EU FP VII PROJECT "VITAL"

STANDARD OPERATING PROCEDURE

SOP VITAL 016

Detection and quantification of bovine polyomavirus 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 detect and quantify bovine polyomavirus present in environmental and food samples using quantitative real-time (Q-)PCR.

PRINCIPLE

This protocol is based in the information provided by the Prof. Girones's group from the University of Barcelona (Spain).

Standard curves used in QPCR are generated by using serial dilutions of known amounts of Internal Amplification Control (IAC). Different controls are needed to guarantee the quality of the assay: a NTC (non template control) and an IAC (see Appendix 1).

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

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.
    - Forward primer:

      QB-F1-1 (5'- CTA GAT CCT ACC CTC AAG GGA AT -3')
    - Reverse primer:

      QB-R1-1 (5'- TTA CTT GGA TCT GGA CAC CAA C -3')
- Bovine polyomavirus TaqMan Probe:

  QB-P1-2 (5'- FAM- GAC AAA GAT GGT GTG TAT CCT GTT GA -BHQ*-3').
- 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.

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_2O	Final volume*	Molarity
Primer QB-F1-1	100 µl	400 µl	500 µl	20 µM
Primer QB-R1-1	100 µl	400 µl	500 µl	20 µM
Probe QB-P1-2	30 µl	470 µl	500 µl	6 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 QB-F1-1	20 µM	400 nM	0.50
Primer QB-R1-1	20 µM	400 nM	0.50
Probe QB-P1-2	6 µM	120 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 15 µl 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 original sample and a ten-fold dilution) in a separate area.
4. Add 10 µl of the DNA standard plasmid of bovine polyomavirus in duplicate.
5. Add 10 µl of nuclease-free dd-water in the NTC wells
6. Close wells with adhesive cover or caps.
7. Perform the QPCR in a real-time PCR platform, selecting the appropriate parameters (considering 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 for the dilution factor.

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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