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Protocol

Simultaneous detection of enteric viruses by multiplex real-time RT-PCR

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Abstract

A multiplex real-time RT-PCR protocol for the simultaneous detection of noroviruses ("Norwalk-like viruses") of genogroups I and II, human astroviruses and enteroviruses is described. The protocol was developed and evaluated using the LightCycler™ and corresponding SYBR Green reagents. New primers were designed within conserved genome regions to optimize the detection range of virus subtypes of each genus. To enable the development of a multiplex PCR assay within one tube (capillary), similar mastermix- and cycling-conditions were respected for each individual primer system. Subsequent melting curve analysis allowed the determination of possible dual-contaminations of entero- and noro- or astroviruses by the formation of dual peaks. Special care was taken to minimize the loss of sensitivity, since the detection of small viral contaminations is a crucial parameter especially for food analysis. The multiplex assay was compared successfully to the single SYBR Green assay, and revealed to be at least 10 times more sensitive than the one obtained with an endpoint PCR thermocycler protocol published previously.

Keywords: Noroviruses; Enteroviruses; Astroviruses; Multiplex; Real-time PCR; Melting curve; RT-PCR

1. Type of research

Noroviruses (NV: former NLV) are a genus belonging to the family of Caliciviridae and are transmitted by the fecal-oral and the aerosol route, or by contaminated food, resulting in the most common cause of non-bacterial gastroenteritis. NVs are responsible for an estimated 67% of illnesses caused by known food-borne pathogens and for 96% of non-bacterial gastroenteritis in the United States. Other enteric viruses like entero- or astroviruses can also be transmitted by food, but their importance has to be evaluated using recent detection methods (Fleet et al., 2000; Greenberg and Matsui, 1992; Mead et al., 1999; Oishi et al., 1994; Wheeler et al., 1999).

The importance of enteric viruses as "emerging" agents of foodborne disease has become evident with the development of molecular techniques like (RT-) PCR and sequencing, which enable the identification of common contamination sources. More research needs to be done to evaluate the importance of viruses in foodborne transmissions (Cliver, 1997; Fleet et al., 2000; Jaykus, 2000; Koopmans et al., 2002).

Virus transmission by food has been described in many publications (Beller et al., 1997; Belliot et al., 1997; Beuret et al., 2000, 2002, 2003; Gaulin et al., 1999; Parashar et al., 1998), often using (RT-) PCR and agarose gel electrophoresis to detect the agent. A crucial parameter, especially for food control laboratories and the field of medical diagnostics, is to minimize the time required to have the viral agent identified. Real-time (RT-) PCR represents a versatile tool to reach this goal (Higuchi et al., 1992, 1993; Locatelli et al., 2000; Morrison, 1998).

Analysis time for real-time (RT-) PCR can additionally be reduced by using the LightCycler™, minimizing the time required for cDNA amplification by efficient heat transfer to small samples (Wittwer et al., 1990, 1997). The subsequent identification of amplicons by melting curve analysis (for example SYBR Green assays) replaces time-consuming procedures such as agarose gel electrophoresis or hybridizations (Ririe et al., 1997).

2. Time required

Reverse transcription of previously extracted viral RNA to cDNA takes 1.5 h (Beuret et al., 2002). Detection by

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single- or multiplex RT-PCR takes another 1.5 h. The whole protocol can easily be carried out within 3 h.

3. Materials

3.1. General considerations

Since contamination risks exist for sensitive molecular biological methods like real-time RT-PCR, some precautions have to be followed regarding the laboratory equipment and the good laboratory practice. Separate working rooms or areas have to be used for the preparation of positive controls, extraction of viral RNA, mastermix preparations for RT and real-time PCR. Separate sets of micropipettes have to be used, especially for the extraction of RNA and the preparation of mastermixes, disposable laboratory gloves have do be changed between every step.

3.2. Controls

Each test is accompanied by two overall controls. A positive control consisting of a spiked sample containing a viral concentration near the detection limit of the method and a "no template control (NTC)" consisting of sterile water and mastermix in order to detect possible contaminations.

Positive control samples for human enteroviruses (EV) consisted of poliomyelitis virus (Sabine 1) with a stock concentration of 1000 plaque forming units (PFU)/ml, the positive control sample for astroviruses (AV) consisted of human astrovirus of serotype 1 with a similar concentration as used for EV. Noroviruses of genogroups I and II were isolated from contaminated human stool specimens using a method described previously (Beuret, 2003, dilution of 1:10 of the stool specimen with sterile water, using the supernant after a centrifugation step of 60 s at 16,000 × *g* and room temperature for the RNA extraction). The NV gg I positive control sample contained Southampton/91/UK-related viruses (L07418); Lordsdale viruses (X86557) were present in the positive control sample for NV gg II. Dilutions of 10–10^6 of each positive stock solution were tested to compare the detection limits of real-time multiplex PCR versus the previously published thermocycler RT-PCR protocol (Beuret, 2003).

3.3. Oligonucleotides

All oligonucleotides were newly designed. After optimization of the single detection protocol for noroviruses belonging to genogroups I and II, human entero- and astroviruses by real-time RT-PCR, a protocol for a multiplex assay was developed using the same oligonucleotides. Table 1 shows sequences and localizations of all oligonucleotides used. The primers (HPLC grade) had been synthesized by Amplimmun AG (Madulain, Switzerland) and stored freeze-dried at −40 °C until first use.

3.4. Special equipment

Essential equipment includes: a programmable thermocycler for the reverse transcription of viral RNA (protocol described was performed on a Biometra UNO II thermocycler, Biometra, Germany) and the LightCycler™ (ROCHE, Germany) with corresponding software (Version 3.5) for real-time PCR analysis and subsequent melting curve analysis.

3.5. Optional equipment

The risk of contamination during the preparation of mastermixes for RT or PCR can be minimized by using a

Table 1
Oligonucleotides used for real-time RT-PCR analysis

Virus and oligonucleotides	Region	Sequence 5′–3′	Polarity	Localization[a]
(1) Genogroup-specific norovirus (NV) oligonucleotides for genogroups I and II				
NV gg I				
BN11 (PCR)	RNA pol	ATGTTCCGYTGGATGC	Sense	5285–5300
BN12 (RT-PCR)	RNA pol	CGTCCTTAGACGCCATCA	Antisense	5379–5262
MGB TaqMan gg I	RNA pol	TGGACAGGAGATCG	Sense	5321–5334
NV gg II				
BN21 (PCR)	RNA pol	TCAGGTGGATGAGGTTCTCAGA	Sense	5016–5037
BN22 (RT-PCR)	RNA pol	CGACGCCATCWTCATTCACA	Antisense	5099–5080
MGB TaqMan gg II	RNA pol	TGGGAGGGCGATCG	Sense	5048–5062
(2) Enterovirus-specific oligonucleotides				
BE1 (PCR)	5′-UTR	CCCTGAATGCGGCTAA	Sense	452–476
BE2 (RT-PCR)	5′-UTR	TGTCACCATAAGCAGCCA	Antisense	596–576
(3) Astrovirus-specific oligonucleotides				
BA1 (PCR)	Mature	TGTGCTTCATGGAAGACT	Sense	1074–1091
BA2 (RT-PCR)	Protein 2	ATTARCRTCACGGATCTCG	Antisense	1270–1252

Mixed bases in degenerate primers: W = A/T, Y = C/T, R = A/G.

[a] Localizations are in reference to: norovirus gg I (NV/8FiiA/68/US: M87661); norovirus gg II (Lordsdale virus: X86557); enterovirus (poliovirus Sabin 1: AY184219); astrovirus (human astrovirus type 1: L23513).

PCR-hood including a UV-transilluminator. Cross-contaminations of templates can be reduced by pipetting within a flow chart with optional decontamination by UV-transillumination using separate pipettes. The centrifugation of the SYBR Green mastermix-template-mix in the LightCycler™ capillaries can be simplified by the use of an adequate centrifuge where the carousel (with the capillaries) can be placed directly.

3.6. Reagents

- QIAamp viral RNA mini kit (QIAGEN, Hilden, Germany: Cat. No. 52906);
- reverse transcription: Sensiscript-RT kit (QIAGEN, Hilden, Germany: Cat. No. 205211), other reagents with similar efficiencies can be used;
- RNasin ribonuclease inhibitor (Promega, Madison, WI, USA: Cat. No. N2111);
- LightCycler™—FastStart DNA Master SYBR Green I (Roche, Germany: Cat. No. 30032300);
- sterile water;
- primers (HPLC quality, see Table 1).

4. Detailed procedures

We describe single and multiplex real-time RT-PCR protocols for the detection of noroviruses of genogroups I and II, human astro- and enteroviruses by using a SYBR Green assay on the LightCycler™.

4.1. RNA extraction

RNA extraction was performed according to manufacturer's vacuum protocol of the QIAamp viral RNA mini kit (QIAGEN, Germany) for small volumes. One hundred and forty microliters of each positive control sample (concentrations are mentioned in 3.2 controls) were used. Resulting 60 μl of RNA (for each sample) were frozen at −40 °C until further use.

4.2. RT-PCR

Reverse transcription (RT) and polymerase chain reaction (PCR) were performed subsequently in two steps to reach a maximal sensitivity.

RT-mastermix was assembled on ice or in a cooling block according to manufacturer's protocol for RNA volumes of 10 μl. Primers BN12 and BN22 were separately used for two independent genogroup-specific norovirus systems, primer BE2 for the enterovirus- and primer BA2 for the astrovirus detection.

Ten microliters of RT-mastermix and 10 μl of isolated RNA were mixed in a 200 μl PCR tube on ice within a laminar flow. In a final volume of 20 μl, the reaction conditions were 1× RT-Sensiscript buffer, 0.5 mM each dNTP, 1 μM primer each (BN12, BN22, BE2 or BA2), 10 units RNase inhibitor (RNasin Ribonuclease Inhibitor; Promega, Madison, WI, USA), 1 μl RT-Sensiscript and 10 μl template-RNA. Strip caps were vigorously pressed on PCR tubes.

Tubes were immediately placed in a preheated thermocycler and the RNA was reverse transcribed by incubation for 60 min at 37 °C followed by 5 min at 95 °C to inactivate the reverse transcriptase. Samples were frozen at −40 °C until further use.

Before preparing the PCR-mastermix, the LightCycler™ software was configured to start a run. Two protocols are described, one for multiplex detection (A) and of for single (B) use:

(A) Real-time PCR-mastermix for multiplex detection: final concentrations for a 20 μl volume: 1× SYBR Green mastermix (ROCHE, Germany), 0.3 μM of BN11/12 primers, 0.2 μM of BN21/22 primers, 0.4 μM of BA1/2- and BE1/2 primers and 1 U of Faststart polymerase; prepared during the RT incubation step.

(B) Real-time PCR-mastermix for single detection: final concentrations for a 20 μl volume: 1× SYBR Green mastermix (ROCHE, Germany), 0.5 μM of BN11/12, BN21/22, BA1/2 or BE1/2 and 1 U of Faststart polymerase; prepared during the RT incubation step.

Five microliters of cDNA were mixed with 15 μl PCR mastermix in a LightCycler™ capillary in a laminar flow. 2.5 μl of two different cDNAs were used for the simulation of dual-contaminations.

Capillaries were placed in the carousel and centrifuged according to manufacturer's protocol (corresponding centrifuge) for 5 s at 700 × *g* and room temperature.

Cycling and melting curve analysis were performed with a LightCycler™ using the following conditions; a two- and a three-steps protocol were optimized to obtain similar results:

- Amplification for SYBR Green-analysis with either a two- or a three-step protocol after an initial denaturation-step for 8 min at 95 °C to activate the "Faststart (hotstart) polymerase" (cycles: 40; type: quantification)

	Parameter				
	Two steps		Three steps		
	Segment 1	Segment 2	Segment 1	Segment 2	Segment 3
Target temperature (°C)	95	60	95	60	72
Incubation time (s)	10	30	10	5	8
Temperature transition rate (°C/s)	20	20	20	20	20
Acquisition mode	None	Single	None	None	Single

- Melting curve analysis (cycle: 1; type: melting curves)

	Segment 1	Segment 2	Segment 3
Target temperature (°C)	95	65	90
Incubation time (s)	0	15	0
Temperature transition rate (°C/s)	20	20	0.1
Acquisition mode	None	None	Contamination

5. Results

In order to confirm the sensitivity improvement of our new real-time PCR assay, we first compared single real-time PCR-analysis with the results of a previously published detection protocol for conventional thermocyclers (Beuret, 2003). Both protocols were performed by using the same dilutions of viruses (10^3 to 10^{-2} PFU of poliovirus (PV) Sabin 1 and human astrovirus of serotype 1, 10^{-1} to 10^{-6}-fold dilutions of the norovirus positive control sample of genogroups I and II). Results in Fig. 1 show an improvement in sensitivity of a factor of 10. An improvement in Sensitivity of a factor of 10^2 could be measured for the detection of poliovirus (Fig. 2) and astrovirus.

The newly developed multiplex assay was then compared to the single analysis protocols. As shown in Table 2, the C_t-values (threshold cycle or crossing points) for single and multiplex analysis are approximately similar, confirming a negligible loss of sensitivity by combining all four primer pairs. There is a beneficial effect measurable for the

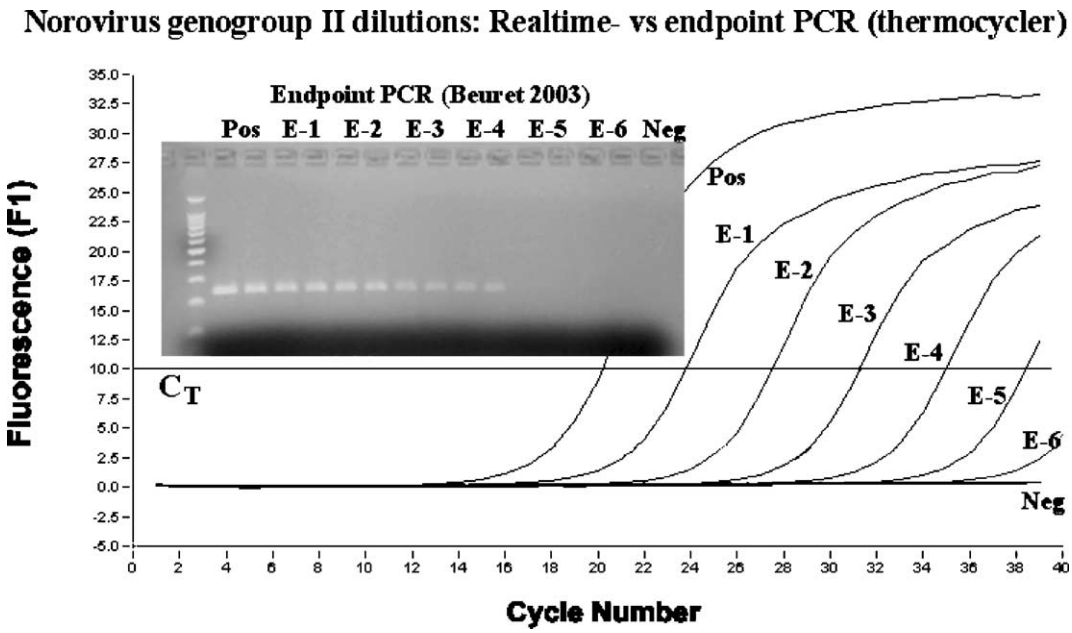

Fig. 1. Comparison of sensitivity for multiplex norovirus genogroup II (NV gg II) detection between real-time SYBR Green- and a conventional RT-PCR protocol (Beuret, 2003): the amplicon size is 85 and 203 bp, respectively. Samples are 10^{-1} to 10^{-6} fold dilutions of NV gg II positive control samples (in duplets for the conventional RT-PCR protocol): E-1 to E-6. The assay by real-time RT-PCR using the LightCycler™ shows an increased sensitivity of a factor of 10 compared to the conventional RT-PCR protocol after 40 cycles each.

Table 2
The crossing points (C_t: threshold cycles) of single and multiplex RT-PCR SYBR Green assays are compared with equal viral concentrations

Virus	Single contaminations				Dual contaminations			
	NV I	NV II	EV	AV	NV gg I + II	NV gg I + EV	NV gg II + EV	AV + EV
Single RT-PCRs	26.5	24.6	28.5	28.1	Sensitivity enhancement			
Multiplex RT-PCRs	27.1	24.1	29.3	30.2	22.4	21.8	22.2	29.1

The C_t-values for single and multiplex analysis are approximately similar and the detection of noroviruses in multiplex is even enhanced for genogroup II. A similar effect is measurable for the simultaneous detection of dual contaminations compared to single ones. There is a little loss of sensitivity for the detection of astroviruses.

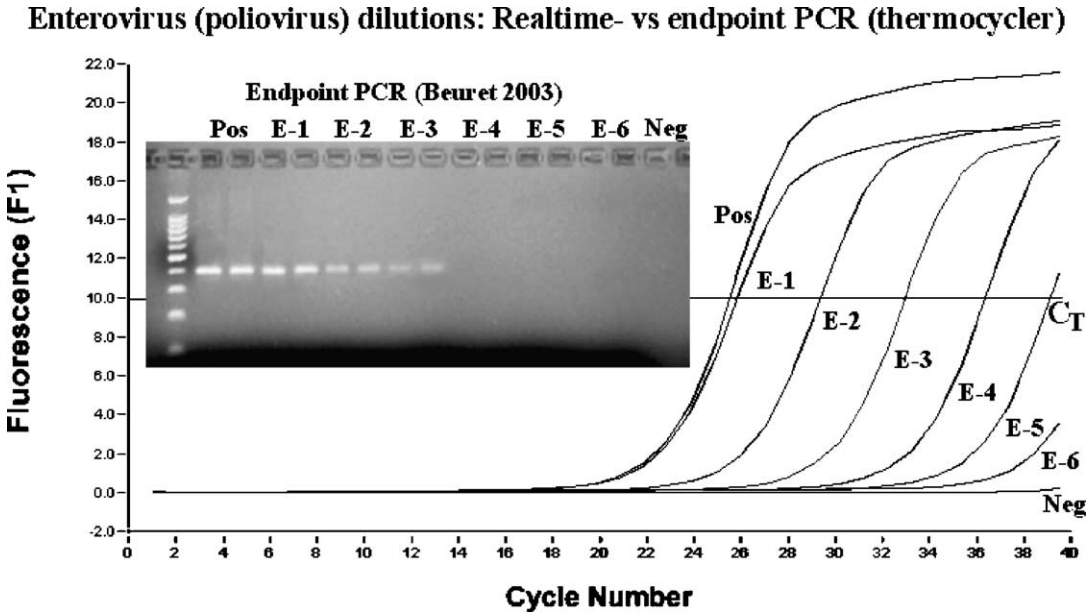

Fig. 2. Comparison of sensitivity for multiplex poliovirus (type 1) detection between real-time SYBR Green- and a conventional RT-PCR protocol (Beuret, 2003): the amplicon size is 145 and 401 bp, respectively. Samples are 10^{-1} to 10^{-6} dilutions of a poliomyelitis virus (Sabine 1) stock solution with a concentration of 1000 PFU/ml (Plaque forming units; in duplets for the conventional RT-PCR protocol): E-1 to E-6. The assay by real-time RT-PCR using the LightCycler™ shows an increased sensitivity of a factor of 10^2 compared to the conventional RT-PCR protocol after 40 cycles each.

detection of NV gg II, but there is also a little loss of sensitivity for the detection of astroviruses. Crossing points of the simultaneous detection of two types of cDNA (for example norovirus versus enterovirus) also show an enhanced sensitivity compared to the multiplex-detection of a single cDNA. However, as described in many publications related to disadvantages of multiplex PCR (Elnifro et al., 2000; Exner and Lewinski, 2002), a slightly decreased efficiency of combined detection systems could only be observed for the detection of astroviruses. The differentiation of low single viral contaminations by melting curve analysis of a multiplex assay was performed and is shown in Fig. 3. Fluorescence signals down

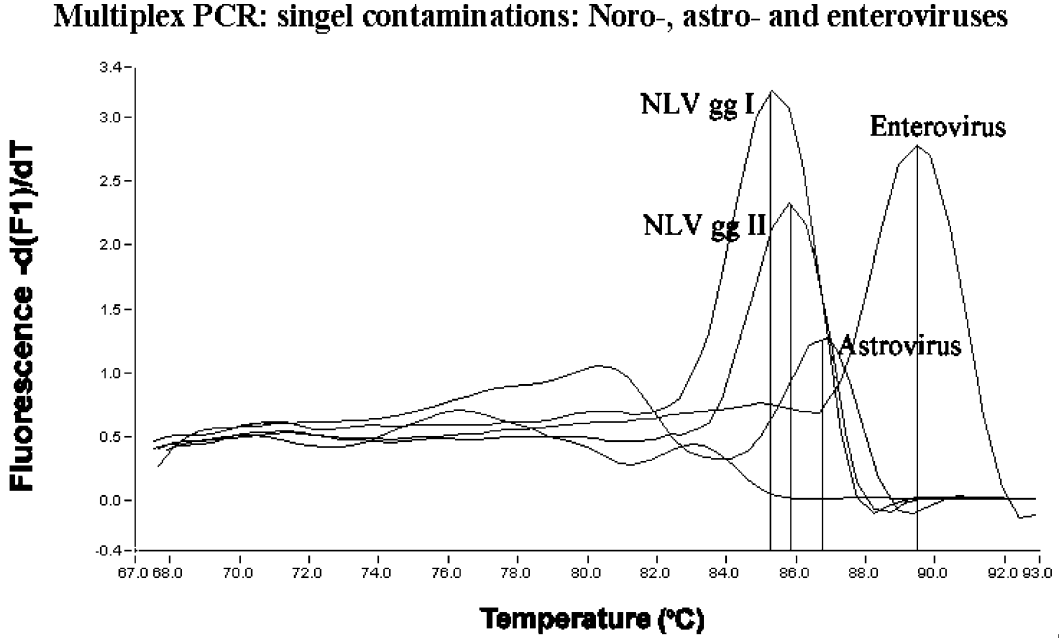

Fig. 3. Melting curve analysis for the separate detection of four different viral pathogens by multiplex real-time RT-PCR (SYBR Green analysis) using the LightCycler™. Separate peaks for noroviruses of genogroups I and II, human astrovirus (type 1) and enteroviruses (poliovirus SABIN 1) can be distinguished. Melting temperatures between 85 and 90 °C enable the identification of possible primer-dimer formations as shown for the detection of astroviruses.

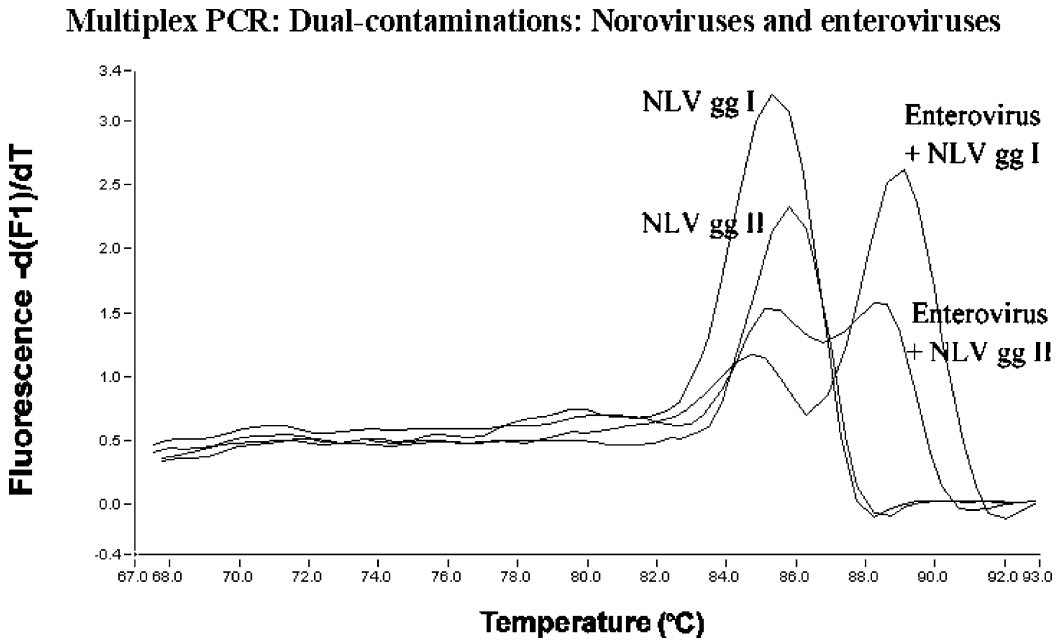

Fig. 4. Melting curve analysis for the simultaneous detection of dual contaminations for noroviruses of genogroups I and II and enteroviruses (poliovirus SABIN 1) by multiplex RT-PCR using a SYBR Green assay. Separate peaks for noroviruses of genogroups I and II and enteroviruses represent single contaminations, integrated peaks (dual peaks) represent dual contaminations. Half the cDNA concentration had to been used for dual contaminations, resulting in a lower fluorescence signal.

to $1.5 - \mathrm{d(F1)/d}T$ for each virus genera could be differentiated by melting curve analysis. An example for the detection of dual contaminations by melting curve analysis is shown in Fig. 4 for NVs and enteroviruses by the formation of dual-peaks. The usual template volume of 5 μl was thereby replaced by two volumes of 2.5 μl for each virus genera. Melting temperatures (T_M) for each genera are: 84–85 °C for NV gg I, 85.5–86.5 °C for NV gg II, 87–88 °C for AV and 88.5–90 °C for EV. Temperature shifts are subtype specific, depending on point mutations within target regions of viral genomes.

6. Discussion

This protocol article describes a simple method for the detection of enteric viruses by multiplex real-time RT-PCR. Each step of the protocol is discussed separately in the following sections.

6.1. Trouble shooting

6.1.1. Primer design

Many primers have been published for the detection of enteric viruses by RT-PCR. As these virus-genera share few consensus sequences within their subtype genomes, it is rather difficult to find appropriate primers to detect the widest range of these viruses. The design of degenerate primers in highly conserved regions of the viral genomes represents an effective way to increase the detection range of a primer system. We tested successfully the specificity of the newly designed primers by the analysis of 12 species of both genogroups (I and II) (results not shown). Also sequence analysis with actual Genebank entries confirmed a theoretical 100% detection rate with every published norovirus sequence. An attempt was made to enhance the specificity of the real-time detection by designing additional probes to the primer pairs mentioned. As consensus regions within each virus genera are rare, it was impossible to use hybridization- or TaqMan probes with a sequence length of 20–40 nt. It was therefore decided to design minor groove binder (MGB; Kutyavin et al., 2000) TaqMan probes for NV gg I and II with a minimal length of 14 nt and to test their suitability. Sensitivities for the detection of both NV genogroups were slightly lower for MGB TaqMan probes compared to the SYBR Green assays. We tried in vain to optimize the sequence of the MGB TaqMan probe and the PCR conditions. Although we could not adapt our primer systems to the MGB TaqMan probes, they remain a powerful tool for the detection of short consensus regions within different sequences. Sequences of both probes are shown in Table 1.

6.1.2. RT

We compared different reverse transcription reagents (AmpliTaq Gold, Perkin-Elmer; RT AMV and M-MuLV, Roche; Omniscript, QIAGEN) and measured the best sensitivity by using the Sensiscript-RT kit (QIAGEN, Hilden, Germany) combined with the SYBR Green Faststart kit (ROCHE, Germany). The type of RT kit has to be chosen with respect to the primers and the PCR reagents used.

6.1.3. Real-time PCR

The use of an intercalating dies like SYBR Green enables the differentiation of multiplex amplicons by melting curve analysis. The specificity of the amplification is a crucial factor regarding the absence of additional probes, since dies like SYBR Green bind to every possible double-stranded (c)DNA. The use of a hotstart ("Faststart" for the manufacturer's vocabulary) polymerase and optimized primers are important strategies to improve the specificity. The recommended heating step at 95 °C for the activation of the polymerase was reduced to 8 min without any loss of sensitivity or specificity. Another crucial factor is the volume of templates that determines the sensitivity of the detection system. We used the maximal template volume proposed by the manufacturer for a SYBR Green assay, 5 μl of cDNA-template for 15 μl of mastermix. We also paid attention to develop two separate PCR-protocols with two or three PCR steps to enable the use of different real-time PCR formats. Both protocols were optimized to reach the same sensitivity.

6.2. Alternative and support protocols

The sensitivity of the developed multiplex RT-PCR assay was also tested by spiking drinking water samples with positive control samples. Analysis (isolation, RNA extraction and reverse transcription) of spiked water samples were performed according to one of our latest publications (Beuret, 2003).

Reagents and materials described for the detection of enteric viruses by RT-PCR could also be replaced by or combined with other available systems to improve the sensitivity. We obtained best results by using the reagents described. As mentioned under Section 6.1.1, the newly developed primer pairs have been adapted to all actual known virus strains of corresponding genera within the NCBI Genbank, but future viral sequences with different conserved genome regions could favor the design of new primers.

Typing of positive results by external sequencing can be performed easily. Amplicons in capillaries have to be "reverse"-centrifuged in a 1.5 ml tube for 5 s at 1000 × *g*. Blot/liquid hybridization or enzyme immunoassay could also be performed for typing, but since the sample volumes in a LightCycler™'s capillary is as little as max. 20 μl, the choice of another typing method as sequencing would be difficult.

7. Essential literature

The following literature provides essential information for the detection of viruses by real-time (RT-) PCR (Higuchi et al., 1992, 1993; Locatelli et al., 2000; Morrison, 1998; Wittwer et al., 1990, 1997; Read et al., 2001; Ririe et al., 1997; Sanchez and Storch, 2002).

RNA extraction was undertaken according to manufacturer's vacuum protocol of the QIAamp viral RNA mini kit (QIAGEN, Germany) for small volumes. One hundred and forty microliters of each positive control sample (concentrations are mentioned in 3.2 controls) were used. Resulting 60 μl of RNA (for each sample) were frozen at −40 °C until further use.

8. Quick procedure

8.1. RNA extraction

RNA extraction was performed according to manufacturer's vacuum protocol of the QIAamp viral RNA mini kit (QIAGEN, Germany) for small volumes by using 140 μl of each positive control sample (concentrations are mentioned in 3.2 controls). Resulting 60 μl of RNA were immediately frozen at −40 °C until further use.

8.2. Reverse transcription (RT)

RT-mastermix was prepared on ice. 10 μl of RT-mastermix and 10 μl of RNA were pipetted into a 200 μl PCR tube on ice. Tubes were immediately placed in a preheated thermocycler, RT was carried out and samples were immediately frozen at −40 °C.

8.3. Real-time PCR

The LightCycler™ software was configured to start a run. Faststart SYBR Green mastermix was prepared on ice. Fifteen microliters of mastermix were mixed with 5 μl of cDNA-template within LightCycler™ capillaries. Capillaries were centrifuged according to the manufacturer's protocol (700 × *g* for 5 s at room temperature) and immediately placed in the LightCycler™. Melting curve analysis was performed to identify the amplicons.

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