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Review

Detection and monitoring of virus infections by real-time PCR

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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

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Contents

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 amplicons 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.

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

Manufacturer/supplier	Nucleic acid test (NAT) system	Virus target
Roche diagnostics (Basel, Switzerland)	COBAS TaqMan Test	HCV, HBV, HIV-1, respectively
	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 (Lyndhurst, NJ, USA)	Hepatitis C viral RNA, quantitative real-time PCR	HCV
	BK and JC virus DNA, real-time PCR	BKV + JCV
Digene (Gaitersburg, MD, USA)	Digene® HPV test	HPV
Abbott (North Chicago, IL, USA)	Abbott m2000rt RealTime™ assay	HCV, HIV-1, respectively
Artus (Hamburg, Germany)	RealArt™ RT PCR Kit	DENV, EV, HAV, HIV 1, Inf, Inf/H5, SARS-CoV, WNV
	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).

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

Table 2
Recently reported approaches to virus quantification by real-time PCR using different detection formats

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)	Stocher et al. (2003), Schaade et al. (2000) and Jebbink et al. (2003)		Jebbink et al. (2003)		
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)	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)		Jebbink et al. (2003)		

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), Stocher et al. (2003), Whiley et al. (2004) and Ramaswamy et al. (2005)	Ramaswamy et al. (2005), Schalasta et al. (2000) and Issa et al. (2005)			Eclipse Stevenson et al. (2005)
Hepatitis virus A (HAV)	Costa-Mattioli et al. (2002) and Jothikumar et al. (2005a)		Brooks et al. (2005)			
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 Niesters (2002), Kohmoto et al. (2003), Jun-Bin et al. (2003) and Weiss et al. (2004)	Jardi et al. (2001), Jursch et al. (2002), Paraskevis et al. (2002), Ho et al. (2003), Aliyu et al. (2004) and Leb 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)	Yates et al. (2001), Abravaya et al. (2003), Sum et al. (2004), Wightman et al. (2004) and Waltz et al. (2005)	Geng et al. (2005), Pang et al. (2004a) and Zhao et al. (2005)	
Hepatitis virus C (HCV)	Martell et al. (1999), Kleiber 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)	Komurian-Pradel et al. (2001), Nozaki and Kato (2002), White et al. (2002), Hazari et al. (2004) and Komurian-Pradel et al. (2004)	Abravaya et al. (2003), Komurian-Pradel et al. (2004), Yang et al. (2002) and Prince et al. (2004)	Castelain 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)					Eclipse Hymas et al. (2005)

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Table 2 (*continued*)

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 Fernandez et al. (2002)
Human herpes virus 8 (HHV-8)	Watzinger et al. (2004), Lallemand et al. (2000), Tedeschi et al. (2001) and Su et al. (2005)	Boivin et al. (2002)				
Human immunodeficiency viruses 1 + 2 (HIV-1, HIV-2)	Candotti et al. (2004), Schutten et al. (2000), Desire et al. (2001), Luo et al. (2005) and Katsoulidou et al. (2006)		Laskus et al. (2004)	Abravaya et al. (2003), Vet et al. (1999) and Summerer and Marx (2002)	Bergroth et al. (2005)	Scorpion primer Saha et al. (2001)
Human metapneumovirus (hMPV)	Mackay et al. (2003), Maertzdorf et al. (2004), Bouscambert-Duchamp et al. (2005), Deffrasnes et al. (2005) and Sumino et al. (2005)		Boivin et al. (2003) and Cote et al. (2003)	Scheltinga et al. (2005)	Kuypers et al. (2005)	
Human rhinovirus (hRV)	Nijhuis et al. (2002), Scheltinga et al. (2005), Lai et al. (2003), Deffernez et al. (2004) and Sanders et al. (2004)	Kares et al. (2004)	Dagher et al. (2004) and Chen et al. (2006)	Scheltinga et al. (2005)		
Human T-lymphotropic viruses 1 + 2 (HTLV-1, HTLV-2)	Dehee et al. (2002), Pennington et al. (2002), Yamano et al. (2002), Estes and Sevall (2003), Matsuda et al. (2005) and Montanheiro et al. (2005)	Kamihira et al. (2000, 2003) and Sonoda et al. (2004)	Lee et al. (2004) and Murphy et al. (2004)	Vet et al. (1999)		
Influenza viruses A + B (Inf-A, -B)	Watzinger et al. (2004), Herrmann et al. (2001), van Elden et al. (2001), Spackman et al. (2002), Lee and Suarez (2004), Ward et al. (2004), Hindiyeh et al. (2005), Krafft et al. (2005) and Ng et al. (2005)	Smith et al. (2003) and Stone et al. (2004a)	Krafft et al. (2005) and Boivin et al. (2004)	Templeton et al. (2004)	Chi et al. (2005)	

Measles virus	Ozoemena et al. (2004), El Mubarak et al. (2005) and Hummel et al. (2005)	Schalk et al. (2004)	Plumet and Gerlier (2005)			LAMP primer Fujino et al. (2005)
Mumps virus	Uchida et al. (2005) and Kubar et al. (2004)					
Newcastle disease virus (NDV)	Wise et al. (2004)		Crossley et al. (2005), Tan et al. (2004) and Pham et al. (2005)			
Norovirus type 1 + 2	Kageyama et al. (2003), Hohne and Schreier (2004), Jothikumar et al. (2005b) and Pang et al. (2005)		Beuret (2004), Gunson et al. (2003), Myrmel et al. (2004), Pang et al. (2004b), Richards et al. (2004a,b) and Schmid et al. (2004)			
Parainfluenza viruses 1–4 (PIV 1, 2, 3, 4)	Watzinger et al. (2004)			Templeton et al. (2004)	Hu et al. (2005a)	
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)	Harder et al. (2001)	Manaresi et al. (2002) and Gallinella et al. (2004)			
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), Ryschkewitsch et al. (2004), Bressollette-Bodin et al. (2005), Randhawa et al. (2005), Si-Mohamed et al. (2006) and Stolt et al. (2005)	Whiley et al. (2001) and Zaragoza et al. (2005)	Randhawa et al. (2004), Beck et al. (2004) and Rollison et al. (2005)		Herman et al. (2004) and McNees et al. (2005)	
Poxvirus						LUX primer Aitichou et al. (2005) and Varga and James (2005)

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Table 2 (*continued*)

Virus	Hydrolysis probe (Refs.)	Hybridization probe (Refs.)	Melting curve (Refs.)	Molecular beacons (Refs.)	MGB™ 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 Whiley 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), Stocher et al. (2003), Hawrami and Breuer (1999), Espy et al. (2000b), Loparev 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), Eisler 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 fluorigenic 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

Table 3
Examples of robotic systems for (semi)automated isolation of viral nucleic acids using proprietary chemistries

Manufacturer	Instrument	Basic principle	Max. capacity/run	Time/run[a]	Hands on time
Applied Biosystems[c]	ABI PRISM™ 6100	Membrane system	96	30 min	<10 min
	ABI PRISM™ 6700	Membrane system	96	30 min	<10 min
bioMérieux[c]	NucliSens® extractor	Silica particles	10	45 min	20 min
	NucliSens® easyMAG™	Magnetic silica particles	24	60 min	<15 min
Qiagen[c]	BioRobot 9604	Silica gel membrane	96	140 min	20 min
	BioRobot EZ1	Magnetic silica particles	6	35 min	<5 min
	BioRobot 8000	Silica gel membrane	96	n.a.[b]	<15 min
	BioRobot M48	Magnetic silica particles	48	230 min	<15 min
	BioRobot MDX	Magnetic silica particles	96	210 min	<15 min
Roche[c]	MagNA pure LC instrument	Magnetic glass beads	32	85 min	15 min
	MagNA pure compact instrument	Magnetic glass beads	1–8	30 min	5 min
	Cobas AmpliPrep	Magnetic silica particles	72	70 min	30 min

[a] Time required to run a virus DNA/RNA-isolation protocol with max. number of samples.
[b] n.a. = not available—no standard virus isolation protocol available.
[c] Applied Biosystems, AB, Foster City, CA, USA; bioMérieux sa, Marcy l'Etoile, France; Qiagen, Hilden, Germany; Roche, Basel, Switzerland.

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 (Najioullah 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.

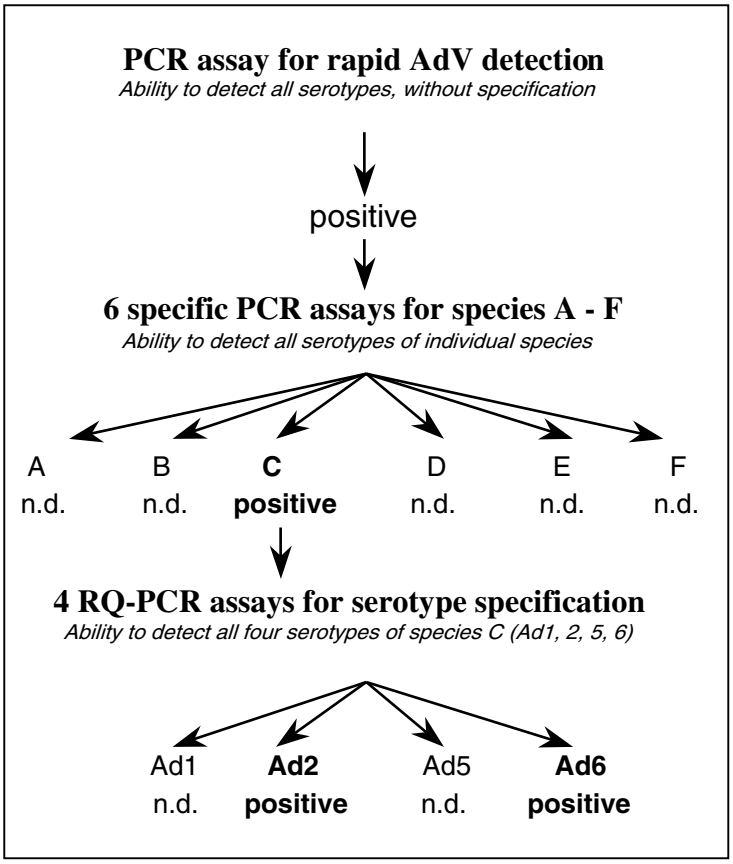

Fig. 1. Example of an algorithm for AdV serotyping by RQ-PCR, revealing presence of two different serotypes of the virus in the sample investigated.

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.

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.

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