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Profile of the VERSANT HCV Genotype 2.0 Assay

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Abstract

Introduction: Hepatitis C virus (HCV) is divided into seven genotypes and 67 subtypes. HCV genotype studies reflect the viral transmission patterns as well as human migration routes. In a clinical setting, HCV genotype is a baseline predictor for the sustained virological response (SVR) in chronic hepatitis C patients treated with peginterferon or some direct acting antivirals (DAAs). The Versant HCV genotype 2.0 assay has been globally used for HCV genotyping over a decade.

Areas covered: The assay is based on reverse hybridization principle. It is evolved from its former versions, and the accuracy and success of genotyping/subtyping rate are substantially improved. It shows an accuracy of 99-100% for genotypes 1-6. It can also reliably identify subtypes 1a and 1b. However, the assay does not allow a high resolution for many other subtypes. Reasons for indeterminate or inaccurate genotyping/subtyping results are discussed in this manuscript.

Expert commentary: Genotyping helps to find the most efficacious and cost-effective treatment regimen. The rapid development of anti-HCV treatment regimens, however, is greatly simplifying laboratory tests. In the near future, the need for HCV genotyping and frequent serial on-treatment HCV RNA tests will decrease along with the wide use of the more potent and pan-genotypic DAA regimens.

Keywords  Hepatitis C virus (HCV), genotype, subtype, recombinant, direct acting antivirals (DAAs), next-generation sequencing (NGS), Versant HCV genotype 2.0 assay
1. Introduction

Hepatitis C virus (HCV) belongs to the Flaviviridae family, Hepacivirus genus. HCV has a positive-strand 9,600-nucleotide genome, containing the 5' untranslated region (5'-UTR), the open reading frame encoding the structural and nonstructural (NS) proteins, and the 3'-UTR (Figure 1). The structural proteins include the core and envelope (E1 and E2) proteins, which form the viral particle. The NS proteins include the p7, NS2, NS3, NS4, and NS5 proteins, which are essential for viral replication [1]. Comparison of HCV isolates demonstrates a high variability in the E and NS sequences, whereas the 5'-UTR and, to a lesser extent, the core sequences are conserved [2, 3].

HCV affects 115 million people worldwide (i.e. 1.6% global anti-HCV seroprevalence) [5], and the viremic prevalence is estimated to be 1.1%. HCV infection is more prevalent in special populations such as intravenous drug users, hemodialysis patients, cancer patients, and paid blood donors [6]. Chronic hepatitis C (CHC) is the major cause of liver cirrhosis and hepatocellular carcinoma in the Western countries. In many developing countries where HCV receives little attention, the disease burden seems much higher [7].

2. HCV genotype & subtype classification

HCV has been divided into seven genotypes and 67 confirmed subtypes through phylogenetic analysis of the viral genome or representative subgenomic regions [8]. Genotypes differ from each other by approximately 30% at the nucleotide level, while subtypes differ by 20-25% [9]. Intergenotypic or intragenotypic recombination is sporadically reported, although the clinical and epidemiological importance remains to be further studied [10-12]. Furthermore, more cases of multi-genotype mixed infection have been discovered recently using the high-throughput deep-sequencing technology, i.e. the next generation sequencing (NGS) technology [13-15]. Mixed infection was reported to be responsible for direct acting antiviral (DAA) treatment failure [14].

3. Worldwide HCV genotype distribution

Genotypes 1, 2 and 3 are distributed worldwide. Genotype 1 is predominant (46%), followed by genotype 3 (22%) and genotype 2 (13%) [5]. Genotype 4 is the most prevalent genotype in Arab countries in the Middle East region and in some African countries, accounting for 56-91% of the HCV infected patients [16, 17]. Genotype-5 infection is mainly restricted in South Africa but sporadic cases are also reported in several other countries [18, 19]. In the south and southwest of China and Southeast Asian countries, genotype-6 infection is endemic [20-23].
4. Epidemiological and clinical importance of HCV genotype

Study on HCV genotype and subtype is of epidemiological importance. The distribution and prevalence of HCV genotypes reflect the viral transmission patterns as well as human migration routes [24, 25]. Our previous report showed that the main transmission route for genotype-3 HCV in China is through intravenous drug use and tattoo, while blood transfusion remains as the main transmission route for genotypes 1 and 2 [20]. Subtype-3a HCV in the United Kingdom might originated in India and was later transmitted in the time of Indian independence [26]. Another intriguing finding is that genotype 1a is predominant in HCV infected patients in the Philippines. The genotypic distribution is unique compared with that of other Asian neighbors, but more similar to that of the Western countries. Special human migration patterns or viral transmission mode is worth investigating [27]. Genotype analysis also helps reveal the etiology of the localized outbreaks of HCV infection [19, 28].

In the time of peginterferon-based anti-HCV treatment (standard of care), HCV genotype is one of the strongest baseline predictors for sustained virological response (SVR) [29]. Patients with genotype-1 or -4 HCV infection are more difficult to treat than those with genotype 2, 3 or 6 [29, 30]. SVR rate is only approximately 50% for genotype 1 or 4 while over 80% for genotype 2, 3 or 6. Genotyping are needed to tailor the treatment duration and dose of ribavirin [29].

In the early stage of DAAs (around the year of 2011), triple therapy using the NS3/4A protease inhibitors (telaprevir or boceprevir) in combination with peginterferon and ribavirin necessitated the differentiation of the subtypes of genotype 1, because 1b was associated with a lower rate of the occurrence of resistance associated substitutions (RASs) and a more favorable response than 1a. Similar subtype-specific patterns were also observed in NS5A inhibitors [31]. Since most DAAs were originally developed using genotype-1 specific replicon models, genotype 3, at one time, was regarded to be more difficult to treat [32]. Shortly afterwards, interferon-free regimens were available with the rapid development of other more potent DAAs. Sofosbuvir, an NS5B inhibitor, has excellent antiviral efficacy and servers as an excellent backbone in the majority of DAAs regimens [33]. Genotype testing, especially for genotype 3, is still needed to achieve the best efficacy [34]. Pre-existing A30K/L31M were found in all subtypes-3b/3g patients. The subtypes seemed inherently resistant to all NS5A inhibitors [35]. Our recent research also shows that over 90% of treatment-naive subtype-3b patients have A30K/L31M while such RASs are rarely observed among subtype-3a patients [36]. This partially explains why the genotype-3b patients are predominant in those with DAA treatment failure in China. Moreover, an in vitro experiment demonstrated that the strain of genotype 6a could
compromise sofosbuvir treatment through the rapid development of S282T, the RAS against NS5B inhibitors, emphasizing the importance of DAAs combination therapy for genotype 6a and reflecting the fact that the efficacy of many all-oral regimens is genotype/subtype dependent [37].

Since 2016, pan-genotypic DAA regimens such as the sofosbuvir/velpatasvir and glecaprevir/pibrentasvir combinations have been available [38-43]. It is possible to cure CHC patients without genotyping HCV. It is especially useful for patients in areas where the virological testing is not available [40, 43].

5. Current HCV genotyping approaches

Serological and molecular technologies are used for genotyping. Compared with molecular assay, serotyping assay is time and labor saving but not accurate due to the cross-reactivity of the anti-HCV antibodies among different genotypes [44, 45]. Current genotyping assays are mainly based on molecular technology.

Whole genome sequence analysis is the “gold standard” for genotyping HCV. It is generally time consuming and labor intensive, although the optimized method was reported to achieve the near full-length genome [46]. The vast majority of genotyping assays focus on the representative subgenomic fragments. In most commercial assays, for instance, the 5'-UTR is included. This region is highly conserved and easily PCR amplified, and a well-characterized set of polymorphisms can indicate the genotype using assays such as the line probe assay (LiPA) [47-51], dot blotting [52], genotype-specific real-time PCR [53-60], and sequencing based assays [14, 61, 62].

There are also many laboratory developed tests widely used in clinical laboratories. Assays based on the restriction fragment length polymorphism analysis of the 5'-UTR is being replaced, because it is complicated, genotyping-information limited and prone to carryover contamination caused by the PCR amplicon aerosol [63]. In-house Sanger sequencing methods usually select the core or NS5B in addition to the 5'-UTR for genotype analysis [11, 64-70]. The core sequence is less conserved than the 5'-UTR sequence, providing enough information for phylogenetic analysis to differentiate genotypes and subtypes; besides, it is easier to be PCR amplified just using a pan-genotypic primer pair. Therefore, it is recommended for genotype and subtype analysis [47, 69, 71-75]. By phylogenetic analysis of a 222-nucleotide fragment of the NS-5 sequence, Simmonds et al. first classified HCV into six major genotypes and a series of subtypes in 1993 [65]. This method serves as the reference genotyping and subtyping methods in many later studies. The region, however, shows higher variety than
the core region. The amplification is more challenging, with a failure rate of 20% or higher and various primer pairs should be used [67, 69, 70, 76]. Therefore, NS5B sequencing assay seems less cost-effective than the core sequencing assay for clinical laboratories. It is noteworthy that since most of the recombination breakpoints exist between the two regions [10], more recombined genotypes will be found if the core and NS5B regions are sequenced in combination [68].

The NGS technology has also been applied for genotypic analysis over the past decade. It can also detect the minor mixed genotypes and potential DAA RASs through ultra-deep sequencing [13, 14, 63, 77-79]. NGS technology provides a more detailed profile of the global HCV subtype and RAS distribution [80]. Table 1 lists the characteristics of currently available genotyping assays.

6. Principle of the Versant HCV genotype 2.0 assay

The Versant HCV Genotype 2.0 Assay (LiPA) is based on the reverse hybridization principle, as shown in Figure 1A. Twenty-two genotype-specific oligonucleotide DNA probes (Figure 1C) are immobilized on LiPA nitrocellulose strip by a poly(dT) tail. First, HCV RNA is extracted from serum or plasma samples, reverse transcribed and PCR amplified with the biotinylated primers for the 5'-UTR and core regions. Second, the biotinylated amplicons are hybridized with the genotype-specific probe bands on the strip. The unbound amplicons are washed away. Third, the alkaline phosphatase labeled streptavidin is added and bound to the biotinylated hybrids. The alkaline phosphatase hydrolyses the substrate, 5-bromo-4-chloro-3-indolylphosphate (BCIP), then BCIP generates purple or brown precipitates by the oxidization of the nitroblue tetrazolium (NBT). The post-PCR procedure can be performed manually or automatically on the Auto-LiPA machine (Figure 1B). The banding patterns and their corresponding genotyping results are listed on the Interpretation Chart. There are more than 360 possible patterns. In our experience, the number of the patterns is approximately 100 in China, and the most commonly encountered patterns are listed in Figure 1D.

7. Evolution of the LiPA HCV genotyping assay

The prototype of the LiPA was developed 25 years ago. In 1996, a modified version, the INNO-LiPA HCV II assay, became widely used along with the gradual establishment of the HCV genotype nomenclature [81]. The reported accuracy was 100% for genotypes 1, 2, 3, 4 and 6a [81, 82]. But later studies showed that it misclassified non-6a/6b subtypes of genotype 6 as 1 [47, 48]. Generally, subtypes 6a/6b can be distinguished easily by the unique dual nucleotide insertion “CA” between positions 197 and 198 in the 5’-UTR.
Non-6a/6b subtypes, however, share almost the same 5'-UTR sequence as genotype 1, so they could hardly be distinguished only using the 5'-UTR [47, 48]. Moreover, the assay was not accurate to differentiate subtypes 1a from 1b [83, 84]. Polymorphisms at positions 107, 204 and/or 243 were responsible for the mistyping [83]. Such limitations promoted the development of the Versant HCV Genotype 2.0 assay. Core sequence was added for determining the non-subtype-6a/b genotype 6 and differentiating between subtypes 1a and 1b [48, 49, 51]. The genotypes 2-5 and 6a/6b are still determined by analysis of the 5'-UTR sequence.

8. Performance of the Versant HCV genotype 2.0 assay

8.1 Accuracy at the genotype level

The Versant HCV Genotype 2.0 assay (LiPA) yields highly accurate results at the genotype level. The overall consistency ranged from 99.2% to 100% for samples which were successfully typed [48, 49, 85, 86]. Although there were relatively small number of genotype-4 and -5 strains involved in the comparison studies, the consistency was also very high (97% to 100%) [87, 88]. It could also differentiate most of the non-6a/6b genotype-6 samples from the genotype-1 samples using the core sequence instead of the 5'-UTR sequence; therefore, the accuracy for genotype-6 determination was also substantially improved [12, 48]

8.2 Accuracy at the subtype level

At the level of subtype, however, the accuracy varies. As mentioned above, the assay performs pretty well for subtyping 1a and 1b. A comparison study from France showed that the accuracy for determining subtypes 1a and 1b was 99.6%, 93.2%, 77.9% and 70.8% for the Versant 2.0, Realtime HCV GT II, Trugene HCV 5'NC genotyping and INNO-LiPA II assays, respectively [83]. Two studies from Italy and China also showed a high concordance between the Versant HCV genotype 2.0 and Realtime HCV GT II assays for subtyping 1a and 1b (95.6% and 96.1%, respectively) [53, 86].

But the assay cannot always provide clear subtype results. The overall rate of concordance at the subtype level was only 64.7% [85]. Especially for genotype-4 samples, the assay fails to generate reliable subtyping results. Ntagirabiri et al. found that the Versant HCV Genotype 2.0 assay failed to subtype 115 (69.3%) genotype-4 samples [89]. It is conceivable. First, high sequence heterogeneity exists among various subtypes, and it is not possible to differentiate them based on sole analysis of the 5'-UTR sequence [9]. Second, the performance of the assay is influenced by the origin of the samples. It is not likely to operate to the published level of accuracy in high-diversity regions [9]. Third, the
assay might not cover the newly designated HCV subtypes in advance [90, 91]. Fourth, most uncommon subtypes are endemically distributed in resource-limited regions and receive little attention from the sponsors of clinical trials and manufacturers of the commercial assays.

8.3 Reasons for the indeterminate or inconsistent results

Existence of the indeterminate genotyping results is inevitable using the Versant HCV genotype 2.0 assay. Larrat et al. found that the indeterminate results were more frequently observed in samples from the Arabian Peninsula than in those from Europe, and more indeterminate results were found in samples with genotypes 4-6 [50]. There are two reasons for such results. First, as mentioned above, single or multiple polymorphisms of the 5'-UTR and core sequences might result in banding patterns unlisted on the Interpretation Chart [12, 50]. Generally speaking, existence of the indeterminate results is one of the inherent limitations for all genotyping assays based on the hybridization principle, such as LiPA, dot blotting, and probe-specific real-time PCR assays [92]. Second, for cases with low viral load, inadequate PCR product might cause inadequate hybridization and unexplainable band patterns as well.

The assay was not intentionally designed to detect the recombinant genotype, which is responsible for many inconsistent cases. In our previous study, two samples with genotype 6/1 recombinant were identified as genotype 6 by the Versant HCV genotype 2.0 assay and Realtime HCV GT II assay, but both were classified as genotype 1 through NS5B sequencing [12]. Intergenotypic 2/1 recombination strains were reported by several study groups. They were classified as genotype 2 using the Versant HCV genotype 2.0 assay [93-95].

8.4 Limit of detection (LOD)

LOD is dependent on the lowest HCV RNA level at which RNA can be successfully reverse transcribed, amplified and used for hybridization. According to the manufacturer’s instruction, the LOD is 2106 IU/mL serum or plasma HCV RNA. LOD can be improved by using an extraction kit of high yield and high quality of RNA, or by an additional concentration step prior to RNA extraction. Samples with low viral load, however, sometimes yield unreliable or indeterminate results. In some cases, multiple “ghost” bands appear on the strip, leading to the misinterpretation of multi-genotype mixed infections [20]. Therefore, for the minority of CHC patients with RNA level far below LOD, it is suggested to refer to other more sensitive assays such as the Realtime HCV genotype II, the Cobas HCV GT, or in-house assays based on nested-PCR.
9. Conclusion

As a very accurate genotyping tool, the Versant HCV genotype 2.0 has played an important role in the era of peginterferon. In the era of DAAs, however, its role is being challenged by the pan-genotypic anti-HCV treatment regimens and the emerging sequencing technology, described as follows.

10. Expert commentary

The LiPA genotyping assay has been widely used over two decades. It has many merits. It is instrument independent (in the manual mode). The band pattern is usually clear to be interpreted, and patients can get results within six hours. But it should be noted that molecular genotyping assay, not just limited to the HCV related experiment, belongs to the experiment of high complexity. Processing samples with high viral load, and handling PCR amplicons in the open air are prone to carryover aerosol contamination. Therefore, special care must be taken. Separated experimental materials and equipment in the appropriate work areas for the RNA extraction, PCR and hybridization are strongly recommended. Samples, equipment, or reagents should not be returned to the area where the previous step has been performed. In addition, PCR amplicons and the final product of the hybridization should be disposed as soon as possible. The negative quality control is indispensable throughout the whole process for each run. Moreover, the accurate temperature control for DNA hybridization is vital. Lower temperature might lead to false positive signals, and higher temperature will result in weak or negative signals.

The Versant HCV genotype 2.0 assay is highly accurate for determining genotypes 1 to 6 and subtype 1a and 1b. But its inherent weakness is the existence of indeterminate genotyping results due to the sequence heterogeneity, especially for samples from the high-diversity areas. Besides, it do not allow the detection of recombinant strains. Genotype recombination may be of clinical importance with regard to the tailored choice of anti-HCV treatment and overall prognosis [94, 95]. In these cases, analysis of at least two different target regions at both ends of the HCV genome is preferable.

From a methodological point of view, genotyping methods based on Sanger sequencing is more potent than those based on hybridization, allowing the simultaneous determination of genotype/subtype, pre-existing RASs or those selected by DAAs treatment, and other potential useful viral sequence information which might benefit the personalized antiviral therapy. The price of the Sanger sequencing is also very affordable now. The biggest limitation for Sanger sequencing is that it is instrument dependent. Sanger sequencing assays are more suitable for large clinical laboratories where the
instrument is affordable, or the sequencing service is easily accessible from the sequencing companies.

Coincidentally, rapid growth of the high-throughput deep-sequencing NGS technology overlaps the revolutionary development of anti-HCV treatment. NGS is an even more powerful tool than Sanger sequencing for molecular studies, but it requires highly skilled personnel with bioinformatics knowledge for accurate and reliable interpretation of results. The emerging on-line genotypic interpretation services, for example, the geno2pheno, simplifies the time-intensive step of processing raw data generated by NGS [96]. Cost is another major concern before NGS can be used in clinical settings. It is still expensive to test a single sample and seems not yet cost-effective for genotyping such a pathogen without a huge genome.

Clinical significance of HCV genotype is changing dramatically in the recent 10 years, as shown in Figure 3. But nonetheless, as a cornerstone biomarker for HCV, genotyping test might contribute its remaining energy in the era of DAAs. First, genotyping is essential for special CHC populations. For instance, even the potent pan-genotypic sofosbuvir/velpatasvir regimen is not recommended to treat genotype-3 patients with compensated cirrhosis. Another DAA, voxilaprevir, should be added [40]. Second, genotype detection helps optimize the treatment duration of the pan-genotypic DAA regimen. For instance, glecaprevir/pibrentasvir can be used for only 8 weeks for non-cirrhosis CHC patients. But for treatment-experienced genotype-3 patients, treatment duration should be extended to 12 weeks. For treatment-experienced genotype-3 patients with compensated cirrhosis, treatment duration should be extended to 16 weeks compared to 12 weeks for patients with other genotype. Third, when a DAA regimen is not efficacious, genotyping and RASs testing may be still crucial for the selection of an alternative regimen. In summary, HCV genotyping remains useful to create a more personalized therapeutic pathway, especially for those with complicated clinical conditions.

Figure 3.

11. Five-year view

Currently, HCV genotype testing is still needed, because in the real world, there are a large number of CHC patients who are receiving the genotype-specific DAA or even the peginterferon-based treatment. For them, the pan-genotypic antiviral regimens have not yet been available, or the cost is not affordable. Genotyping helps to find the most efficacious and cost-effective regimen.
However, the rapid development of antiviral treatment is greatly simplifying the laboratory testing for HCV. The need for HCV genotyping as well as the serial on-treatment HCV RNA tests will gradually decrease along with the more potent and pan-genotypic DAA regimens. As a result, the market for HCV genotyping assays will shrink.

HCV strains with new genotype or subtype are still being reported [97, 98], whereas they do not seem to be a barrier to achieving SVR. Borgia et al. identified a new genotype, the genotype 8, in four Indian patients. All achieved SVR under different DAAs treatment regimens [97]. This new genotype was previously classified as genotype 5 by the Versant HCV genotype 2.0 and other commercial assays. However, efforts to improve the inclusivity for the new genotype or subtype seems not necessitated in the coming years of the pan-genotypic DAAs.

In the next five years, with the aid of the advanced molecular tools such as the NGS, the concept of “HCV genotyping” might be extended to the whole genome level, including genotype, subtype, genotype mixture, recombination, RASs, potential substitutions and even the quasispecies profile that might affect the virological response. The panoramic genotyping result might benefit the retreatment of the difficult-to-treat patients who fails to be cured using the potent DAAs combination regimen, and promote the goal of global HCV elimination.

Key issues

- The Versant HCV genotype 2.0 assay is a classical genotyping assay, which has been widely used in laboratories and clinical trials. It provides highly accurate genotype results. For some subtypes, however, it cannot provide clear and reliable subtyping results based on sole analysis of the 5’-UTR sequence.
- Sequence heterogeneity of the 5’-UTR and core regions, genotype recombination, multi-genotype mixed infection, and testing samples with very low viral load may lead to indeterminate and inaccurate results.
- In the era of interferon-based treatment for CHC, baseline genotype test is used to predict SVR and tailor the treatment duration and dosage. In the early development stage of DAAs, subtypes 1a and 1b should also be identified for interferon-based triple antiviral regimens. Furthermore, efficacy of some of the current all-oral DAA regimens is still genotype dependent.
- The usefulness of the HCV genotype assay, however, is being fiercely challenged by the rise of the pan-genotypic DAA regimens.
- The usefulness of the Versant HCV genotype 2.0 assay is also being challenged by
the revolutionary development of the NGS technology, a more powerful tool providing more detailed information for precise and personalized antiviral therapy.

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Declaration of Interest

The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

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Abbreviations: LiPA, the line probe assay; 5’-UTR, 5’ untranslated region; CHC, chronic hepatitis C; SVR, sustained virological response; DAAs, direct acting antivirals; NGS, next-generation sequencing; RAS, resistance associated substitution; BCIP, 5-bromo-4-chloro-3-indolylphosphate; NBT, nitroblue tetrazolium; RT-PCR, reverse transcription-polymerase chain reaction; LOD, limit of detection
References

Reference annotations

* Of interest
** Of considerable interest


* Demonstrating the use of the representative subgenomic sequences for epidemiological and clinical studies.


** The updated classification of seven HCV genotypes and 67 subtypes.


** Systematic nomenclature of hepatitis C genotypes and subtypes.


** HCV genotype is an important predictor for peginterferon and ribavirin therapy.


** The era of the pan-genotypic anti-HCV treatment is coming, and the clinical significance of HCV genotyping is being weakened.


** The Versant HCV Genotype 2.0 assay (LiPA) could detect genotypes 6 more accurately.


** Reasons for the indeterminate results released by the Versant HCV Genotype 2.0 assay (LiPA).


** Classification of HCV into 6 major genotypes using 222 base-pair fragment of the NS5B region.


* The modified version of the HCV genotyping assay, the INNO-LiPA HCV II assay (in 1996).


* The Versant HCV Genotype 2.0 assay (LiPA) could identify subtype 1a and 1b more accurately.


Table 1. Currently available assays for HCV genotyping

<table>
<thead>
<tr>
<th>Target region(s)</th>
<th>Versant HCV Genotype 2.0 (LIPA)</th>
<th>Realtime HCV GT II</th>
<th>Cobas HCV GT</th>
<th>Sentosa HCV Genotyping assay</th>
<th>SQ Sanger sequencing HCV genotyping assay</th>
<th>HISCL HCV Genotyping assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manufacturer</td>
<td>Siemens</td>
<td>Abbott</td>
<td>Roche</td>
<td>Vela Diagnostics</td>
<td>Sanger laboratory developed test</td>
<td>Sysmex</td>
</tr>
<tr>
<td>Principle</td>
<td>reverse line probe hybridization</td>
<td>real-time PCR with genotype/subtype specific primers and fluorescent-labeled oligonucleotide probes</td>
<td>real-time PCR with genotype/subtype specific primers and fluorescent-labeled oligonucleotide probes</td>
<td>next-generation sequencing (deep sequencing) and data analysis</td>
<td>Sanger population sequencing and phylogenetic analysis</td>
<td>genotype-specific antibodies against NS4A</td>
</tr>
<tr>
<td>Workflow</td>
<td>RNA extraction, one-step RT-PCR, DNA hybridization and result interpretation</td>
<td>RNA extraction, RT-PCR and result interpretation</td>
<td>RNA extraction, RT-PCR and result interpretation</td>
<td>RNA extraction, library preparation, template preparation, deep sequencing and data quality control/analysis partially automated on the Sentosa platform</td>
<td>RNA extraction, RT-PCR, Sanger sequencing and phylogenetic analysis</td>
<td>competitive chemiluminescent immunoassay</td>
</tr>
<tr>
<td>Automation</td>
<td>DNA hybridization performed manually or automatically on the Auto-LIPA</td>
<td>completely automated on the m2000sp/rt platform</td>
<td>completely automated on the Cobas 4800 platform</td>
<td>manually</td>
<td>completely automated on the HISCL platform</td>
<td></td>
</tr>
<tr>
<td>Turnaround time</td>
<td>6 hours</td>
<td>5 hours</td>
<td>5 hours</td>
<td>48 hours or longer</td>
<td>24 hours or longer</td>
<td>17 minutes</td>
</tr>
<tr>
<td>Genotyping result</td>
<td>1-6</td>
<td>1-6</td>
<td>1-6</td>
<td>6-12</td>
<td>4-6</td>
<td>1 and 2</td>
</tr>
<tr>
<td>Subtyping result</td>
<td>1a,1b, 2a/c, 2b, 3a, 3b, 4a/o/d, 5a, 6a/b, 6c/l</td>
<td>1a, 1b</td>
<td>1a, 1b</td>
<td>all subtypes</td>
<td>all subtypes</td>
<td>incapable of subtyping HCV</td>
</tr>
<tr>
<td>Lower limit of detection</td>
<td>2106 IU/mL HCV RNA</td>
<td>500 IU/mL HCV RNA</td>
<td>125 IU/mL HCV RNA</td>
<td>100 IU/mL HCV RNA</td>
<td>100-5000 IU/mL HCV RNA</td>
<td>detectable anti-HCV antibodies</td>
</tr>
<tr>
<td>Strength and limitation</td>
<td>instrument independent (in manual mode); clear result interpretation; short turnaround time; indeterminate results due to the sequence heterogeneity which leads to indeterminate results; limited in the determination of recombinant strains</td>
<td>complete automation; labor-saving; short turnaround time; more resistant to potential aerosol contamination; subject to the sequence heterogeneity which leads to indeterminate results; limited in the determination of recombinant strains</td>
<td>complete automation; labor-saving; short turnaround time; more resistant to potential aerosol contamination; subject to the sequence heterogeneity which leads to indeterminate results; limited in the determination of recombinant strains</td>
<td>more efficient for subtyping; providing quantitative results of the minor mixed genotypes and potential RASs; sequencing instrument dependent; complicated experiment and more expertise needed; turnaround time usually over 24 hours</td>
<td>more efficient for HCV subtyping test and RASs detection; sequencing instrument dependent; complicated experiment and more expertise needed; turnaround time usually over 24 hours</td>
<td>simple and rapid; can be used in patients with very low viral load, or patients with SVR or spontaneous viral clearance; not very accurate due to the cross-reaction among anti-HCV antibodies unable to determine genotypes 3-6 and subtypes</td>
</tr>
</tbody>
</table>

- Genotyping assay targets: Genotypes 1-6
- Lower limit of detection: 50 IU/mL HCV RNA
- Strength and limitation:
  - Instrument independent (in manual mode)
  - Clear result interpretation
  - Short turnaround time
  - Indeterminate results due to the sequence heterogeneity
  - Prone to aerosol contamination
  - Limited in the determination of recombinant strains

- Limitation:
  - Prone to contamination
  - Heterogeneity
  - Sequence results due to time, turnaround short, interpretation clear results

- Limitation of Genotyping assay:
  - Results limited in the region(s)
  - More resistant to potential aerosol contamination
  - Heterogeneity

- Subtyping result:
  - Genotypes 1a, 1b, 2, 3, 4, 6
  - Genotypes 5
  - Genotypes 5a, 5b
  - Genotypes 5c, 5d

- Lower limit of detection:
  - HCV RNA 50 IU/mL
  - HCV RNA 125 IU/mL
  - HCV RNA 1000 IU/mL
  - HCV RNA 5000 IU/mL

- Strength and limitation:
  - More efficient for subtyping
  - Providing quantitative results of the minor mixed genotypes and potential RASs
  - Sequencing instrument dependent
  - Complicated experiment and more expertise needed
  - Turnaround time usually over 24 hours

- Strength and limitation (in manual mode):
  - The test is slightly more labor-saving
  - The test is slightly more accurate

- Limitation:
  - The test is limited in the determination of recombinant strains
  - The test is prone to aerosol contamination
  - The test is prone to heterogeneity
of mixed genotypes and recombinant strains expertise needed; turnaround time usually over 48 hours

Figure 1. Schematic of HCV genome and the representative regions for genotype/subtype test, treatment response or resistance study, or epidemiological survey. The conversation score demonstrates the degree of sequence variation (adapted from Qiu et al. [3] with modification). Numbers under the genomic structure represent the start number of each region, and 9646 is the ending number of the whole genome [4]. Sequence of the isolate H77 (GenBank: AF009606) serves as a reference sequence.
Figure 2. Schematic of the principle of the Versant HCV genotype 2.0 assay (A.), the Auto-LiPA hybridization machine (B.), the genotype and subtype-specific probes used for genotyping HCV (C.), and the most commonly encountered band patterns and their proportions in 1a, 1b, 2a/c, 3a, 3b and 6a result patterns [20] (D.)

BCIP, 5-bromo-4-chloro-3-indolylphosphate; NBT, nitroblue tetrazolium
Figure 3. Clinical significance of HCV genotyping changing with the evolution of antiviral treatment regimens. Grayscale of the time axis represents the SVR rate. DAA, direct acting antiviral