biosellal

HANDBOOK

Bio-T kit® PCV2 & PCV3

Cat. N° BIOTK072 - 50 reactions
Cat. N° BIOTK073 - 100 reactions

Detection and quantification of
Porcine Circovirus type 2 (PCV2) and Porcine Circovirus type 3 (PCV3)
by real-time PCR (qPCR)
with exogenous internal positive control (IPC)

SWINE

Sample Types

- Whole blood (on EDTA), serum
- Oral Fluids
- Organs
- Individual analysis or by pool up to 10 according to the matrix

Recommended nucleic acids (NA) extractions

- Magnetic beads extraction (e.g.: BioSellal – BioExtract® SuperBall® Cat. N° BES384)
- Silica membrane columns extraction (e.g.: BioSellal – BioExtract® Column Cat. N° BEC050 or BEC250)

Veterinary use only

DOCUMENTS MANAGEMENT

The Bio-T kit® PCV2 & PCV3 has two technical handbooks:

- The extraction handbook shared between the Bio-T kit® PCV2 & PCV3, PCV3, PRRSV and PRRSV DIVA, displaying BioSellal's recommended extraction protocols for each type of sample.
- The Bio-T kit® PCV2 & PCV3 qPCR handbook, presenting the instruction information to perform the qPCR.

The last versions in use for each handbook are indicated on the certificate of analysis (CA) provided with the Bio-T kit® PCV2 & PCV3.

Besides these two handbooks, a summary report of the validation and a performances confirmation handbook are available on request, contact BioSellal (contact@biosellal.com).

MODIFICATIONS MANAGEMENT

BioSellal indicates modifications done to this document by highlighting them using the rules presented in the Table below:

MODIFICATIONS MANAGEMENT			
Type of modification Highlighting color	**Minor modifications**	**Type 1 Major modifications**	**Type 2 Major modifications**
Impact on revision / version	**Change of revision date** **No change of version**	**Change of revision date** **+ change of version**	**Change of revision date** **+ change of version**
Examples of modifications	Corrections: typographical, grammatical or turns of phrase	EPC reference modification	Modification of Master Mix composition
	Addition of new sample type for extraction	Exogenous IPC reference modification	Modification of validated extraction protocol
	Addition of information giving more details or alternative protocol		
	Addition/Suppression of optional information		

PRESENTATION

Recommendations for sampling, shipping and storage of samples

Real-time PCR is a powerful technique allowing the detection of few amounts of pathogen genome. Genome can be rapidly degraded depending on the pathogen nature (bacteria / parasites, enveloped viruses...), the genome nature (DNA / RNA) and the sample type (presence of DNase / RNase). Thus, BioSellal recommends the following instructions to guarantee an optimal diagnosis.

Sampling

To prevent cross-contamination between samples leading to false positive results, it is mandatory to use disposable materials for single use and to avoid direct contact between specimens.

Shipping

For circoviruses diagnosis:
It is recommended to ship soon as possible after sampling, under cover of positive cold.
In case of differential diagnosis for circoviruses and PRRSV on the same sample:
It is mandatory to ship immediately after sampling or by default to store it at ≤ -16°C. Shipment has to be done within 24h under cover of positive cold.

Storage after reception

For circoviruses diagnosis:
Recommended storage of samples at 5°C ± 3 for a maximum of 7 days and ≤ -16°C beyond.
In case of differential diagnosis for circoviruses and PRRSV on the same sample:
It is recommended to immediately analyze samples after receipt or freezing at ≤ -16 ° C for a few months and ≤ -65°C beyond 1 year.

PIG Line

This kit belongs to the PIG line which gather a set of kits sharing common extraction and qPCR protocols. It is compatible with BioSellal's other kits belonging to the AVIAN line. (information available on www.biosellal.com).

Description of the Bio-T kit® PCV2 & PCV3

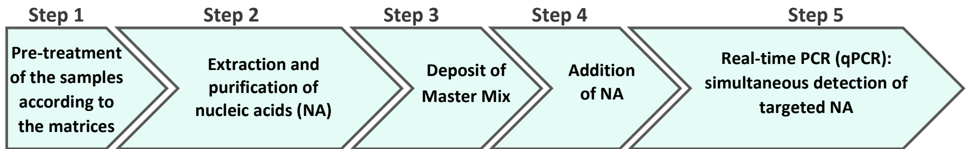
The Bio-T kit® PCV2 & PCV3 (Cat. N° BIOTK072/BIOTK073) contains a ready to use **PCR Master Mix** allowing the detection **in the same reaction well of**:

- **Porcine Circovirus of type 2 (PCV2)** using a 6-FAM labelling,
- **Porcine Circovirus of type 3 (PCV3)** using a VIC labelling,
- **An exogenous positive control DNA IPC** (Cy5 labelling), to add during the extraction step in order to validate the quality of the nucleic acids during the extraction and the absence of PCR inhibitors.

This kit, based on the detection and the quantification of PCV2 and PCV3 (relative quantification toward a reference material set at the interpretation threshold or MRSI) from blood, serum, oral fluids or organs, was developed and validated according to the **French regulatory standard NF U47-600-2 edited by AFNOR for the PCR part.**

Extraction protocols recommended by BioSellal are described in the extraction handbook shared between the Bio-T kit® PCV2 & PCV3, PCV3, PRRSV and PRRSV DIVA.
The pool up to 10 is possible for whole blood (on EDTA) and serum matrices with all Bio-T kit® mentioned before.

Description of the whole process



Extraction handbook for Bio-T kit® PRRSV, PRRSV DIVA, PCV3, PCV2 & PCV3		Bio-T kit® PCV2 & PCV3 qPCR handbook		
Blood, serum Oral Fluids * Organs*	BioExtract® SuperBall® BioExtract® Column	Master Mix ready-to-use MMPCV2&3-A	Samples NC/NCS Process Positive control or MRSI EPC (EPCPCV2&3-A)	Detectors: FAM/VIC/Cy5 Passive Reference: ROX Programs: PIG/AVIAN without RT PIG/AVIAN with RT using Fast or Standard ramping

*** pre-treatment mandatory**

Kit content and storage conditions

Table 1. Kit content description

Description	Reference	Volume / tube BIOTK072 50 reactions	Volume / tube BIOTK073 100 reactions	Presentation	Storage
Master Mix (MM) Ready-to-use	MMPCV2&3-A	1000 µl	2x1000 µl	**White** cap tube Bag A	≤-16°C In a dark place, « MIX » area
Exogenous Internal Control (IPC) Exogenous amplification control	IPC-A	250 µl	2x250 µl	**Pink** cap tube Bag B	≤-16°C « Extraction » area
External Positive Control (EPC) PCV2 & PCV3 amplification positive control	EPCPCV2&3-A	110 µl		**Orange** cap tube Bag C	≤-16°C « Nucleic Acids (NA) addition » area
Water RNase/DNase free	Aqua-A	1 ml		**Blue** cap tube Bag C	5°C ±3 or ≤-16°C « Nucleic Acids (NA) addition » area

Kit reagents are stable until the expiration date stated on the label, subject to compliance with good storage conditions.

List of consumables and reagents not provided with the kit

Table 2. Consumables and reagents not provided with the kit

Consumable / Reagent	Description	Provider	Cat. N°
ATL Buffer	Lysis buffer	BioSellal	ATL19076
BioExtract® Column	DNA/RNA column Extraction kit (50)	BioSellal	BEC050
BioExtract® Column	DNA/RNA column Extraction kit (250)	BioSellal	BEC250
BioExtract® SuperBall®	DNA/RNA Extraction kit Magnetic beads (4 x 96)	BioSellal	BES384

For thermal cycler's consumables: refer to the device instruction of use.

List of reagents for performances validation

To confirm performances of your thermal cycler(s), for absolute quantification, or to constitute the reference material set at the interpretation threshold (MRSI), PCV2 or PCV3 DNA (quantified in copies/qPCR, cADN-PCV2-001 / cADN-PCV3-001) used by BioSellal in the validation file, are required. Ready to use MRSI may also be provided. These MRSI consist of a PCV2 and PCV3 negative blood sample that has been supplemented with a titrated DNA of PCV2 or PCV3 at a level of 10^6 GE / ml of blood, a level considered to be an indicator of high viremia. BioSellal commercializes this reagents under the references listed below:

Table 3. Optional Reagents *

Reagent	Description	Provider	Cat. N°
PCV2 DNA	Quantified PCV2 DNA (1×10^6 copies/qPCR)	BioSellal	cADN-PCV2-001
PCV3 DNA	Quantified PCV3 DNA (1×10^6 copies/qPCR)	BioSellal	cADN-PCV3-001
PCV2 MRSI for blood sample	Blood positive for PCV2 at 10^6 GE / ml	BioSellal	MRSI-PCV2-001
PCV3 MRSI for blood sample	Blood positive for PCV3 at 10^6 GE / ml	BioSellal	MRSI-PCV3-001

*These reagents are available on request only, contact BioSellal (contact@biosellal.com).

Main critical points

- Wear appropriate personal protective equipment (lab coat, disposable gloves frequently changed).
- Work in dedicated and separate areas to avoid contamination: "Extraction" (unextracted samples storage, extraction equipment area), "MIX" (ready to use Master Mix storage, qPCR plates preparation), "Nucleic acids (NA) Addition" (Nucleic Acid storage and addition of extracted Nucleic Acid and controls in the qPCR plate), "PCR" (final area containing the thermal cycler(s)).
- Use dedicated equipment for each working area (gloves, lab coat, pipettes, vortex, ...).
- Use filter tips.
- Before use, thaw all components at room temperature.
- Vortex and spin briefly (mini-centrifuge) all reagents before use.
- It is recommended to not exceed 3 freeze-thawing cycles for samples, lysates, reagents and extracted nucleic acids. Depending on the use, we recommend to make aliquots using appropriate volume.
- Genomes of pathogens detected by the **PIG line** kits can be DNA or RNA. **Working with RNA is more demanding than working with DNA** (RNA instability and omnipresence of the RNases). For these reasons, special precautions must be taken:
  - Always wear gloves.
  - Treat all surfaces and equipment with RNases inactivation agents (available commercially).
  - When wearing gloves and after material decontamination, minimize the contact with surfaces and equipment in order to avoid the reintroduction of RNases.
  - Use "RNase free" consumable.
  - It is recommended to store the RNA at +5°C±3°C during the manipulation and then freeze it as soon as possible, preferably at ≤-65°C or by default at ≤-16°C.
  - Open and close tubes one by one in order to limit the opening times and avoid any contact with RNases present in the environment (skin, dust, working surfaces…).

qPCR DETECTION OF PCV2 AND PCV3 USING BIOTK072/BIOTK073 KITS

Global procedure

1) **Establish plate setup** defining each sample position and **including the following controls** described below:

- **Negative Control Sample (NCS)**: water or PBS replaces the sample from the initial extraction step to the end of the process. This control is mandatory for each run of extraction.

- **Negative PCR Control (NC)**: 5 µl of RNase/DNase free water replaces the 5 µl of extracted nucleic acids in the qPCR plate. The provided tube Aqua-A (**blue** cap tube) can be used. This control is recommended when using the kit for the first time or to verify the absence of Master Mix contamination.

- **PCR positive control amplification for PCV2 and PCV3** (**EPC,** tube **EPCPCV2&3-A**, **orange** cap): synthetic DNA, titrated, containing the targeted sequences specifics of PCV2 and PCV3. This control is mandatory except in the case of the use of a positive control sample (e.g.: MRSI).

- **For absolute quantification:** Standard curves for PCV2 and PCV3. Quantified PCV2 and PCV3 DNA, (cADN-PCV2-001, cADN-PCV3-001) must be diluted in a serial manner. See page 14 or please refer to the performance confirmation handbook for more details.

  ⚠ CAUTION: *EPC and standards tube handling represents a nucleic acid contamination hazard, it is thus recommended to open and handle it in a restricted area, away from other PCR components and to take precautions to avoid cross-contamination with nucleic acids extracts during deposit on the PCR plate.*

- If available, **a Process Positive Control (MRI),** a weak positive sample of oral fluids or organs is extracted in parallel with tested samples. After qPCR, MRI Ct value will be monitored on a Shewhart control card. Obtaining conform Ct values validate the whole process. In this case, the use of the EPC, provided with the kit, is not mandatory.

- For relative quantification on blood and serum samples: **MRSI** (reference material set at the interpretation threshold) constituted by a negative blood sample for PCV2 and PCV3 supplemented at **10^6 GE / ml of blood** with a titrated DNA of PCV2 or PCV3**.** This viral load in blood and serum was determined based on published data (eg Olvera *et al.,* J Virol Methods. 2004 Apr; 117(1):75-80.) and is considered as a high viral load for PCV2. BioSellal has extrapolated this level to PCV3. These MRSI samples must be extracted in parallel with tested samples. After qPCR, MRSI Ct values will be monitored on a Shewhart control card. Obtaining conform Ct values validate the whole process. In this case, the use of the EPC provided with the qPCR kit is not mandatory. BioSellal sells these MRSI (MRSI-PCV2-001; MRSI-PCV3-001).

2) qPCR plate preparation

In the "MIX" dedicated area

1. After thawing, vortex and rapid centrifugation, **transfer 20 µl Master Mix MMPCV2&3-A** (white cap) in each well of interest (samples and controls).

In the "Nucleic Acid addition" dedicated area

2. **Add 5 µl of extracted nucleic acids (or NCS, water, MRI, MRSI**, **standards or EPC**: **EPCPCV2&3-A** orange cap tube) in each well of interest. Make sure to pipet out in the bottom of the well, in the Master Mix, and to avoid the formation of bubbles.

3. **Seal the plate with an optically clear sealer** or **close the strip caps**.

In the "PCR" amplification dedicated area

4. **Define the thermal cycler parameters** (see Table 4, Table 5, Table 6)

5. It is recommended to **spin the plate down prior to place it in the thermal cycler**, to prevent drops in the well pit walls.

6. Start the qPCR program. Approximate run time: 70 min.

3) Thermal cycler settings

This kit was developed and validated on ABI PRISM® 7500 Fast (Applied Biosystems) in standard ramping and confirmed on AriaMx™ (Agilent Technologies, Fast ramping by default) and ABI PRISM® 7500 Fast (Applied Biosystems) in Fast ramping. For other thermal cycler(s), contact our technical support.

Table 4. Thermal cycler configuration

	ABI PRISM® 7500 Fast	AriaMx™
Mode	Quantitation – Standard curve	Quantitative PCR, Fluorescence Probe
Ramping	Standard or Fast Ramping	Fast Ramping by default
Passive Reference	**ROX**	**ROX**

Table 5. Thermal cycler Settings

Target	Detectors		Final Volume / well
	Reporter	Quencher	
PCV2	FAM	NFQ-MGB or None*	**25 µl** = 20 µl Master Mix + 5 µl extracted nucleic acids or controls†
PCV3	VIC	NFQ-MGB or None*	
Exogenous IPC	Cy5	NFQ-MGB or None*	
To assign to samples and controls†			

* Depends on the thermal cycler model. Do not hesitate to contact the BioSellal Technical Support (tech@biosellal.com)
† Controls are NC (water), NCS (extracted water) and/or EPC/ extracted MRI or MRSI.

Table 6. PIG/AVIAN Amplification program settings without RT †

Standard or Fast Ramping		
Cycles	Time	Temperature
1 cycle	5 min	95°C
40 cycles	10 sec	95°C
	45 sec + data acquisition	60°C

† optional step, in case of simultaneous detection of RNA genomes. Achieving a reverse-transcription (RT) step prior to PCR for the amplification of RNA genomes has no impact on the performances of the Bio-T kit® PCV2 & PCV3 (see the validation file).
NB: This amplification program is compatible with all Bio-T kit® of the PIG and AVIAN LINES.

RESULTS INTERPRETATION

To analyze and interpret the signals obtained by qPCR, the Threshold must be set up.
The threshold must be assigned carefully in order to obtain the most reproducible result between different manipulations according to the requirements defined in Annex C of the French Standard **NF U47-600 (part 1)**. A consistent set of positives controls, usually an In-house Reference Material (MRI) or the EPC, is used to set the threshold value above the baseline and in the exponential amplification phase of the plot.
The Threshold Cycle, named « Ct » or « Cq » (depending on thermal cycler(s), corresponds to the intersection between the amplification curves and the threshold line. It allows the relative measurement of the concentration of the target in the PCR reaction when a calibrated extract is analyzed in the same series.
The qPCR series is validated if the controls (EPC, MRI, MRSI, NCS and NC) present valid results, then the result of each sample can be interpreted.

Main Scenarios

Controls Reading

<table>
<tr><th colspan="5">Table 7. PCR Controls results interpretation</th></tr>
<tr><th rowspan="2"></th><th colspan="3">Targets</th><th rowspan="2">Interpretation</th></tr>
<tr><th>PCV2 (FAM)</th><th>PCV3 (VIC)</th><th>Exogenous IPC (Cy5)</th></tr>
<tr><td rowspan="3">NCS
Negative Control Sample
MANDATORY</td><td>Neg</td><td>Neg</td><td>Pos</td><td>Valid</td></tr>
<tr><td colspan="2">At least one of the two targets
Pos</td><td>Pos</td><td>Contamination with a positive sample during extraction step or during qPCR plate preparation.</td></tr>
<tr><td>Neg</td><td>Neg</td><td>Neg</td><td>Omission of exogenous IPC DNA addition?
Defective extraction ?</td></tr>
<tr><td rowspan="2">NC
Negative PCR Control
OPTIONAL</td><td>Neg</td><td>Neg</td><td>Neg</td><td>Valid</td></tr>
<tr><td colspan="3">At least one of the three targets
Pos</td><td>Contamination with a negative or a positive sample during PCR plate preparation?
or Master Mix / Water contamination?</td></tr>
<tr><td rowspan="3">EPC
PCV2 and PCV3 PCR external positive control
MANDATORY
IN ABSENCE OF MRSI</td><td>Pos*</td><td>Pos*</td><td>Neg</td><td>Valid</td></tr>
<tr><td>Neg</td><td>Neg</td><td>Neg</td><td>Problem during qPCR plate preparation:
Master Mix error? EPC omission?</td></tr>
<tr><td>Pos*</td><td>Pos*</td><td>Pos</td><td>Contamination with a positive sample or control during qPCR plate preparation?</td></tr>
<tr><td rowspan="3">Sample process positive Control MRI/MRSI
RECOMMENDED
IF AVAILABLE</td><td>Pos†</td><td>Pos†</td><td>Pos¥</td><td>Valid</td></tr>
<tr><td>Neg</td><td>Neg</td><td>Neg</td><td>Problem during qPCR plate preparation:
Master Mix error? Nucleic acid extract omission or extract not in contact with Master Mix?
Process drift: extraction and/or qPCR ?</td></tr>
<tr><td>Neg</td><td>Neg</td><td>Pos¥</td><td>Process drift: extraction (in case of exogenous IPC addition directly into qPCR plate and not during extraction)
Problem with MRSI/MRI preparation?
Degradation of the sample process positive control?</td></tr>
</table>

* The Ct value obtained must be conform with the value indicated on the Certificate of Analysis (CA).

† The Ct value must be included within control card limits.

¥ The Ct value obtained depends on the thermal cycler(s), on the sample type and on the extraction protocol used. This value must be, at least, included within the range specified on the certificate of analysis (CA). Ct values for IPC using the recommended extraction protocols are available upon request. BioSellal recommends you to determine your own maximal IPC Ct value depending on your own extraction method and thermal cycler.

Samples Reading

Table 8. Different types of results obtained for the samples

Targets			Interpretation
PCV2 (FAM)	**PCV3 (VIC)**	**Exogenous IPC (Cy5)**	
Neg	**Neg**	**Pos***	**Negative or Undetected**
Pos	**Pos**		**Positive or Detected** **Relative Quantification possible** **(cf Table 9)**
At least one of two targets **Pos**			**Positive or Detected for the positive target** **Negative or Undetected for the negative target** **Relative Quantification possible** **(cf Table 9)**
Pos	**Pos**	**Neg or Ct>35**	**Positive or Detected** **Relative quantification not possible** Problem during the IPC addition? Presence of inhibitors‡? Competition with the main target?
One of the targets is **Neg**		**Neg or Ct>35**	**Positive or Detected** **for the positive target** **Relative quantification not possible** **Uninterpretable for the negative target :** **Risk of low positive sample non- detection** = Repeat the analysis for the negative target IPC exogenous omission during the extraction and/or qPCR Presence of inhibitors‡? Nucleic acids degradation in the sample? Extraction problem? Competition with the main target?
Neg	**Neg**	**Neg or Ct>35**	**Uninterpretable** **Risk of low positive sample non- detection** = Repeat the analysis Nucleic acids extract omission or extract not in contact with Master Mix ? Presence of inhibitors‡? Nucleic acids degradation in the sample? Problem during the IPC addition? Extraction problem?

* The Ct value obtained depends on the thermal cycler(s), on the sample type and on the extraction protocol used. This value must be, at least, included within the range specified on the certificate of analysis (CA). Ct values for IPC using the validated extraction protocols are available upon request. BioSellal recommends you to determine your own maximal IPC Ct value depending on your own extraction method and thermal cycler(s).

‡ If an inhibition is suspected, 1) Repeat the qPCR with a pre-dilution of the extracted NA at 1/10 or 1/100 in DNase/RNase free water or 2) Repeat the analysis from the extraction.

Relative Quantification of positive samples toward MRSI Ct value (MRSI 10^6 GE/ml blood) – individual analysis

Using relative quantification, samples Ct values are compared to the MRSI Ct values extracted in the same series. This comparison enables to estimate the relative PCV2 or PCV3 viral load (GE) per ml of blood relative to that of MRSI.
The MRSI standard corresponds to PCV2 and PCV3 negative blood sample supplemented at 10^6 GE/ml of blood with a titrated DNA of PCV2 or PCV3. This viral load in blood and serum was determined based on published data (eg Olvera *et al.,* J Virol Methods. 2004 Apr; 117(1):75-80.) and is considered as a high viral load for PCV2. BioSellal has extrapolated this level to PCV3.

1) Follow samples reading rules presented in Table 8 (qualitative analysis).
2) Interpret the results relative to the MRSI Ct values obtained, as indicated in Table 9.

Table 9. PCV2 or PCV3 qPCR Results Interpretation using relative quantification		
qPCR Results		**Interpretation**
Negative		**Not detected** **PCV2 or PCV3 GE quantity < LD_{PCR}**
Positive	Ct > Ct MRSI*	**Positive: PCV2 or PCV3 load < MRSI* load**
	Ct = Ct MRSI*	**Positive: PCV2 or PCV3 load is equal to the MRSI* load**
	Ct < Ct MRSI*	**Strong Positive: PCV2 or PCV3 load > MRSI* load**

* : including the uncertainty interval.

It is recommended to monitor the MRSI Ct values on Shewhart control card to insure the accuracy of the analytical process.

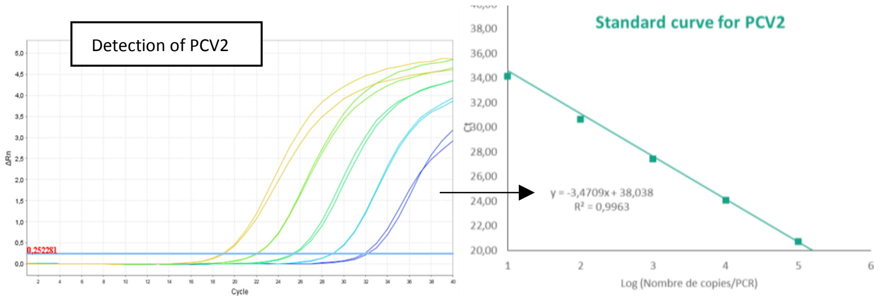
Absolute Quantification

This kit enables an absolute quantification (determination of the exact PCV2 and/or PCV3 viral load into the sample). To do this, quantified PCV2 and PCV3 DNA, that could be supply by BioSellal (cADN-PCV2-001, cADN-PCV3-001), must be diluted in a serial manner to perform a 6-points calibration line for each target linking viral load (GE/ml) to Ct values (see table 10). PCV2 and PCV3 DNA are titrated at 1 x 10^6 copies/qPCR. Using extraction methods recommended by BioSellal, these quantities can be extrapolated to 1.2 x 10^8 GE/ml of blood by estimating an extraction yield of 100% and considering one target sequence copy by genome (GE). Under these conditions, the MRSI equivalent levels correspond approximately to 1/100 dilutions of the titrated DNA.
For more details, please refer to the performances confirmation handbook.

Table 10. absolute quantification using quantified DNA			
Name	**Concentration level (GE/ml of blood)**	**Concentration level (copies/PCR)**	**Dilutions to achieve**
Std A	**1.2 x 10^8**	**10^6**	Corresponds to the synthetic DNA of PCV2 or PCV3 tube
Std B	**1.2 x 10^7**	**10^5**	Dilute 10 µL of synthetic DNA of PCV2 or PCV3 in 90 µL water or TE (=1/10 dilution)
Std C	**1.2 x 10^6**	**10^4**	Dilute 10 µL of the previous tube in 90 µL water or TE (=1/100 dilution)
Std D	**1.2 x 10^5**	**10^3**	Dilute 10 µL of the previous tube in 90 µL water or TE (=1/1 000 dilution)
Std E	**1.2 x 10^4**	**10^2**	Dilute 10 µL of the previous tube in 90 µL water or TE (=1/1 000 dilution)
Std F	**1.2 x 10^3** (=LQ_{PCR})	**10** (=LQ_{PCR})	Dilute 10 µL of the previous tube in 90 µL water or TE (=1/10 000 dilution)

In order to obtain the standard curve, the 6 wells corresponding to 6 points calibration line must be qualified as "standard" when setting the thermal-cycler sowftware, and assign them a concentration value of PCV2 or PCV3.

Example of standard curve obtained for absolute quantification:



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