# Quantitative PCR SOPs for MST indicators

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).

| 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 |
| Enzim Mix | ------ | 1.25 µl |
| $H_2O$ | ------ | 11 µl |
| TOTAL VOLUME OF MIX | 15 µl | 20 µl |
| Sample | 10 µl | 5 µl |

| Virus | Ref | Name | Sequence (5'➔3') | Working solutions* | Final PCR conc |
|---|---|---|---|---|---|
| **HAdV** | 1, 2 | AdF | CWTACATGCACATCKCSGG | 22.5 µl | 0.9 µM |
| | | AdR | CRCGGGCRAAYTGCACCAG | 22.5 µl | 0.9 µM |
| | | AdP1 | 6-FAM-CCGGGCTCAGGTACTCCGAGGCGTCCT-BHQ1 | 11.25 µl | 0.225 µM |
| **JCPyV** | 3 | JE3F | ATGTTTGCCAGTGATGATGAAAA | 10 µl | 0.4 µM |
| | | JE3R | GGAAAGTCTTTAGGGTCTTCTACCTTT | 10 µl | 0.4 µM |
| | | JE3P | 6-FAM-AGGATCCCAACACTCTACCCCACCTAAAAAGA-BHQ1 | 6 µl | 0.12 µM |
| **PAdV** | 4 | Q-PAdV-F | AACGGCCGCTACTGCAAG | 22.5 µl | 0.9 µM |
| | | Q-PAdV-R | AGCAGCAGGCTCTTGAGG | 22.5 µl | 0.9 µM |
| | | Q-PAdV-P | 6-FAM-CACATCCAGGTGCCGC-BHQ1 | 11.25 µl | 0.225 µM |
| **BPyV** | 5 | QB-F1-1 | CTAGATCCTACCCTCAAGGGAAT | 10 µl | 0.4 µM |
| | | QB-R1-1 | TTACTTGGATCTGGACACCAAC | 10 µl | 0.4 µM |
| | | QB-P1-2 | 6-FAM-GACAAAGATGGTGTGTATCCTGTTGA-BHQ1 | 6 µl | 0.12 µM |
| **OPyV** | 6 | Ov_qFI | TTCTCACAGCTGCAGACATTG | 22.5 µl | 0.9 µM |
| | | Ov_qRI | TCCAATCTGGGCATAAGATTTG | 22.5 µl | 0.9 µM |
| | | Ov_qPI | 6-FAM-ATGATTACCAAGCCAGACAGTGGG-BQH1 | 11.25 µl | 0.225 µM |
| **Ch/TyPV** | 7 | Q-PaV-F | AGTCCACGAGATTGGCAACA | 7.5 µl | 0.3 µM |
| | | Q-PaV-R | GCAGGTTAAAGATTTTCACG | 22.5 µl | 0.9 µM |
| | | Q-PaV-P | 6-FAM-AATTATTCGAGATGGCGCCCACG-BHQ1 | 12.5 µl | 0.25 µM |
| **MS2** | 8 | pecson-2F | AAGGTGCCTACAAGCGAAGT | 25 µl | 1 µM |
| | | pecson-2R | TTCGTTTAGGGCAAGGTAGC | 25 µl | 1 µM |
| | | PecP-2 | 6-FAM-ATCGTGGGGTCGCCCGTACG-BHQ1 | 25 µl | 0.25 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

**Procedure:**

Prepare the mix in a clean separated area, including 0.10x more reaction for inaccuracies during pipetting. Once the mix has been prepared aliquot 15 µl if qPCR or 20 µl if qRT-PCR into each well including a non template control (NTC) to prove mix does not produce fluorescence. Non-template controls shouldn't present signal (Ct). If an amplification signal (distinguishable from background fluorescence) is present, this would indicate cross-contamination or nonspecific amplification.

Add samples (10 µl if qPCR or 5 µl if qRT-PCR) in a separate area. Run direct and a ten-fold dilution in purified water of each sample in duplicate. Cut the adhesive cover in two parts, one for covering the samples, the other for covering the wells containing the standard suspension. Cover the wells containing the samples to avoid contamination.

In a separated area add the reference suspension in triplicate. Use a micropipette exclusively used for this purpose.

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

| 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) |

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

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 Ct values. Decimal dilutions of standard DNA or RNA suspensions should present Ct values within approximately 3 Cts 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 Ct 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. If necessary the analysis of a -2 dilution may be considered. In samples showing Ct>38, if two replicates are positive, we may consider the data as a robust and confirmed result. However, if only one positive signal with Ct>38 is detected in one sample, the result will be considered as negative at least it is possible to confirm the result. For confirming the result it is advisable to repeat the assay or perform nested-PCR assays 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.The slope of the regression curve should be approximately -3.3 and the efficiency close to 100%.

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 inhibition or mistake |
| ~~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.

**Enzymatic inhibition control**

It is advisable to evaluate potential enzymatic inhibition due to inhibitors present in each of the different environmental matrices evaluated:

1 µl of reference suspension containing $10^4$GC/10 µl in 9 µl of water

1 µl of reference suspension containing $10^4$GC/10 µl in 9 µl of sample

1 µl of reference suspension containing $10^4$GC/10 µl in 9 µl of sample diluted 1:10

**Contamination**

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.

**References:**

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