATS

Hytest's HCV recombinant proteins were evaluated on a CLIA platform using a two-step indirect format and double-antigen sandwich method. In summary, HCV-specific recombinant proteins are coated onto magnetic particles, which capture the corresponding antibodies from the patient's serum sample. From there, the captured antibodies are detected using Alkaline phosphatase (ALP)-conjugated anti-human IgG (see Figure 3A) or HCV-specific protein (see Figure 3B).

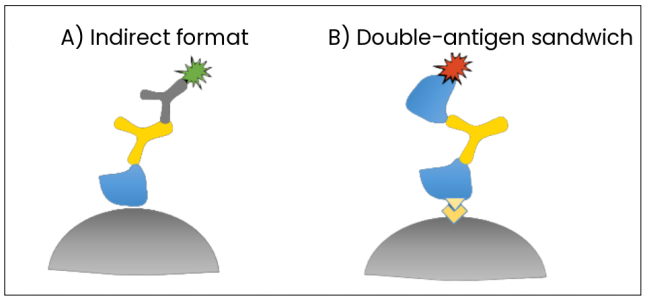

Figure 3. (A) The scheme of two-step indirect format and (B) double-antigen sandwich format chemiluminescent particle-based HCV antigen (CLIA platform).

Table 1.
Clinical sensitivity and specificity evaluation of recombinant HCV core antigen (Cat. # 8HC59).

Anti-HCV core antibody assay		
Performance	Indirect format	
	Particle antigen, Cat. #	8HC59
	Conjugate	Anti-Human IgG-ALP
Sensitivity	Number of positive clinical samples	125
	False negative	0
	Clinical Sensitivity, %	100.0
Specificity	Number of clinical samples tested	4,709
	False reactive	2
	Clinical specificity, %	99.96

Table 2.
Clinical sensitivity evaluation of two recombinant HCV core antigens (Cat. # 8HC55 and Cat. # 8HC56).

Anti-HCV core antibody assay			
Double antigen sandwich format			
Particle antigen		8HC55	8HC56
Conjugate antigen		Synthetic core peptide (M1)	
Type of samples	Number	False negative	
Clinical positive samples	220	0	0
Clinical sensitivity, %		100.0	100.0

Evaluation results under the two-step indirect method

- Recombinant HCV core antigen Cat. # 8HC59 was tested with a positive sample panel (125 clinical positive samples) and demonstrated sensitivity of 100.0%. On the other hand, clinical specificity was assessed against clinical specimens from various hospitals. Hytest's anti-HCV core antibody assay prototype composed of recombinant core antigen (Cat. # 8HC59) met the performance requirement defined in the Commission Implementing Regulation (EU) 2022/1107 (specificity >99.5%) (see Table 1).

Evaluation results under the double-antigen sandwich method

- Recombinant HCV core antigens Cat. # 8HC55 and Cat. # 8HC56 were tested with respective positive sample panels (220 clinical positive samples) and demonstrated sensitivity of 100.0% (see Table 2). Analytical specificity was assessed against various interference samples and clinical specimens. The two HCV core antigen assay prototypes met the performance requirement that is defined in the Commission Implementing Regulation (EU) 2022/1107 (specificity >99.5%) (see Table 3).

Table 3.
Specificity evaluations of two HCV core antigens, Cat. # 8HC55 and Cat. # 8HC56.

Anti-HCV core assay				
Double antigen sandwich format				
Particle antigen			8HC55	8HC56
Conjugate antigen			Synthetic core peptide	
Type of interference		Number	False Reactive	
Interference sample		78	0	1
ANA positive sample		48	1	1
Pregnant population		38	0	0
Dialysis population		42	0	0
RF sample		37	0	0
Total	Sample number	243	243	243
	False reactive	/	1	2
Sample category		**Number**	**False reactive**	
Collected samples		1,178	1	1
Clinical samples		/	0	0
Total	Sample number	1,632	1,632	1,632
	False reactive	/	1	1
Total clinical samples specificity, %		/	99.89	99.84

Table 4.
Clinical sensitivity evaluation of anti-HCV NS3 antibody asssay prototype.

Anti-HCV NS3 antibody assay		
Double antigen sandwich format		
Particle antigen		8HC53
Conjugate antigen		8HC53
Type of samples	Number	False negative
Positive sample	96	0
Clinical sensitivity, %	/	100.0

- Recombinant HCV NS3 antigen, Cat. # 8HC53 was tested with a positive sample panel (96 clinical positive samples) and demonstrated sensitivity of 100% (see Table 4). Analytical specificity was assessed against various interference samples and clinical specimens. Anti-HCV NS3 assay prototype met the performance requirement defined in the Commission Implementing Regulation (EU) 2022/1107 (specificity >99.5%) (see Table 5).
- Anti-HCV NS4 assay prototype composed of Cat. # 8HC51 and Cat. # 8HC52, was evaluated using a positive sample panel consisting of 28 clinical positive samples and demonstrated 100.0% sensitivity. Analytical specificity was assessed against clinical specimens. The anti-HCV NS4 assay prototype met the performance requirement defined in the Commission Implementing Regulation (EU) 2022/1107 (specificity >99.5%) (see Table 6).

Table 6.
Sensitivity and specificity evaluation of HCV, NS4 antibody assay prototype.

Anti-HCV NS4 antibody assay			
Double antigen sandwich format			
Performance	Particle antigen		8HC51
	Conjugate antigen		8HC52
Sensitivity	Type of positive samples	Number	False negative
	Clinical positive sample	28	0
	Sensitivity, %	/	100.0
Specificity	Type of clinical samples	Number	False positive
	Random samples	1,180	1
	Specificity, %	/	99.92

Table 5.
Specificity evaluation of an anti-HCV NS3 assay prototype.

Anti-HCV NS3 antibody assay			
Double antigen sandwich format			
Particle antigen			8HC53
Conjugate antigen			8HC53
Type of interference		Number	False reactive
Interference sample		78	0
ANA positive sample		48	0
Pregnant population		38	1
Dialysis population		42	1
RF sample		37	0
Total	Sample number	243	243
	False reactive	/	2
Sample category		**Number**	**False reactive**
Collected samples	Study 1	482	0
	Study 2	1,244	1
Random clinical samples		684	684
Total	Sample number	2,410	2,410
	False reactive	/	1
Total specificity, %		/	99.89

Hytest Anti-HCV Core/NS3/NS4 Combo Assay Prototypes Evaluation

Using the above-described combinations of HCV core antigen, NS3 antigen and NS4 antigen assay prototypes, two anti-HCV combo assay prototypes were constructed and evaluated on a CLIA platform under the double-antigen sandwich method. The anti-HCV combo assay combines three assays for the detection of antibodies to the HCV core, NS3, and NS4 antigens.

The diagnostic sensitivity evaluation of Hytest's assay prototypes demonstrated a sensitivity of 100.0% across all 97 HCV-positive samples, including different genotypes, matching the performance of the comparator IVD assays with no false-negative results (see Table 7).

For a total of 3,455 clinical samples with a variety of disease status, including interference samples, the anti-HCV combo assay prototype 1 showed a specificity of 99.91%, and the anti-HCV combo assay prototype 2 showed a specificity of 99.88% (see Table 8).

Broad HCV portfolio offers assay developers unparalleled flexibility for anti-HCV antibody assay development and optimization.

Table 7.
Sensitivity evaluation of anti-HCV Core/NS3/NS4 combo assay prototypes.

Assay			Anti-HCV Combo1	Anti-HCV Combo2
Pairs			Core: 8HC55+M1	Core: 8HC56+M1
			NS3: 8HC53+8HC53	NS3: 8HC53+8HC53
			NS4: 8HC51+8HC52	NS4: 8HC51+8HC52
Type of positive samples		Number	False negative	
HCV genotype samples	1a	1	0	0
	1b	48	0	0
	1b/2a	3	0	0
	2a	34	0	0
	2a/1b	1	0	0
	3b	1	0	0
	3b (5'UTR)	1	0	0
	6 (5'UTR)	3	0	0
	6/1b	3	0	0
	6a	1	0	0
	6a/2a	1	0	0
Total	Sample number	97	97	97
	False neactive	0	0	0
Total sensitivity, %		/	100.0	100.0

Table 8.

Specificity evaluation of anti-HCV Core/NS3/NS4 combo assay prototypes.

Assay			Anti-HCV Combo1	Anti-HCV Combo2
Pairs			Core: 8HC55+M1	Core: 8HC56+M1
			NS3: 8HC53+8HC53	NS3: 8HC53+8HC53
			NS4: 8HC51+8HC52	NS4: 8HC51+8HC52
Type of positive samples		Number	False reactive	
Interference sample		78	0	1
ANA positive sample		50	0	0
High IgG interference		50	0	0
High IgM interference		50	0	0
High IgA interference		50	0	0
RF sample		103	1	1
Total	Sample number	381	381	381
	False reactive	/	1	2
Sample category		Number	False reactive	
Collected samples	Study 1	725	0	0
	Study 2	650	0	0
Pregnant population		200	1	1
Dialysis population		75	0	0
Old adults (>60 years old) population		200	0	0
Random clinical samples		1,224	1	1
Total	Sample number	3,074	3,074	3,074
	False reactive	/	2	2
Total specificity, %		/	99.91	99.88

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ORDERING INFORMATION

ANTIGENS

Product name	Cat. #	pI, theoretical	Molecular weight, kDa	Purity, %	Expression system
HCV, NS4 antigen, recombinant	8HC51	5.9	19.2	≥90	prokaryotic cells
HCV, NS4 antigen, recombinant	8HC52	5.9	86.7	≥90	mammalian cells
HCV, NS3 antigen, recombinant	8HC53	6.2	29	≥92	prokaryotic cells
HCV, core antigen, C-terminal Cys, recombinant	8HC55	11.8	17.5	≥90	prokaryotic cells
HCV, core antigen, recombinant	8HC56	5.7	36	≥90	mammalian cells
HCV, core antigen, recombinant	8HC59	11.8	17.4	≥90	prokaryotic cells

HYTEST LTD
Intelligate 1, 6th floor, Joukahaisenkatu 6 • FI-20520 Turku, FINLAND
Tel. +358 2 512 0900 • E-mail: hytest@hytest.fi • **HYTEST.FI**
HYTEST NORTH AMERICA INC.
800 MacArthur Blvd, STE 100, Mahwah, New Jersey 07430
Tel. 833-368-3489 • E-mail: hytestna@hytest.fi • **HYTESTNA.COM**