Quantification of nucleic acids of enteric viruses in concentrated environmental samples

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A B S T R A C T

This method describes a quantitative polymerase chain reaction (qPCR) and two triplex quantitative reverse transcription PCR (qRT-PCR) protocols for the quantitative detection of enteric viruses, namely norovirus GI and GII, sapovirus GI, hepatitis A and E viruses and human adenoviruses. The method is suitable for the detection of viruses in nucleic acid extracts of environmental samples. The assay also targets mengovirus that can be used as a quality control during sample processing prior to q(RT-)PCR. The detection and quantification limit of the assay allows the accurate quantification of low concentrations of viral nucleic acids and hence allows the accurate risk assessment of the target pathogens. The method is suitable for screening nucleic acid extracts derived from difficult matrices (e.g. wastewater, river water, sediment, shellfish, faecal matter).

Introduction

Human pathogenic viruses causing gastroenteritis (i.e. enteric viruses) are often found in the environment due to wastewater discharge. In order to assess and mitigate risks associated with the presence of these viruses in agricultural, recreational water, drinking water sources, aquatic sediment and shellfish, accurate methods for detection and quantification are essential. To date, there are only limited culturing-based methods that can be routinely used to assess virus titres in the environment. Therefore, molecular methods, such as polymerase chain reaction (PCR), are often used to detect and quantify pathogens. These methods target a short segment of the viral genome and hence give no information on the infectivity of the pathogens (Ronnqvist et al., 2014). Furthermore, PCR amplification may be inhibited by organic matter (e.g. humic substances) co-extracted with nucleic acids and hence may give false negative results (Meschke and Sobsey, 1998; Rock et al., 2010). Nonetheless, due to their sensitivity, accuracy and flexibility, PCR-based methods are widely used for the quantification of viruses.

Here we describe a singleplex qPCR and two triplex qRT-PCR assays for the accurate quantification of a range of enteric viruses. The two triplex quantitative reverse transcription PCR (qRT-PCR) assays described here targets enteric viruses (norovirus GI and GII and hepatitis A virus) that have been shown to commonly associate with waterborne and foodborne illnesses (Bosch et al., 2016). Emerging pathogens that have recently been associated with water (sapovirus and hepatitis E virus) were also targeted along with a process control (mengovirus). Mengovirus has very similar structure to hepatitis A virus and *Caliciviruses* and hence is often used to estimate the efficiency of sample processing and detection. Mengovirus is commercially available and easy to culture in vitro and hence a suitable control for RNA quantification and infectivity studies.

The singleplex qPCR assay targets human adenovirus, which has been shown to be a good indicator for wastewater contamination in the environment and may better represent the spread of enteric viruses in the environment that traditional indicators, such as coliform bacteria and bacteriophages (Rames et al., 2016).

These qPCR assays have been shown to be suitable for the quantification of viral nucleic acids in environmental concentrates derived from sediment, shellfish, wastewater, river water and seawater (Farkas et al., 2018). The assays have a limit of detection of 1 genome copy/reaction and limit of quantification of 3-40 genome copies/reaction (Farkas et al., 2017).

The two triplex qRT-PCR assays are based on the method described in Farkas et al. (2017). The adenovirus qPCR method has been published in Farkas et al. (2018).

Equipment

- Real-time PCR machine supporting TaqMan and SYBR Green chemistry
- Vortex
- Microfuge
- Pipettes, size 0.01 ml, 0.1 ml, 1 ml

Materials

- RNA Ultrasense One-Step qRT-PCR system #11732927 (Invitrogen)
- Quantifast SYBR Green master mix #204054 (Qiagen) or KAPA SYBR FAST qPCR Master Mix (2X) Universal #KK4600 (KAPA Biosystems) with ROX dye
- Bovine serum albumin (BSA), 1mg/ml
- PCR-grade water
- Pipette tips
- PCR-grade 1.5 mL centrifuge tubes
- qPCR optical plates with optical caps/ adhesive film
- Primers and probes as described in **Table 1**.
- Quantification standards (STDs) (**Error! Reference source not found.**)
    - Ten-times dilution series (10^5 – 10^0 copies/µl) of plasmid DNA standards incorporating the target sequences for each viral target.
    - Each STD should be used in duplicates.
- RT control (RTC)

- Viral RNA incorporating the target sequences for each RNA target, approx. 10^4 copies/µl. (derive from commercially available viral genomes or use sample mix known to be positive for the target viruses.)
- RTC should be used in duplicates.

- Non-template control (NTC)
  - PCR-grade water.
  - NTC should be used in duplicates.

Table 1. Primers and probes use in the triplex qRT-PCR and singleplex qPCR assays

Target virus	Primer/probe		Reference
	name	sequence	
Norovirus GI	QNIF4-F	CGCTGGATGCGNTTCCAT	Da Silva et al. (2007)
	NV1LC-R	CCTTAGACGCCATCATCATTTAC	Svraka et al. (2007)
	TM9-P	FAM-TGGACAGGAGATCGC-NFQMGB	Hoehne and Schreier (2006)
Sapovirus GI	CU-SV-F	TTGGCCCTCGCCACCTAC	Chan et al. (2006)
	CU-SV-R	CAAATTAGTGTTTGAGATGGAGGG	Chan et al. (2006)
	CU-SV-P	VIC-TGGTTCATAGGTGGTAC-NFQMGB*	Chan et al. (2006)
Hepatitis E virus	JVHEV-F	GGTGGTTTCTGGGGTGAC	Jothikumar et al. (2006)
	JVHEV-R	AGGGGTTGGTTGGATGAA	Jothikumar et al. (2006)
	JVHEV-P	ABY-TGATTCTCAGCCCTTCGC-QSY*	Jothikumar et al. (2006)
Norovirus GII	QNIF2-F	ATGTTCAGATGGATGAGATTCTCAGA	Loisy et al. (2005)
	COG2-R	TCGACGCCATCTTCATTCACA	Kageyama et al. (2003)
	QNIFS-P	FAM-AGCACGTGGGAGGGCGATCG-QSY	Loisy et al. (2005)
Hepatitis A virus	HAV68-F	TCACCGCCGTTTGCCTAG	Costafreda et al. (2006)
	HAV240-R	GGAGAGCCCTGGAAGAAAG	Costafreda et al. (2006)
	HAV150-P	VIC-CCTGGACCTGCAGGAATTAA-QSY*	Costafreda et al. (2006)
Mengovirus	Me110-F	GCGGGTCCTGCCGAAAGT	Pintó et al. (2009)
	Me209-R	GAAGTAACATATAGACAGACGCACAC	Pintó et al. (2009)
	Me147-P	ABY-ATCACATTACTGGCCGAAGC-NFQMGB*	Pintó et al. (2009)
Adenovirus	AdVs	CATGACTTTTGAGGTGGATC	van Maarseveen et al. (2010)
	AdVas	CCGGCCGAGAAGGGTGTGCGCAGGTA	van Maarseveen et al. (2010)

Method details

1. Mix the q(RT-)PCR reaction mix with ROX according to the manufacturers' instructions. ROX can be omitted from the mastermix. For information on the use of ROX, contact the qPCR machine's manufacturer.
2. Prepare the q(RT-)PCR master mixes containing water, the reaction and enzyme mixes, ROX, BSA, primers and probes for all samples and STDs, RTC and NTC according to **Table 2**.
   a. Prepare a mastermix of at least 20 reactions: 6x2 STDs, 1x2 RTC, 1x2 NTC, 1x2 sample and 2x reactions excess volume. Increase the volume of the mastermix by 1 reaction excess volume for every 10 reactions.
   b. If only one or two RNA viruses are targeted in the qRT-PCR reactions the corresponding primers and probes for the excluded targets can be omitted and replaced with water.
3. Distribute 17 µl of the mastermix in a qPCR plate. See **Figure 1** as an example.
4. Add 3 µl nucleic acid sample/STD/RTC/NTC in duplicates to the mastermix. See **Figure 1** as an example.
   a. The RTC is only used in the qRT-PCR reactions.
   b. The volume of samples/controls can vary between 1 and 5 µl. If less/more than 3 µl of the samples/controls are added the water volume in the mastermix should be changed accordingly.
   c. Always use the same volume of the samples, STDs, RTC and NTC in one q(RT-)PCR reaction.
5. Seal and spin/shake down the qPCR plate.

6. Place the plate into the qPCR machine and start the run as detailed in **Table 3**.
7. Analyse the results according to the recommendations of the manufacturer of the qPCR machine.

Table 2. Preparation of the qRT-PCR and qPCR master mix (c: concentration)

qRT-PCR-1	c	1x	qRT-PCR-2	c	1x	qPCR-AdV	c	1x
Water		2 µl	Water		2 µl	Water		2 µl
RNA Ultrasense reaction mix		4 µl	RNA Ultrasense reaction mix		4 µl	qPCR mix		4 µl
RNA Ultrasense enzyme mix		1 µl	RNA Ultrasense enzyme mix		1 µl	BSA	1 mg/ml	1 µl
BSA	1 mg/ml	1 µl	BSA	1 mg/ml		AdVs	10 µM	1 µl
QNIF4-F	10 µM	1 µl	QNIF2-F	10 µM		AdVas	10 µM	1 µl
NV1LC-R	20 µM	1 µl	COG2-R	20 µM				
TM9-P	5 µM	1 µl	QNIFS-P	5 µM				
CU-SV-F	AdVs	AdVs	HAV68-F	10 µM				
CU-SV-R	AdVas	AdVas	HAV240-R	20 µM				
CU-SV-P	5 µM	1 µl	HAV150-P	5 µM				
JVHEV-F	10 µM	1 µl	Me110-F	10 µM				
JVHEV-R	20 µM	1 µl	Me209-R	20 µM				
JVHEV-P	5 µM	1 µl	Me147-P	5 µM				

Sample -1	Sample -1	Sample -2	Sample -2	Sample -3	Sample -3						
RTC	RTC	NTC	NTC								
STD 10^5 copy/µl	STD 10^5 copy/µl	STD 10^4 copy/µl	STD 10^4 copy/µl	STD 10^3 copy/µl	STD 10^3 copy/µl	STD 10^2 copy/µl	STD 10^2 copy/µl	STD 10^1 copy/µl	STD 10^1 copy/µl	STD 10^0 copy/µl	STD 10^0 copy/µl

Figure 1. Example qPCR plate layout.

Table 3. qRT-PCR and qPCR run conditions. *: data capture

qRT-PCR-1		qRT-PCR-2		qPCR-AdV	
55°C – 60 min	1 cycle	55°C – 60 min	1 cycle	95°C – 5 min	1 cycle
95°C – 5 min	1 cycle	95°C – 5 min	1 cycle	95°C – 15 s 55°C – 60 s*	40 cycles
95°C – 15 s 56°C – 60 s 65°C – 60 s*	45 cycles	95°C – 15 s 60°C – 60 s 65°C – 60 s*	45 cycles	Melting curve analysis	
				95°C – 15 s	
				60°C – 60 s	0.5°C/s continuous*
				95°C – 15 s	Step and hold 5 s

Additional information

Quality control

1. Background signal should be minimised by adjusting the threshold manually if necessary.

2. If ROX was used the ROX signal should be very similar in all wells used. Wells with ROX spikes/lack of ROX signal should be omitted from analysis. For information on the use of ROX, contact the qPCR machine's manufacturer.
3. The NTC should be negative. Amplification was noted in the NTC wells indicates cross-contamination during plate preparation and hence he results are not reliable.
4. The RTC should be positive with concentration in the same order of magnitude as expected.
5. The STDs should be positive and the STD calibration curve slope should be 3.1-3.6 with qPCR efficiency of 90-110%.
6. The melting curve of the samples should be similar to the melting curve of the STDs. Samples with lower/higher melting temperature should be considered negative.

Health and safety

Standard microbiology safety precautions should be applied. Laboratories should perform a full risk assessment before performing this procedure.

Appendices

Appendix 1 qPCR standards

Some of the standards used in this protocol are commercially available, however, in some cases the plasmids need to prepared. A general overview of plasmid preparation is provided here with example reagents, which can be replaced with similar, commercially available products.

1. Acquire the target sequence
   Viral genomes can be purchased from the Public Health England (PHE) Culture Collection or the American Type Culture Collection (ATCC).
2. (RT-)PCR amplify the target sequence using the reagents and conditions described above.
3. Isolate the target sequence using 2% agarose gel electrophoresis with 50 bp DNA ladder (GenePilot, 50 bp ladder #239025, Qiagen).
4. Isolate the PCR product from the gel using a gel cleanup kit (ISOLATE II PCR and Gel Kit #BIO-52058, Bioline).
5. Ligate the PCR product to vector suitable for polyA-tailed inserts.
   Example using the pGEM®-T Vector System #A3600 (Promega):
   Mix 3 µl PCR product with 5 µl reaction buffer, 1 µl pGEM®-T Easy Vector and 1 µl T4 DNA ligase (3U/µl) and incubate at room temperature for 3 hours.
6. Transform the plasmid to competent cell.
   Example using the Alpha Select Bronze Competent Cells #BIO-85025 (Bioline):
   i. Mix 50 µl competent cells with 5 µl ligate.
   ii. Incubate on ice for 30 min.
   iii. Heat shock at 40°C for 30s.
   iv. Incubate on ice for 2 min.
   v. Add 200 µl Luria-Bertani (LB) solution
   vi. Incubate at 37°C 40 min.
   vii. Spread the solution on LB agar plates with ampicillin.
   viii. Incubate at 37°C for 14-18 hours.
7. Transfer a colony to LB solution with ampicillin. Incubate at 150-300 rpm at 37°C for 14-18 hours.
8. Purify the plasmid form solution using a plasmid cleanup kit (ISOLATE II Plasmid Mini Kit #BIO-52055 Bioline).
9. Determine the concentration of the plasmid solution using Nanodrop or Qubit. Dilute to a range suitable for qPCR.

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