EU VII FP PROJECT "VITAL"

STANDARD OPERATING PROCEDURE

SOP VITAL 022

Virus detection by (RT-)PCR: details on quality controls, virus detection and quantification

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AIM

To provide a convenient overview of the procedure to set up an (RT-)PCR correctly with respect to the number of replicates, the dilutions to be analysed, the controls to be included and the 96-wells plate layout.

PRINCIPLE

The analyses to be done can be divided into:

1) Implementation and detection of quality controls (Process controls and Internal Amplification Controls (IACs)).
2) Detection of target viruses (pathogenic and index viruses).
3) Quantification of target viruses and controls.

RELATED DOCUMENTS

- SOP 14: General Adenovirus qPCR
- SOP 15: Detection and quantification of porcine adenoviruses by real-time PCR
- SOP 16: Detection and quantification of bovine polyomavirus by real-time reverse transcriptase PCR
- SOP 18: Detection and quantification of norovirus by real-time reverse transcriptase PCR
- SOP 19: Detection and quantification of hepatitis A virus by real-time reverse transcriptase PCR
- SOP 20: Detection and quantification of hepatitis E virus by real-time reverse transcriptase PCR
- SOP 21: Detection and quantification of murine norovirus by real-time reverse transcriptase PCR
- SOP 23: Protocol for the establishment of IAC incorporation, optimized for each laboratory
- Document 'Controls worksheets.xls'
- Guidance documents for sampling concerning the production and processing phase and point of sale.

PROCEDURE

1.- Implementation and detection of quality controls:

- A Sample Process Control Virus (SPCV) is to be added to every sample to verify that the pre-amplification treatment has functioned correctly. In VITAL, muNoV is used as SPCV. Ten microliters of muNoV is added to every test sample prior to sample treatment (for details see SOPs on sampling and virus concentration and nucleic acid extraction).
- A Negative Target Sample Process Control (NTSPC) is to be included with every batch of samples, to detect contamination of the extraction and concentration reagents with

the target viruses or amplicons thereof. This control sample does not include a matrix (i.e. food or environmental material). The SPCV is added to the NTSPC to monitor the efficiency of virus concentration and nucleic acids extraction.

- A Negative (for SPCV) Control (NSPCVC) is to be included with every batch of samples, to detect contamination of any of the reagents with the target viruses, SPCV or amplicons thereof. The NSPCVC does not contain any matrix (*i.e.*, food or environmental material) or SPCV. Contamination with SPCV is important to detect, because the recovery of the method is estimated from the difference in concentration between spiked and detected SPCV per sample.
- A target specific IAC is to be included in each (RT-)PCR to monitor inhibition (see SOP 023 for details of IAC preparation). To guarantee that in all reactions the same amount of IAC is added, the IAC will be added to the master mix. Consequently, negative template controls (NTCs) do contain IAC. To control for contamination of any of the (RT-)PCR reagents with IAC RNA or DNA, a control is to be included which neither contains sample DNA/RNA nor IAC: the NIC (No-IAC Control). This control can be obtained by taking an amount of mastermix for two or more (RT-)PCRs prior to the addition of the IAC and add to this amount of mastermix ultrapure water instead of IAC. Continue the (RT-)PCR analysis together with the other samples. Although this control is important, it is optional as can be seen in the plate layouts for target viruses and sample process control virus (muNoV) (Figure 1 and 2).
- For correct interpretation of (RT-)PCR results and controls, the document 'Controls worksheets.xls' can be consulted.

2.- Detection of target virus:

- (RT-)PCRs have to be performed for all target viruses as duplicates, in at least two dilutions (thus two neat nucleic acid extracts and two 10-fold diluted extracts). Which target viruses to be detected in which samples can be found in the guidance documents provided for the production, processing and point of sale phases.
- If both 10-fold dilutions give a positive (RT-)PCR result, the sample should be tested in further 10-fold dilutions to determine the dilution in which no target is detected (the so-called end-point dilution).
- In all (RT-)PCRs performed to detect one of the target viruses, an IAC has to be included (see SOP 023 for details). As explained above, this control is included to estimate the amount of inhibition. In case of a failed (RT-)PCR reaction (*i.e.*, when no target and no IAC signal is present), further 10-fold dilutions of the nucleic acid extract should be analysed until the dilution is obtained that results in a detectable signal of the IAC or target virus.
- In every (RT-)PCR run which is performed, at least one, but preferably more, Non-Template Controls (NTCs) are to be included. The (RT-)PCR mastermix should be added to the NTC wells without addition of any sample nucleic acid extract. When the NTC (RT-)PCR gives a negative result for the target virus to be detected, then no contamination of primer- or mastermixes had occurred.
- In every (RT-)PCR run which is performed at least two Positive Amplification Controls (PACs) are to be included. These PACs will be the nucleic acids extracted from the

target virus or chimerical standards provided in the project. Nucleic acid sequences of these chimerical standards are identical to the sequence of the target viruses.

- For correct interpretation of (RT-)PCR results and controls, the document 'Controls worksheets.xls' can be consulted.

3.- Quantification of target viruses and controls:

- Virus concentrations will be determined as PCR-Detectable Units (PDU) using most probable numbers (MPN) estimation. Estimation of the number of PDU in the samples will be based on the end-point dilutions of 10-fold serially diluted RNA or DNA samples. PDU MPN-concentrations in undiluted samples are estimated by using the presence or absence of a (RT-)PCR signal in the neat solution and 10-fold dilutions on replicate (RT-)PCRs under the assumption that viral RNA or DNA is distributed homogeneously in samples and that negative samples do not contain viral RNA or DNA. In addition to the presence/absence data, the equivalent volume (EV) that is tested per samples needs to be reported to RIVM. This volume is the actual volume or weight of samples that is tested in a single (RT-PCR reaction. The VITAL tool (available at www.eurovital.org for download) can be used to calculate the EV for samples. **It is therefore essential to note down during sample preparation and RNA/DNA isolation all the parameters required for EV calculation as listed in the tool.**
- To control for inhibition of the (RT-)PCR, a target-specific IAC is included in all reactions (See SOP 023 for details). The IAC will be detected by a probe that targets a different sequence than the target virus probe, and is distinguished from the target probe by a different fluorescent label. Inhibition is usually caused by inhibitors present in the matrix. These inhibitors are usually absent in the negative template control (NTC). If similar Ct values of the IAC are obtained in the NTC and in the sample, then no inhibition of the (RT-)PCR occurred. If the Ct value of the IAC is higher in the sample than in the NTC, then the (RT-)PCR was partially inhibited. Inhibition is taken into account when genome copies are quantified by MPN.
- Recoveries of the whole procedure of virus concentration and nucleic acids extraction are determined based on the recovery of spiked SPCV (muNoV) and IAC. To quantify the number of genome copies of the SPCV-seed, a 1:10 dilution of the SPCV stock is added directly into the muNoV RT-PCR (Fig 2; SPCV-1). This analysis is done in duplicate. The recovery per sample subsequently can be estimated from the difference between number of genome copies of the muNoV stock and after virus concentration and nucleic acids extraction.

Table 1. Summary of the controls.

Abbrev.	Control	Explanation
NTSPC	negative target sample process control	Process control without sample added, with MNoV
NSPCVC	negative sample process control virus control	Process control without sample added, without MNoV
NTC	non-template control	(RT-)PCR control with ultrapure water instead of sample RNA/DNA, with IAC
NIC	no-IAC control	(RT-)PCR control with ultrapure water replacing sample RNA/DNA and IAC
PAC	positive amplification control	target virus RNA/DNA from samples or from plasmid standards produced by ITACyL
SPCV	sample process control virus	MNoV-seed from which MNoV is added to samples prior to the virus isolation process.

Figure 1: Plate layout for target viruses

	1	2	3	4	5	6	7	8	9	10	11	12
A	Neat	-1	Neat	-1	Neat	-1	Neat	-1	Neat	-1	Neat	-1
B	Neat	-1	Neat	-1	Neat	-1	Neat	-1	Neat	-1	Neat	-1
C	Neat	-1	Neat	-1	Neat	-1	Neat	-1	Neat	-1	Neat	-1
D	Neat	-1	Neat	-1	Neat	-1	Neat	-1	Neat	-1	Neat	-1
E	Neat	-1	Neat	-1	Neat	-1	Neat	-1	Neat	-1	Neat	-1
F	Neat	-1	Neat	-1	Neat	-1	Neat	-1	Neat	-1	Neat	-1
G	Neat	-1	Neat	-1	Neat	-1	NTSPC	NTSPC-1	NTC	PAC	NIC†	
H	Neat	-1	Neat	-1	Neat	-1	NTSPC	NTSPC-1	NTC	PAC	NIC†	

*PAC consists of target virus RNA/DNA, or chimerical standards for target viruses prepared by ITACyL, used as positive (RT-)PCR control.

† NIC (**N**o **I**AC **C**ontrol): these are OPTIONAL negative control reactions in which no IAC is included.

PLEASE NOTE: the target virus IAC is used in *every* well, including those containing the standards, the NTC and the NTSPC, **BUT NOT** in the NIC well.

Figure 2: Plate layout for Sample Process Control Virus (muNoV)

	1	2	3	4	5	6	7	8	9	10	11	12
A	Neat	-1	Neat	-1	Neat	-1	Neat	-1	Neat	-1	Neat	-1
B	Neat	-1	Neat	-1	Neat	-1	Neat	-1	Neat	-1	Neat	-1
C	Neat	-1	Neat	-1	Neat	-1	Neat	-1	Neat	-1	Neat	-1
D	Neat	-1	Neat	-1	Neat	-1	Neat	-1	Neat	-1	Neat	-1
E	Neat	-1	Neat	-1	Neat	-1	Neat	-1	Neat	-1	Neat	-1
F	Neat	-1	Neat	-1	Neat	-1	Neat	-1	Neat	-1	Neat	-1
G	Neat	-1	Neat	-1	SPCV -1*	NSPCVC neat	NSPCVC -1	NTSPC neat	NTC	PAC†	NIC‡	
H	Neat	-1	Neat	-1	SPCV -1*	NSPCVC neat	NSPCVC -1	NTSPC-1	NTC	PAC†	NIC‡	

* SPCV-1 is a 1:10 dilution of the MuNoV suspension used to spike samples, added directly to the (RT-)PCR.

† PAC consists of the standard solution of MuNoV RNA used as positive RT-PCR control.

‡NIC (**N**o **I**AC **C**ontrol): these are OPTIONAL negative control reactions in which no IAC is included.

PLEASE NOTE: the target virus IAC is used in *every* well, including those containing the standards, the NTC and the NTSPC, **BUT NOT** in the NIC well.