Quantitative PCR SOPs for quantification of viruses in irrigation water

This SOP describes the performance of specific qPCR for 6 DNA viruses (human adenovirus (HAdV), JC polyomavirus (JCPyV), porcine adenovirus (PAdV), bovine polyomavirus (BPyV), ovine polyomavirus (OPyV) and avian parvovirus (Ch/TyPV)) and 1 RNA virus used as process control, MS2 phage.

All qPCRs have been tested by using the ABI PRISM®HID 7700 SDS from Applied Biosystems and the Mx3000P from Stratagene. However, several equipments can be used for running this SOP. The reactions take place in a 96-well optical reaction plate (Applied Biosystems. Cat. No. 4306737 for 20 units or 43426659 for 500 units) covered with ABI Prism™ optical adhesive covers (Applied Biosystems. Cat. No. 4311971 for 100 units or 4360954 for 25 units) and the optical adhesive cover starter kit (Cat. No 4313663). Optical caps and its corresponding installing tool can also be used (Cat. No 4323032 and 4330015).

Standards curves used in qPCR and qRT-PCR are generated by using serial dilutions of known amounts of a synthetic DNA construct containing the target region of the PCR. We strongly recommend storing the reference suspensions into DNA low binding tubes (Eppendorf Cat. No. 0030108-035 for 0.5 ml and Cat. No. 0030108-051 for 1.5 ml).

Reagents:

For qPCR: TaqMan Environmental PCR Master Mix (Applied Biosystems. Part nº 4396838) supplied in a 2x concentration.

For qRT-PCR: RNA Ultrasense™ One-Step Quantitative RT-PCR System (Invitrogen Cat.No.: 11732-927).

Mastermix preparation:

Prepare the mix in a clean separated area following the instructions provided:

qPCR	DNA	RNA
Environmental Mix 2x	12.5 µl	------
Ultrasense Mix 5x	------	5 µl
Primer Forward	1 µl	1 µl
Primer Reverse	1 µl	1 µl
Probe	0.5 µl	0.25 µl
ROX (dil 1/10)	------	0.5 µl
Enzime Mix	------	1.25 µl
H_2O	------	11 µl
TOTAL VOLUME OF MIX	15 µl	20 µl
Sample	10 µl	5 µl

The inclusion of 0.10x more mastermix reaction is recommended to avoid inaccuracies during pipetting. Once the mix has been prepared aliquot 15 µl of mastermix for DNA viruses and 20 µl of mastermix for RNA viruses. An example of a qPCR template at the end of the SOP is provided.

Primers and probes for qPCR and RT-qPCR

Virus	Ref	Name	Sequence (5'➔3')	Working solutions*	Final PCR conc
		AdF	CWTACATGCACATCKCSGG	22.5 µl	0.9 µM
HAdV	1, 2	AdR	CRCGGGCRAAYTGCACCAG	22.5 µl	0.9 µM
		AdP1	6-FAM-CCGGGCTCAGGTACTCCGAGGCGTCCT-BHQ1	11.25 µl	0.225 µM
		JE3F	ATGTTTGCCAGTGATGATGAAAA	10 µl	0.4 µM
JCPyV	3	JE3R	GGAAAGTCTTTAGGGTCTTCTACCTTT	10 µl	0.4 µM
		JE3P	6-FAM-AGGATCCCAACACTCTACCCCACCTAAAAAGA-BHQ1	6 µl	0.12 µM
		Q-PAdV-F	AACGGCCGCTACTGCAAG	22.5 µl	0.9 µM
PAdV	4	Q-PAdV-R	AGCAGCAGGCTCTTGAGG	22.5 µl	0.9 µM
		Q-PAdV-P	6-FAM-CACATCCAGGTGCCGC-BHQ1	11.25 µl	0.225 µM
		QB-F1-1	CTAGATCCTACCCTCAAGGGAAT	10 µl	0.4 µM
BPyV	5	QB-R1-1	TTACTTGGATCTGGACACCAAC	10 µl	0.4 µM
		QB-P1-2	6-FAM-GACAAAGATGGTGTGTATCCTGTTGA-BHQ1	6 µl	0.12 µM
		Ov_qFI	TTCTCACAGCTGCAGACATTG	22.5 µl	0.9 µM
OPyV	6	Ov_qRI	TCCAATCTGGGCATAAGATTTG	22.5 µl	0.9 µM
		Ov_qPI	6-FAM-ATGATTACCAAGCCAGACAGTGGG-BQH1	11.25 µl	0.225 µM
		Q-PaV-F	AGTCCACGAGATTGGCAACA	7.5 µl	0.3 µM
Ch/TyPV	7	Q-PaV-R	GCAGGTTAAAGATTTTCACG	22.5 µl	0.9 µM
		Q-PaV-P	6-FAM-AATTATTCGAGATGGCGCCCACG-BHQ1	12.5 µl	0.25 µM
		pecson-2F	AAGGTGCCTACAAGCGAAGT	25 µl	1 µM
MS2	8	pecson-2R	TTCGTTTAGGGCAAGGTAGC	25 µl	1 µM
		PecP-2	6-FAM-ATCGTGGGGTCGCCCGTACG-BHQ1	25 µl	0.25 µM
		QNIF4	CGCTGGATGCGNTTCCAT	12.5 µl	0.5 µM
NoVGI	**9,10,11**	NV1LCR	CCTTAGACGCCATCATCATTTAC	22.5 µl	0.9 µM
		TM9	6-FAM-TGGACAGGAGATCGC-MGB	25 µl	0.25 µM
		QNIF2d	ATGTTCAGRTGGATGAGRTTCTCWGA	12.5 µl	0.5 µM
NoVGII	**12,13**	COG2R	TCGACGCCATCTTCATTCACA	22.5 µl	0.9 µM
		QNIFS	6-FAM-AGCACGTGGGAGGGCGATCG-TAMRA	25 µl	0.25 µM
		HAV68	TCACCGCCGTTTGCCTAG	12.5 µl	0.5 µM
HAV	**14**	HAV240	GGAGAGCCCTGGAAGAAAG	22.5 µl	0.9 µM
		HAV150	6-FAM-CCTGAACCTGCAGGAATTAA-MGB	25 µl	0.25 µM
		JVHEVF	GGTGGTTTCTGGGGTGAC	25 µl	0.1 µM
HEV	**15**	JVHEVR	AGGGGTTGGTTGGATGAA	25 µl	0.1 µM
		JVHEVP	6-FAM-TGATTCTCAGCCCTTCGC-BHQ1	5 µl	0.25 µM
		NSP3-F	ACCATCTWCACRTRACCCTCTATGAG	10 µl	0.4 µM
RoV	**16**	NSP3-R	GGTCACATAACGCCCCTATAGC	10 µl	0.4 µM
		NSP3-P	6-VIC-AGTTAAAAGCTAACACTGTCAAA-MGB	10 µl	0.4 µM

* Volume of starting stock solution of primer or probe to prepare 100µl of working solution (adjust to 100ul with DNA-RNA free water). A starting stock solution of 100 µM for both primers and probe is assumed

Analysis of target viruses:

Add 10 µl of the nucleic acid extraction for DNA viruses or 5 µl of the nucleic acid extraction for RNA viruses per well in a separate area. Run direct and a ten-fold dilution in purified water of each extracted sample in duplicate.

Negative process control (NPC):

Add 10 µl of the nucleic acid extraction for DNA viruses or 5 µl of the nucleic acid extraction for RNA viruses per well from the negative process control extraction. If positive results are obtained samples belonging to that concentration batch should be not be used for calculations.

NTC:

Two wells for non-template controls (NTCs) should be included. NTC prove that mix does not produce fluorescence. Add 10 µl of molecular grade water for DNA viruses and 5µl for RNA viruses. Non-template controls shouldn't present signal (Cq). If an amplification signal (distinguishable from background fluorescence) is present, this would indicate cross-contamination or nonspecific amplification.

Negative extraction control:

Two wells containing the negative extraction controls should be included for each batch of extraction performed.

Both NTCs and negative extraction controls should be negative. If positive results are obtained in these controls all positive samples should be retested.

To avoid any further contamination with standard suspension is strongly recommended to add samples and negative controls firstly, cover this part of the microplate, add standard positive control in a separate area and cover this part of the plate . One option is to cut the adhesive cover in two parts, one for covering the samples, the other for covering the wells containing the standard suspension.

Standard suspensions (dsDNA):

In a separated area add 10^1,10^2,10^3,10^4 and 10^5 GC/10 µl per reaction for DNA viruses or $5x10^1$,$5x10^2$,$5x10^3$,$5x10^4$ and $5x10^5$ GC/5 µl for RNA viruses of the dsDNA control materials previously diluted in a suitable buffer (e.g. TE buffer). Use a micropipette exclusively used for this purpose. Add the reference suspension in duplicate ot triplicate.

Enzymatic inhibition control: (suggested)

It is advisable to evaluate potential enzymatic inhibition due to inhibitors present in each of the different environmental matrices evaluated. To do so, in representative samples of each matrix analyzed, an extra inhibition control could be used.

For DNA viruses:

1 µl of reference suspension containing 10^5GC/10 µl in 10 µl of water

1 µl of reference suspension containing 10^5GC/10 µl in 10 µl of sample

1 µl of reference suspension containing 10^5GC/10 µl in 10 µl of sample diluted 1:10

For RNA viruses:

1 µl of reference suspension containing 10^5GC/10 µl in 5 µl of water

1 µl of reference suspension containing 10^5GC/10 µl in 5 µl of sample

1 µl of reference suspension containing 10^5GC/10 µl in 5 µl of sample diluted 1:10

Process control:

Extract 140 µl of the stock suspension of MS2 phage used to spike all processed samples. A quantification of MS2 per sample will be performed to estimate virus recovery/extraction efficiency. If negative results are obtained or concentration efficiency is lower than <0.1% results obtained for this sample should be discarded.

TaqMan Assay parameters:

Perform the QPCR into an adequate system selecting the appropriate parameters:

Virus	Genome	qPCR cycles
HAdV	DNA	10min at 95°C, 40 cycles (15s at 95°C and 1min at 60°C)
JCPyV	DNA	10min at 95°C, 40 cycles (15s at 95°C and 1min at 60°C)
PAdV	DNA	10min at 95°C, 45 cycles (15s at 95°C, 20s at 55ºC and 20s at 60°C)
BPyV	DNA	10min at 95°C, 45 cycles (15s at 95°C, 30s at 60°C)
OPyV	DNA	2min at 50ºC, 10min at 95ºC, 40 cycles (15s at 95ºC, 1min at 60ºC)

Ch/TyPV	DNA	2min at 50ºC, 10min at 95ºC, 40 cycles (15s at 95ºC, 1min at 60ºC)
MS2	RNA	Following the RT-PCR (1h at 55°C), 5min at 95°C, 40 cycles (15s at 95°C, 1min at 60°C and 1min at 65°C)
NoVGI	RNA	Following the RT-PCR (1h at 55ºC), 5 min at 95ºC, 45 cycles (15s at 95ºC, 1 min at 60ºC, 1min at 65ºC)
NOVGII	RNA	Following the RT-PCR (1h at 55ºC), 5 min at 95ºC, 45 cycles (15s at 95ºC, 1 min at 60ºC, 1min at 65ºC)
HAV	RNA	Following the RT-PCR (1h at 55ºC), 5 min at 95ºC, 45 cycles (15s at 95ºC, 1 min at 60ºC, 1min at 65ºC)
HEV	RNA	Following the RT-PCR (30min at 50ºC), 15 min at 95ºC, 45 cycles (10s at 95ºC, 20s at 55ºC, 15s at 72ºC)
RoV	RNA	Following the RT-PCR (30 min at 48ºC), 10 min at 95ºC, 40 cycles (15s at 95ºC, 1 min at 60ºC)
MNV	RNA	

Once the run is completed store data and obtain the results as described in the user's manual of the equipment used.

Quality control

Standard precautions should be applied in all the manipulations in order to reduce the probability of sample contamination by amplified DNA molecules. Decontaminate the pipettes after each use. Use UV or cleaning products that can be obtained from your local pipette suppliers.

Analysis of results:

The amount of DNA will be defined as the median of the data obtained after correcting the dilution factor when needed.

Take into account that DNA suspensions containing less than 1 GC/reaction shouldn't present Cq values. Decimal dilutions of standard DNA or RNA suspensions should present Cq values within approximately 3 Cqs of difference between one dilution and the next ten-fold dilution. However, this may not be the case in environmental samples where direct dilutions sometimes show enzymatic inhibition (higher Cq values than expected considering the 1/10 dilution results); these results presenting inhibition should not be considered for quantification, considering only the assays with 1/10 dilutions.

All amplification plots shall be checked to identify false-positive results (reactions with C_q values not associated with exponential amplification) caused by high or uneven background signal. This shall be noted and results for any reactions affected in this way shall be regarded as negative.

The slope of the regression curve should be between 3.10 – 3.60 (corresponding to amplification efficiencies of ~90-110%). Points of a minimum of 3 dilutions of standard must be included in the regression curve. Curves with r^2 values of <0.98 or with slopes out of the range 3.10-3.60 should not be used for calculations.

Interpretation of results

If higher inhibition is observed/ expected the analysis of a -2 dilution may be considered. In samples showing Cq > 38, if two replicates are positive, we may consider the data as a robust and confirmed result. However, if only one positive signal with Cq > 38 is detected in one sample, the result will be considered as negative if no other assay to confirm the positive result is performed. It is advisable to repeat the assay or perform a nested-PCR assay on these samples to rule out non-specific amplification. If nested-PCR shows negative results the sample should be considered negative since no confirmation has been possible.

Examples:

D_1	**D_2**	**Dil_1**	**Dil_2**	**Conclusion**
35	35.4	38.2	38.7	ok, calculate mean value
35	~~38~~	38.2	38.7	probable mistake, calculate mean of the 3 valid data
~~35~~	~~35.4~~	35.2	36	potential inhibition, consider for quantification only 1/10 dilutions
39	38.6	no Ct	no Ct	ok, calculate mean value
38.2	no Ct	no Ct	no Ct	repeat, or confirm by nested-PCR
No Ct	no Ct	no Ct	39.3	repeat, or confirm by nested-PCR

D: direct, Dil: 1-fold dilution

Expression of results: The results will be expressed in the tables as the row Ct values obtained in each assay, with a blank in the wells not used for the calculations of the mean GC values. An additional column will show the mean value as GC/100 ml.

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METAWATER

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Example plate layout (HAdV)

HAdV dsDNA 10^1	HAdV dsDNA 10^1	HAdV dsDNA 10^2	HAdV dsDNA 10^2	HAdV dsDNA 10^3	HAdV dsDNA 10^3	HAdV dsDNA 10^4	HAdV dsDNA 10^4	HAdV dsDNA 10^5	HAdV dsDNA 10^5		
Cut with the scissors the optical adhesive and cover the sample wells to avoid any further contamination											
										CNP	CNP
Test sample1 (neat)	Test sample 1 (neat)	Test sample 1 (-1)	Test sample 1 (-1)	Test sample 2 (neat)	Test sample 2 (neat)	Test sample 2 (-1)	Test sample 2 (-1)	Test sample 3 (neat)	Test sample 3 (neat)	Test sample 3 (-1)	Test sample 3 (-1)
Test sample 4 (neat)	Test sample 4 (neat)	Test sample 4 (-1)	Test sample 4 (-1)	Test sample 5 (neat)	Test sample 5 (neat)	Test sample 5 (-1)	Test sample 5 (-1)	Test sample 6 (neat)	Test sample 6 (neat)	Test sample 6 (-1)	Test sample 6 (-1)
Test sample 7 (neat)	Test sample 7 (neat)	Test sample 7 (-1)	Test sample 7 (-1)	Test sample 8 (neat)	Test sample 8 (neat)	Test sample 8 (-1)	Test sample 8 (-1)	NTC	NTC	CNE	CNE

Standard suspension: 10µl for DNA viruses, 5µl for RNA viruses, 15 µl mastermix for DNA viruses and 20 µl for RNA viruses
Nucleic acid extraction: 10µl for DNA viruses, 5µl for RNA viruses, 15 µl mastermix for DNA viruses and 20 µl for RNA viruses
CNP: 10µl for DNA viruses, 5µl for RNA viruses, 15 µl mastermix for DNA viruses and 20 µl for RNA viruses using the control process NA extraction.
NTC: 10µl of molecular grade water for DNA viruses, 5µl of molecular grade water for RNA viruses, 15 µl mastermix for DNA viruses and 20 µl for RNA viruses
CNE: 10µl of negative extraction control for DNA viruses, 5µl of negative extraction control for RNA viruses, 15 µl mastermix for DNA viruses and 20 µl for RNA viruses

Example plate layout for process control (suggested process control MS2 phage)

MS2 dsDNA 10^1	MS2 dsDNA 10^1	MS2 dsDNA 10^2	MS2 dsDNA 10^2	MS2 dsDNA 10^3	MS2 dsDNA 10^3	MS2 dsDNA 10^4	MS2 dsDNA 10^4	MS2 dsDNA 10^5	MS2 dsDNA 10^5		
Test sample 1 (neat)	Test sample 1 (-1)	Test sample 2 (neat)	Test sample 2 (-1)	Test sample 3 (neat)	Test sample 3 (-1)	Test sample 4 (neat)	Test sample 4 (-1)	Test sample 5 (neat)	Test sample 5 (-1)	Test sample 6 (neat)	Test sample 6 (-1)
Test sample 7 (neat)	Test sample 7 (-1)	Test sample 8 (neat)	Test sample 8 (-1)	NTC	NTC	CNE	CNE				

Standard suspension: 5µl for dsDNA MS2 phage standard suspension and 20 µl of mastermix
Nucleic acid extraction: 5µl for extracted RNA viruses and 20 µl of mastermix
NTC: 5µl of molecular grade water for RNA viruses and 20 µl for RNA viruses
CNE: 5µl of negative extraction control for RNA viruses and 20 µl for RNA viruses