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**Multiplex qPCR for serodetection and serotyping of hepatitis viruses: A brief review**

Irshad M *et al*. Multiplex qPCR for hepatitis viruses

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**Abstract**

Present review describes the current status of multiplex quantitative real time PCR (qPCR) assays developed and used globally for detection and subtyping of hepatitis viruses in body fluids. Several studies reported the use of multiplex qPCR for detection of hepatitis viruses including HAV, HBV, HCV, HDV and HEV. Simultaneously, multiplex qPCR was also developed for genotyping of HBV, HCV, and HEV subtypes in last few years. Although, a single step multiplex qPCR assay for all six hepatitis viruses *i.e.,* A to G viruses, is not yet reported, however, it appears quite likely in near future with increasing advancement in technologies. All studies demonstrate the use of conserved region on viral genome as the basis of amplification and hydrolysis probes as the preferred chemistries for better detection in the assays developed. Based on standard plot prepared using varying concentration of template and the observed threshold cycle (Ct) value, it is possible to find out linear dynamic range and calculate an exact copy number of virus in the specimen. Moreover, the advantage of multiplex qPCR assay over singleplex or other molecular techniques lies in fast results, low cost and single step investigation process in samples from patients with co-infection.

**Key words:** Viral genome; Hepatitis Viruses; Quantitative Real-Time PCR; Genotyping Techniques; Co-infection; Serotyping

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**Core tip:** Present review describes the worldwide application and the significance of multiplex quantitative real time PCR (qPCR) for simultaneous detection of hepatitis viruses and their subtypes in serum. The published literature demonstrates that multiplex qPCR assay is a fast, easy, cost-effective and sensitive technique for early diagnosis of hepatitis co-infections. This technique in comparison to other diagnostic procedures is finding more use in the diagnostic laboratories.

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**INTRODUCTION**

Viral hepatitis is a serious public health problem requiring its early diagnosis and timely treatment. There are a number of hepatitis viruses that have already been characterized for their molecular structure and named alphabetically as hepatitis viruses A, B, C, D, E, and G (HAV, HBV, HCV, HDV, HEV, HGV), respectively. These are hepatotropic and non-cytopathic in nature and cause liver damage by immune mediated cell lysis[1]. Besides, there is an additional group of viruses causing hepatitis but not yet characterized. These have been put under the category of non A-G hepatitis viruses. HAV infects mainly pediatric age group, occurs both sporadically as well as in epidemics and accounts for an estimated 1.4 million cases annually[2]. Two billion people are supposed to be infected with HBV globally and approximately 350 million of them, suffer from chronic hepatitis B infection[3]. About 25% of adults infected with HBV during childhood are reported to die from hepatocellular carcinoma (HCC) or liver cirrhosis[4]. Also, 3-4 million people are infected with HCV each year, and a high proportion of them develop chronic HCV infection. A large population infected with HCV dies from serious liver diseases annually[5]. Similarly, reports are available on HEV infection also. In addition to individual viral infection, there are cases of co-infections reported from various parts of the world. Hepatitis A and E infections usually run benign course of disease and resolve in due course of time without developing chronic diseases. On the contrary, hepatitis B and C infections cause severe liver diseases developing chronicity in significant number of patients. Another interesting finding is that hepatitis A and E infections in patients with pre-existing HBV or HCV infections, develop serious diseases with significant rise in morbidity and mortality[6].

 The diagnosis of hepatitis viral infections is usually done with serological markers in blood. However, there are situations where serology loses its credibility. For example, serological markers can not differentiate between past and present infections. Also, serological tests do not address the problem of antigenic variations in viruses, infections with different genotypes, presence of silent carriers and absence of antibody in early phase of infection[7]. Moreover, the presence of maternal antibodies makes it impossible to detect infections in newborns[8]. In order to have an alternate system, the nucleic acid tests (NAT) based methods were developed for detecting viral genome in serum for the diagnosis of viral hepatitis. The NAT based methods have the benefit of direct examination of the infectious agent’s genome in serum[9,10].

 The conventional PCR is one such NAT based method which has been a practice in some laboratories for diagnosis of viral hepatitis in last few years[11]. However, the conventional PCR still remains a lengthy procedure with several technical and operational problems and so, finds a selective use. Also each marker has to be investigated separately by PCR and it takes very long time before reaching final diagnosis. With all these limitations of conventional PCR, the use of real time PCR was supposed to be a better option for an earliest diagnosis of viral hepatitis both in sporadic and epidemic cases. Real time PCR is one of the latest techniques frequently used for diagnosis of various infectious diseases including viral hepatitis. It is based on the detection of causative pathogen-related nucleic acid in body fluids in a very short time period. It is also used to determine different molecular forms and variant molecular species of pathogens including bacteria, viruses and several parasites *etc*.[12,13]. This is a specific and sensitive technique and uses specific probes and primers to detect target sequences on genome. Moreover, this technique is performed on an automated machine without need of post PCR procedures, thus minimizing cross contamination between samples, simultaneously accelerating the analysis[14].

The development of molecular technologies in last few years has passed a strong message to medical researchers to explore ways for further improvement of diagnostic procedures. Those working in the area of medical virology have switched from traditional approaches of virus detection in clinical samples to multiplexing for simultaneous detection of multiple pathogens in a single assay[15]. In recent past, several PCR based assays coupled with oligonucleotide microarray technology have been designed to allow simultaneous detection and genotyping of several viruses including blood borne pathogens[16], respiratory viruses[17] and adenoviruses[18]. These assays show a significant increase in the sensitivity of detection reaching to 10-100 copies of target RNA/DNA in a sample[19]. Simultaneously, easy performance, fast reaction, low cost and monitoring of results on screen, has made these assays an attraction for all diagnostic laboratories, furnished with minimal essential facilities. After survey of literature on use of PCR based multiplex assays for detection and genotyping of hepatitis viruses, we could notice several attempts made for the development of multiplex real time PCR assays in the area of viral hepatitis in last few years. To compile up all such informations for their use to readers working in this area, present review gives an update on the development, use and significance of multiplex qPCR in the field of viral hepatitis.

**EXPERIMENTAL APPROACH FOR MULTIPLEX qPCR**

***Search for conserved regions***

In order to develop a multiplex qPCR assay for multiple pathogens, the first and foremost step is to explore and locate the target region on each pathogen’s genome for amplification purpose. Since variation in genome is a dynamic process, it is necessary that multiplex assay uses the most conserved region representing all the strains/variants for detection of the pathogen in body fluid. In case of hepatitis viral infections, the available studies report a distinct conserved region used as a target for amplification of each individual viral genome[4,20,21]. Table-1 shows the list of target regions used in various studies on multiplex qPCR assays developed for hepatitis viruses. The 5’ UTR region is reported to be a main target template in case of HAV, HCV and HGV[20,22]. It is based on the availability of most conserved sequence in 5’ UTR for amplification purpose. Similarly, S-gene or X-gene were used for HBV, ribozyme-1 gene for HDV and ORF2 or ORF-3 region for HEV. Different studies have reported different sequences as template in these selected conserved regions, though at the same time, there is very little information provided about the exact location of sequences used.

***Designing of primers and probes***

After a decision of conserved region and location of sequence to be used as template, the next important step is to design the primers and probes for their use in the development of qPCR[23]. The selection of primer is based on its specificity with target template. At the same time, its length, melting temperature, GC content, 3’ end stability, sequence complexity and location in the target sequence determine the length and melting temperature of amplicon produced and also the amplification efficiency of assay[23,24]. This is worth to note here that the choice of chemistry and probe design are at the liberty of user’s interest with numerous options available to them[24]. During selection of chemistry and probe, one needs to see whether to quantify DNA, profile mRNA or perform allelic discrimination assays[25].

 Real-time PCR and melting curve analysis (MCA) are good techniques for quantifying nucleic acids, detection of mutation and conduct genotyping analysis. These methods often use TaqMan probes[26], Molecular beacons[27], Sunrise primers[28], Scorpion primers[29] and Light-up probes[30]. An alternative to probe-based methods is the use of DNA intercalating dyes that bind to double-stranded DNA. These include ethidium brominde[31] and SYBR Green I[32,33]. However, certain drawbacks limit the use of SYBR Green I for resolving multiplex PCR based on MCA[34]. Other alternative dyes such as BEBO[35], YO-PRO-1[36], LC Green[37] and SYTO-9[38,39] have also been tried for use in real time PCR. Table-2 gives a brief review of various chemistries/dyes offering several options for their use in qPCR assay developed for different puposes. The studies on qPCR for detection and genotyping of hepatitis viruses have reported different sets of dyes based on choice and their availibilty[40]. However, most of the studies conducted have reported a frequent use of hydrolysis probes despite many options available. These informations are available in the data[41-71] compiled in Table-3.

 The probe-based assays (*e.g.,* TaqMan assays)[72] began to gain attention in mid-1990s with the development of quenched, fluorescent probes[73,74] and the commercialization of real-time thermal cyclers[26,75]. TaqMan (also known as Fluorogenic 5’ nuclease assay) probes contain two dyes, a reporter dye (*e.g.,* 6-FAM) at the 5’ end and acceptor dye at 3’ end, usually tetramethyl rhodamine (TAMRA). Recently, TAMRA fluorescent acceptor quencher dye is substituted with non-fluorescent quencher, e.g. Black Hole Quencher[76]. The proximity of the quencher to the reporter in an intact probe quenches the fluorescence signal of the reporter dye through fluorescence resonance energy transfer (FRET). During amplification, the 5’ to 3’ nucleolytic activity of Taq polymerase cleaves the probe between the reporter and the quencher only if the probe hybridizes to the target. The probe fragments get displaced from the target, separating the reporter dye from the quencher dye resulting in increased emission of fluorescence. Floating TaqMan probes are quenched due to random coiling in solution, where fluorophore- and quencher-labeled ends come together[77]. In contrast, Molecular Beacon probes are oligonucteotides designed in a way to induce hairpin formation and produce the quenched state[78]. TaqMan and Molecular Beacon probes prove less effective in discriminating closely related targets, as in single nucleotide polymorphisms (SNPs), drug-resistant mutants and somatic cancer mutations[79,80]. However, molecular beacons are useful in situations where it is not possible to isolate probe-target hybrids from an excess of the hybridization probes, for example in sealed tubes or within living cells[81]. An effective probe requires a careful balancing act based on melting temperature (Tm) and so, repeated design and testing are needed to develop an effective probe[82]. It appears as if use of TaqMan probes in qPCR assay for hepatitis viruses provides a good balancing act.

***Designing tools***

Today, several designing tools are available to guide the design of qPCR assays and analyze resulting quantitative data. Many of them are available online and at the same time, some are provided with qPCR instruments from different manufacturers[83]. Some important tools include Primer3, Primer-BLAST, PerlPrimer, FastPCR software, IDTSciTools and UniPrime[84-89]. Also, some of them have programming to analyze the secondary structure of primers. MP primer is used for primer designing for multiplex PCR assays[90]. The Minimum Information for Publication of qPCR Experiments (MIQE) guidelines also provide clear instructions on the steps that are important for qPCR assay design[91]. Several research companies offer help for designing primers and probes with the help of their designing tools. The studies reported in this article demonstrate a liberal use of tools without any specific need or choice affecting the results.

***Instruments used in multiplex qPCR assay***

Today, a number of advanced technology based equipments used for multiplex qPCR assays simultaneous with analysis of amplified products are available globally. Table-3 enlists the instruments used with their brands in various studies conducted on qPCR for viral hepatitis. With increasing advances in technology, more number of filters used and significant increase in resolution of amplification curve during PCR assay, now it is possible to detect/discriminate more number of pathogens or allelic/mutational changes[92,93] in a single step multiplex assay. The choice of instrument is more a function of availability without much difference in their analytical qualities. Multiplex qPCR assays developed for hepatitis viruses may use any one brand depending on a match between the number of component pathogens to be detected and the filters available for detection. Other features of equipment do not seem to affect the results.

***Optimization of protocol***

Multiplex assay needs a carefully developed singleplex assay for each individual pathogen used as a component in multiplex assay. The designing of primer and probes is dictated purely by the nature of target template and clear guidelines for amplification. This exercise is followed to prepare a record of common amplification conditions noted in singleplex assays, for their application as such in multiplex assays. The multiplex protocol is reframed in a way to have minimum possible deviations from the working protocol of singleplex assay. During multiplex assay, the possibility of cross interaction/interference among different molecules is quite likely and poses the fear of unsuccessful amplification. This may or may not be the case, however, it has to be worked out cautiously in each multiplex assay.

 There are reports available on singleplex as well as multiplex assays developed for detection of some hepatitis viruses and their genotypes[16] (Table-3). Such a study was conducted at our research center also where multiplex assay was developed for simultaneous detection of hepatitis virus A, B, C and E[15]. These viruses are frequently prevalent in India posing a serious problem causing incidences of both sporadic and epidemic hepatitis from time to time[94,95]. The use of singleplex followed by development of multiplex assay in all these cases, does not show much changes in the experimental protocol. This implies that amplification protocol of individual viruses are not influenced during multiplex assays. We noted a clear amplification curve on the screen during multiplex assay for each virus exactly on the pattern noted during singleplex assay[15].

 Table-3 shows a global status of multiplex assays used for analyzing hepatitis viruses with or without other pathogens[41-71]. In all these assays, there is indication of viral amplification by the simultaneous presence of other pathogenic genomes. An overall survey of the experimental designs reported in multiplex assays indicated that standard conditions of reverse transcription, denaturation, annealing and extension temperature were followed without a highlight of much deviation from singleplex protocol.

***MIQE guidelines***

The guidelines published by Bustin *et al*[91] in 2009 clearly define the terms used and steps taken in designing of the experiment during development of qPCR assay. Many published reports in the area of viral hepatitis on multiplex qPCR in years later than 2009 were found to follow these guidelines and give interpretation of results referring to terminology and definitions outlined there. The guidelines state that multiplexing expands power of qPCR analysis but needs documentation for accurate quantification of multiple targets without an impairment in a single tube assay.

**ASSESSMENT OF SENSITIVITY AND SPECIFICITY**

***Generation of standard curve***

In order to generate a standard curve for each hepatitis virus, the standard control that include the conserved region, targeted for amplification/ detection, is synthesized artificially and cloned into a suitable vector (*e.g.,* pUC 57)[15] using cloning kits. These standards are used as template for standardization of amplifications. The copy number of standard plasmid are calculated using their concentration and the size of linearized plasmids. Each standard template is added to PCR mix (Tris-HCL, KCl, MgCl2, 4 dNTPs, primers and Taq DNA polymerase in a suitable concentration ratio) and PCR is performed under the standardized conditions. For generation of standard curve, a 10-fold serial dilution of each standard plasmid (101 – 108 copies/μL) is prepared and run in triplicate. At the end, data are analyzed by automatic system generating standard curve[21]. The standard curves are used to quantify the amplification product and assess the linear dynamic range using 10-fold dilution series of standard plasmid of each individual virus. One specimen standard plot is shown in figure 1 that was prepared during development of quadruplex qPCR for hepatitis virus A, B, C and E. Such plots are used to calculate copy number of individual template using correlation coefficient and Y-intercept value based on regression analysis.

Standard curve showing amplification plots of 10-fold serial dilution of HAV template using standard cloned plasmids. Such standard curves are generated from the amplification plots run in triplicate and showing linear dynamic range. The correlation coefficient and the slope of each standard plot have been shown in figure.

***Assessment of sensitivity***

Using the standard curve prepared above, now it is possible to assess the sensitivity and find out the linear dynamic range of individual virus. Moreover, observed Ct values may be used to calculate the exact copy number of virus in unknown specimen used[96,97]. Based on the data collected from various studies including our study[15], it has been noticed that the linear dynamic range of each individual hepatitis virus usually falls in the range 101 – 108 copies/μL.

***Assessment of specificity***

The specificity of qPCR assay is assessed by its use in sera from healthy controls and patients with unrelated diseases negative for hepatitis markers by serology and all other NAT based techniques. The negative results by qPCR in all these sera and clear positive signal in serologically positive hepatitis sera demonstrate high level of specificity. All studies on qPCR till date demonstrate the assay to be specific[15,22]. In case of reports available on viral hepatitis, qPCR assays demonstrated high specificity with very little chance of false positive results[19,71].

**MULTIPLEX qPCR IN RELATION WITH OTHER ASSAYS**

The multiplex qPCR assays were developed and used both for comparison as well as in combination of few other molecular technologies to improve the sensitivity for detection of viral genome[16,98]. Various other assay systems were also developed for simultaneous detection of HBV, HCV and HIV in addition to multiplex qPCR. The status of multiplex qPCR assay was assessed in comparison to all such molecular techniques used for detection and genotyping of viruses including hepatitis viruses. The other assay systems included flowcytometric microsphere based hybridization assay[99], transcription-mediated amplification (TMA)[100] and nucleic acid sequence based amplification (NASBA)[101]. Comparatively, TMA was reported to be an equally sensitive technique. However, comparison of qPCR with NASBA and TMA, used separately for detection of hepatitis viruses, could demonstrate the level of sensitivity of TMA to be closely associated with qPCR[100]. Of course, qPCR assay was reported to be comparatively fast, economic and easy to perform in comparison to all other assays.

**FUTURE AND LIMITATIONS OF MULTIPLEX qPCR**

Multiplex qPCR assays are proving very good analytical and diagnostic procedures in medicine. Recently, these assays have been successfully used for both basic research and clinical applications[42,102]. Although, there is still a practice to do separate assays for separate pathogens including hepatitis viral markers, however, the use of multiplex assay is seen much beneficial in respect of time and overall cost involved. Moreover multiplex assays, when used for quantification of HCV- RNA, were found to solve many problems with real time monitoring of amplification process. In fact, in multiplex qPCR assays, real time PCR makes quantification of DNA and RNA of different organism, more precisely and with better reproducibility as it depends on threshold cycle value determined during the exponential phase of PCR rather than on end points[103]. Also these assays report direct relationship between starting template copy number and the number of cycles required to get a positive signal. In this manner real time qPCR appears a good option for laboratory diagnosis of viral hepatitis, both for screening as well as final diagnosis of cases suspected with viral hepatitis infections.

**CONCLUSION**

Based on the informations compiled in the present write-up, it may be concluded that there is an increasing trend/interest in diagnostic area towards the development and use of multiplex qPCR assay for the simultaneous detection of hepatitis viruses or their subtypes in sera samples. There are several studies conducted in last few years that clearly demonstrate the preferable use of qPCR over other techniques in the area of viral hepatitis. This technique has been used to detect hepatitis viruses in combination with various other viral and non-viral pathogens and reported to be a sensitive, fast and cost-effective technique when compared with other multi-step assay procedures. The use of multiplex qPCR in genotyping of hepatitis viral subtypes is also providing great help in serotype detection. Till date, multiplex qPCR has been successfully tried for simultaneous detection of hepatitis virus A, B, C, D and E and genotyping of their strains. It appears as a good tool for screening of blood donor samples in blood banks for hepatitis viruses. Moreover, a single step multiplex qPCR assays saves a lot of time for an early diagnosis and timely treatment of the patients with viral hepatitis. A regular input from literature shows that several studies are in progress with more important informations to be available till the next such an update is ready in this field.

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**Figure 1 Standard curve showing amplification[15].**

**Table 1 Conserved genomic regions used as template for amplification of hepatitis viruses in qPCR assays**

|  |  |  |
| --- | --- | --- |
| **Virus** | **Conserved region** | **Reference** |
| HAV | 5’ UTR | [4, 15, 20, 22]  |
| HBV | S-geneX-gene | [4, 19, 20,21][15] |
| HCV | 5’ UTR | [4, 15, 19, 20, 21] |
| HDV | Ribozyme-1 | [20] |
| HEV | ORF2ORF3 | [15, 22][20] |
| HGV | 5’ UTR | [20] |

**Table 2 Chemistries / Dyes used in qPCR assays**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **S. NO.** | **Class** | **Types** | **Structure** | **Mechanism of action** | **Advantages** | **Applications** |
| 1 | DNA binding dyes | Ethidium Bromide, SYBR Green, SYBR Gold, YO-PRO-1, SYTO, BEBO, BOXTO, EvaGreen | Intercalating dyes | Bind to the minor groove of dsDNA during amplification | InexpensiveEasily available | Pathogen detection Gene expressionSNP detectionGenotyping |
| 2. | Fluorophore labeled oligonucleotide | Primer probes |
| Hairpins: Scorpions, Ampliflour, LUX | Loop based oligonucleotides | Bind to target during denaturation with emission of fluorescence | Inexpensive, Prevent formation of primer dimer, Less background signals | Pathogen detectionGenotypingSNP allelic discriminationMutation detection |
| Cyclicons :  | Cyclic structure with reporter at 3’ end and quencher at 5’ end. | Reporter & quencher in close proximity with energy transfer via FRET quenching. Their separation results in fluorescence emission during amplification | InexpensiveLess contaminationLess background signals | Pathogen detectionGenotypingSNP allelic discriminationMutation detection. |
| Angler : | Probe with DNA sequence bound to reverse primer through a HEG linker. | During annealing step, DNA polymerase does extension of 3’ end reverse primer. Later on, SYBR Gold dye intercalates in dsDNA emitting fluorescence. | Highly specific | Gene expressionPathogen detection SNP detectionGenotyping |
| **Probes** |
| Hydrolysis Probes:TaqMan probes,MGB-TaqMan,Snake assay | Oligonucleotide with reporter at 5’and quencher at 3’ end | Probe is degraded by 5’ to 3’ exonuclease activity of DNA polymerase generating fluorescence during extension | Design & synthesis easy | Microarray validationPathogen detectionSNP allelic discriminationMutation detection |
| Hybridization Probes :Hybprobes, Molecular Beacon, HyBeacon, MGB Probes | A pair of oligonucleotides having reporter dye on first and quencher on second oligonucleotide | Binding to target during hybridization & annealing brings fluorophore into proximity producing fluorescence by FRET | Design and synthesis quick and easy | Microarray validationPathogen detectionViral/ Bacterial genotypingSNP allelic discriminationMutation detection |
| **Nucleic acid analogues** |
| PNAs, LNAs, ZNAsNon-natural bases | Intercalating/ inserting dyes  | Identical to conventional oligonucleotides  | Resistant to nuclease and proteases activity | Discriminate between DNA & cDNA in prokaryotes |

All above details were collected from report published in *Clinica Chimica Acta* 2015; 439: 231-250[25].

**Table 3 Global status of multiplex qPCR developed for hepatitis viral infections with and without other pathogens**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **No.** | **Assay systems** | **Instruments****used** | **Group of pathogens detected** | **Types of chemistries / detection methods used** | **ref.** |
| **Hepatitis viruses** | **Other pathogens** |
| 1 | Multiplex real time PCR | Mx4000(Stratagene) | HBV, HCV | HIV type-1, *T. pallidum* | TaqMan-LNA probe | [21] |
| 2 | Multiplex real time PCR | Light cycler 480 (Roche) | HEV genotypes | \_\_ | N.A. | [41] |
| 3 | Real time PCR assay | ABI 7500(Applied Biosystems) |  HAV, HBV, HCV, HDV, HEV | \_\_ | TaqMan Array card | [42] |
| 4 | Multiplex qPCR assay | Light cycler 480 (Roche) | HBV, HDV | \_\_ | TaqMan probe | [43] |
| 5 | Multiplex qPCR assay | ABI 7500(Applied Biosystems) | HAV, HEV | \_\_ | Hydrolysis probe | [22] |
| 6 | Multiplex qRT-PCR | N.A. | HAV | Norovirus genotypes 1 and 2 | TaqMan probe  | [44] |
| 7 | Multiplex ligation dependant probe real time PCR | Rotor-GeneQ (Qiagen) | HBV mutants | \_\_ | TaqMan probeMLPA probe | [45] |
| 8 | Multiplex real time RT-PCR | N.A. | HEV genotypes | \_\_ | N.A. | [46] |
| 9 | Multiplex qPCR | N.A. | HBV genotypes | \_\_ | SYBR Green | [47] |
| 10 | Multiplex Real time PCR | N.A. | HAV | Norovirus, Rotavirus, Coxsackievirus | TaqMan probe | [48] |
| 11 | Multiplex Real time PCR | Light cycler 2.0(Roche) | HAB, HBV, HCV & HEV | \_\_ | FRET probe | [15] |
| 12 | Multiplex RT-PCR | ABI 2720 (Applied Biosystems) | HCV | HIV type-1 | SYBR Green I | [8] |
| 13 | Multiplex qPCR | N.A. | HAV, HEV | Entero and Adeno-viruses | N.A. | [49] |
| 14 | Multiplex Real-Time PCR Assay | CFX96(Bio-Rad) | HAV, HBV, HCV | \_\_ | READ technology based fluorophore | [4] |
| 15 | RT PCR assay | Smart cycler II (Cepheid) | HBV, HCV | \_\_ | TaqMan probe | [50] |
| 16 | Duplex real time PCR | ABI 7500(Applied Biosystems) | HBV variants | \_\_ | Hydrolysis probe | [51] |
| 17 | Multiplex RT PCR | N.A. | HCV subtyping | \_\_ | Electrophoresis | [52] |
| 18 | Multiplex qPCR | N.A. | HBV genotypes | \_\_ | N.A. | [53] |
| 19 | Multiplex qPCR | N.A. | HCV | HIV type-1 | SYBR Green I | [54] |
| 20 |  Duplex real-time RT-PCR | ABI Prism system (Applied Biosystems) | HCV variants | \_\_ | Hydrolysis probe | [55] |
| 21 | Multiplex real time PCR | N.A. | HAV | Norovirus genotypes 1 and 2 | N.A. | [56] |
| 22 | Duplex real-time qRT-PCR | ABI Prism 7000 (Applied Biosystems) | HAV | MS2 bacteriophage | MGB-TaqMan probe | [57] |
| 23 | Multiplex TaqMan RT-qPCR system | MX30005P(Stratagene) | HEV | FCV | TaqMan probe | [58] |
| 24 | Multiplex realtime PCR | ABI 7300(Applied Biosystems) | HBV genotypes | \_\_ | TaqMan probe | [59] |
| 25 | Real time PCR | N.A. | HBV genotypes | \_\_ | TaqMan probe | [60] |
| 26 | Multiplex real time PCR | Mx3005P(Stratagene) | HEV | FCV  | TaqMan probe | [61] |
| 27 | Multiplex RT PCR assay | ABI Prism 7500(Applied Biosystems) | HCV  | PDV | MGB hybridization probe | [62] |
| 28 | Multiplex qPCR assay | N.A. | HBV | B19, HHV-8,EBV, CMV, VZV | N.A. | [63] |
| 29 | Multiplex qPCR | N.A. | HBV, HCV | HIV type-1 | SYBR Green I | [16] |
| 30 | Multiplex Real Time PCR | ABI 7500(Applied Biosystems) | HBV mutants | \_\_ | LNA probes with SYBR Green I | [64] |
| 31 | Microarray multiplex assay | ABI Prism 7700 (Applied Biosystems) | HBV, HCV | HIV type-1 | Oligonucleotide array labeled with Cy5 & Cy3 | [65] |
| 32 | Real time multiplexPCR | N.A. | HAV | Entero & Adeno-viruses | Probes labeled with FAM, R6G, ROX, Cy5 | [66] |
| 33 | Multiplex real time RT-PCR | LightCycler(Roche) | HCV | HIV type-1 | SYBR Green | [67] |
| 34 | Real time multiplex PCR | icycler iQ(Bio-Rad) | HCV variants | \_\_ | TaqMan probes | [68] |
| 35 | Multiplex real-time RT PCR | ABI 7000(Applied Biosystems) | HCV genotypes | \_\_ | Primer probes | [69] |
| 36 | Multiplex real-time qPCR | Mx4000 (Stratagene) | HBV, HCV | HIV type-1 | TaqMan probes | [70] |
| 37 | Automated multiplex PCR | ABI Prism 7700 (Applied Biosystems) | HBV, HCV | HIV type-1 | TaqMan probes | [71] |