

ViTAL	STANDARD OPERATING PROCEDURE	SOP VITAL 015
		Version: 3
	Detection and quantification of porcine adenoviruses by real-time PCR	Date: 18/02/2010
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EU FP VII PROJECT “VITAL”

STANDARD OPERATING PROCEDURE

SOP VITAL 015

Detection and quantification of porcine adenoviruses by real-time PCR

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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 detect and quantify porcine adenoviruses present in environmental samples using quantitative real-time (Q)PCR.

PRINCIPLE

This protocol is based in the information provided by the “Virus transmesos per l'ambient i els aliments” research group led by Prof. Rosina Girones¹

Standard curves used in QPCR are generated by using serial dilutions of known amounts of the Internal Amplification Control (IAC). Different controls are needed to guarantee the quality of the assay: the use of UNG (uracil N-glycosylase) as a component of the PCR mix, a NTC (non template control), and an IAC (see controls section below).

This SOP has been tested by using a Mx3000P (Stratagene) equipment. However, several alternative 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/pebi docs/00105622.pdf>

¹ Hundesa et al. Development of a qPCR assay for the quantification of porcine adenoviruses as an MST tool for swine fecal contamination in the environment . Submitted for publication

EQUIPMENT

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

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REAGENTS AND PLASTICWARE


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

- TaqMan Universal PCR Master Mix (Applied Biosystems. Part n°.: 4304437): supplied in a 2× concentration. Contains AmpliTaq Gold DNA Polymerase, AmpErase UNG, dNTPs with dUTP, passive Reference and optimized buffer components.
- Tris-EDTA pH 8 (Ambion, Applied Biosystems).
- Nuclease-free water.
- *EcoRI* restriction enzyme (New England Biolabs) or similar from other companies
- 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.
- Micropipette tips of a range of sizes, 1000µl, 200µl and 20µl
- *E. coli* JM109 competent cells (Promega, L2001) or similar from other companies.
- QIAGEN Plasmid Midi kit (Cat. No. 12143) or similar from other companies
- QIAGEN QIAquick PCR Purification kit

OLIGONUCLEOTIDES

- Primers at a final concentration of 0.9 µM each.
 - o Forward primer: PAdV-F (5'-AACGGCCGCTACTGCAAG-3') at
 - o Reverse primer: PAdV-R (5'- AGCAGCAGGCTCTTGAGG-3')
- Porcine Adenovirus TaqMan Probe: PAdV-P (5'- 6FAM-CACATCCAGGTGCCGC-BHQ1-3') at a final concentration of 0.225 µM.
- IAC MGB TaqMan probe: IACP (5'-VIC- CCA TAC ACA TAG GTC AGG -MGB-NFQ- 3' at a final concentration of 0.100 µM.

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

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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.

NOTE:

The reaction takes place in a 96-well optical reaction plate covered with optical adhesive covers and the optical adhesive cover starter kit. Optical caps and their corresponding installing tool can also be used.

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 ₂ O	Final volume*	Molarity
Primers	225 µl	275 µl	500 µl	45 µM
Probe PAdV-P	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 PAdV-F	45 µM	900 nM	0.50
Primer PAdV-R	45 µM	900 nM	0.50
Probe PAdV-P	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 25µl (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 with adhesive cover and take care not to touch the cover since finger prints could interfere with the fluorescent signal register by the thermocycler.
7. Perform the QPCR in a real-time PCR platform, selecting the appropriate parameters (considering the use of adhesive cover and the total volume in each well, etc). Following activation of the uracil N-glycosylase (2 min, 50°C) and activation of the AmpliTaq Gold for 10 min at 95°C, 45 cycles (15 s at 95 °C, 20 s at 55 °C and 20 s 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.

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9. The amount of DNA will be defined as the mean of the data obtained after correcting for the dilution factor (10^{-1}).

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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

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.