EU FP VII PROJECT "VITAL"

STANDARD OPERATING PROCEDURE

SOP VITAL 014

General protocol for the quantification of adenovirus by real-time PCR

CREATED:	REVISED:	APPROVED:
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— WARNING —

All samples and controls shall be handled by trained staff in a laboratory with appropriate equipment. Staff must be fully vaccinated against Hepatitis A and poliovirus. Persons using this SOP must be familiar with normal virology laboratory practice. This SOP does not presume to address fully all of the safety issues associated with its use. It is the responsibility of the user to establish appropriate health and safety practices and to ensure compliance with any national regulatory conditions.

AIM

To quantify adenovirus from food and environmental samples using quantitative real-time (Q)PCR.

PRINCIPLE

This protocol is based in the Standard Operating Procedure "Adenovirus QPCR" of the FP 6 Project VIROBATHE Contract 513648 (www.virobathe.org). The quantitative real-time PCR is based on the method described by Hernroth et al., 2002[1]. Standard curves used in the QPCR are generated by using purified viral genomic DNA. Different controls are needed to guarantee the quality of the assay: the use of uracil *N*-glycosylase (UNG) as a component of the PCR mix, a NTC (non template control) and an internal amplification control (IAC) for the presence of amplification inhibitors.

The original VIROBATHE SOP was tested using the ABI PRISM®HID 7700 SDS and the ABI PRISM®HID 7000 SDA from Applied Biosystems. However, other real-time PCR platforms can be used for performing this SOP.

For the basic terminology used in this SOP and further information on the principles on which QPCR (TaqMan assay) is based please go to:
http://docs.appliedbiosystems.com/pebiodocs/00105622.pdf

[1] Hernroth BE, Conden-Hansson AC, Rehnstam-Holm AS, Girones R, Allard AK. (2002) Environmental factors influencing human viral pathogens and their potential indicator organisms in the blue mussel, *Mytilus edulis*: the first Scandinavian report. Appl. Environ. Microbiol. 68: 4523-4533.

EQUIPMENT

- Real-time PCR platform
- Micropipettes of a range of sizes, 1000µl, 200µl and 20µl
- Gloves
- Vortex mixer
- Microcentrifuge
- Refrigerator
- Freezer

REAGENTS AND PLASTICWARE

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles.

- 96-well optical reaction plates (Applied Biosystems. Cat. No. 4306737 for 20 units or 43426659 for 500 units) or similar from other companies.
- Optical adhesive covers (Applied Biosystems. Cat. No. 4311971 for 100 units or 4360954 for 25 units) or similar from other companies.
- Optical adhesive cover starter kit (Cat. No 4313663) or similar from other companies
- Optical caps and their corresponding installing tool (Cat. No 4323032 and 4330015) or similar from other companies.
- DNA low binding tubes (Eppendorf Cat. No. 0030108-035 for 0.5 ml and Cat. No. 0030108-051 for 1.5 ml).
- Micropipette tips of a range of sizes, 1000µl, 200µl and 20µl
- TaqMan Universal PCR Master Mix (Applied Biosystems. Part nº.: 4304437): supplied in a 2× concentration. Contains Ampli*Taq* Gold DNA Polymerase, AmpErase UNG, dNTPs with dUTP, passive Reference and optimized buffer components.

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- Primers at a final concentration of 0.9 µM each.
    - Forward primer: AdF (5'- CWT ACA TGC ACA TCK CSG G-3')
    - Reverse primer: AdR (5'- CRC GGG CRA AYT GCA CCA G-3')
- Adenovirus TaqMan Probe: AdP1 (5'- FAM- CCG GGC TCA GGT ACT CCG AGG CGT CCT-BHQ*-3') at a final concentration of 0.225 µM.
- IAC MGB TaqMan probe: IACP (5'-VIC- CCA TAC ACA TAG GTC AGG –MGB-NFQ- 3'

* Black hole quencher or non-fluorescent quenchers are strongly recommended instead of TAMRA

PROCEDURE

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

1. Prepare the mix including 0.10× more reaction for inaccuracies during pipetting. Prepare mix in a clean separate area following the tables 1 and 2.

Table 1. Working solutions of primers and probe.
(A starting stock solution of 100 µM for both primers and probes is assumed)

	Stock volume	H_2O	Final volume*	Molarity
Primers	225 µl	275 µl	500 µl	45 µM
Probe AdP1	56.25 µl	443.75 µl	500 µl	11.25 µM
Probe IAC	12.5 µl	487.5 µl	500 µl	2.5 µM

* Distribute the final volume solution in 50 µl-aliquots

Table 2. QPCR mix (for one reaction)

Reagent	Working concentration	Final concentration	Volume (µl)
Mix	2×	1×	12.5
Primer AdF	45 µM	900 nM	0.50
Primer AdR	45 µM	900 nM	0.50
Probe AdP1	11.25 µM	225 nM	0.50
IAC probe	2.5 µM	50 nM	0.50
IAC	*	*	0.50
Total volume of mix			15
Sample			10
Final volume			25

*see SOP 023 for details on IAC preparation

2. Once the mix has been prepared aliquot 15µl into each well including the NTC (see Appendix 1). The total volume for one reaction after addition of target will be 25 µl (15 µl mix + 10 µl sample or standard).
3. Add samples (10 µl of the ten-fold dilution of the original sample) in a separate area.
4. Add DNA standard as positive control in duplicate.
5. Add 10 µl of nuclease-free dd-water in the NTC wells
6. Close wells with adhesive cover.
7. Perform the QPCR in a real-time PCR platform (ABI sequence detector system -Applied Biosystems- or equivalent), selecting the appropriate parameters (considering the use of adhesive cover and the total volume in each well, etc). Following activation of the UNG (2 min, 50°C) and activation of the Ampli*Taq* Gold for 10 min at 95°C, 45 cycles (15 s at 95°C and 1 min at 60°C) are performed.
8. Once the reaction is completed, store results and data as described in the user's manual of the equipment used.
9. The amount of DNA will be defined as the mean of the data obtained after correcting the dilution factor (10^{-1}).

APPENDIX 1: CONTROLS

Non-template control (NTC)

The assay must include a NTC to prove mix does not produce fluorescence. This control comprises 15 µl of QPCR mix and 10 µl of nuclease-free dd-water.

Internal amplification control (IAC)

The IAC must be added to every well, to verify that the reaction has worked and has not failed, e.g. through inhibition or instrument failure or operator error. Please see SOP no. 023 for details on the preparation of the IAC.

Contamination

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