Table 2. Commercially available kits for manual extraction of viral nucleic acids from clinical samples.

Manufactures	Kit	Principle for extraction	Target	Type of specimen	Comments
Roche Diagnostics	High Pure Viral Nucleic Acid Kit	Nucleic acid capture by glass fiber fleece immobilized in a special plastic filter tube and subjected to centrifugation	DNA/RNA	Serum, plasma, total blood, CSF, urine, stool, respiratory tract specimens, swabs (throat, genital, dermal)	Ethanol required. Protease K treatment of samples is required before the purification step
	High Pure Viral RNA kit		RNA	Serum, pplasma, cell and precipitate-free specimens	Ethanol required. Poly A is used for binding of viral RNA
Qiagen	QIAamp Viral RNA Mini kit	Nucleic acid capture by silica gel membrane placed in tube column and subjected to centrifugation of vacuum conditions	RNA	Serum, plasma, cell and precipitate-free specimens	Extraction by using organic solubent not required
	QIAamp MinElute Virus Vacuum Kit/QIAamp MinElute Virus Spin Kit		DNA/RNA	Serum, plasma, cell and precipitate-free specimens	Extraction by using organic solubent not required
	QIAamp Ultrasens Virus kit		DNA/RNA	Serum, plasma, cell and precipitate-free specimens	The viral nucleic acids are concentrated and eluted
	QIAamp DNA Blood Kit		DNA	Specimens that contain cells such as total blood and swabs (throat, genital, dermal)	Ethanol not required
	QIAamp DNA Mini Kit		DNA		Ethanol not required
	QIAamp RNA Blood Mini		RNA		Ethanol required

controls must be treated in the same way as the test samples throughout the entire process, from extraction of the virus genome, to the aliquoting of the purified samples to the reaction tube, to amplification and detection. To validate the quality of the assay for each batch, the inclusion of a weakly positive control, in which the concentration of the virus genome is slightly above the detection threshold, is recommended. The negative control should be processed last at each step, after any positive controls. Once clusters of positive results, particularly following a strongly positive sample, are observed, the likelihood of false positives should be considered, requiring retest to confirm the result, even when the performance of controls is adequate. In cases in which the target genome is RNA, treatment of the purified RNA with DNase can reduce the risk of false-positive results due to amplicon contamination. The PCR reagent must be properly stored and the master-mix must be properly prepared. Each step of the master-mix preparation, virus genome extraction from samples, addition of purified virus genome to PCR mixes, PCR amplification, postreaction manipulation and sequencing or cloning of products, should be conducted in physically separated rooms with independent airflows. Each room must be equipped with its own dedicated pipettors, tips and other consumables, as well as protective equipment for staff. Most importantly, to minimize the risk of contamination, staff must be well trained in the handling of materials using sterile techniques and the entire real-time qPCR procedure. PCR-graded materials, such as filtered tips, reagents (including water) and PCR tubes, must be used at all stages. Work areas, in which samples and reagents are manipulated, and equipment such as pipettes should be treated with 2% sodium hypochlorite disinfectants or irradiated with UV light.

4.3 Minimization of the risk of false-negative results

False-negative results are also a great concern in real-time qPCR assays. False-negative results usually stem from the following factors: inhibitory elements (inhibitors) in the reaction mixes, poor management and stock of reagents, and mismatches in the nucleotide sequence between the designed primers and probes and the target genome. The reagents used in the real-time qPCR should be properly managed and stored, as poorly maintained equipment and reagents can introduce errors, resulting in test failure. The performance of equipment such as pipettors and theromocyclers must be routinely validated to ensure accuracy. The presence of inhibitors to genome amplification in the reaction mixture often leads to false-negative results [136,137]. To remove the inhibitors, appropriate nucleic acid extraction reagents for both DNA and RNA should be used. Several manufacturers provide high-quality nucleic acid purification kits. Most commercially available nucleic acid extraction systems are highly efficient in removing inhibitors from the samples by washing the silica gel membrane or magnetic beads combined with the virus genome followed by elution. Among the types of sample collected from patients, feces may be the most problematic because it contains high concentrations of inhibitors. To monitor the problem of false-negative results due to inhibitors, inhibitory controls should be included in each assay. The concentration of the surrogate virus genome should be at a level just above the detection threshold. The inhibitory control, the surrogate virus genome spiked through the addition of either the virus or the virus genome, should be tested for the reactivity in the real-time qPCR. In the case that the inhibitory control shows positive reactions, the level of the inhibitor's removal should be satisfactory. However, it must be noted that the multiplex assay for the amplification of both the surrogate control and the target sometimes results in a reduction in sensitivity. The method for the extraction of viral genome for real-time qPCR should be optimized according to sample type. Selection of an appropriate polymerase, sample volume, and dilution of the purified virus genome are important factors in the reduction of false-negative results due to the presence of inhibitors. The quality of the reagents used should also be maintained at an appropriate level. Repeated freeze-and-thaw treatment of reagents should be avoided as such treatment reduces the DNA polymerase activity. Primers and probes should be properly stored and managed. Mismatches in the nucleotide sequence between the primers, including the probes, and the target sequence increases the detection threshold, resulting in a decrease in the sensitivity of the amplification of the target genome. The diversity in nucleotide sequence is generally much greater in RNA viruses than in DNA viruses. Although the primers and probes used in the real-time qPCR are usually designed according to the nucleotide sequence conserved among the target virus strains, there is always a potential risk of false-negative results owing to a mismatch in the nucleotide sequence between the primers and probes and the target sequence of the circulating viruses in different regions and/or at different times.

4.4 Comparison of characteristics between real-time qPCR and other virus detection diagnostics

The advantages and disadvantages of each diagnostic procedure are summarized in Table 3. The greatest advantage of real-time qPCR is that this assay can be applied to all viruses for which the nucleotide sequence is available. Theoretically, conventional PCR, including nested PCR, can also be applied to the diagnosis of all virus infections, but conventional PCR is inferior to real-time qPCR in terms of quantification capacity, sensitivity and rapidity. Enzyme immunoassay for the detection of virus antigens is practically useful in the diagnosis of RSV, influenza virus, rotavirus and adenovirus infections in a clinical setting. However, this procedure is only available for such infections. It is noteworthy that the sensitivity of virus isolation techniques for the detection of viruses from clinical samples is very high if the samples are properly collected and managed until the inoculation of samples to the

Table 3. Characteristics of diagnostic methods: real-time PCR, conventional PCR including nested PCR, enzyme immunoassay and antigen-detection ELISA, and virus isolation.

Factors	Real-time PCR	Conventional PCR	Nested PCR	EIA/antigen-detection	ELISA/antigen-detection	Virus isolation
Handout time	Very short*	Short*	Short	Short	Short	Long*
Sensitivity	Very high	High	Very high	High	High	High
Requirement of expensive equipment	Yes	Yes	Yes	No	Yes	Yes
Application capacity to virus species	Wide	Wide	Wide	Limited	Limited	Limited
Capacity of quantification	Positive	Negative	Negative	Negative/Positive	Positive	Negative
Risk of contamination	Very high	High	Very high	Negative	Negative	Negative
Usefulness in diagnosis of CNS infections	Very useful	Useful	Very useful	Limited	Limited	Limited
Usefulness in assessment of antiviral therapy	Very useful	Useful	Useful	Useful	Useful	Useful

*Very short, short and long indicate the periods of time to have results of < 6 h, 6 – 24 h and > 24 h, respectively.

designated cells for virus isolation. Unfortunately, virus isolation procedures can be applied only to the diagnosis of a limited number of virus infections, and there are many kinds of viruses that cannot be isolated using cell culture-based techniques. Furthermore, identification of the isolated virus requires complicated procedures and is time-consuming and expensive. Therefore, the use of virus isolation techniques in the diagnosis of virus infections is becoming less popular. However, we must recognize that the value of cell-culture based virus-isolation techniques for the diagnosis of virus infections remains.

5. Summary

This review presents the basic mechanism, applications and characteristics of the recently developed real-time qPCR. There is no doubt that the development and application of real-time qPCR offers great advantages in the diagnosis, treatment and assessment of infectious diseases. It is believed that this assay system will become the gold standard. The procedure for real-time qPCR should be carried out in compliance with the principles of good laboratory practice. Quality assurance among institutes in which real-time qPCR is performed for diagnostic purposes is certain to become an important issue in the future. In order to maintain the quality of the assay in each institute, inter-institutional collaboration for its assessment will be required.

6. Expert opinion

Real-time qPCR should be applied not only to the determination of causative agents but also to the determination of changes in viral load. There is no doubt that real-time qPCR offers great advantages in the management of infectious diseases. However, the value of traditional and conventional assays, including virus isolation-based procedures, will remain. Each assay must be selected dependent upon the relevant circumstances. For example, enzyme immunoassay for the detection of respiratory syncytial virus and rotavirus in nasopharyngeal aspirate and stool samples, respectively, is the most practical technique for the diagnosis of these virus infections in out-patients and hospitalized children. Virus isolation-based diagnostics are sufficiently sensitive, if the samples are properly collected and managed. This technique is still generally necessary for the diagnosis and epidemiological survey of infectious diseases. Virus isolation techniques are the most powerful tool for the identification of unknown agents. Although, real-time qPCR offers important information on diagnosis and assessment of infectious diseases, we should not rely excessively only on real-time qPCR to have proper diagnosis and management. We should recognize the nature of real-time qPCR, and the nature should be taken into account for interpretation of the results by real-time qPCR. Owing to considerations of false-positive and false-negative results in real-time qPCR, quality assurance should be

659 undertaken routinely and good management practices should 660 be strictly applied. Furthermore, the quality of the real-time qPCR conducted in each laboratory should be validated using designated samples, each of which contains the target virus of a different genotype and at a different concentration. To achieve validation, one option is that designated 665 samples should be prepared and provided to participating institutions by a leading institute. The validation program among multiple institutions has already been conducted in the real-time qPCR-based diagnosis of HIV, viral hepatitis, and other viral infections. When the efficacy of the real-time 670 qPCR-based diagnostics is confirmed to be high enough, the kit becomes commercially available. Sharing the results from this type of validation program among multiple institutions would make it possible to improve the standard of the assay.

In the US, the congress passed the Clinical Laboratory 675 Improvement Amendments (CLIA) in 1988 to establish quality standards for all laboratories' testing to ensure the 676 accuracy, reliability and timeliness of patient test results regardless of where the test was performed. In such a framework, the development of guidelines for real-time qPCR that envelop all the necessary verification and validation 680 by all accreditation agencies would be necessary. However, the methods in real-time qPCR, for example the nucleic acid extraction, are changing rapidly. Frequent revision of such guidelines is also required. It is expected that real-time qPCR will become more functional and offer greater benefits 685 for patients, particularly those with life-threatening or emerging and re-emerging infections.

Declaration of interest

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