Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.
Review

Detection and monitoring of virus infections by real-time PCR

F. Watzinger, K. Ebner, T. Lion *

Children’s Cancer Research Institute, St. Anna Kinderspital, A-1090 Vienna, Austria

Abstract

The employment of polymerase chain reaction (PCR) techniques for virus detection and quantification offers the advantages of high sensitivity and reproducibility, combined with an extremely broad dynamic range. A number of qualitative and quantitative PCR virus assays have been described, but commercial PCR kits are available for quantitative analysis of a limited number of clinically important viruses only. In addition to permitting the assessment of viral load at a given time point, quantitative PCR tests offer the possibility of determining the dynamics of virus proliferation, monitoring of the response to treatment and, in viruses displaying persistence in defined cell types, distinction between latent and active infection. Moreover, from a technical point of view, the employment of sequential quantitative PCR assays in virus monitoring helps identifying false positive results caused by inadvertent contamination of samples with traces of viral nucleic acids or PCR products. In this review, we provide a survey of the current state-of-the-art in the application of the real-time PCR technology to virus analysis. Advantages and limitations of the RQ-PCR methodology, and quality control issues related to standardization and validation of diagnostic assays are discussed.

Keywords: RQ-PCR; Virology; Clinical; Comparison; Method

* Corresponding author. Tel.: +43 1 40470 489; fax: +43 1 40470 437.
E-mail address: thomas.lion@ccri.at (T. Lion).
1. Introduction

The polymerase chain reaction (PCR) is a powerful tool for detection of minute amounts of nucleic acids. Due to the exponential amplification of the target sequence, it has exquisite sensitivity. Less than 10 copies of any transcript can be readily detected, even against a high background of unrelated nucleic acids. The establishment of PCR-based detection methods has provided the basis for rapid and reliable detection of viral nucleic acids in the clinical setting. It was shown that
the monitoring of virus load and the kinetics of virus proliferation have prognostic relevance for the course of disease and clinical outcome (Whalley et al., 2001; Humar et al., 2002; Snijders et al., 2003; Biedermann et al., 2004; Wagner et al., 2004; Watzinger et al., 2004). The availability of quantitative virus detection tests is therefore of paramount importance for the clinical management of virus infections.

Quantification of target sequences by real-time quantitative PCR (RQ-PCR) is based on the continuous measurement of the accumulation or reduction of fluorescence signals during the amplification reaction. In contrast to quantitative PCR approaches based on data analysis at the end of PCR (end-point quantitative PCR), the real-time PCR technology permits the detection of the number of amplification cycles generated during each amplification cycle in a real-time mode (=kinetic quantitative PCR). This technique has eliminated the need of post-amplification handling of the samples and has paved the way towards fully automated detection systems. Appropriate calibration of RQ-PCR assays and the use of standard curves permit the assessment of absolute copy numbers of the target of interest. Results are typically displayed as amplification plots resulting from a series of fluorescence measurements taken at defined time points during the amplification process. One of the important features of the real-time technology is the ability to monitor the increasing amount of product at early time points during the PCR reaction. This facilitates quantification of the target in the exponential phase of PCR, when the amplification product first becomes detectable. Quantification by real-time PCR is therefore neither affected by limiting concentrations of reagents, nor by other variables, such as cycling conditions, which affect quantification in endpoint analysis-based PCR assays. Optimized RQ-PCR tests display very high sensitivity, with detection limits between 1 and 10 target molecules per reaction. The RQ-PCR technology offers a broad dynamic capacity facilitating quantification across a more than seven-log range of target molecules (Chen et al., 2001; Jebbink et al., 2003; Lindh and Hannoun, 2005b).

Owing to these features, RQ-PCR has become the most important technique for the detection and monitoring of virus infections (Middeldorp, 2002; Niesters, 2002, 2004). The surveillance of viral load is a useful indicator of active infection, virus-host interaction and the response to antiviral therapy (Watzinger et al., 2004; Hausler et al., 2003). Commercial RQ-PCR assays are available only for a limited number of viral pathogens including e.g. the human immunodeficiency virus (HIV 1), the hepatitis viruses B and C (HBV, HCV), the cytomegalovirus (CMV), the human papilloma virus (HPV), and the SARS-associated coronavirus (SARS-CoV) (see Table 1). Due to the fact that quantitative monitoring of virus infections has become indispensable for patient and disease management in different clinical settings, home-brew real-time assays have been established for a number of other viral targets and implemented in clinical diagnosis (see Table 2). Within the foreseeable future, it can be expected that diagnostic laboratories will have to continue developing in-house methods for the quantification of many clinically relevant viral targets.
2. Detection formats and chemistries

2.1. Detection and quantification without target-specific probes

2.1.1. Intercalating dyes

DNA-binding dyes like ethidium bromide (EtBr) (Higuchi et al., 1992), YO-PRO 1 (Ishiguro et al., 1995), SYBR® Green I (FMC Bioproducts, Rockland, ME, USA) (Wittwer et al., 1997; Zipper et al., 2004), or BEBO (Bengtsson et al., 2003) intercalate in a non-specific manner into double-stranded DNA molecules and, in the bound state, emit fluorescence when excited by an appropriate light source. During the annealing and extension steps, an increasing amount of dye binds to the newly synthesized DNA strands leading to maximum fluorescence emission at the end of the elongation phase. As soon as the DNA is denatured again during PCR cycling, intercalated dye molecules are released into the solution resulting in a drop of fluorescence. The fluorescence is recorded after each cycle at the end of the elongation phase.

Table 1
Examples of commercial RQ-PCR kits for virus detection

| Manufacturer/supplier | Nucleic acid test (NAT) system | Virus target |
|-----------------------|-------------------------------|--------------|
| Roche diagnostics     | COBAS TaqMan Test             | HCV, HBV, HIV-1, respectively |
| (Basel, Switzerland)  | TaqScreen West Nile virus test (in clinical trial) | WNV |
| LightCycler®—quantification kit | PVB19, HAV, HSV 1, HSV 2, EBV, respectively |
| LightCycler®—SARS-CoV quantification kit (in clinical trial) | SARS-CoV |
| Quest diagnostics     | Hepatitis C viral RNA, quantitative real-time PCR | HCV |
| (Lyndhurst, NJ, USA)  | BK and JC virus DNA, real-time PCR | BKV + JCV |
| Digene                | Digene® HPV test              | HPV |
| (Gaitersburg, MD, USA) |                               |              |
| Abbott                | Abbott m2000rt RealTime™ assay | HCV, HIV-1, respectively |
| (North Chicago, IL, USA) |                               |              |
| Artus                 | RealArt™ RT PCR Kit           | DENV, EV, HAV, HIV 1, Inf, Inf/H5, SARS-CoV, WNV |
| (Hamburg, Germany)    | RealArt™ PCR kit              | EBV, HBV, HIV-1 + 2, Orthopox, PVB 19, VZV, |

Examples of other commercially available quantitative virus detection kits based on amplification technologies other than RQ-PCR: PCR/ELISA (HCV, HBV, HIV-1, CMV; Roche diagnostics), branched DNA (HIV-1, HBV, HCV; Bayer Diagnostics, Tarrytown, NY, USA; HCV, HIV-1; quest diagnostics); solution hybridization antibody capture (HBV, HPV, CMV; Digene), transcription mediated amplification (HIV-1 + HCV, HIV-1 + HBV + HCV, WNV; Chiron, Emeryville, CA, USA; HCV; quest diagnostics), competitive PCR (HIV; Abbott Laboratories), enzyme immunoassays (HIV; Innogenetics, Gent, Belgium) and a combination of (RT)PCR-Electrophoresis-Southern Blot, and densitometric quantitation (HBV, HCV, HIV-1; National Genetics Institute, Los Angeles, CA, USA).
| Virus          | Hydrolysis probe (Refs.) | Hybridization probe (Refs.) | Melting curve (Refs.) | Molecular beacons (Refs.) | MGB™ probe (Refs.) | Other system (Refs.) |
|---------------|--------------------------|-----------------------------|----------------------|--------------------------|-------------------|----------------------|
| Adenovirus (AdV) | Lion et al. (2003), Houng et al. (2002), Gu et al. (2003), Heim et al. (2003) and He and Jiang (2005) | Ko et al. (2005) and Koidl et al. (2005) | Watanabe et al. (2005) | Claas et al. (2005) | Ebner et al. (2005), Claas et al. (2005) and Leruez-Ville et al. (2004) |
| Coronavirus (SARS) | Gut et al. (1999), Bressler and Nolte (2004), Chantratita et al. (2004), Emery et al. (2004), Mahony et al. (2004), Chui et al. (2005) and Huang et al. (2005) | Chantratita et al. (2004) | Mahony et al. (2004) | Chui et al. (2005), Lin et al. (2004) and Hu et al. (2005b) | LAMP primer Poon et al. (2005) |
| Cytomegalovirus (CMV = HHV5) | Watzinger et al. (2004), Machida et al. (2000), Guiver et al. (2001) and Greenlee et al. (2002) | Watzinger et al. (2004), Monpoeho et al. (2000), Verstrepen et al. (2001), Corless et al. (2002), Donaldson et al. (2002), Katayama et al. (2002), Monpoeho et al. (2002), Nijhuis et al. (2002), Rabenau et al. (2002), Verstrepen et al. (2002), Watkins-Riedel et al. (2002), Brilot et al. (2004), Mohamed et al. (2004), Cinek et al. (2006), Donia et al. (2005) and Petitjean et al. (2005) | Stocher et al. (2003), Schaade et al. (2000) and Jebbink et al. (2003) | Donia et al. (2005), Kares et al. (2004) and Archimbaud et al. (2004) | LUX primer Donia et al. (2005) |
| Enterovirus (EV) | Watzinger et al. (2004), Monpoeho et al. (2000), Verstrepen et al. (2001), Corless et al. (2002), Donaldson et al. (2002), Katayama et al. (2002), Monpoeho et al. (2002), Nijhuis et al. (2002), Rabenau et al. (2002), Verstrepen et al. (2002), Watkins-Riedel et al. (2002), Brilot et al. (2004), Mohamed et al. (2004), Cinek et al. (2006), Donia et al. (2005) and Petitjean et al. (2005) | Krumbholz et al. (2003) and Kares et al. (2004) | Stocher et al. (2003) and Jebbink et al. (2003) | Donia et al. (2005), Kares et al. (2004) and Archimbaud et al. (2004) | LUX primer Donia et al. (2005) |
| Epstein–Barr virus (EBV) | Watzinger et al. (2004), Kimura et al. (1999), Jabs et al. (2001), Leung et al. (2002a) and Le et al. (2005) | Stocher et al. (2003) and Jebbink et al. (2003) | Stocher et al. (2003) and Jebbink et al. (2003) | Donia et al. (2005), Kares et al. (2004) and Archimbaud et al. (2004) | LUX primer Donia et al. (2005) |
| Human herpes viruses 1 + 2 (HSV-1, HSV-2) | Watzinger et al. (2004) and Weidmann et al. (2003) | Ryncarz et al. (1999), Espy et al. (2000a), Kessler et al. (2000), O’Neill et al. (2003), Stoich et al. (2003), Whitey et al. (2004) and Ramaswamy et al. (2005) |
| --- | --- | --- |
| Hepatitis virus A (HAV) | Costa-Mattioli et al. (2002) and Joithkumar et al. (2005a) | Jardi et al. (2001), Jursch et al. (2002), Paraskevis et al. (2002), Aliyu et al. (2004) and Leb et al. (2004) |
| Hepatitis virus B (HBV) | Lindh and Hannoun (2005b), Abe et al. (1999), Candotti et al. (2004), Garson et al. (2005), Loeb et al. (2000), Pas et al. (2000), Weinberger et al. (2000), Chen et al. (2001), Pas and Nieters (2002), Kohmoto et al. (2003), Jun-Bin et al. (2003) and Weiss et al. (2004) | Zhang et al. (2002), Rodriguez-Frias et al. (2003), Schaefer et al. (2003), Payungporn et al. (2004), Yamashiro et al. (2004) and Yeh et al. (2004) |
| Hepatitis virus C (HCV) | Martell et al. (1999), Kleber et al. (2000), Candotti et al. (2004), Enomoto et al. (2001), Kishimoto et al. (2001), Komurian-Pradel et al. (2001), Mitsunaga et al. (2002), Puig et al. (2002), Zanella et al. (2002), Cook et al. (2004) and Lindh and Hannoun (2005a) | Ratge et al. (2000), Pham et al. (2004) and Schuttler et al. (2004) |
| Hepatitis virus E (HEV) | Mansuy et al. (2004), Narayanan et al. (2006) | Orru et al. (2004) |
| Human herpes virus 6 (HHV-6) | Watzinger et al. (2004), Nitsche et al. (2001) and Zerr et al. (2000) | Ramaswamy et al. (2005), Schalasta et al. (2000) and Issa et al. (2005) |

Eclipse Stevenson et al. (2005)

(continued on next page)
| Virus                              | Hydrolysis probe (Refs.) | Hybridization probe (Refs.) | Melting curve (Refs.) | Molecular beacons (Refs.) | MGB™ probe (Refs.) | Other system (Refs.) |
|-----------------------------------|--------------------------|-----------------------------|-----------------------|---------------------------|---------------------|---------------------|
| Human herpes virus 7 (HHV-7)      | Watzinger et al. (2004)  | and Zerr et al. (2000)      |                       |                           |                     | Eclipse             |
| Human herpes virus 8 (HHV-8)      | Watzinger et al. (2004), | Lallemand et al. (2000),   |                       |                           | Bergroth et al. (2005) | Fernandez et al. (2002) |
|                                   | Ledeschi et al. (2001),  | and Su et al. (2005)        |                       |                           |                     |                     |
| Human immunodeficiency viruses 1 + 2 (HIV-1, HIV-2) | Candotti et al. (2004), | Schutten et al. (2000),   |                       | Laskus et al. (2004)     | Abravaya et al. (2003), | Scorpion primer Saha et al. (2001) |
|                                   | Desire et al. (2001),    | and Katsoulidou et al. (2006) |                       |                           | Vet et al. (1999) and |                     |
|                                   | Luo et al. (2005)        |                            |                       |                           | Summerer and Marx (2002) |                     |
| Human metapneumovirus (hMPV)      | Mackay et al. (2003),    | Boivin et al. (2003)       |                       | Scheltinga et al. (2005) | Kuypers et al. (2005) |                     |
|                                   | Maertzdorf et al. (2004),| and Cote et al. (2003)     |                       |                           |                     |                     |
|                                   | Bouscambert-Duchamp et al. (2005), |                  |                       |                           |                     |                     |
|                                   | Deffrasnes et al. (2005),| and Sumino et al. (2005)   |                       |                           |                     |                     |
| Human rhinovirus (hRV)            | Nijhuis et al. (2002),   | Scheltinga et al. (2005),  |                       | Dagher et al. (2004)     | Scheltinga et al. (2005) |                     |
|                                   | Lai et al. (2003),       | and Deffnerz et al. (2004) |                       | and Chen et al. (2006)   |                     |                     |
|                                   | and Sanders et al. (2004) |                          |                       |                           |                     |                     |
| Human T-lymphotropic viruses 1 + 2 (HTLV-1, HTLV-2) | Dehee et al. (2002),    | Pennington et al. (2002),  |                       | Kamihira et al. (2000, 2003) and | Lee et al. (2004) and | Vet et al. (1999) |
|                                   | Yamano et al. (2002),    | Estes and Sevall (2003),   |                       | Sonoda et al. (2004)     | Murphy et al. (2004) |                     |
|                                   | Matsuda et al. (2005) and| Montanheiro et al. (2005)  |                       |                           |                     |                     |
| Influenza viruses A + B (InfA, -B) | Watzinger et al. (2004),| Herrmann et al. (2001),   |                       | Smith et al. (2003)      | Krafft et al. (2005) | Templeton et al. (2004) |
|                                   | Herrmann et al. (2001),  | van Elden et al. (2001),   |                       | and Boivin et al. (2004) | and Boivin et al. (2004) | Chi et al. (2005) |
|                                   | Spackman et al. (2002),  | Lee and Suarez (2004),     |                       |                           |                     |                     |
|                                   | Ward et al. (2004),      | Hidryjeh et al. (2005),    |                       |                           |                     |                     |
|                                   | and Ng et al. (2005)     |                            |                       |                           |                     |                     |
| Virus Type                  | Authors and Year(s)                                                                 |
|----------------------------|----------------------------------------------------------------------------------|
| Measles virus              | Ozoemena et al. (2004), El Mubarak et al. (2005) and Hummel et al. (2005)        |
| Mumps virus                | Uchida et al. (2005) and Kubar et al. (2004)                                     |
| Newcastle disease virus (NDV) | Wise et al. (2004)                                                              |
| Norovirus type 1 + 2       | Kageyama et al. (2003), Hohne and Schreier (2004), Jothikumar et al. (2005b) and Pang et al. (2005) |
| Parainfluenza viruses 1–4  | Watzinger et al. (2004)                                                          |
| Parvovirus B19 (PVB 19)    | Watzinger et al. (2004), Aberham et al. (2001), Gruber et al. (2001), Knoll et al. (2002), Bultmann et al. (2003), Saito et al. (2003), Boschetti et al. (2004), Donoso et al. (2004) and Liefeldt et al. (2005) |
| Polyoma viruses (BK, JC, SV 40) | Watzinger et al. (2004), Shi et al. (1999), Biel et al. (2000), Hirsch et al. (2001), Leung et al. (2002b), Randhawa et al. (2002), Priftakis et al. (2003), Ryschekwitsch et al. (2004), Bressollette-Bodin et al. (2005), Randhawa et al. (2005), Si-Mohamed et al. (2006) and Stolt et al. (2005) |
| Poxvirus                  |                                                                                   |

*Continued on next page*
| Virus                      | Hydrolysis probe (Refs.)          | Hybridization probe (Refs.) | Melting curve (Refs.) | Molecular beacons (Refs.) | MGB<sup>TM</sup> probe (Refs.) | Other system (Refs.) |
|---------------------------|----------------------------------|-----------------------------|-----------------------|--------------------------|--------------------------------|----------------------|
| Respiratory syncytial virus (RSV) | Hu et al. (2003), Mentel et al. (2003), van Elden et al. (2003) and Perkins et al. (2005) | Gueudin et al. (2003) | Boivin et al. (2004) | Templeton et al. (2004) and O’Shea and Cane (2004) | Kuypers et al. (2004) and Whiteley and Sloots (2006) |
| Varicella zoster virus (VZV = HHV 3) | Watzinger et al. (2004), Furuta et al. (2001), Weidmann et al. (2003) and Ishizaki et al. (2003) | O’Neill et al. (2003), Scocher et al. (2003), Hawrami and Breuer (1999), Espy et al. (2000b), Logarev et al. (2000) and Tipples et al. (2003) | Campsall et al. (2004) |
| West Nile virus (WNV) | Lanciotti et al. (2000), Hadfield et al. (2001), Shi et al. (2001), Eiser et al. (2004), Stone et al. (2004b), Tewari et al. (2004), Vanlandingham et al. (2004) and Cameron et al. (2005) | Papin et al. (2004) | Shirato et al. (2005) | LAMP primer Parida et al. (2004) |
phase, and reflects the number of PCR products generated during the amplification process (Morrison et al., 1998). It is important to consider that the number of intercalating dye molecules, and thus, the amount of fluorescence signal emitted is greatly influenced by the length of the PCR amplicons. If the absolute amount of initial target copies is to be determined, a correction factor must be used to correctly assess the number of target molecules (Piatak et al., 1993).

Compared to other real-time detection formats, systems based on the use of intercalating dyes are easier to establish and less expensive, because no target-specific fluorogenic probes (see below) are required. Moreover, signal intensities and quantification results are not affected by mutations within the sequence encompassed by the primers. This property may be advantageous in the PCR analysis of viruses displaying a high mutation rate (e.g. different RNA viruses). Since point mutations in the target sequence can have an adverse effect on the hybridization efficiency of fluorogenic probes, detection and quantitative analysis of viral target sequences prone to mutation may be compromised in this detection format. On the other hand, the lack of probes in the assay results in lower sensitivity and specificity. Amplification and detection of specific PCR products are determined solely by the amplification primers. Dye molecules binding to non-specific PCR products or primer dimers contribute to overall fluorescence signal intensity, and may therefore lead to inaccurate quantification of target transcripts. These artifacts may also result in the generation of fluorescence in the “No Amplification (NAC) and No Template (NTC) Controls” (see below), thus affecting the interpretation of results. It is essential therefore to control the specificity of amplified fragments at the end of PCR by melting curve analysis (see below).

2.2. Detection and quantification by using target-specific probes

Detection formats based on specific hybridization of one or two fluorescence-labelled oligonucleotide probes to the target sequence during amplification are the most frequently reported formats for virus detection in diagnostic assays. Depending on the chemistry used, different types of fluorogenic probes have been introduced.

2.2.1. Hydrolysis probes

Most assays described for the detection of viral DNA or RNA are based on the use of hydrolysis probes (see Table 2). Hydrolysis probes (Applied Biosystems, AB, Foster City, CA, USA), also referred to as TaqMan® or 5′ nuclease probes, are non-extendible target-specific oligonucleotide probes that bind to the target strand between the PCR primers. They are dually labelled with a fluorescent reporter dye (e.g. FAM or VIC) covalently attached to the 5′-end, and a quencher dye (e.g. TAMRA), covalently attached to the 3′-end. When the reporter molecule on the TaqMan probe is stimulated by an appropriate light source to emit fluorescence, the energy is transferred to the quencher, thereby suppressing the emission of fluorescence by the reporter. This physical principle is known as the fluorescence resonance energy transfer (FRET) (Selvin, 1995). The transfer of energy works efficiently only across very short distances, and decreases rapidly when the reporter
and quencher molecules move apart. During PCR, when the DNA polymerase extends the primers, the hybridized probes are cleaved by the 5′ exonuclease activity of the enzyme and the corresponding quencher and reporter molecules are separated. The energy transfer to the quencher molecule is thus abrogated, and the reporter starts emitting fluorescence which can be measured at the end of each extension step (Holland et al., 1991).

In well established assays, there is a linear correlation between the number of released reporter molecules and the number of amplicons synthesized during each PCR cycle. This correlation serves as a basis for calculation of initial copy numbers of the target transcript.

The main advantages of this chemistry include easy probe design and only minor restrictions that apply to the selection of appropriate target sequences. These facts are apparently the main reasons for the reported 80% success rate of new RQ-PCR assays based on the use of hydrolysis probes (Kubista, 2004). However, short amplicons (80–130 bp) are required to achieve optimal amplification efficiencies and, since the TaqMan® probe molecules are cleaved after each cycle, no melting curve analysis of the amplicons is possible. Another disadvantage is the reduced temperature of strand extension required for the 5′-nuclease activity to displace and cleave the probe. This temperature is suboptimal for the processing activity of the Taq DNA polymerase enzyme and may therefore affect the amplification efficiency of the PCR reaction.

2.2.2. Hybridization probes

Another detection format frequently used for the detection of DNA viruses is based on hybridization probes (HybProbe) (Caplin et al., 1999). This method relies on the use of two oligonucleotide probes that hybridize next to each other to a sequence located between the amplification primers. One of these probes is labelled with a donor dye at the 3′ end (e.g. Fluorescein, emitting green light), the other is labelled with an acceptor dye at the 5′ end (e.g. LC Red 640 or LC Red 705, emitting red light). The probes are designed to hybridize during the annealing step to the same strand in a head-to-tail arrangement, at a distance of 1–5 nucleotides to bring the two dyes in close proximity (hence the name “kissing” probes). The donor dye is stimulated by an appropriate light source to emit fluorescence. If both probes are bound to the specific target sequences, the fluorescence energy is transferred from the donor to the acceptor molecules (FRET), and the excited fluorophore emits a fluorescent signal, which is detected and measured at the end of each annealing step. After the annealing step, the temperature in the cycler is raised for strand extension, and the hybridization probes detach from the target. In solution, the hybridization probes are not close enough to permit relevant energy transfer. The amount of red fluorescence emitted during each annealing step is proportional to the number of PCR products generated. Measurement of the fluorescence kinetics during PCR permits calculation of the initial target copies (Nitsche et al., 1999).

An advantage of this method is that the hybridization probes are not hydrolyzed, thereby facilitating the generation of amplicon-specific melting curves (see below). A potential disadvantage is the requirement to design two oligonucleotide probes
capable of hybridizing between the amplification primers. Depending on the sequence of the targeted region, it may be difficult to find the appropriate space for placing two primers and two probes in close proximity to each other. In virus analysis, this problem may arise when a consensus target region for the detection of multiple strains of a virus needs to be identified.

2.2.3. Molecular beacons

A less commonly used detection format is based on molecular beacons (Tyagi and Kramer, 1996). Molecular beacons are oligonucleotide probes containing flanking sequences of 5–7 nucleotides designed to be complementary to each other, and an intervening sequence complementary to the target of interest. The ends of the probe are labelled with a fluorescent and a non-fluorescent quenching dye (DABCYL), respectively. The term “molecular beacon” is derived from the fact that in solution the complementary sequences of the probe anneal to each other forming a stem-like structure, whereas the intervening sequence remains single-stranded and loops out. The result is a hairpin structure that brings the fluorescent dye and the quencher in close proximity, leading to efficient quenching of the fluorophore. The energy is released from the quencher dye in the form of heat (collisional quenching). In the presence of specific template, the intervening loop sequence of the molecular beacon binds to the target. This leads to a conformational transition from the hairpin structure to a linear structure, resulting in the separation of fluorophore and quencher. Energy transfer no longer occurs, and the fluorescence emitted can be detected at the end of each annealing step. Due to the fluorogenic mechanism, molecular beacons can be used for the generation of melting curves after the final extension step at the end of PCR, as a means of controlling the specificity of target amplification.

According to earlier reports, well designed molecular beacons are capable of discriminating single base-pair mismatches more accurately than any other probe (Tyagi et al., 1998). The reason for the higher specificity is the high thermal stability of the hairpin structure. This conformation is favored over the linear structure if the target sequence is not perfectly matched to the complementary region of the probe. If the probe is not linearized by hybridizing to its target, no fluorescence signal is generated.

The real challenge of working with molecular beacons is the design of the stem and the loop. If the stability of the stem is too high, the hairpin structure is retained even in the presence of complementary target. If the stability is too low, the molecular beacon may fold into alternate conformations that do not place the fluorophore in the vicinity of the quencher, leading to an increase in background signals. To estimate the melting temperature of the stem, specific software, such as the Zuker DNA folding program, available on the internet at URL <http://www.bioinfo.rpi.edu/applications/mfold/old/dna/form1.cgi> can be used. A PC version of this program can also be downloaded from <http://128.151.176.70/RNAstructure.html>. Molecular beacons can be designed with the help of a dedicated software package termed ‘Beacon Designer,’ which is available from Premier Biosoft International (URL <www.premierbiosoft.com>).
2.2.4. **Melting curve analysis**

This approach to assessing the specificity of generated PCR products is applicable to real-time assays based on the use of intercalating dyes, hybridization probes or molecular beacons. In melting curve analysis, measurements of fluorescence are performed at the end of the PCR reaction. The temperature in the reaction tubes is gradually raised until complete denaturation of double-stranded DNA molecules occurs ($T_{D}$) ($T_{D}$ = denaturing temperature = temperature at which the double stranded DNA amplicons are denatured). The target–probe hybrids melt at a specific melting temperature ($T_{M}$) ($T_{M}$ = melting temperature (temperature at which 50% of the probe–target hybrids have dissociated)) according to their sequence, length, and GC content, thus leading to a characteristic pattern of fluorescence kinetics. The melting curve analysis is done by plotting the intensity of fluorescence against the temperature gradient on a logarithmic scale.

Melting temperature profiles can be used to discriminate full length amplicons from shorter products, such as primer dimers, by their reduced $T_{D}$. Moreover, non-specific PCR products displaying a different sequence can be differentiated from specific amplicons. Even single-base differences can be identified by different $T_{M}$ and can be exploited to identify and genotype highly homologous viruses. With appropriately validated melting curve analysis, it is also possible to determine the quantity of specific amplicons as a basis for the calculation of initial target copy numbers (Safronetz et al., 2003; Payungporn et al., 2004).

3. **Recent technical developments of primer and probe modification for RQ-PCR analysis**

A new generation of modified RQ-PCR probes or primers revealing increased thermal duplex stability and improved specificity for their target sequences has recently been launched by different vendors. The employment of modified oligonucleotides as primers or probes can provide more accurate target discrimination and quantification, which may be particularly useful for the detection and quantitative analysis of traditionally problematic target sequences, like GC- or AT-rich regions.

3.1. **PNA-probes**

Peptide nucleic acids (PNAs) (Nielsen, 2001) are nucleic acid analogs in which the phosphate/sugar backbone is replaced by an uncharged polyamine backbone. The side groups consist of nitrogenous purine and pyrimidine bases, identical to biological nucleic acids. The binding to complementary base sequences is stronger and more specific than that achieved with DNA or RNA probes (Uhlmann, 1998). The greater stability is reflected by a higher $T_{M}$ as compared to the corresponding DNA/DNA or DNA/RNA duplexes. PNA/DNA hybridization is significantly more affected by base mismatches than DNA/DNA hybridization. A single PNA/DNA mismatch reduces the $T_{M}$ by an average of 15 °C, compared with 11 °C in a
DNA/DNA duplex. This allows shorter lengths of PNA probes to be used in situations where longer DNA probes are normally employed. However, the specificity of the probes requires careful consideration: while a 15-mer PNA probe will have roughly the same melting temperature as a standard 25-mer DNA probe, the former will not display an equally high specificity.

PNA probes show excellent chemical and biological stability, they are completely resistant to all nucleases, proteases, and peptidases commonly used to degrade DNA and peptides. Because of their resistance to enzymatic degradation, the lifetime of PNAs is extended both in vivo and in vitro (Pellestor and Paulasova, 2004). The consequence for the application of PNA chemistry in RQ-PCR assays is that PNA probes cannot be designed as hydrolysis probes. Moreover, PNAs are not recognized by polymerases and thus cannot be used as primers in RQ-PCR assays (Orum et al., 1993). A practical problem with PNA is the insolubility in water of oligomers greater in length than 15–18 bases. Probes of more than 18 bases in length tend to aggregate, a problem that also occurs with purine-rich PNA probes. Hence, for best performance, PNA probes should be no longer than 18 nucleotides and should display a balanced nucleotide composition.

The PNA backbone has been exploited for the development of LightUp probes (Wolffs et al., 2001; Svanvik et al., 2000; Isacsson et al., 2000). The principle of LightUp probe is based on the presence of a single reporter dye, which becomes luminescent upon regular hybridization, without the requirement of any conformational change. In contrast to some other detection formats, this principle permits target quantification by the measurement of increasing rather than fluctuating fluorescence intensity. Due to the presence of a PNA backbone, the probes are not degraded by the Taq DNA polymerase during the extension phase. In comparison to oligonucleotide-based probes, LightUp probes reveal faster hybridization, stronger binding to target DNA and efficient competition with re-annealing of heat-denatured template. Based on these properties, LightUp probes can be readily implemented in standardized real-time PCR protocols.

3.2. MGB™ probes

TaqMan Minor Groove Binding (MGB™) probes (Applied Biosystems, AB, Foster City, CA, USA) are short oligonucleotides characterized by the conjugation of minor groove binders, such as dihydrocyclopyrroloindole, at the 3′ or, less commonly, at the 5′-end (Kutyavin et al., 2000). This chemical modification increases the $T_M$ of the hybridized probe and facilitates highly specific binding to the targeted sequence at the minor groove of the DNA helix. The difference in $T_M$-values between completely and incompletely matched probes is pronounced, thus providing a basis for reliable discrimination between sequences displaying minimal differences in the base pair composition. Moreover, these probes contain a quencher dye that does not emit fluorescence within the detectable wavelength range. This improvement eliminates spectral overlaps with fluorescence emitted by the reporter dye, and results in greater accuracy in the measurement of reporter-specific signals.
For the discrimination between viral serotypes or strains differing even by a single nucleotide within the region amplified, two differentially labelled TaqMan MGB probes, each specific for one virus type, can be used in a PCR reaction driven by a single set of primers (Geng et al., 2005). Our experience with the use of two MGB probes in a single RQ-PCR reaction on the ABI Prism 7700 instrument revealed their excellent capability of differentiating between minimally mismatched sequences (unpublished observations). However, precise quantification of the two targets was hampered by spectral interference (cross-talk) of the reporter dye fluorescence signals (FAM and VIC) in the detection channels.

3.3. LNA® primers and probes

The term locked nucleic acid (LNA®) describes oligonucleotide modifications characterized by the presence of one or more bicyclic ribose analogs (Braasch and Corey, 2001). The structural resemblance to native nucleic acids leads to very good solubility in water and easy handling. In contrast to PNA and MGB chemistry, LNA modifications are applicable to both primers and probes in RQ-PCR assays. LNA substitutions to DNA oligonucleotides confer exceptional biological stability and significantly increased affinity to their complementary DNA targets. The increased thermal stability is dependent on the number of LNA monomers present in the sequence. LNA modifications greatly increase the melting temperature of oligonucleotides and the differences in $T_M$ between perfectly and imperfectly matched nucleic acid duplexes, thus facilitating the discrimination even between single base mutations. Owing to these properties, LNA-containing oligonucleotides used in RQ-PCR assays have a length ranging between 13 and 20 nucleotides (nt), which is significantly shorter than unmodified primers and probes displaying the same $T_M$. This feature provides greater flexibility in designing consensus primers and probes for the detection of partially homologous target sequences, such as related viral species and serotypes. Finally, our experience with LNA-modified oligonucleotides in RQ-PCR assays revealed a specificity and sensitivity superior to other types of primers and probes (unpublished observations).

3.4. Other primer modifications

If the relatively high cost of the probe is an impediment to the implementation of RQ-PCR approaches, it is possible to use detection systems based on modified primers generating fluorescence signals upon target amplification. Examples of such detection systems include Scorpion primers (Whitcombe et al., 1999), Amplifour primers (Uehara et al., 1999), LUX™ primers (Invitrogen, Carlsbad, CA, USA) (Nazarenko et al., 2002), or QZyme system (BD, 2003). Another recently launched primer-based probe-less real-time system is based on the measurement of gradual reduction of fluorescence signal (Plexor™, Promega, Mannheim, FRG) (Johnson et al., 2004; Sherrill et al., 2004), which leads to an inversion of the characteristic exponential amplification curve.
All these systems are less expensive than probe-based detection formats and may provide an attractive alternative, especially if multiple targets have to be detected simultaneously by multiplex reactions. In these instances, the high cost of synthesis of several specific fluorogenic probes can be avoided. However, it is necessary to determine for each probe-less detection system whether the sensitivity and specificity are adequate.

3.5. Comments on technical issues

The detection formats and chemistries outlined above have both advantages and specific limitations. Intercalating dyes are significantly cheaper than probes, but bind also to primer dimers and other spurious PCR products. Subsequent melting curve analysis increases the discriminative power of dye-based assays, but adds to the complexity of data analysis. Other probe-less RQ-PCR detection systems also lack the intrinsic specificity control offered by the inclusion of target-specific probe and are therefore less commonly used in clinical diagnostics. The employment of specifically binding fluorogenic probes offers not only greater specificity of target detection, but the inclusion of a hybridization step in the PCR reaction also increases the sensitivity of detection, which is comparable to that achieved by nested PCR or Southern blot hybridization of PCR products. Another benefit of probe-based assays over intercalating dyes is the possibility to combine two or even more probes with different labels in one PCR reaction (multiplex reaction). In these instances, it is essential to select fluorescent dyes with minimal spectral overlap in excitation and emission in order to avoid the phenomenon of “cross-talk”.

It is important, however, to bear in mind that none of the fluorescence-based detection systems eliminate non-specific amplification emerging from mispriming or primer dimer formation. Unless fluorescence-labelled primers are used, the non-specific products can remain undetected, but may affect the amplification efficiency of the specific target and the final quantification result. It is recommended therefore to optimize the reaction conditions in the initial phase of assay development by employing intercalating dyes and subsequent gel electrophoresis or melting curve analysis, in order to ensure the absence of amplification artefacts prior to implementing a specific probe in the assay. In our hands, the use and LNA-modified hydrolysis probes has been particularly successful in establishing highly specific RQ-PCR detection assays displaying a sensitivity superior to other systems. Moreover, several LNA-modified probes can be combined in multiplex assays, without relevant losses of sensitivity.

4. Application of the RQ-PCR technology to the detection and surveillance of virus infections

4.1. Specimens for virus analysis

The real-time PCR technology permits the detection and quantification of viral targets derived from a large variety of different types of clinical samples. Upon
isolation of viral DNA or RNA by different commercially available nucleic acid extraction kits, viral targets can be detected in primary human materials including plasma, serum, peripheral blood (PB), bone marrow (BM), mononuclear cells isolated from PB or BM, saliva, buccal and nasopharyngeal swabs, sputum, tracheal aspirate, bronchoalveolar lavage, cerebrospinal fluid, urine, stool, and various solid tissues. Moreover, molecular virus detection by RQ-PCR can be performed in tissue culture, paraffin-embedded tissue, water, urban sludge, plants, and animals.

4.2. Purification of viral DNA and RNA for subsequent RQ-PCR analysis

Virus-containing specimens can be kept at ambient temperature or stored at +4 °C for several days without loss of yield and integrity, because viruses are protected from nuclease attack as long as the protein (+/- lipid) coat is not disrupted (Anderson et al., 2003).

For many years, the guanidinium thiocyanate method described by Chomczynski (Chomczynski and Sacchi, 1987) was among the most sensitive and reproducible protocols for the extraction of viral nucleic acids (Verhofstede et al., 1996). However, the procedure is time-consuming, labor-intensive, and susceptible to contamination. Moreover, safety issues associated with the required use of phenol–chloroform–isoamylalcohol extraction rendered this organic extraction a less convenient nucleic acid purification method for clinical applications. In an effort to eliminate the use of hazardous solvents for the extraction of impurities, and in order to save time and labor, more rapid and automated nucleic acid extraction protocols with fewer manipulation steps were established and commercialized. The majority of these “ready-to-use” sample preparation kits are based on the capture of DNA/RNA on silica matrices or on paramagnetic beads coated with target specific ligands. The elimination of organic solvents and the development of appropriate technical instruments have paved the way to the implementation of robotic workstations for automated sample processing. Systems for automated sample preparation include, for example, the AmpliPrep or the MagNA Pure LC system (Roche Diagnostics) or workstations like the BioRobots M48 or 9604 (QIAGEN) (see Table 3). Despite the relatively high cost of acquisition, maintenance and required consumables, these instruments can be recommended for implementation in clinical virology laboratories. The obvious advantages of largely automated systems for virus analysis include increased throughput and less hands-on time for the staff, in addition to improved reproducibility, higher specificity and lower inter-laboratory variation. Moreover, the risk of pre-PCR cross-contamination can be significantly reduced (Mifflin et al., 2000). Irrespective of the extraction procedure applied, manual or automated, and the chemistry used, home-brew or approved (e.g. as “analytic specific reagents” (ASR) or “CE analytical”), potential problems affecting the efficiency and reliability of viral nucleic acid purification require consideration:

1. Impurities and contaminations present after nucleic acid isolation may cause false negative results of PCR analysis owing to the presence of nucleases or enzyme inhibitors. An adequate control for the purity of nucleic acid
preparations is the performance of over-time stability studies using well defined DNA or RNA spikes added after the nucleic acid isolation step.

(2) The detection limit of virus analysis may be affected by restriction of the sample volume that can be processed by a given nucleic acid extraction protocol. A number of commercially available products for isolation of nucleic acids suffer from this limitation. For the detection of very low virus copy numbers, virus-enrichment (Roth et al., 1999; Shyamala et al., 2004) or high-volume extraction methods (Hourfar et al., 2005) may be required.

### 4.3. Reverse transcription of RNA viruses

The reverse transcription (RT) step is a highly underestimated source of variability in the quantification of RNA viruses. Depending on the complexity of the RNA
target, the choice of primer, enzyme and reaction conditions are essential for the efficiency and fidelity of the RT reaction (Bustin and Nolan, 2004; Bustin et al., 2005). The most commonly used reverse transcriptases include the retroviral enzymes AMV-RT (purified from Avian Myeloblastosis Virus-infected chicks), and MMLV-RT (product of the pol gene of Moloney Murine Leukemia Virus). First strand cDNA synthesis can be initiated with non-specific primers, such as poly-T or random hexamer oligonucleotides, or with target sequence-specific primers. The activity of RT enzymes is adversely affected by residual blood components such as heme, by the presence of heparin, alcohol, phenol or high salt concentrations. Moreover, contaminations carried over from the RNA precipitation step can affect the measurable amount of RNA template, leading to inter-tube and inter-experimental variability (Freeman et al., 1999). Controls for the reverse transcription step are addressed below (see Section 5.2).

4.4. Qualitative and semi-quantitative virus detection by end-point PCR analysis using the real-time technology

Owing to its high sensitivity and rapid availability of test results, the use of PCR or RT-PCR has become the technique of choice for the detection of many pathogenic DNA and RNA viruses. In most instances, “home-brew” detection assays must be employed, because only few tests are available as commercial kits (see Table 1). Moreover, diagnostic approaches based on PCR are increasingly used to monitor the response to treatment, and assess progression of viral infections. However, as outlined below, the occurrence of false positive results, attributable to inadvertent contamination of test tubes by amplification products from previous reactions or by viral nucleic acids present at high levels in a different specimen, has remained an impediment to the diagnostic use of PCR assays.

Potential drawbacks of using real-time PCR in comparison to conventional PCR include the inability to monitor amplicon size without opening the system and the incompatibility of individual platforms with some fluorogenic chemistries. Moreover, the initial expense of real-time PCR may be prohibitive for low-throughput laboratories, but real-time PCR has proven cost effective when implemented in a high-throughput laboratory (Martell et al., 1999). Once established, the real-time technology offers a number of important advantages over conventional PCR including a decreased chance of contamination, because the systems are closed and do not require handling of the reaction contents after completion of PCR. An additional advantage is the monitoring of product accumulation in the reaction tube, which eliminates the requirement of separate detection methods, such as gel electrophoresis, thus shortening the effective assay time considerably. Since the fluorescence generated during the amplification reaction is proportional to the amount of PCR product, semi-quantitative estimation of virus copies in the specimen investigated is feasible even without precise calibration of the assay, which is required for truly quantitative analysis (see Section 5.1). A large number of real-time PCR assays for the detection of viral pathogens, including both DNA and RNA viruses, have been described (Abe et al., 1999; Kimura et al., 1999; Ryncarz et al., 1999; Kleiber
et al., 2000; Lallemand et al., 2000; Monpoeho et al., 2000; Tanaka et al., 2000; Ohyashiki et al., 2000; Furuta et al., 2001; Watzinger et al., 2004). The latter require special care in specimen processing because of the susceptibility of RNA to the digestion by ribonucleases that may be present in clinical samples. The efficiency of the reverse transcription step, which is required prior to PCR amplification, is critical for the sensitivity of RNA virus detection and must be monitored by appropriate controls (see Section 5.2). Reverse transcription real-time PCR has been applied to the detection of numerous RNA viruses including, for example, the human immunodeficiency virus (HIV), the hepatitis C virus (HCV), the human T-cell lymphotropic viruses HTLV-1 and HTLV-2, enteroviruses (EV), the respiratory syncytial virus (RSV), the influenza viruses (Inf-A, Inf-B), the rotavirus and other viral pathogens that cause gastroenteritis, pulmonary and other infections (see Table 2). RT-PCR can also be employed to detect viral messenger RNA, which may be particularly useful in the diagnosis of DNA viruses displaying a latent phase in their life cycle. Detection of viral DNA in such cases may not permit distinction between latent and productive infection. In these instances, the detection of mRNA expressed only during productive infection would provide evidence of active viral infection. Apart from viral mRNA analysis, the presence or absence of an actively proliferating virus can be determined by serial quantitative measurement of relative or absolute viral load (see Section 4.6).

In addition to the detection of individual viral pathogens, real-time PCR facilitates the performance of multiplex reactions, with simultaneous amplification of more than one target, by using multiple primers and probes labelled with fluorescent dyes displaying different emission wavelengths.

4.5. Virus analysis by multiplex real-time PCR

The possibility to combine several primers and fluorescent probes in a real-time assay is an efficient means for detecting multiple viruses within a single reaction. Recent improvements in the design of probes and novel combinations of fluorophores, such as in the LightUp probe system (see Section 3.1), have improved the ability to discriminate an increasing number of targets. The discovery and application of non-fluorescent quenchers has rendered some wavelengths available that were previously occupied by the emissions from the early quenchers themselves. This improvement has permitted the inclusion of a greater number of spectrally discernable oligoprobes per reaction, and highlighted the need for a single non-fluorescent quencher capable of absorbing a broad range of emission wavelengths.

Recently described multiplex real-time virus detection assays include, for example, the hepatitis viruses B and C (HBV, HCV) and the human immunodeficiency virus HIV-1 (Candotti et al., 2004), enteric viruses (Beuret, 2004) or influenza viruses (Inf-A, Inf-B), parainfluenza viruses (PIV1–4) and the respiratory syncytial virus (RSV) (Templeton et al., 2004). We have recently introduced a multiplex assay permitting the detection of all species and serotypes of human adenoviruses (see Section 4.7) (Ebner et al., 2005). Future developments of novel chemistries, such as combinatorial fluorescence energy transfer tags (Tong et al., 2001), and improvements to the
design of real-time instrumentation and software can be expected to broaden the applicability of multiplex real-time PCR assays.

4.6. Quantitative virus analysis by real-time PCR: measurement of absolute virus load at individual time-points and monitoring of proliferation kinetics

Determining the amount of template by real-time PCR can be performed either by relative or absolute quantification. Absolute quantification determines the exact number of nucleic acid targets present in the sample in relation to a specific unit, and relative quantification describes changes in the amount of a target sequence compared with its level in a related sample (Freeman et al., 1999). Commonly, relative quantification provides sufficient information and is easier to establish. However, when monitoring the course of an infection, absolute quantification is useful in order to express the results in units that are common to both scientists and clinicians and across different platforms. Absolute quantification is also helpful when no sequential specimens are available to demonstrate changes in virus levels or when the viral load is used to differentiate active from latent infection.

Moreover, measurement of viral load by real-time-PCR has become a widely used approach to studying the effect of antiviral therapies or the emergence of drug-resistant variants (Clarke, 2002; Pas et al., 2005). Well known examples include the hepatitis viruses C and B (HCV; HBV), or human immune deficiency virus (HIV) infections, in which clinical management is based on the monitoring of viral load in peripheral blood (Najjoullah et al., 2001; Garson et al., 2005; Palmisano et al., 2005).

The importance of quantitative virus analysis is further underscored by the fact that different viruses may persist in a latent state after primary infection in healthy immunocompetent individuals as well as in asymptomatic patients, and cause universally positive results in PCR assays. Mere detection of these viral pathogens by PCR may not be relevant for the clinical outcome in these individuals. By contrast, consecutive assessment of the virus load seems to play an important role for the diagnosis and prognosis in patients with viral reactivation, by providing a basis for timely initiation of appropriate treatment (Yoshikawa, 2003; Zhong et al., 2004; Cesaro et al., 2005). A clinically important example of such viruses is the cytomegalovirus (CMV), a member of the human herpes virus family, which commonly persists in peripheral blood leukocytes after primary infection and is often reactivated in severely immunodeficient patients. It is the most common cause of opportunistic infection in patients with AIDS and in individuals who have received either solid organ or allogeneic stem cell transplantation. In view of the fact that CMV disease is life-threatening in these patients, early diagnosis of reactivation is important for therapeutic management. Mere detection of CMV in peripheral blood by PCR cannot distinguish between latent and active virus infection and has therefore a low predictive value for impending disease. Measurement of CMV load by quantitative PCR may, however, provide clinically more relevant information (Limaye et al., 2001). Detection of high levels of CMV DNA or the documentation of increasing viral load in serial blood specimens were reported to herald systemic disease and provide a basis for timely onset of preemptive antiviral therapy (i.e. treatment before the
occurrence of symptoms). Moreover, quantitative surveillance of CMV load may be helpful in providing a measure of response to therapy, with rising levels potentially permitting an early identification of antiviral drug resistance (Roberts et al., 1998).

For some viruses, threshold levels have been defined as parameters for the initiation of antiviral therapy and for determining the efficacy and required duration of treatment (Van Esser et al., 2002; Lipman and Cotler, 2003; Wagner et al., 2004). For example, in patients undergoing organ or allogeneic stem cell transplantation, reactivation of the Epstein–Barr virus (EBV) can lead to the syndrome of post-transplantation lymphoproliferative disorder (PTLD). The risk of PTLD has been related to defined cut-off values of EBV load (Baiocchi et al., 2004). However, the monitoring of EBV in peripheral blood (mononuclear cells and/or plasma) and the detection of rising viral loads permit early recognition of impending PTLD, thus providing a basis for timely initiation of antiviral treatment and for the clinical management of this syndrome (Orii et al., 2000; Wagner et al., 2001).

Less well known examples of other viruses where the measurement of viral load by quantitative PCR appears to be of clinical importance include the human polyoma virus BK (BKV) and the human papilloma virus 16 (HPV16): quantification of BKV load in serum appears to facilitate the diagnosis of BK virus-associated nephropathy in renal transplant recipients (Limaye et al., 2001) and the amount of HPV16 DNA in cervical smears has been reported to be a useful predictor of progression to carcinoma in situ (Josefsson et al., 2000).

However, the assessment of absolute viral load at a given time point does not necessarily reflect the risk of viral disease in all instances. In a recent study in pediatric patients after allogeneic stem cell transplantation, we have investigated the potential of serial real-time PCR analysis to facilitate diagnosis of invasive adenovirus (AdV) infection early in its pre-clinical stage. Some of the patients who ultimately developed fatal disseminated disease had very high levels of AdV copies in peripheral blood (>10^7/ml), while others displayed relatively low peak levels, ranging between 10^3 and 10^4 copies/ml. Regardless of the maximum AdV levels reached, quantitative monitoring of virus load by real-time PCR usually revealed rising virus copy numbers several weeks before the onset of clinical symptoms (Lion et al., 2003), indicating that the detection of virus proliferation kinetics has a better predictive value in this instance. For clinically relevant evaluation of dynamic changes determined by serial RQ-PCR analyses, we have shown previously for different quantitative PCR assays that a tenfold increase of the respective target copy number is well beyond the intrinsic variability of the method, and provides reliable evidence of an expanding process (Lion et al., 1993, 2003). The detection of rising viral load by serial real-time PCR analysis not only demonstrates the presence of an active infection, but also eliminates a technical problem inherent in PCR diagnosis, the occurrence of false positive PCR tests due to inadvertent contamination with extraneous nucleic acids.

4.7. RQ-PCR assay design and target specification

Limited homology among different strains, serotypes or even species belonging to a viral genus can be a major challenge for the establishment real-time PCR assays, if
detection of the entire spectrum of these viruses is of potential clinical relevance. The human adenoviruses (AdV), which are divided into six species (A–F) and 51 serotypes, where individual serotypes may contain a number of different strains, are an important example. Different approaches have been described for detecting and quantifying all human adenoviruses. An AdV species-specific real-time PCR assay requiring six separate reactions, each of which contains individual primer and probe systems, permitting quantitative analysis of all serotypes within a species, has provided a clinically useful, but relatively laborious approach (Lion et al., 2003). In a recent report, a more economic pan-adenovirus detection assay, a multiplex PCR combining different primer/probe systems in two reactions, has been described (Ebner et al., 2005). The detection of all human adenoviruses in one RQ-PCR reaction using a single consensus primer/probe system is feasible (Heim et al., 2003), but requires lower stringency conditions of target amplification, thus potentially compromising the specificity of the assay. Other clinically important examples of viruses requiring quantitative analysis of different genotypes and mutated variants include the blood-borne viruses HIV, HCV, and HBV. For HIV infected patients, genotype assays are available facilitating the identification of mutations in the viral genome associated with resistance to particular drugs (Clarke, 2002; Pas et al., 2005). This type of analysis permits the selection of an appropriate antiretroviral regimen and provides an indication at which time-point treatment needs to be changed. In patients suffering from HCV infection, information on the presence of specific genotypes is used in addition to the viral load (see above) for determining the duration of antiviral therapy (EASL, 1999). Similarly, the success of antiviral treatment in HBV infected patients seems to correlate with the genotype detected (Sugauchi et al., 2002). A common procedure for virus genotyping is target amplification using consensus primer-mediated PCR, followed by sequencing of the amplified fragments (Germer et al., 1999; Chow et al., 2000; Vernon et al., 2000; Plantier et al., 2004). In the presence of mixed virus populations, this approach has serious limitations owing to preferential amplification of the dominant virus strain, which prevents the identification of subdominant strains by sequencing, if they account for less than 10–20% of the entire viral load. Known virus variants can be directly targeted and quantified by specifically designed RQ-PCR assays, exploiting particularly the high discriminating capacity of primers or probes carrying one of the modifications described above. To identify a particular virus variant of interest for subsequent quantitative analysis, PCR-based algorithms may be employed, as exemplified in Fig. 1 for selective quantification of a human AdV serotype. This approach permits highly sensitive and specific quantification, even in the presence of multiple AdV serotypes of the same or different species.

5. Quality controls in RQ-PCR virus detection assays

Reliable quantification of viral pathogens in clinical specimens by real-time PCR requires normalization against defined standards and the implementation of various controls.
5.1. Normalization

The indication of the number of viruses detected in clinical specimens depends on the type of material investigated: in liquid specimens, such as serum, plasma or cerebrospinal fluid, viral load is usually indicated per volume unit (e.g. milliliter) of the sample; extracellular viruses in solid specimens, such as stool, are commonly reported per mass unit (e.g. gram), and the number of intracellular viruses in specimens such as peripheral blood leukocytes, buccal swabs or biopsy material is generally indicated per number of cells (e.g. \(10^6\)) of the tissue investigated. The cell number in clinical specimens can be easily assessed by co-amplification of a single-copy housekeeping gene, but for extracellular virus detection the size of the clinical sample used for virus isolation needs to be measured to permit accurate assessment of viral load.

---

Fig. 1. Example of an algorithm for AdV serotyping by RQ-PCR, revealing presence of two different serotypes of the virus in the sample investigated.
The quantification of viral targets and control genes is generally performed against external standards (Watzinger et al., 2004). Standard curves used as a reference for quantitative analysis are established by titration of a precisely defined, identically amplified template, in a related sample matrix. Well established real-time PCR assays provide remarkably reproducible results with low intra- and inter-assay variability. Internal controls, which are an essential prerequisite for quantitative target analysis in the competitive PCR format (Niesters, 2004), are therefore not a common feature of the real-time PCR platform. However, despite the high reproducibility of real-time PCR assays, amplification of replicates of each sample is regarded as instrumental for reliable quantification.

5.2. Controls for false positive and negative results

In conventional PCR assays, false positive results are most commonly caused by contamination of PCR reactions with amplification products from previous tests or by carryover of homologous genomic DNA. Alternatively, false positive results may arise from non-specific binding of primers to irrelevant sequences. The latter problem is greatly reduced in real-time PCR assays implementing specific hybridization probes that lead to the generation of fluorescence signals only upon binding to the amplified products. Although in real-time PCR assays performed on fluorescence-based instruments, the absence of post-amplification handling greatly reduces the risk of contamination by PCR products, it is important to bear in mind that this problem is not entirely eliminated. Contamination may occur by leakage from tubes or microtiter plates with lids not tightly closed or by breakage of glass capillaries leading to spillage of the amplification mixture. If the nucleotide dTTP is substituted by dUTP in all PCR reactions, amplicons will differ from genomic DNA by the presence of dUTP. In this instance, the problem of contamination with PCR products can be easily reduced by implementing a digestion step with Uracil-DNA-glycosylase (UNG) prior to each amplification reaction. When using this pretreatment in PCR reactions, digestion of newly synthesized PCR product is prevented by heat-mediated inactivation of the enzyme before the start of amplification (Watzinger and Lion, 2003). Although the routine use of UNG is highly effective, it does not completely eradicate the problem of contamination. In virus analysis, an important source of carryover contamination is patient material containing high titers of a viral pathogen, which is not affected by enzymatic digestion using UNG. The risk of contamination by extraneous viral nucleic acids can be reduced by extremely careful handling of clinical samples by skilled personnel, strict separation of working areas and other well established precautions recommended for PCR work (Kwok and Higuchi, 1989; Sarkar and Sommer, 1990; Scherczinger et al., 1999; Bustin, 2002). Moreover, multiple negative controls, i.e. reactions lacking any template, the so-called “no-template controls (NTCs)”, and reactions including non-homologous template, the “no-amplification controls (NACs)”, must be included in each assay to permit the identification of contamination and prevent false interpretation of positive results.

In view of the great sensitivity of PCR, the occurrence of false negative results is a highly underestimated problem. The absence of positive signals in samples
containing the viral target may result from inhibitory substances present in the clinical specimen analyzed, from low efficiency or failure of viral nucleic acid extraction, from inadequate PCR amplification or, in case of RNA virus analysis, from inefficient reverse transcription. The efficiency of nucleic acid isolation, reverse transcription and amplification can be assessed by adding a defined standard to the samples before each of these steps. Artificial constructs generated by cloning of the specific viral target sequence into a plasmid are often used as external controls for the amplification step. However, the handling of plasmids harboring the targeted nucleic acid sequence is a dangerous source of contamination. In order to avoid the risk of cross-contamination and to include well defined internal controls with physical properties similar to those of the target of interest, it is recommended to spike the specimens with non-human, naturally occurring viruses (see below). Alternatively, the so-called “armored RNA” technology can be implemented as an internal control, where the samples investigated are spiked with a known concentration of a synthetic RNA standard protected from degradation by packaging in pseudoviral particles (Pasloske et al., 1998). In our laboratory, we use the phocine (=seal) herpes virus type 1 (PhHV) as a DNA virus control and the phocine distemper virus (PDV) as an RNA virus control (both were kindly provided to us by Niesters, Department of Virology, University Hospital Rotterdam, the Netherlands). Clinical specimens to be tested for the presence and quantity of DNA viruses are spiked with a defined amount of PhHV prior to processing. The presence or absence of the expected level of fluorescent signal obtained upon real-time amplification of the control virus permits the assessment of the efficiency of nucleic acid isolation and possible effects of inhibitory agents on the amplification step. Similarly, clinical specimens to be investigated by real-time PCR for RNA viruses are spiked with defined quantities of PDV (Niesters, 2002; Watzinger et al., 2004). After RNA extraction and reverse transcription, the amount of control virus is determined by real-time PCR analysis. The presence of the expected result indicates adequate extraction and reverse transcription of viral RNA and the lack of any inhibitory effects on amplification. The results of quantitative virus analysis in clinical specimens are deemed eligible for evaluation only if quantitative analysis of the respective control virus reveals a readout in the expected range.

6. Future prospects

The rapidly growing number of publications on the quantification of viral targets demonstrates the increasing importance of quantitative virus detection in different areas of research and clinical diagnosis. Detection of the viral load and the dynamics of proliferation of the infectious pathogen have prognostic relevance in a number of clinical situations, and serve as a basis for guiding therapeutic interventions (Orum et al., 1993; Whitcombe et al., 1999; Uehara et al., 1999). The variety of predominantly home-brew methods used for quantification of viral targets and the lack of comprehensive information on the quality control measures in some published reports render the comparison of data generated by different diagnostic laboratories
rather difficult. It is desirable therefore to establish standardized technical approaches to quantitative virus analysis. Automation of the entire process, from isolation of viral nucleic acids to quantification, would provide the most convenient way of eliminating inter-laboratory variation.

Necessary prerequisites for the generation of diagnostic RQ-PCR results comparable between laboratories include the use of quality control reagents and calibrated standards, and participation in multi-center quality control programs. Due to the lack of standardized reference material and the absence of international accrediting bodies, different programs for quality control in clinical virology, such as the Quality Control for Molecular Diagnostics (QCMD) (URL: <http://www.qcmd.org/>), have been established. This activity was supported by the European Commission and endorsed by the European Society for Clinical Virology (ESCV) and the European Society for Clinical Microbiology and Infectious Disease (ESCMID). The QCMD is an independent, non-profit organization that designs, develops and provides QC materials and proficiency programs for an increasing number of viral targets. However, the number of currently available internationally accepted controls and standardized QC reagents is limited and restricted mainly to blood-borne viruses, such as HIV-1, HBV, and HCV. Certification and accreditation of diagnostic laboratories are becoming increasingly important in order to document the expertise and quality of work. Widely accepted criteria for quality assurance and the certification of clinical laboratories include the ISO standards 9001 and 15189. According to these guidelines it is necessary “to apply other methods to calibrate the measuring systems and to participate in inter-laboratory comparison programs or alternative mechanisms that can assure the quality of analytical procedures”. Examples of such collaborative efforts in Europe supported by the European Commission include Concerted Actions focusing on the standardization and clinical implementation of different diagnostic approaches. This kind of international collaboration may serve as a good example for future efforts aiming at the development of widely applicable concepts for quantitative virus diagnostics based on the real-time PCR technology.

References

Abe, A., Inoue, K., Tanaka, T., Kato, J., Kajiyama, N., Kawaguchi, R., Tanaka, S., Yoshiha, M., Kohara, M., 1999. Quantitation of hepatitis B virus genomic DNA by real-time detection PCR. J. Clin. Microbiol. 37 (9), 2899–2903.
Aberham, C., Pendl, C., Gross, P., Zerlauth, G., Gessner, M., 2001. A quantitative, internally controlled real-time PCR Assay for the detection of parvovirus B19 DNA. J. Virol. Methods 92 (2), 183–191.
Abravaya, K., Huff, J., Marshall, R., Merchant, B., Mullen, C., Schneider, G., Robinson, J., 2003. Molecular beacons as diagnostic tools: technology and applications. Clin. Chem. Lab. Med. 41 (4), 468–474.
Aitichou, M., Javorschi, S., Ibrahim, M.S., 2005. Two-color multiplex assay for the identification of orthopox viruses with real-time LUX-PCR. Mol. Cell. Probe. 19 (5), 323–328.
Aliyu, S.H., Aliyu, M.H., Salihu, H.M., Parmar, S., Jalal, H., Curran, M.D., 2004. Rapid detection and quantitation of hepatitis B virus DNA by real-time PCR using a new fluorescent (FRET) detection system. J. Clin. Virol. 30 (2), 191–195.
Anderson, N.G., Gerin, J.L., Anderson, N.L., 2003. Global screening for human viral pathogens. Emerg. Infect. Dis. 9 (7), 768–774.
Archimbaud, C., Mirand, A., Chambron, M., Regagnon, C., Bailly, J.L., Peigne-Lafeuille, H., Henquell, C., 2004. Improved diagnosis on a daily basis of enterovirus meningitis using a one-step real-time RT-PCR assay. J. Med. Virol. 74 (4), 604–611.

Baiocchi, O.C., Colleoni, G.W., Caballero, O.L., Vettore, A.L., Andrade, A.L., Pestana, J.O., 2004. Quantification of Epstein–Barr viral load and determination of a cut-off value to predict the risk of post-transplant lymphoproliferative disease in a renal transplant cohort. Haematologica 89 (3), 366–368.

BD, 2003. QZyme™ assays for quantitative PCR. Clontechniques 18 (4), 2–3.

Beck, R.C., Kohn, D.J., Tuohy, M.J., Prayson, R.A., Yen-Lieberman, B., Procop, G.W., 2004. Detection of polyoma virus in brain tissue of patients with progressive multifocal leukoencephalopathy by real-time PCR and pyrosequencing. Diagn. Mol. Pathol. 13 (1), 15–21.

Bengtsson, M., Karlsson, H.J., Westman, G., Kubista, M., 2003. A new minor groove binding asymmetric cyanine reporter dye for real-time PCR. Nucleic Acids Res. 31 (8), e45.

Bergroth, T., Sonnerborg, A., Yun, Z., 2005. Discrimination of lamivudine resistant minor HIV-1 variants by selective real-time PCR. J. Virol. Methods 127 (1), 100–107.

Beuret, C., 2004. Simultaneous detection of enteric viruses by multiplex real-time RT-PCR. J. Virol. Methods 115 (1), 1–8.

Biedermann, K., Dandachi, N., Trattner, M., Vogl, G., Doppelmayer, H., More, E., Staudach, A., Dietze, O., Hauser-Kronberger, C., 2004. Comparison of real-time PCR signal-amplified in situ hybridization and conventional PCR for detection and quantification of human papillomavirus in archival cervical cancer tissue. J. Clin. Microbiol. 42 (8), 3758–3765.

Biel, S.S., Held, T.K., Landt, O., Niedrig, M., Gelderblom, H.R., Siegert, W., Nitsche, A., 2000. Rapid quantification and differentiation of human polyomavirus DNA in undiluted urine from patients after bone marrow transplantation. J. Clin. Microbiol. 38 (10), 3689–3695.

Boivin, G., Cote, S., Cloutier, N., Abed, Y., Maguigad, M., Routy, J.P., 2003. Human herpesvirus 8 by real-time PCR in blood fractions of AIDS patients with Kaposi’s sarcoma and multicentric Castleman’s disease. J. Med. Virol. 68 (3), 399–403.

Boivin, G., De Serres, G., Cote, S., Gilca, R., Abed, Y., Rochette, L., Bergeron, M.G., Dery, P., 2003. Human metapneumovirus infections in hospitalized children. Emerg. Infect. Dis. 9 (6), 634–640.

Boivin, G., Cote, S., Dery, P., De Serres, G., Bergeron, M.G., 2004. Multiplex real-time PCR assay for detection of influenza and human respiratory syncytial viruses. J. Clin. Microbiol. 42 (1), 45–51.

Boschetti, N., Niederhauser, I., Kempf, C., Stuhler, A., Lower, J., Blumel, J., 2004. Different susceptibility of B19 virus and mice minute virus to low pH treatment. Transfusion 44 (7), 1079–1086.

Bouscambert-Duchamp, M., Lina, B., Trompette, A., Moret, H., Motte, J., Andreouletti, L., 2005. Detection of human metapneumovirus RNA sequences in nasopharyngeal aspirates of young French children with acute bronchiolitis by real-time reverse transcriptase PCR and phylogenetic analysis. J. Clin. Microbiol. 43 (3), 1411–1414.

Braasch, D.A., Corey, D.R., 2001. Locked nucleic acid (LNA): fine-tuning the recognition of DNA and RNA. Chem. Biol. 8 (1), 1–7.

Bressler, A.M., Nolte, F.S., 2004. Preclinical evaluation of two real-time, reverse transcription-PCR assays for detection of the severe acute respiratory syndrome coronavirus. J. Clin. Microbiol. 42 (3), 987–991.

Bressollette-Bodin, C., Coste-Burel, M., Hourmant, M., Sebille, V., Andre-Garnier, E., Imbert-Marcille, B.M., 2005. A prospective longitudinal study of BK virus infection in 104 renal transplant recipients. Am. J. Transplant. 5 (8), 1926–1933.

Briot, F., Geneen, V., Hofer, D., Stoddart, C.A., 2004. Coxackievirus B4 infection of human fetal thymus cells. J. Virol. 78 (18), 9854–9861.

Brooks, H.A., Gersberg, R.M., Dhar, A.K., 2005. Detection and quantification of hepatitis A virus in seawater via real-time RT-PCR. J. Virol. Methods 127 (2), 109–118.

Bultmann, B.D., Klingel, K., Sotlar, K., Bock, C.T., Baba, H.A., Sauter, M., Kandolf, R., 2003. Fatal parvovirus B19-associated myocarditis clinically mimicking ischemic heart disease: an endothelial cell-mediated disease. Hum. Pathol. 34 (1), 92–95.

Bustin, S.A., 2002. Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. J. Mol. Endocrinol. 29 (1), 23–39.
Bustin, S.A., Nolan, T., 2004. Pitfalls of quantitative real-time reverse-transcription polymerase chain reaction. J. Biomol. Technol. 15 (3), 155–166.
Bustin, S.A., Benes, V., Nolan, T., Pfaffl, M.W., 2005. Quantitative real-time RT-PCR—a perspective. J. Mol. Endocrinol. 34 (3), 597–601.
Cameron, C., Reeves, J., Antonishyn, N., Tilley, P., Alport, T., Euirich, B., Towns, D., Lane, D., Saldanha, J., 2005. West Nile virus in Canadian blood donors. Transfusion 45 (4), 487–491.
Campsall, P.A., Au, N.H., Prendiville, J.S., Speert, D.P., Tan, R., Thomas, E.E., 2004. Detection and genotyping of varicella-zoster virus by TaqMan allelic discrimination real-time PCR. J. Clin. Microbiol. 42 (4), 1409–1413.
Candotti, D., Temple, J., Owusu-Ofori, S., Allain, J.P., 2004. Multiplex real-time quantitative RT-PCR assay for hepatitis B virus, hepatitis C virus, and human immunodeficiency virus type 1. J. Virol. Methods 118 (1), 39–47.
Caplin, B.D., Rasmussen, R.P., Bernard, P.S., Wittwer, C.T., 1999. LightCycler™—Hybridization probes. The most direct way to monitor PCR amplification for quantification and mutation detection. Biomedica 1, 5–8.
Castelain, S., Descamps, V., Thibault, V., Francois, C., Bonte, D., Morel, V., Izopet, J., Capron, D., Zawadzki, P., Duverdely, G., 2004. TaqMan amplification system with an internal positive control for HCV RNA quantitation. J. Clin. Virol. 31 (3), 227–234.
Cesaro, S., Murrone, A., Mengoli, C., Pillon, M., Biasolo, M.A., Calore, E., Tridello, G., Varotto, S., Alaggio, R., Zanesco, L., Palu, G., Messina, C., 2005. The real-time polymerase chain reaction-guided modulation of immunosuppression enables the pre-emptive management of Epstein–Barr virus reactivation after allogeneic haematopoietic stem cell transplantation. Brit. J. Haematol. 128 (2), 224–233.
Chantratita, W., Pongtanapisit, W., Srichunrasmi, C., Seesu, S., 2004. Development and comparison of the real-time amplification based methods—NASBA-Beacon, RT-PCR taqman and RT-PCR hybridization probe assays—for the qualitative detection of sars coronavirus. Southeast Asian J. Trop. Med. Public Health 35 (3), 623–629.
Chen, R.W., Piiparinen, H., Seppanen, M., Koskela, P., Sarna, S., Lappalainen, M., 2001. Real-time PCR for detection and quantitation of hepatitis B virus DNA. J. Med. Virol. 65 (2), 250–256.
Chen, Y., Hamati, E., Lee, P.K., Lee, W.M., Wachi, S., Schnurr, D., Yagi, S., Dolganov, G., Boushey, H., Avila, P., Wu, R., 2006. Rhinovirus induces airway epithelial gene expression through dsRNA and interferon-dependent pathways. Am. J. Resp. Cell. Mol. Biol. 34 (2), 192–203.
Chi, X.S., Hu, A., Bolar, T.V., Al Rimawi, W., Zhao, P., Tam, J.S., Rappaport, R., Cheng, S.M., 2005. Detection and characterization of new influenza B virus variants in 2002. J. Clin. Microbiol. 43 (5), 2345–2349.
Chomczynski, P., Sacchi, N., 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162 (1), 156–159.
Chow, V.T., Loh, E., Yeo, W.M., Tan, S.Y., Chan, R., 2000. Identification of multiple genital HPV types and sequence variants by consensus and nested type-specific PCR coupled with cycle sequencing. Pathology 32 (3), 204–208.
Chui, L., Drebosat, M., Andonov, A., Betrich, A., Glushek, M., Mahony, J., 2005. Comparison of 9 different PCR primers for the rapid detection of severe acute respiratory syndrome coronavirus 2 RNA extraction methods. Diagn. Microbiol. Infec. Dis. 53 (1), 47–55.
Cinek, O., Witso, E., Jeansson, S., Rasmussen, T., Drevinek, P., Wetlesen, T., Vavrincec, J., Grinde, B., Ronningen, K.S., 2006. Longitudinal observation of enterovirus and adenovirus in stool samples from Norwegian infants with the highest genetic risk of type 1 diabetes. J. Clin. Virol. 35 (1), 33–40.
Claas, E.C., Schilham, M.W., de Brouwer, C.S., Hubacek, P., Echavarria, M., Lankester, A.C., van Tol, M.J., Kroes, A.C., 2005. Internally controlled real-time PCR monitoring of adenovirus DNA load in serum or plasma of transplant recipients. J. Clin. Microbiol. 43 (4), 1738–1744.
Clarke, J.R., 2002. Molecular diagnosis of HIV. Expert. Rev. Mol. Diagn. 2 (3), 233–239.
Cook, L., Ng, K.W., Bagabag, A., Corey, L., Jerome, K.R., 2004. Use of the MagNA pure LC automated nucleic acid extraction system followed by real-time reverse transcription-PCR for ultrasensitive quantitation of hepatitis C virus RNA. J. Clin. Microbiol. 42 (9), 4130–4136.
Corless, C.E., Guiver, M., Borrow, R., Edwards-Jones, V., Fox, A.J., Kaczmarski, E.B., Mutton, K.J., 2002. Development and evaluation of a ‘real-time’ RT-PCR for the detection of enterovirus and parechovirus RNA in CSF and throat swab samples. J. Med. Virol. 67 (4), 555–562.

Costa-Mattioli, M., Monpoeho, S., Nicand, E., Aleman, M.H., Billaudel, S., Ferre, V., 2002. Quantification and duration of viraemia during hepatitis A infection as determined by real-time RT-PCR. J. Viral. Hepatitis 9 (2), 101–106.

Cote, S., Abed, Y., Boivin, G., 2003. Comparative evaluation of real-time PCR assays for detection of the human metapneumovirus. J. Clin. Microbiol. 41 (8), 3631–3635.

Crossley, B.M., Hietala, S.K., Shih, L.M., Lee, L., Skowronski, E.W., Ardans, A.A., 2005. High-throughput real-time RT-PCR assay to detect the exotic Newcastle disease virus during the California 2002–2003 outbreak. J. Vet. Diagn. Invest. 17 (2), 124–132.

Dagher, H., Donninger, H., Hutchinson, P., Ghildyal, R., Bardin, P., 2004. Rhinovirus detection: comparison of real-time and conventional PCR. J. Virol. Methods 117 (2), 113–121.

Deffernez, C., Wunderli, W., Thomas, Y., Yerly, S., Perrin, L., Kaiser, L., 2004. Amplicon sequencing and improved detection of human rhinovirus in respiratory samples. J. Clin. Microbiol. 42 (7), 3212–3218.

Deffrasnes, C., Cote, S., Boivin, G., 2005. Analysis of replication kinetics of the human metapneumovirus in different cell lines by real-time PCR. J. Clin. Microbiol. 43 (1), 488–490.

Dehee, A., Cesaire, R., Desire, N., Lezin, A., Bourdonne, O., Bera, O., Plumeau, Y., Smadja, D., Nicolas, J.C., 2002. Quantitation of HTLV-I proviral load by a TaqMan real-time PCR assay. J. Virol. Methods 102 (1–2), 37–51.

Desire, N., Dehee, A., Schneider, V., Jacomet, C., Goujon, C., Girard, P.M., Rozenbaum, W., Nicolas, J.C., 2001. Quantification of human immunodeficiency virus type 1 proviral load by a TaqMan real-time PCR assay. J. Clin. Microbiol. 39 (4), 1303–1310.

Donaldson, K.A., Griffin, D.W., Paul, J.H., 2002. Detection, quantification and identification of enteroviruses from surface waters and sponge tissue from the Florida keys using real-time RT-PCR. Water Res. 36 (10), 2505–2514.

Donald, D., Divizia, M., Pana’, A., 2005. Use of armored RNA as a standard to construct a calibration curve for real-time RT-PCR. J. Virol. Methods 126 (1–2), 157–163.

Donoso, M.O., Nitsche, A., Meyer, R., Klingel, K., Niedrig, M., 2004. Analysing myocardial tissue from explanted hearts of heart transplant recipients and multi-organ donors for the presence of parvovirus B19 DNA. J. Clin. Virol. 31 (1), 32–39.

EASL, 1999. International Consensus Conference on Hepatitis C. Paris, 26–27 February 1999. Consensus statement. J. Hepatol. 31 (Suppl. 1), 3–8.

Ebner, K., Suda, M., Watzinger, F., Lion, T., 2005. Molecular detection and quantitative analysis of the entire spectrum of human adenoviruses by a two-reaction real-time PCR assay. J. Clin. Microbiol. 43 (7), 3049–3053.

Eisler, D.L., McNabb, A., Jorgensen, D.R., Isaac-Renton, J.L., 2004. Use of an internal positive control in a multiplex reverse transcription-PCR to detect West Nile virus RNA in mosquito pools. J. Clin. Microbiol. 42 (2), 841–843.

El Mubarak, H.S., De Swart, R.L., Osterhaus, A.D., Schutten, M., 2005. Development of a semi-quantitative real-time RT-PCR for the detection of measles virus. J. Clin. Virol. 32 (4), 313–317.

Emery, S.L., Erdman, D.D., Bowen, M.D., Newton, B.R., Winchell, J.M., Meyer, R.F., Tong, S., Cook, B.T., Holloway, B.P., McAustad, K.A., Rota, P.A., Bankamp, B., Lowe, L.E., Ksiazek, T.G., Bellini, W.J., Anderson, L.J., 2004. Real-time reverse transcription-polymerase chain reaction assay for SARS-associated coronavirus. Emerg. Infect. Dis. 10 (2), 311–316.

Enomoto, M., Nishiguchi, S., Shiomi, S., Tanaka, M., Fukuda, K., Ueda, T., Tamori, A., Hubu, D., Takeda, T., Yano, Y., Otani, S., 2001. Comparison of real-time quantitative polymerase chain reaction with three other assays for quantitation of hepatitis C virus. J. Gastroenterol. Hepatol. 16 (8), 904–909.

Espy, M.J., Ross, T.K., Teo, R., Svien, K.A., Weld, A.D., Uhl, J.R., Smith, T.F., 2000a. Evaluation of LightCycler PCR for implementation of laboratory diagnosis of herpes simplex virus infections. J. Clin. Microbiol. 38 (8), 3116–3118.
Espy, M.J., Teo, R., Ross, T.K., Svien, K.A., Wold, A.D., Uhl, J.R., Smith, T.F., 2000b. Diagnosis of varicella-zoster virus infections in the clinical laboratory by LightCycler PCR. J. Clin. Microbiol. 38 (9), 3187–3189.

Estes, M.C., Sevall, J.S., 2003. Multiplex PCR using real time DNA amplification for the rapid detection and quantitation of HTLV I or II. Mol. Cell. Probe. 17 (2–3), 59–68.

Fernandez, C., Boutotelleau, D., Manichanh, C., Mangeney, N., Agut, H., Gautheeret-Dejean, A., 2002. Quantitation of HHV-7 genome by real-time polymerase chain reaction assay using MGB probe technology. J. Virol. Methods 106 (1), 11–16.

Freeman, W.M., Walker, S.J., Vrana, K.E., 1999. Quantitative RT-PCR: pitfalls and potential. Biotechniques 26 (1), 112–115.

Fujino, M., Yoshida, N., Yamaguchi, S., Hosaka, N., Ota, Y., Notomi, T., Nakayama, T., 2005. A simple method for the detection of measles virus genome by loop-mediated isothermal amplification (LAMP). J. Med. Virol. 76 (3), 406–413.

Furuta, Y., Ohtani, F., Sawa, H., Fukuda, S., Inuyama, Y., 2001. Quantitation of varicella-zoster virus DNA in patients with Ramsay Hunt syndrome and zoster sine herpete. J. Clin. Microbiol. 39 (8), 2856–2859.

Gallinella, G., Bonvicini, F., Filippone, C., Delbarba, S., Manaressi, E., Zerbini, M., Musiani, M., 2004. Calibrated real-time PCR for evaluation of parovirus b19 viral load. Clin. Chem. 50 (4), 759–762.

Garson, J.A., Grant, P.R., Alyliffe, U., Ferns, R.B., Tedder, R.S., 2005. Real-time PCR quantitation of hepatitis B virus DNA using automated sample preparation and murine cytomegalovirus internal control. J. Virol. Methods 126 (1–2), 207–213.

Geng, H., Hua, B., Wang, H., Cao, Y., Sun, Y., Yu, A., 2005. Dual-probe assay for detection of lamivudine-resistance hepatitis B virus by real-time PCR. J. Virol. Methods, Epub-ahead of print.

Germer, J.J., Rys, P.N., Thorvilson, J.N., Persing, D.H., 1999. Determination of hepatitis C virus genotype by direct sequence analysis of products generated with the Amplicor HCV test. J. Clin. Microbiol. 37 (8), 2625–2630.

Greenlee, D.J., Fan, H., Lawless, K., Harrison, C.R., Gulley, M.L., 2002. Quantitation of CMV by real-time PCR in transfusable RBC units. Transfusion 42 (4), 403–408.

Gu, Z., Belzer, S.W., Gibson, C.S., Bankowski, M.J., Hayden, R.T., 2003. Multiplexed, real-time PCR applying duplex amplification, internal standardization, and two-color fluorescence detection. Appl. Environ. Microbiol. 67 (6), 2837–2839.

Gunson, R.N., Miller, J., Carman, W.F., 2003. Comparison of real-time PCR and EIA for the detection of outbreaks of acute gastroenteritis caused by norovirus. Commun. Dis. Public Health 6 (4), 297–299.

Gut, M., Leutenegger, C.M., Huder, J.B., Pedersen, N.C., Lutz, H., 1999. One-tube fluorogenic reverse transcription-polymerase chain reaction for the quantitation of feline coronaviruses. J. Virol. Methods 77 (1), 37–46.

Hawrami, K., Breuer, J., 1999. Development of a fluorogenic polymerase chain reaction assay (TaqMan) for the detection and quantitation of varicella zoster virus. J. Virol. Methods 79 (1), 33–40.
Hazari, S., Acharya, S.K., Panda, S.K., 2004. Development and evaluation of a quantitative competitive reverse transcription polymerase chain reaction (RT-PCR) for hepatitis C virus RNA in serum using transcribed thio-RNA as internal control. J. Virol. Methods 116 (1), 45–54.

He, J.W., Jiang, S., 2005. Quantification of enterococci and human adenoviruses in environmental samples by real-time PCR. Appl. Environ. Microbiol. 71 (5), 2250–2255.

Heim, A., Ebnet, C., Harste, G., Pring-Akerblom, P., 2003. Rapid and quantitative detection of human adenovirus DNA by real-time PCR. J. Med. Virol. 70 (2), 228–239.

Herman, J., Van Ranst, M., Snoeck, R., Beuselinck, K., Lerut, E., Damme-Lombaerts, R., 2004. Polyomavirus infection in pediatric renal transplant recipients: evaluation using a quantitative real-time PCR technique. Pediatr. Transplant. 8 (5), 485–492.

Herrmann, B., Larsson, C., Zwegberg, B.W., 2001. Simultaneous detection and typing of influenza viruses A and B by a nested reverse transcription-PCR: comparison to virus isolation and antigen detection by immunofluorescence and optical immunoassay (FLU OIA). J. Clin. Microbiol. 39 (1), 134–138.

Higuchi, R., Dollinger, G., Walsh, P.S., Griffith, R., 1992. Simultaneous amplification and detection of specific DNA sequences. Biotechnology (NY) 10 (4), 413–417.

Hindiyeh, M., Levy, V., Azar, R., Varsano, N., Regev, L., Shaley, Y., Grossman, Z., Mendelson, E., 2005. Evaluation of a multiplex real-time reverse transcriptase PCR assay for detection and differentiation of influenza viruses A and B during the 2001–2002 influenza season in Israel. J. Clin. Microbiol. 43 (2), 589–595.

Hirsch, H.H., Mohaupt, M., Klimkait, T., 2001. Prospective monitoring of BK virus load after discontinuing sirolimus treatment in a renal transplant patient with BK virus nephropathy. J. Infect. Dis. 184 (11), 1494–1495.

Ho, S.K., Yam, W.C., Leung, E.T., Wong, L.P., Leung, J.K., Lai, K.N., Chan, T.M., 2003. Rapid quantification of hepatitis B virus DNA by real-time PCR using fluorescent hybridization probes. J. Med. Microbiol. 52 (Pt 5), 397–402.

Hohne, M., Schreier, E., 2004. Detection and characterization of norovirus outbreaks in Germany: application of a one-tube RT-PCR using a fluorogenic real-time detection system. J. Med. Virol. 72 (2), 312–319.

Holland, P.M., Abramson, R.D., Watson, R., Gelfand, D.H., 1991. Detection of specific polymerase chain reaction product by utilizing the 5′-3′ exonuclease activity of thermus aquaticus DNA polymerase. Proc. Natl. Acad. Sci. USA 88 (16), 7276–7280.

Houng, H.S., Liang, S., Chen, C.M., Keith, J., Echavarria, M., Sanchez, J.L., Kolavic, S.A., Vaughn, D.W., Binn, L.N., 2002. Rapid type-specific diagnosis of adenovirus type 4 infection using a hexon-based quantitative fluorogenic PCR. Diagn. Microbiol. Infec. Dis. 42 (4), 227–236.

Houfar, M.K., Schmidt, M., Seifried, E., Roth, W.K., 2005. Evaluation of an automated high-volume extraction method for viral nucleic acids in comparison to a manual procedure with preceding enrichment. Vox Sang. 89 (2), 71–76.

Hu, A., Colella, M., Tam, J.S., Rappaport, R., Cheng, S.M., 2003. Simultaneous detection, subgrouping, and quantitation of respiratory syncytial virus A and B by real-time PCR. J. Clin. Microbiol. 41 (1), 149–154.

Hu, A., Colella, M., Zhao, P., Li, F., Tam, J.S., Rappaport, R., Cheng, S.M., 2005a. Development of a real-time RT-PCR assay for detection and quantitation of parainfluenza virus 3. J. Virol. Methods 130 (1–2), 145–148.

Hu, W., Bai, B., Hu, Z., Chen, Z., An, X., Tang, L., Yang, J., Wang, H., Wang, H., 2005b. Development and evaluation of a multitarget real-time Taqman reverse transcription-PCR assay for detection of the severe acute respiratory syndrome-associated coronavirus and surveillance for an apparently related coronavirus found in masked palm civets. J. Clin. Microbiol. 43 (5), 2041–2046.

Huang, J.L., Lin, H.T., Wang, Y.M., Yeh, Y.C., Peck, K., Lin, B.L., Liu, H.W., Chen, A., Lin, C.S., 2005. Rapid and sensitive detection of multiple genes from the SARS-coronavirus using quantitative RT-PCR with dual systems. J. Med. Virol. 77 (2), 151–158.

Humar, A., Kumar, D., Boivin, G., Caliendo, A.M., 2002. Cytomegalovirus (CMV) virus load kinetics to predict recurrent disease in solid-organ transplant patients with CMV disease. J. Infect. Dis. 186 (6), 829–833.
Hummel, K.B., Lowe, L., Bellini, W.J., Rota, P.A., 2005. Development of quantitative gene-specific real-time RT-PCR assays for the detection of measles virus in clinical specimens. J. Virol. Methods, Epub-ahead of print.

Hymas, W., Stevenson, J., Taggart, E.W., Hillyard, D., 2005. Use of lyophilized standards for the calibration of a newly developed real time PCR assay for human herpes type six (HHV6) variants A and B. J. Virol. Methods 128 (1–2), 143–150.

Isacsson, J., Cao, H., Ohlsson, L., Nordgren, S., Svanvik, N., Westman, G., Kubista, M., Sjoback, R., Sehlstedt, U., 2000. Rapid and specific detection of PCR products using light-up probes. Mol. Cell. Probe. 14 (5), 321–328.

Ishiguro, T., Saitoh, J., Yawata, H., Yamagishi, H., Iwasaki, S., Mitoma, Y., 1995. Homogeneous quantitative assay of hepatitis C virus RNA by polymerase chain reaction in the presence of a fluorescent intercalater. Anal. Biochem. 229 (2), 207–213.

Ishizaki, Y., Tezuka, J., Ohga, S., Nomura, A., Suga, N., Kuromaru, R., Kusuhaara, K., Mizuno, Y., Kasuga, N., Haru, T., 2003. Quantification of circulating varicella zoster virus-DNA for the early diagnosis of visceral varicella. J. Infect. 47 (2), 133–138.

Issa, N.C., Espy, M.J., Uhl, J.R., Smith, T.F., 2005. Sequencing and resolution of amplified herpes simplex virus DNA with intermediate melting curves as genotype 1 or 2 by LightCycler PCR assay. J. Clin. Microbiol. 43 (4), 1843–1845.

Jabs, W.J., Hennig, H., Kettler, M., Pethig, K., Smets, F., Buczynski, P., Kirchner, H., Wagner, H.J., 2001. Normalized quantification by real-time PCR of Epstein–Barr virus load in patients at risk for posttransplant lymphoproliferative disorders. J. Clin. Microbiol. 39 (2), 564–569.

Jardi, R., Rodriguez, F., Buti, M., Costa, X., Cotrina, M., Valdes, A., Galimany, R., Esteban, R., Guardia, J., 2001. Quantitative detection of hepatitis B virus DNA in serum by a new rapid real-time fluorescence PCR assay. J. Viral. Hepatitis 8 (6), 465–471.

Jebbink, J., Bai, X., Rogers, B.B., Dawson, D.B., Scheuermann, R.H., Domiati-Saad, R., 2003. Development of real-time PCR assays for the quantitative detection of Epstein–Barr virus and cytomegalovirus, comparison of TaqMan probes, and molecular beacons. J. Mol. Diagn. 5 (1), 15–20.

Johnson, S.C., Sherrill, C.B., Marshall, D.J., Moser, M.J., Prudent, J.R., 2004. A third base pair for the polymerase chain reaction: inserting isoC and isoG. Nucleic Acids Res. 32 (6), 1937–1941.

Joseffson, A.M., Magnusson, P.K., Ylitalo, N., Sorensen, P., Warforth-Tubbin, P., Andersen, P.K., Melbye, M., Adam, H.O., Gyllensten, U.B., 2000. Viral load of human papilloma virus 16 as a determinant for development of cervical carcinoma in situ: a nested case-control study. Lancet 355 (9222), 2189–2193.

Jothikumar, N., Cromeans, T.L., Sobsey, M.D., Robertson, B.H., 2005a. Development and evaluation of a broadly reactive TaqMan assay for rapid detection of hepatitis A virus. Appl. Environ. Microbiol. 71 (6), 3359–3363.

Jothikumar, N., Lowther, J.A., Henshilwood, K., Lees, D.N., Hill, V.R., Vinje, J., 2005b. Rapid and sensitive detection of noroviruses by using TaqMan-based one-step reverse transcription-PCR assays and application to naturally contaminated shellfish samples. Appl. Environ. Microbiol. 71 (4), 1870–1875.

Jun-Bin, S., Zhi, C., Wei-Qin, N., Jun, F., 2003. A quantitative method to detect HBV cccDNA by chimeric primer and real-time polymerase chain reaction. J. Virol. Methods 112 (1–2), 45–52.

Jursch, C.A., Gerlich, W.H., Glebe, D., Schaefer, S., Marie, O., Traehnert, O., 2002. Molecular approaches to validate disinfectants against human hepatitis B virus. Med. Microbiol. Immunol. (Berl.) 190 (4), 189–197.

Kageyama, T., Kojima, S., Shinohara, M., Uchida, K., Fukushima, F.B., Takeda, N., Katayama, K., 2003. Broadly reactive and highly sensitive assay for Norwalk-like viruses based on real-time quantitative reverse transcription-PCR. J. Clin. Microbiol. 41 (4), 1548–1557.

Kamihira, S., Dateki, N., Sugahara, K., Yamada, Y., Tomonaga, M., Maeda, T., Tahara, M., 2000. Real-time polymerase chain reaction for quantification of HTLV-1 proviral load: application for analyzing aberrant integration of the proviral DNA in adult T-cell leukemia. Int. J. Hematol. 72 (1), 79–84.
Kamihira, S., Dateki, N., Sugahara, K., Hayashi, T., Harasawa, H., Minami, S., Hirakata, Y., Yamada, Y., 2003. Significance of HTLV-1 proviral load quantification by real-time PCR as a surrogate marker for HTLV-1-infected cell count. Clin. Lab. Haematol. 25 (2), 111–117.

Kares, S., Lonrov, M., Vuorinen, P., Olkarinen, S., Taurianen, S., Hyoty, H., 2004. Real-time PCR for rapid diagnosis of entero- and rhinovirus infections using LightCycler. J. Clin. Virol. 29 (2), 99–104.

Katayama, H., Shimasaki, A., Ohgaki, S., 2002. Development of a virus concentration method and its application to detection of enterovirus and norwalk virus from coastal seawater. Appl. Environ. Microbiol. 68 (3), 1033–1039.

Katsoulidou, A., Petrodaskalaki, M., Sypsa, V., Papachristou, E., Anastassopoulou, C.G., Gargalianos, P., Karafoulidou, A., Lazanas, M., Kordossia, T., Andoniadou, A., Hatzakis, A., 2006. Evaluation of the clinical sensitivity for the quantification of human immunodeficiency virus type 1 RNA in plasma: comparison of the new COBAS TaqMan HIV-1 with three current HIV-RNA assays-LCx HIV RNA quantitative, VERSANT HIV-1 RNA 3.0 (bDNA) and COBAS AMPLICOR HIV-1 Monitor v1.5. J. Virol. Methods 131 (2), 168–174.

Kessler, H.H., Muhlbauer, G., Rinner, B., Stelzl, E., Berger, A., Dorr, H.W., Santner, B., Matr, E., Rabenau, H., 2000. Detection of herpes simplex virus DNA by real-time PCR. J. Clin. Microbiol. 38 (7), 2638–2642.

Kimura, H., Morita, M., Yabuta, Y., Kuzushima, K., Kato, K., Kojima, S., Matsuyama, T., Morishima, T., 1999. Quantitative analysis of Epstein–Barr virus load by using a real-time PCR assay. J. Clin. Microbiol. 37 (1), 132–136.

Kishimoto, H., Yoshioka, K., Yano, M., Ukai, K., Ito, H., Watanabe, K., Kawamata, O., Kakumu, S., 2001. Real-time detection system for quantitation of hepatitis C virus RNA: a comparison with the other three methods. Hepatol. Res. 19 (1), 12–21.

Kleiber, J., Walter, T., Haberhausen, G., Tsang, S., Babiul, R., Rosenstraus, M., 2000. Performance characteristics of a quantitative, homogeneous TaqMan RT-PCR test for HCV RNA. J. Mol. Diagn. 2 (3), 158–166.

Knoll, A., Louwen, F., Kochanowski, B., Plentz, A., Stussel, J., Beckenlehner, K., Jilg, W., Modrow, S., 2002. Parvovirus B19 infection in pregnancy: quantitative viral DNA analysis using a kinetic fluorescence detection system (TaqMan PCR). J. Med. Virol. 67 (2), 259–266.

Ko, G., Jothiskumar, N., Hill, V.R., Sobsey, M.D., 2005. Rapid detection of infectious adenoviruses by mRNA real-time RT-PCR. J. Virol. Methods 127 (2), 148–153.

Kohimoto, M., Enomoto, M., Yano, Y., Otani, S., Minamitani, S., Tamori, A., Habu, D., Takeda, T., Shiomi, S., Seki, S., Arakawa, T., Nishiguchi, S., 2003. Detection of serum hepatitis B virus DNA by real-time quantitative polymerase chain reaction (TaqMan PCR) during lamivudine treatment: comparison with three other assays. Hepatol. Res. 26 (2), 125–133.

Koidl, C., Bozic, M., Miosbock, G., Muhlbauer, G., Berg, J., Stoche, M., Dehnhardt, J., Mart, E., Kessler, H.H., 2005. Rapid diagnosis of adenoviral keratoconjunctivitis by a fully automated molecular assay. Ophthalmology 112 (9), 1521–1528.

Komurian-Pradel, F., Paranpos-Baccala, G., Sodoyer, M., Chevallier, P., Mandrand, B., Lotteau, V., Andre, P., 2001. Quantitation of HCV RNA using real-time PCR and fluorimetry. J. Virol. Methods 95 (1–2), 111–119.

Komurian-Pradel, F., Perret, M., Deiman, B., Sodoyer, M., Lotteau, V., Paranpos-Baccala, G., Andre, P., 2004. Strand specific quantitative real-time PCR to study replication of hepatitis C virus genome. J. Virol. Methods 116 (1), 103–106.

Krafft, A.E., Russell, K.L., Hawksworth, A.W., McCall, S., Irvine, M., Daum, L.T., Connolly, J.L., Reid, A.H., Gaydos, J.C., Taubenberger, J.K., 2005. Evaluation of PCR testing of ethanol-fixed nasal swab specimens as an augmented surveillance strategy for influenza virus and adenovirus identification. J. Clin. Microbiol. 43 (4), 1768–1775.

Krubnikhoff, A., Wurm, R., Scheck, O., Birch-Hirschfeld, E., Egerer, R., Henke, A., Wutzler, P., Zell, R., 2003. Detection of porcine teschoviruses and enteroviruses by LightCycler real-time PCR. J. Virol. Methods 113 (1), 51–63.

Kubar, A., Yaper, M., Besirbelloiglu, B., Avc, I.Y., Guney, C., 2004. Rapid and quantitative detection of mumps virus RNA by one-step real-time RT-PCR. Diagn. Microbiol. Infec. Dis. 49 (2), 83–88.
Kubista, M., 2004. Nucleic acid-based technologies: application amplified. Pharmacogenomics 5 (7), 767–773.

Kutyavin, I.V., Afonina, I.A., Mills, A., Gorn, V.V., Lukhtanov, E.A., Belousov, E.S., Singer, M.J., Walburger, D.K., Lokhov, S.G., Gall, A.A., Dempcy, R., Reed, M.W., Meyer, R.B., Hedgpeth, J., 2000. 3′-minor groove binder-DNA probes increase sequence specificity at PCR extension temperatures. Nucleic Acids Res. 28 (2), 655–661.

Kuypers, J., Wright, N., Morrow, R., 2004. Evaluation of quantitative and type-specific real-time RT-PCR assays for detection of respiratory syncytial virus in respiratory specimens from children. J. Clin. Virol. 31 (2), 123–129.

Kuypers, J., Wright, N., Corey, L., Morrow, R., 2005. Detection and quantification of human metapneumovirus in pediatric specimens by real-time RT-PCR. J. Clin. Virol. 33 (4), 299–305.

Kwok, S., Higuchi, R., 1989. Avoiding false positives with PCR. Nature 339 (6221), 237–238.

Lai, K.K., Cook, L., Wendt, S., Corey, L., Jerome, K.R., 2003. Evaluation of real-time PCR versus PCR with liquid-phase hybridization for detection of enterovirus RNA in cerebrospinal fluid. J. Clin. Microbiol. 41 (7), 3133–3141.

Lallemand, F., Desire, N., Rozenbaum, W., Nicolas, J.C., Marechal, V., 2000. Quantitative analysis of human herpesvirus 8 viral load using a real-time PCR assay. J. Clin. Microbiol. 38 (4), 1404–1408.

Lanciotti, R.S., Kerst, A.J., Nasci, R.S., Godsey, M.S., Mitchell, C.J., Savage, H.M., Komar, N., Panella, N.A., Allen, B.C., Volpe, K.E., Davis, B.S., Roehrig, J.T., 2000. Rapid detection of west Nile virus from human clinical specimens, field-collected mosquitoes, and avian samples by a TaqMan reverse transcriptase-PCR assay. J. Clin. Microbiol. 38 (11), 4066–4071.

Lee, C.W., Suarez, D.L., 2004. Application of real-time RT-PCR for the quantitation and competitive replication study of H5 and H7 subtype avian influenza virus. J. Virol. Methods 119 (2), 151–158.

Lee, T.H., Chafets, D.M., Busch, M.P., Murphy, E.L., 2004. Quantitation of HTLV-I and II proviral load using real-time quantitative PCR with SYBR Green chemistry. J. Clin. Virol. 31 (4), 275–282.

Leruez-Ville, M., Minard, V., Lacaille, F., Buzyn, A., Abachin, E., Blanche, S., Freymuth, F., Rouzioux, C., 2004. Real-time blood plasma polymerase chain reaction for management of disseminated adenovirus infection. Clin. Infect. Dis. 38 (1), 45–52.

Leung, E., Shenton, B.K., Jackson, G., Gould, F.K., Yap, C., Talbot, D., 2002a. Use of real-time PCR to measure Epstein–Barr virus genomes in whole blood. J. Immunol. Methods 270 (2), 259–267.

Leung, A.Y., Chan, M., Tang, S.C., Liang, R., Kwong, Y.L., 2002b. Real-time quantitative analysis of polyoma BK viremia and viruria in renal allograft recipients. J. Virol. Methods 103 (1), 51–56.

Liefeldt, L., Plentz, A., Klemper, B., Kershaw, O., Endres, A.S., Raab, U., Neumayer, H.H., Meisel, H., Modrow, S., 2005. Recurrent high level parvovirus B19/genotype 2 viremia in a renal transplant recipient analyzed by real-time PCR for simultaneous detection of genotypes 1 to 3. J. Med. Virol. 75 (1), 161–169.

Limaye, A.P., Huang, M.L., Leisenring, W., Stensland, L., Corey, L., Boeckh, M., 2001. Cytomegalovirus (CMV) DNA load in plasma for the diagnosis of CMV disease before engraftment in hematopoietic stem-cell transplant recipients. J. Infect. Dis. 183 (3), 377–382.

Lin, H.H., Wang, S.J., Liu, Y.C., Lee, S.S., Hwang, C.K., Chen, Y.S., Wann, S.R., Shih, Y.L., 2004. Quantitation of severe acute respiratory syndrome coronavirus genome by real-time polymerase chain reaction assay using minor groove binder DNA probe technology. J. Microbiol. Immunol. Infect. 37 (5), 258–265.
Lindh, M., Hannoun, C., 2005a. Genotyping of hepatitis C virus by Taqman real-time PCR. J. Clin. Virol. 34 (2), 108–114.
Lindh, M., Hannoun, C., 2005b. Dynamic range and reproducibility of hepatitis B virus (HBV) DNA detection and quantification by Cobas Taqman HBV, a real-time semiautomated assay. J. Clin. Microbiol. 43 (8), 4251–4254.
Lion, T., Henn, T., Gaiger, A., Kalhs, P., Gadner, H., 1993. Early detection of relapse after bone marrow transplantation in patients with chronic myelogenous leukaemia. Lancet 341 (8840), 275–276.
Lion, T., Baumgartinger, R., Watzinger, F., Matthes-Martin, S., Suda, M., Preuner, S., Futterknecht, B., Lawitschka, A., Peters, C., Potschger, U., Gadner, H., 2003. Molecular monitoring of adenovirus in peripheral blood after allogeneic bone marrow transplantation permits early diagnosis of disseminated disease. Blood 102 (3), 1114–1120.
Lipman, M.M., Cotler, S.J., 2003. Antiviral therapy for hepatitis C. Curr. Treat. Options Gastroenterol. 6 (6), 445–453.
Loeb, K.R., Jerome, K.R., Goddard, J., Huang, M., Cent, A., Corey, L., 2000. High-throughput quantitative analysis of hepatitis B virus DNA in serum using the TaqMan fluorogenic detection system. Hepatology 32 (3), 626–629.
Loparev, V.N., McCastland, K., Holloway, B.P., Krause, P.R., Takayama, M., Schmid, D.S., 2000. Rapid genotyping of varicella-zoster virus vaccine and wild-type strains with fluorophore-labeled hybridization probes. J. Clin. Microbiol. 38 (12), 4315–4319.
Luo, W., Yang, H., Rathbun, K., Pau, C.P., Ou, C.Y., 2005. Detection of human immunodeficiency virus type 1 DNA in dried blood spots by a duplex real-time PCR assay. J. Clin. Microbiol. 43 (4), 1851–1857.
Machida, U., Kami, M., Fukui, T., Kazuyama, Y., Kinoshita, M., Tanaka, Y., Kanda, Y., Ogawa, S., Honda, H., Chiba, S., Mitani, K., Muto, Y., Osumi, K., Kimura, S., Hirai, H., 2000. Real-time automated PCR for early diagnosis and monitoring of cytomegalovirus infection after bone marrow transplantation. J. Clin. Microbiol. 38 (7), 2536–2542.
Mackay, I.M., Jacob, K.C., Woolhouse, D., Waller, K., Syrmis, M.W., Whiley, D.M., Siebert, D.J., Nissen, M., Sloots, T.P., 2003. Molecular assays for detection of human metapneumovirus. J. Clin. Microbiol. 41 (1), 100–105.
Maertzdorf, J., Wang, C.K., Brown, J.B., Quinto, J.D., Chu, M., de Graaf, M., van den Hoogen, B.G., Spaeet, R., Osterhaus, A.D., Fouchier, R.A., 2004. Real-time reverse transcriptase PCR assay for detection of human metapneumoviruses from all known genetic lineages. J. Clin. Microbiol. 42 (3), 981–986.
Mahony, J.B., Petrich, A., Louie, L., Song, X., Chong, S., Smieja, M., Chernesky, M., Loeb, M., Richardson, S., 2004. Performance and cost evaluation of one commercial and six in-house conventional and real-time reverse transcription-PCR assays for detection of severe acute respiratory syndrome coronavirus. J. Clin. Microbiol. 42 (4), 1471–1476.
Manaresi, E., Gallinella, G., Zuffi, E., Bonvicini, F., Zerbini, M., Musiani, M., 2002. Diagnosis and quantitative evaluation of parvovirus B19 infections by real-time PCR in the clinical laboratory. J. Med. Virol. 67 (2), 275–281.
Mansuy, J.M., Peron, J.M., Abravanel, F., Poirson, H., Dubois, M., Miedouge, M., Vischi, F., Alric, L., Vinel, J.P., Izopet, J., 2004. Hepatitis E in the south west of France in individuals who have never visited an endemic area. J. Med. Virol. 74 (3), 419–424.
Martell, M., Gomez, J., Esteban, J.I., Sauleda, S., Quer, J., Cabot, B., Esteban, R., Guardia, J., 1999. High-throughput real-time reverse transcription-PCR quantitation of hepatitis C virus RNA. J. Clin. Microbiol. 37 (2), 327–332.
Matsuda, T., Tomita, M., Uchihara, J.N., Okudaira, T., Ohshiro, K., Tomoyose, T., Ikema, T., Masuda, M., Saito, M., Osame, M., Takasu, N., Ohta, T., Mori, N., 2005. Human T cell leukemia virus type I-infected patients with Hashimoto's thyroiditis and Graves' disease. J. Clin. Endocrinol. Metab. 90 (10), 5704–5710.
McNees, A.L., White, Z.S., Zanwar, P., Vilchez, R.A., Butel, J.S., 2005. Specific and quantitative detection of human polyomaviruses BKV, JCV, and SV40 by real time PCR. J. Clin. Virol. 34 (1), 52–62.
Mentel, R., Wegner, U., Bruns, R., Gurtler, L., 2003. Real-time PCR to improve the diagnosis of respiratory syncytial virus infection. J. Med. Microbiol. 52 (Pt 10), 893–896.

Middeldorp, J.M., 2002. Molecular diagnosis of viral infections in renal transplant recipients. Curr. Opin. Nephrol. Hypertens. 11 (6), 665–672.

Mifflin, T.E., Estey, C.A., Felder, R.A., 2000. Robotic automation performs a nested RT-PCR analysis for HCV without introducing sample contamination. Clin. Chim. Acta 290 (2), 199–211.

Mitsunaga, S., Fujimura, K., Matsumoto, C., Shiozawa, R., Hirakawa, S., Nakajima, K., Tadokoro, K., Juji, T., 2002. High-throughput HBV DNA and HCV RNA detection system using a nucleic acid purification robot and real-time detection PCR: its application to analysis of posttransfusion hepatitis. Transfusion 42 (1), 100–106.

Mohamed, N., Elfaitouri, A., Fohlman, J., Friman, G., Blomberg, J., 2004. A sensitive and quantitative single-tube real-time reverse transcriptase-PCR for detection of enteroviral RNA. J. Clin. Virol. 30 (2), 150–156.

Monpoeho, S., Dehee, A., Mignotte, B., Schwartzbrod, L., Marechal, V., Nicolas, J.C., Billaudel, S., Ferre, V., 2000. Quantification of enterovirus RNA in sludge samples using single tube real-time RT-PCR. Biotechniques 29 (1), 88–93.

Monpoeho, S., Coste-Burel, M., Costa-Mattioli, M., Besse, B., Chomel, J.J., Billaudel, S., Ferre, V., 2002. Application of a real-time polymerase chain reaction with internal positive control for detection and quantification of enterovirus in cerebrospinal fluid. Eur. J. Clin. Microbiol. Infect. Dis. 21 (7), 532–536.

Montanheiro, P.A., Penalva de Oliveira, A.C., Posada-Vergara, M.P., Milagres, A.C., Tauli, C., Marchiori, P.E., Duarte, A.J., Casseb, J., 2005. Human T-cell lymphotropic virus type I (HTLV-I) proviral DNA viral load among asymptomatic patients and patients with HTLV-I-associated myelopathy/tropical spastic paraparesis. Braz. J. Med. Biol. Res. 38 (11), 1643–1647.

Morrisson, T.B., Weis, J.J., Wittwer, C.T., 1998. Quantification of low-copy transcripts by continuous SYBR Green I monitoring during amplification. Biotechniques 24 (6), 954–962.

Murphy, E.L., Lee, T.H., Chafets, D., Nass, C.C., Wang, B., Loughlin, K., Smith, D., 2004. Higher human T lymphotropic virus (HTLV) provirus load is associated with HTLV-I versus HTLV-II, with HTLV-II subtype A versus B, and with male sex and a history of blood transfusion. J. Infect. Dis. 190 (3), 504–510.

Myrmel, M., Berg, E.M., Rimstad, E., Grinde, B., 2004. Detection of enteric viruses in shellfish from the Norwegian coast. Appl. Environ. Microbiol. 70 (5), 2678–2684.

Najjoullah, F., Touvenot, D., Lina, B., 2001. Development of a real-time PCR procedure including an internal control for the measurement of HCMV viral load. J. Virol. Methods 92 (1), 55–64.

Narayanan, J., Crommeans, T.L., Robertson, B.H., Meng, X.J., Hill, V.R., 2006. A broadly reactive one-step real-time RT-PCR assay for rapid and sensitive detection of hepatitis E virus. J. Virol. Methods 131 (1), 65–71.

Nazarenko, I., Lowe, B., Darfler, M., Ikonomi, P., Schwester, D., Rashtchian, A., 2002. Multiplex quantitative PCR using self-quenched primers labeled with a single fluorophore. Nucleic Acids Res. 30 (9), e37.

Ng, E.K., Cheng, P.K., Ng, A.Y., Hoang, T.L., Lim, W.W., 2005. Influenza A H5N1 detection. Emerg. Infect. Dis. 11 (8), 1303–1305.

Nielsen, P.E., 2001. Peptide nucleic acid: a versatile tool in genetic diagnostics and molecular biology. Curr. Opin. Biotechnol. 12 (1), 16–20.

Niesters, H.G., 2002. Clinical virology in real time. J. Clin. Virol. 25 (Suppl 3), S3–S12.

Niesters, H.G., 2004. Molecular and diagnostic clinical virology in real time. Clin. Microbiol. Infect. 10 (1), 5–11.

Nijhuis, M., van Maarseveen, N., Schuurman, R., Verkuiljen, S., de Vos, M., Hendriksen, K., van Loon, A.M., 2002. Rapid and sensitive routine detection of all members of the genus enterovirus in different clinical specimens by real-time PCR. J. Clin. Microbiol. 40 (10), 3666–3670.

Nitsche, A., Steuer, N., Schmidt, C.A., Landt, O., Siegert, W., 1999. Different real-time PCR formats compared for the quantitative detection of human cytomegalovirus DNA. Clin. Chem. 45 (11), 1932–1937.
Nitsche, A., Muller, C.W., Radonic, A., Landt, O., Ellerbrok, H., Pauli, G., Siegert, W., 2001. Human herpesvirus 6A DNA is detected frequently in plasma but rarely in peripheral blood leukocytes of patients after bone marrow transplantation. J. Infect. Dis. 183 (1), 130–133.

Nozaki, A., Kato, N., 2002. Quantitative method of intracellular hepatitis C virus RNA using LightCycler PCR. Acta. Med. Okayama. 56 (2), 107–110.

Ohayashi, J.H., Suzuki, A., Aritaki, K., Nagate, A., Shoji, N., Ohyashiki, K., Ojima, T., Abe, K., Yamamoto, K., 2000. Use of real-time PCR to monitor human herpesvirus 6 reactivation after allogeneic bone marrow transplantation. Int. J. Mol. Med. 6 (4), 427–432.

O’Neill, H.J., Wyatt, D.E., Coyle, P.V., McCaughey, C., Mitchell, F., 2003. Real-time nested multiplex PCR for the detection of herpes simplex virus types 1 and 2 and varicella zoster virus. J. Med. Virol. 71 (4), 557–560.

Orii, T., Ohkohchi, N., Kikuchi, H., Koyamada, N., Chubachi, S., Satomi, S., Kimura, H., Hoshino, Y., Morita, M., 2000. Usefulness of quantitative real-time polymerase chain reaction in following up patients with Epstein–Barr virus infection after liver transplantation. Clin. Transplant. 14 (4 Pt 1), 308–317.

Orru, G., Masia, G., Orru, G., Romano, L., Piras, V., Coppola, R.C., 2004. Detection and quantitation of hepatitis E virus in human faeces by real-time quantitative PCR. J. Virol. Methods 118 (2), 77–82.

Orum, H., Nielsen, P.E., Egholm, M., Berg, R.H., Buchardt, O., Stanley, C., 1993. Single base pair mutation analysis by PNA directed PCR clamping. Nucleic Acids Res. 21 (23), 5332–5336.

O’Shea, M.K., Cane, P.A., 2004. Development of a highly sensitive semi-quantitative real-time PCR and molecular beacon probe assay for the detection of respiratory syncytial virus. J. Virol. Methods 118 (2), 101–110.

Ozoemenma, L.C., Minor, P.D., Afzal, M.A., 2004. Comparative evaluation of measles virus specific TaqMan PCR and conventional PCR using synthetic and natural RNA templates. J. Med. Virol. 73 (1), 79–84.

Palmisano, L., Giuliano, M., Nicola, E., Pirillo, M.F., Andreotti, M., Galluzzo, C.M., Bucciardini, R., Fragola, V., Andreoni, M., Vella, S., 2005. Residual viraemia in subjects with chronic HIV infection and viral load <50 copies/ml: the impact of highly active antiretroviral therapy. AIDS 19 (16), 1843–1847.

Pang, A., Yuen, M.F., Yuan, H.J., Lai, C.L., Kwong, Y.L., 2004a. Real-time quantification of hepatitis B virus core-promoter and pre-core mutants during hepatitis E antigen seroconversion. J. Hepatol. 40 (6), 1008–1017.

Pang, X., Lee, B., Chui, L., Preiksaitis, J.K., Monroe, S.S., 2004b. Evaluation and validation of real-time reverse transcription-PCR assay using the LightCycler system for detection and quantitation of norovirus. J. Clin. Microbiol. 42 (10), 4679–4685.

Pang, X.L., Preiksaitis, J.K., Lee, B., 2005. Multiplex real time RT-PCR for the detection and quantitation of norovirus genogroups I and II in patients with acute gastroenteritis. J. Clin. Virol. 33 (2), 168–171.

Papin, J.F., Vahrson, W., Dittmer, D.P., 2004. SYBR green-based real-time quantitative PCR assay for detection of West Nile Virus circumsents false-negative results due to strain variability. J. Clin. Microbiol. 42 (4), 1511–1518.

Parskevis, D., Haida, C., Tassopoulos, N., Raptopoulos, M., Tsantoulas, D., Papachristou, H., Syspa, V., Hatzakis, A., 2002. Development and assessment of a novel real-time PCR assay for quantitation of HBV DNA. J. Virol. Methods 103 (2), 201–212.

Parida, M., Posadas, G., Inoue, S., Hasebe, F., Morita, K., 2004. Real-time reverse transcription loop-mediated isothermal amplification for rapid detection of West Nile virus. J. Clin. Microbiol. 42 (1), 257–263.

Pas, S.D., Niesters, H.G., 2002. Detection of HBV DNA using real time analysis. J. Clin. Virol. 25 (1), 93–94.

Pas, S.D., Fries, E., de Man, R.A., Osterhaus, A.D., Niesters, H.G., 2000. Development of a quantitative real-time detection assay for hepatitis B virus DNA and comparison with two commercial assays. J. Clin. Microbiol. 38 (8), 2897–2901.

Pas, S.D., Noppornpanth, S., van der Eijk, A.A., de Man, R.A., Niesters, H.G., 2005. Quantification of the newly detected lamivudine resistant YSDD variants of Hepatitis B virus using molecular beacons. J. Clin. Virol. 32 (2), 166–172.
Pasloske, B.L., Walkerpeach, C.R., Obermoeller, R.D., Winkler, M., DuBois, D.B., 1998. Armored RNA technology for production of ribonuclease-resistant viral RNA controls and standards. J. Clin. Microbiol. 36 (12), 3590–3594.

Payungporn, S., Tangkijvanich, P., Jantaradsamee, P., Theamboonlers, A., Poovorawan, Y., 2004. Simultaneous quantitation and genotyping of hepatitis B virus by real-time PCR and melting curve analysis. J. Virol. Methods 120 (2), 131–140.

Pellestor, F., Paulasova, P., 2004. The peptide nucleic acids, efficient tools for molecular diagnosis (Review). Int. J. Mol. Med. 13 (4), 521–525.

Pennington, J., Taylor, G.P., Sutherland, J., Davis, R.E., Sengatchev, J., Allain, J.P., Williamson, L.M., 2002. Persistence of HTLV-I in blood components after leukocyte depletion. Blood 100 (2), 677–681.

Perkins, S.M., Webb, D.L., Torrance, S.A., El Saleebey, C., Harrison, L.M., Aitken, J.A., Patel, A., DeVincenzo, J.P., 2005. Comparison of a real-time reverse transcriptase PCR assay and a culture technique for quantitative assessment of viral load in children naturally infected with respiratory syncytial virus. J. Clin. Microbiol. 43 (5), 2356–2362.

Petitjean, J., Vabret, A., Dina, J., Gourarin, S., Freymuth, F., 2005. Development and evaluation of a real-time RT-PCR assay on the LightCycler for the rapid detection of enterovirus in cerebrospinal fluid specimens. J. Clin. Virol., Epub-ahead of print.

Pflug, M., Mihalik, K., Yu, M.Y., Feinstone, S.M., Major, M.E., 2003. BK virus (BKV) quantification in urine samples of bone marrow transplanted patients is helpful for diagnosis of hemorrhagic cystitis, although wide individual variations exist. J. Clin. Virol. 26 (1), 71–77.

Prince, A.M., Pawlotsky, J.M., Soulier, A., Tobler, L., Brozman, B., Pfahler, W., Lee, D.H., Li, L., Shata, M.T., 2004. Hepatitis C virus replication kinetics in chimpanzees with self-limited and chronic infections. J. Viral. Hepatitis 11 (3), 236–242.

Puig, M., Mihalik, K., Yu, M.Y., Feinstone, S.M., Major, M.E., 2002. Sensitivity and reproducibility of HCV quantitation in chimpanzee sera using TaqMan real-time PCR assay. J. Virol. Methods 105 (2), 253–263.

Rabenau, H.F., Clarici, A.M., Muhlbaier, G., Berger, A., Vince, A., Muller, S., Daghofer, E., Santner, B.I., Marth, E., Kessler, H.H., 2002. Rapid detection of enterovirus infection by automated RNA extraction and real-time fluorescence PCR. J. Clin. Virol. 25 (2), 155–164.

Ramaysaym, M., Smith, M., Geretti, A.M., 2005. Detection and typing of herpes simplex DNA in genital swabs by real-time polymerase chain reaction. J. Virol. Methods 126 (1–2), 203–206.

Randhawa, P.S., Vats, A., Zymgunt, D., Swalsky, P., Scantlebury, V., Shapiro, R., Finkelsstein, S., 2002. Quantitation of viral DNA in renal allograft tissue from patients with BK virus nephropathy. Transplantation 74 (4), 485–488.

Randhawa, P., Ho, A., Shapiro, R., Vats, A., Swalsky, P., Finkelsstein, S., Uhrmacher, J., Weck, K., 2004. Correlates of quantitative measurement of BK polyomavirus (BKV) DNA with clinical course of BKV infection in renal transplant patients. J. Clin. Microbiol. 42 (3), 1176–1180.
Randhawa, P., Shapiro, R., Vats, A., 2005. Quantitation of DNA of polyomaviruses BK and JC in human kidneys. J. Infect. Dis. 192 (3), 504–509.

Ratge, D., Scheibhuber, B., Nitsche, M., Knabbe, C., 2000. High-speed detection of blood-borne hepatitis C virus RNA by single-tube real-time fluorescence reverse transcription-PCR with the LightCycler. Clin. Chem. 46 (12), 1987–1989.

Richards, G.P., Watson, M.A., Fankhauser, R.L., Monroe, S.S., 2004a. Genogroup I and II noroviruses detected in stool samples by real-time reverse transcription-PCR using highly degenerate universal primers. Appl. Environ. Microbiol. 70 (12), 7179–7184.

Richards, G.P., Watson, M.A., Kingsley, D.H., 2004b. A SYBR green, real-time RT-PCR method to detect and quantitate Norwalk virus in stools. J. Virol. Methods 116 (1), 63–70.

Roberts, T.C., Brennan, D.C., Buller, R.S., Gaudreault-Keener, M., Schnitzler, M.A., Sternhell, K.J., Garlock, K.A., Singer, G.G., Storch, G.A., 1998. Quantitative polymerase chain reaction to predict occurrence of symptomatic cytomegalovirus infection and assess response to ganciclovir therapy in renal transplant recipients. J. Infect. Dis. 178 (3), 626–635.

Rodriguez-Frias, F., Jardi, R., Buti, M., Esteban, R., 2003. Applicability of real-time PCR combined with melting curve analysis for the study of hepatitis B virus genome mutations. Hepatology 37 (2), 478–479.

Rollison, D.E., Utaipat, U., Ryschkewitsch, C., Hou, J., Goldthwaite, P., Daniel, R., Helzlsouer, K.J., Burger, P.C., Shah, K.V., Major, E.O., 2005. Investigation of human brain tumors for the presence of polyomavirus genome sequences by two independent laboratories. Int. J. Cancer 113 (5), 769–774.

Roth, W.K., Weber, M., Seifried, E., 1999. Feasibility and efficacy of routine PCR screening of blood donations for hepatitis C virus, hepatitis B virus, and HIV-1 in a blood-bank setting. Lancet 353 (9150), 359–363.

Ryncarz, A.J., Goddard, J., Wald, A., Huang, M.L., Roizman, B., Corey, L., 1999. Development of a high-throughput quantitative assay for detecting herpes simplex virus DNA in clinical samples. J. Clin. Microbiol. 37 (6), 1941–1947.

Ryschkewitsch, C., Jensen, P., Hou, J., Fahlé, G., Fischer, S., Major, E.O., 2004. Comparison of PCR-southern hybridization and quantitative real-time PCR for the detection of JC and BK viral nucleotide sequences in urine and cerebrospinal fluid. J. Virol. Methods 121 (2), 217–221.

Safronetz, D., Humar, A., Tipple, G.A., 2003. Differentiation and quantitation of human herpesviruses 6A, 6B and 7 by real-time PCR. J. Virol. Methods 112 (1–2), 99–105.

Saha, B.K., Tian, B., Bucy, R.P., 2001. Quantitation of HIV-1 by real-time PCR with a unique fluorogenic probe. J. Virol. Methods 93 (1–2), 33–42.

Saiko, T., Munakata, Y., Fujii, H., Kodera, T., Miyagawa, E., Ishii, K., Sasaki, T., 2003. Evaluation of anti-parvovirus B19 activity in sera by assay using quantitative polymerase chain reaction. J. Virol. Methods 107 (1), 81–87.

Sanders, S.P., Proud, D., Permutt, S., Siekierski, E.S., Yachechko, R., Lui, M.C., 2004. Role of nasal nitric oxide in the resolution of experimental rhinovirus infection. J. Allergy. Clin. Immunol. 113 (4), 697–702.

Sarkar, G., Sommer, S.S., 1990. Shedding light on PCR contamination. Nature 343 (6253), 27.

Schaede, L., Kockelkorn, P., Ritter, K., Kleines, M., 2000. Detection of cytomegalovirus DNA in human specimens by LightCycler PCR. J. Clin. Microbiol. 38 (11), 4006–4009.

Schaefer, S., Glebe, D., Wend, U.C., Oyunbileg, J., Gerlich, W.H., 2003. Universal primers for real-time amplification of DNA from all known Orthohepadnavirus species. J. Clin. Virol. 27 (1), 30–37.

Schalasta, G., Arends, A., Schmid, M., Braun, R.W., Enders, G., 2000. Fast and type-specific analysis of herpes simplex virus types 1 and 2 by rapid PCR and fluorescence melting-curve-analysis. Infection 28 (2), 85–91.

Schalk, J.A., van den, E.C., Ovelgonne, H., Baas, C., Jongen, P.M., 2004. Estimation of the number of infectious measles viruses in live virus vaccines using quantitative real-time PCR. J. Virol. Methods 117 (2), 179–187.

Scheltinga, S.A., Templeton, K.E., Beersma, M.F., Claas, E.C., 2005. Diagnosis of human metapneumovirus and rhinovirus in patients with respiratory tract infections by an internally controlled multiplex real-time RNA PCR. J. Clin. Virol. 33 (4), 306–311.
Scherczinger, C.A., Ladd, C., Bourke, M.T., Adamowicz, M.S., Johannes, P.M., Scherczinger, R., Beesley, T., Lee, H.C., 1999. A systematic analysis of PCR contamination. J. Forensic. Sci. 44 (5), 1042–1045.

Schmid, M., Oehme, R., Schalasta, G., Brockmann, S., Kimmig, P., Enders, G., 2004. Fast detection of Noroviruses using a real-time PCR assay and automated sample preparation. BMC Infect. Dis. 4 (1), 15.

Schutten, M., van den, H.B., van der Ende, M.E., Gruters, R.A., Osterhaus, A.D., Niesters, H.G., 2000. Development of a real-time quantitative RT-PCR for the detection of HIV-2 RNA in plasma. J. Virol. Methods 88 (1), 81–87.

Schuttl, C.G., Thomas, C., Discher, T., Friese, G., Lohmeyer, J., Schuster, R., Schaefer, S., Gerlich, W.H., 2004. Variable ratio of hepatitis C virus RNA to viral core antigen in patient sera. J. Clin. Microbiol. 42 (5), 1977–1981.

Selvin, P.R., 1995. Fluorescence resonance energy transfer. Methods Enzymol. 246, 300–334.

Sherrill, C.B., Marshall, D.J., Moser, M.J., Larsen, C.A., Daude-Snow, L., Prudent, J.R., 2004. Nucleic acid analysis using an expanded genetic alphabet to quench fluorescence. J. Am. Chem. Soc. 126 (14), 4550–4556.

Shi, L., Ho, J., Norling, L.A., Roy, M., Xu, Y., 1999. A real time quantitative PCR-based method for the detection and quantification of simian virus 40. Biologicals 27 (3), 241–252.

Shi, P.Y., Kauffman, E.B., Ren, P., Felton, A., Tai, J.H., Dupuis, A.P., Jones, S.A., Ngo, K.A., Nicholas, D.C., Maffei, J., Ebel, G.D., Bernard, K.A., Kramer, L.D., 2001. High-throughput detection of West Nile virus RNA. J. Clin. Microbiol. 39 (4), 1264–1271.

Shirato, K., Miyoshi, H., Kariwa, H., Takashima, I., 2005. Detection of West Nile virus and Japanese encephalitis virus using real-time PCR with a probe common to both viruses. J. Virol. Methods 126 (1–2), 119–125.

Shyamala, V., Arcangel, P., Cottrell, J., Coit, D., Medina-Selby, A., McCoin, C., Madriaga, D., Chien, D., Phelps, B., 2004. Assessment of the target-capture PCR hepatitis B virus (HBV) DNA quantitative assay and comparison with commercial HBV DNA quantitative assays. J. Clin. Microbiol. 42 (11), 5199–5204.

Si-Mohamed, A., Goff, J.L., Desire, N., Maylin, S., Glotz, D., Belec, L., 2006. Detection and quantitation of BK virus DNA by real-time polymerase chain reaction in the LT-ag gene in adult renal transplant recipients. J. Virol. Methods 131 (1), 21–27.

Smith, A.B., Mock, V., Melear, R., Colarusso, P., Willis, D.E., 2003. Rapid detection of influenza A and B viruses in clinical specimens by Light Cycler real time RT-PCR. J. Clin. Virol. 28 (1), 51–58.

Snijders, P.J., van den Brule, A.J., Meijer, C.J., 2003. The clinical relevance of human papillomavirus testing: relationship between analytical and clinical sensitivity. J. Pathol. 201 (1), 1–6.

Sonoda, J., Koriyama, C., Yamamoto, S., Kozako, T., Li, H.C., Lema, C., Yashiki, S., Fujiyoshi, T., Yoshinaga, M., Nagata, Y., Akiba, S., Takezaki, T., Yamada, K., Sonoda, S., 2004. HTLV-1 provirus load in peripheral blood lymphocytes of HTLV-1 carriers is diminished by green tea drinking. Cancer Sci. 95 (7), 596–601.

Spackman, E., Senne, D.A., Myers, T.J., Bulaga, L.L., Garber, L.P., Perdue, M.L., Lohman, K., Daum, L.T., Suarez, D.L., 2002. Development of a real-time reverse transcriptase PCR assay for type A influenza virus and the avian H5 and H7 hemagglutinin subtypes. J. Clin. Microbiol. 40 (9), 3256–3260.

Stevenson, J., Hymas, W., Hillyard, D., 2005. Effect of sequence polymorphisms on performance of two real-time PCR assays for detection of herpes simplex virus. J. Clin. Microbiol. 43 (5), 2391–2398.

Stocher, M., Leb, V., Bozic, M., Kessler, H.H., Halwachs-Baumann, G., Landt, O., Stekel, H., Berg, J., 2003. Parallel detection of five human herpes virus DNAs by a set of real-time polymerase chain reactions in a single run. J. Clin. Virol. 26 (1), 85–93.

Stolt, A., Kjellin, M., Sasnaukas, K., Luostarinen, T., Koskela, P., Lehtinen, M., Dillner, J., 2005. Maternal human polyomavirus infection and risk of neuroblastoma in the child. Int. J. Cancer 113 (3), 393–396.

Stone, B., Burrows, J., Schepetiuk, S., Higgins, G., Hampson, A., Shaw, R., Kok, T., 2004a. Rapid detection and simultaneous subtype differentiation of influenza A viruses by real time PCR. J. Virol. Methods 117 (2), 103–112.
Stone, W.B., Okoniewski, J.C., Therrien, J.E., Kramer, L.D., Kauffman, E.B., Eidson, M., 2004b. VecTest as diagnostic and surveillance tool for West Nile virus in dead birds. Emerg. Infect. Dis. 10 (12), 2175–2181.

Su, C.C., Li, C.F., Liao, Y.L., Lin, C.N., Lu, J.J., 2005. Immunohistochemical and molecular assessment of human herpesvirus type 8 in gastrointestinal tumours. J. Clin. Pathol. 58 (8), 856–859.

Sugauchi, F., Orito, E., Ichida, T., Kato, H., Sakugawa, H., Kakumu, S., Ishida, T., Chutaputti, A., Lai, C.L., Ueda, R., Miyakawa, Y., Mizokami, M., 2002. Hepatitis B virus of genotype B with or without recombination with genotype C over the precore region plus the core gene. J. Virol. 76 (12), 5985–5992.

Sum, S.S., Wong, D.K., Yuen, M.F., Yuan, H.J., Yu, J., Lai, C.L., Ho, D., Zhang, L., 2004. Real-time PCR assay using molecular beacon for quantitation of hepatitis B virus DNA. J. Clin. Microbiol. 42 (8), 3438–3440.

Sumino, K.C., Agapov, E., Pierce, R.A., Trulock, E.P., Pfeifer, J.D., Ritter, J.H., Gaudreault-Keener, M., Storch, G.A., Holtzman, M.J., 2005. Detection of severe human metapneumovirus infection by real-time polymerase chain reaction and histopathological assessment. J. Infect. Dis. 192 (6), 1052–1060.

Summerer, D., Marx, A., 2002. A molecular beacon for quantitative monitoring of the DNA polymerase reaction in real-time. Angew. Chem. Int. Ed. Engl. 41 (19), 3620–3622, 3516.

Svanvik, N., Stahlberg, A., Sehlstedt, U., Sjöback, R., Kubista, M., 2000. Detection of PCR products in real time using light-up probes. Anal. Biochem. 287 (1), 179–182.

Tan, S.W., Omar, A.R., Ain, I., Yusof, K., Tan, W.S., 2004. Detection of Newcastle disease virus using a SYBR Green I real time polymerase chain reaction. Acta Virol. 48 (1), 23–28.

Tanaka, N., Kimura, H., Iida, K., Saito, Y., Tsuge, I., Yoshimi, A., Matsuyama, T., Morishima, T., 2000. Quantitative analysis of cytomegalovirus load using a real-time PCR assay. J. Med. Virol. 60 (4), 455–462.

Tedeschi, R., Enbom, M., Bidoli, E., Linde, A., De Paoli, P., Dillner, J., 2001. Viral load of human herpesvirus 8 in peripheral blood of human immunodeficiency virus-infected patients with Kaposi’s sarcoma. J. Clin. Microbiol. 39 (12), 4269–4273.

Templeton, K.E., Scheltinga, S.A., Beersma, M.F., Kroes, A.C., Claas, E.C., 2004. Rapid and sensitive method using multiplex real-time PCR for diagnosis of infections by influenza a and influenza B viruses, respiratory syncytial virus, and parainfluenza viruses 1, 2, 3, and 4. J. Clin. Microbiol. 42 (4), 1564–1569.

Tewari, D., Kim, H., Feria, W., Russo, B., Acland, H., 2004. Detection of West Nile virus using formalin fixed paraffin embedded tissues in crows and horses: quantification of viral transcripts by real-time RT-PCR. J. Clin. Virol. 30 (4), 320–325.

Tipples, G.A., Safronetz, D., Gray, M., 2003. A real-time PCR assay for the detection of varicella-zoster virus DNA and differentiation of vaccine, wild-type and control strains. J. Virol. Methods 113 (2), 113–116.

Tong, A.K., Li, Z., Jones, G.S., Russo, J.J., Ju, J., 2001. Combinatorial fluorescence energy transfer tags for multiplex biological assays. Nat. Biotechnol. 19 (8), 756–759.

Tyagi, S., Kramer, F.R., 1996. Molecular beacons: probes that fluoresce upon hybridization. Nat. Biotechnol. 14 (3), 303–308.

Tyagi, S., Bratu, D.P., Kramer, F.R., 1998. Multicolor molecular beacons for allele discrimination. Nat. Biotechnol. 16 (1), 49–53.

Uchida, K., Shinohara, M., Shimada, S., Segawa, Y., Doi, R., Gotoh, A., Hondo, R., 2005. Rapid and sensitive detection of mumps virus RNA directly from clinical samples by real-time PCR. J. Med. Virol. 75 (3), 470–474.

Uehara, H., Nardone, G., Nazarenko, I., Hohman, R.J., 1999. Detection of telomerase activity utilizing energy transfer primers: comparison with gel- and ELISA-based detection. Biotechniques 26 (3), 552–558.

Uhlmann, E., 1998. Peptide nucleic acids (PNA) and PNA-DNA chimeras: from high binding affinity towards biological function. Biol. Chem. 379 (8–9), 1045–1052.

van Elden, L.J., Nijhuis, M., Schipper, P., Schuurman, R., van Loon, A.M., 2001. Simultaneous detection of influenza viruses A and B using real-time quantitative PCR. J. Clin. Microbiol. 39 (1), 196–200.
van Elden, L.J., van Loon, A.M., van der, B.A., Hendriksen, K.A., Hoepelman, A.I., van Kraaij, M.G., Schipper, P., Nijhuis, M., 2003. Applicability of a real-time quantitative PCR assay for diagnosis of respiratory syncytial virus infection in immunocompromised adults. J. Clin. Microbiol. 41 (9), 4378–4381.

Van Esser, J.W., Niesters, H.G., van der, H.B., Meijer, E., Osterhaus, A.D., Gratama, J.W., Verdonck, L.F., Lowenberg, B., Cornelissen, J.J., 2002. Prevention of Epstein–Barr virus-lymphoproliferative disease by molecular monitoring and preemptive rituximab in high-risk patients after allogeneic stem cell transplantation. Blood 99 (12), 4364–4369.

Vanlandingham, D.L., Schneider, B.S., Klingler, K., Fair, J., Beasley, D., Huang, J., Hamilton, P., Higgs, S., 2004. Real-time reverse transcriptase-polymerase chain reaction quantification of West Nile virus transmitted by Culex pipiens quinquefasciatus. Am. J. Trop. Med. Hyg. 71 (1), 120–123.

Varga, A., James, D., 2005. Detection and differentiation of Plum pox virus using real-time multiplex PCR with SYBR Green and melting curve analysis: a rapid method for strain typing. J. Virol. Methods 123 (2), 213–220.

Verhoefstede, C., Fransen, K., Marissens, D., Verhelst, R., van der, G.G., Lauwers, S., Zissis, G., Plum, J., 1996. Isolation of HIV-1 RNA from plasma: evaluation of eight different extraction methods. J. Virol. Methods 60 (2), 155–159.

Vernon, S.D., Unger, E.R., Williams, D., 2000. Comparison of human papillomavirus detection and typing by cycle sequencing, line blotting, and hybrid capture. J. Clin. Microbiol. 38 (2), 651–655.

Verstrepen, W.A., Kuhn, S., Kockx, M.M., Van De Vyvere, M.E., Mertens, A.H., 2001. Rapid detection of enterovirus RNA in cerebrospinal fluid specimens with a novel single-tube real-time reverse transcription-PCR assay. J. Clin. Microbiol. 39 (11), 4093–4096.

Verstrepen, W.A., Bruyneels, P., Mertens, A.H., 2002. Evaluation of a rapid real-time RT-PCR assay for detection of enterovirus RNA in cerebrospinal fluid specimens. J. Clin. Virol. 25 (Suppl. 1), S39–S43.

Vet, J.A., Majithia, A.R., Marras, S.A., Tyagi, S., Dube, S., Poesz, B.J., Kramer, F.R., 1999. Multiplex detection of four pathogenic retroviruses using molecular beacons. Proc. Natl. Acad. Sci. USA 96 (11), 6394–6399.

Wagner, H.J., Wessel, M., Jabs, W., Smets, F., Fischer, L., Offner, G., Bucsky, P., 2001. Patients at risk for development of posttransplant lymphoproliferative disorder: plasma versus peripheral blood mononuclear cells as material for quantification of Epstein–Barr viral load by using real-time quantitative polymerase chain reaction. Transplantation 72 (6), 1012–1019.

Wagner, H.J., Cheng, Y.C., Huls, M.H., Gee, A.P., Kuehnle, I., Krance, R.A., Brenner, M.K., Rooney, C.M., Heslop, H.E., 2004. Prompt versus preemptive intervention for EBV lymphoproliferative disease. Blood 103 (10), 3979–3981.

Waltz, T.L., Marras, S., Rochford, G., Nolan, J., Lee, E., Melegari, M., Pollack, H., 2005. Development of a molecular-beacon assay to detect the G1896A precore mutation in hepatitis B virus-infected individuals. J. Clin. Microbiol. 43 (1), 254–258.

Ward, C.L., Dempsey, M.H., Ring, C.J., Kempson, R.E., Zhang, L., Gor, D., Snowden, B.W., Tisdale, M., 2004. Design and performance testing of quantitative real time PCR assays for influenza A and B viral load measurement. J. Clin. Virol. 29 (3), 179–188.

Watanabe, M., Kohdera, U., Kino, M., Haruta, T., Nukuzuma, S., Suga, T., Akiyoshi, K., Ito, M., Suga, S., Komada, Y., 2005. Detection of adenovirus DNA in clinical samples by SYBR Green real-time polymerase chain reaction assay. Pediatr. Int. 47 (3), 286–291.

Watkins-Riedel, T., Woegerbauer, M., Hollemann, D., Hufnagl, P., 2002. Rapid diagnosis of enterovirus infections by real-time PCR on the LightCycler using the TaqMan format. Diagn. Microbiol. Infec. Dis. 42 (2), 99–105.

Watzinger, F., Lion, T., 2003. Approaches to Quantification of RNA Targets by PCR Based Techniques. Curr. Genom. 4 (2), 185–204.

Watzinger, F., Suda, M., Preuner, S., Baumgartinger, R., Ebner, K., Baskova, L., Niesters, H.G., Lawitschka, A., Lion, T., 2004. Real-time quantitative PCR assays for detection and monitoring of pathogenic human viruses in immunosuppressed pediatric patients. J. Clin. Microbiol. 42 (11), 5189–5198.

Weidmann, M., Meyer-Konig, U., Hufert, F.T., 2003. Rapid detection of herpes simplex virus and varicella-zoster virus infections by real-time PCR. J. Clin. Microbiol. 41 (4), 1565–1568.
Zaragoza, C., Li, R.M., Fahle, G.A., Fischer, S.H., Raffeld, M., Lewis Jr., A.M., Kopp, J.B., 2005. Squirrel monkeys support replication of BK virus more efficiently than simian virus 40: an animal model for human BK virus infection. J. Virol. 79 (2), 1320–1326.

Zerr, D.M., Huang, M.L., Corey, L., Erickson, M., Parker, H.L., Frenkel, L.M., 2000. Sensitive method for detection of human herpesviruses 6 and 7 in saliva collected in field studies. J. Clin. Microbiol. 38 (5), 1981–1983.

Zhang, M., Gong, Y., Osiowy, C., Minuk, G.Y., 2002. Rapid detection of hepatitis B virus mutations using real-time PCR and melting curve analysis. Hepatology 36 (3), 723–728.

Zhao, J.R., Bai, Y.J., Zhang, Q.H., Wan, Y., Li, D., Yan, X.J., 2005. Detection of hepatitis B virus DNA by real-time PCR using TaqMan-MGB probe technology. World J. Gastroenterol. 11 (4), 508–510.

Zhong, S., Yeo, W., Schroder, C., Chan, P.K., Wong, W.L., Ho, W.M., Mo, F., Zee, B., Johnson, P.J., 2004. High hepatitis B virus (HBV) DNA viral load is an important risk factor for HBV reactivation in breast cancer patients undergoing cytotoxic chemotherapy. J. Viral. Hepatitis 11 (1), 55–59.

Zipper, H., Brunner, H., Bernhagen, J., Vitzthum, F., 2004. Investigations on DNA intercalation and surface binding by SYBR Green I, its structure determination and methodological implications. Nucleic Acids Res. 32 (12), e103.