ViroReal® Kit PCV2

Manual

For use with the

- **ABI PRISM® 7500 (Fast)**
- **Mx3005P®**
- **LightCycler® 480**

For veterinary use only

REF **DVEV00511, DVEV00513** Σ **100**

REF **DVEV00551, DVEV00553** Σ **50**

ingenetix GmbH
Arsenalstraße 11
1030 Vienna, Austria
T +43(0)1 36 198 0 198
F +43(0)1 36 198 0 199
office@ingenetix.com
www.ingenetix.com

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1. Product description

ViroReal® Kit PCV2 is a real-time PCR assay for detection of DNA of the porcine circovirus type 2 (PCV2) including the three genotypes A, B and C known so far. This test was developed and validated for the ABI PRISM® 7500 (Fast) instrument (Thermo Fisher Scientific), LightCycler® 480 (Roche) and Mx3005P® (Agilent), but is also suitable for other real-time PCR instruments. This test allows the rapid and sensitive detection of DNA of PCV2 from samples purified from serum, urine, faeces and tracheobronchial swab specimens (e.g. with the QIAamp DNA Mini Kit or QIAamp DSP Virus Kit).

ViroReal® Kit PCV2 detects the ORF1 of the porcine circovirus type 2. A probe-specific amplification-curve at 530 nm (FAM channel) indicates the amplification of PCV2 specific DNA.
The assay is designed to detect very small amounts of PCV2 but does not detect PCV1 or PPV. The assay allows determination of PCV2 genomic copy numbers in sample material. A quantitative real-time PCR standard can be ordered separately (order no. PC100). Pigs with 10^7 PCV2 genomic copies/ml serum or greater are likely to have PCV2-associated lymphoid lesions and disease (Brunborg et al., 2004).
An internal positive control system for detection in VIC/HEX channel, (554 nm, order no. DVEV00511 or DVEV00551) or Cy5 channel (667 nm; order no. DVEV00513 or DVEV00553) excludes false-negative interpretation of results due to inhibition of real-time PCR (see 8. Interpretation of PCR-data).
When using PCR-platforms not validated by ingenetix, an evaluation of the multiplex-PCR is recommended. Please be aware that some PCR-platforms have to be calibrated with the corresponding dye before performing multiplex-PCR.

BactoReal®, MycoReal, ParoReal and ViroReal® Kits are optimized to run under the same thermal cycling conditions. RNA and DNA material can be analysed in one run.

2. Pathogen information

The porcine circovirus is a non-enveloped virus with single-stranded and circular DNA. It can be classified into two types (PCV1 and PCV2) according to its antigenicity, pathogenicity and genomic difference. PCV1 is considered non-pathogenic while PCV2 infects pigs. PCV2-infection is widespread and essentially all pig herds are infected with PCV2 but relatively few have PCV2-associated disease (PCVAD) including postweaning multisystemic wasting syndrome (PMWS), porcine dermatitis and nephropathy syndrome (PDNS), and porcine proliferative and necrotizing pneumonia (PNP). From piglets showing PCVAD signs PCV2 can be isolated at high titres.

References:
Gillespie J, Opriessnig T, Meng XJ, Pelzer K, Buechner-Maxwell V. 2009. Porcine circovirus type 2 and porcine circovirus-associated disease. J. Vet. Intern. Med. 23:1151-1163.

3. Principle of real-time PCR

A specific DNA sequence of the pathogen genome is amplified and the generated PCR-product is detected by an oligonucleotide-probe labelled with a fluorescent dye. This technology allows for a sequence-specific detection of PCR amplificates.

4. General Precautions

The user should always pay attention to the following:
- Always include a negative control per PCR-run (water instead of sample).
- Optional: for valid interpretation of results, a negative control should be included during DNA-extraction (for example extraction of water instead of sample material), in order to exclude false-positive results due to contamination with PCV2 DNA during extraction.
- Be careful when handling the positive control.
- Store and extract positive material (specimens, controls and amplicons) separately from all other reagents and add it to the reaction mix in a spatially separated workspace.
- Periodically decontaminate benches and devices.
- Use sterile pipette tips with filters.
- Thaw all components thoroughly at room temperature before starting an assay. When thawed, mix the components and centrifuge briefly.
- For MSDS, see www.ingenetix.com.

5. Contents of the Kit

5.1. ViroReal® Kit PCV2 order no. DVEV00511 or DVEV00551

Labelling	Content	Amount		Storage
		DVEV00511	DVEV00551	
PCV2 Assay Mix (green cap)	Primer and probe (FAM) for detection of PCV2	2 x 50 µl	1 x 50 µl	-20°C
CR-1 Assay Mix (yellow cap)	Primer, probe (VIC/HEX) and target for detection of IPC	2 x 50 µl	1 x 50 µl	-20°C
PCV2 Positive Control (red cap)	Control-DNA (approx. 10,000 target copies/µl)	1 x 25 µl	1 x 25 µl	-20°C
DNA Reaction Mix (white cap)#	Reaction Mix	2 x 500 µl	1 x 500 µl	**-20°C until first use, then at +4°C**
Water (blue cap)	Water	1 x 1000 µl	1 x 1000 µl	-20°C to +4°C

#DNA Reaction Mix contains uracil-N glycosylase (UNG)

5.2. ViroReal® Kit PCV2 order no. DVEV00513 or DVEV00553

Labelling	Content	Amount		Storage
		DVEV00513	DVEV00553	
PCV2 Assay Mix (green cap)	Primer and probe (FAM) for detection of PCV2	2 x 50 µl	1 x 50 µl	-20°C
CR-3 Assay Mix (yellow cap)	Primer, probe (Cy5) and target for detection of IPC	2 x 50 µl	1 x 50 µl	-20°C
PCV2 Positive Control (red cap)	Control-DNA (approx. 10,000 target copies/µl)	1 x 25 µl	1 x 25 µl	-20°C
DNA Reaction Mix (white cap)#	Reaction Mix	2 x 500 µl	1 x 500 µl	**-20°C until first use, then at +4°C**
Water (blue cap)	Water	1 x 1000 µl	1 x 1000 µl	-20°C to +4°C

#DNA Reaction Mix contains uracil-N glycosylase (UNG)

The components of ViroReal® Kit PCV2 are stable until the expiry date stated on the label. Repeated thawing and freezing should be avoided. Please protect kit components from light.

6. Additionally required materials and devices

- Reagents and devices for DNA-extraction
- PCR-grade water
- Disposable powder-free gloves
- Pipettes (adjustable)
- Sterile pipette tips with filters
- Vortex mixer
- Desktop centrifuge with rotor for 2 ml reaction tubes
- Real-time PCR instrument which is able to detect and differentiate fluorescence in FAM and VIC/HEX or Cy5 channel
- Appropriate 96 well reaction plates or reaction tubes with corresponding (optical) closing material

7. Preparation of real-time PCR

Please make sure that at least one negative control (water, blue cap), as well as one positive control (red cap) and one extraction negative control (optional, recommended) are included per PCR run.
Ingenetix highly recommends performing PCR analyses in duplicates, which increases the probability of detection of the pathogen and facilitates interpretation of results.

7.1. Pipetting scheme

		Per sample
Preparation of Master Mix (mix well)	Water*	3.0 µl
	DNA Reaction Mix (2x)	10.0 µl
	PCV2 Assay Mix	1.0 µl
	CR Assay Mix	1.0 µl
	Total volume Master Mix	**15.0 µl**
Preparation of PCR	Master Mix	15.0 µl
	Sample*	5.0 µl
	Total volume	**20.0 µl**

*1-8 µl of the sample can be used. When using an amount < 5 µl of the sample, the amount of H_2O has to be changed accordingly.

Positive Control: As positive control please use 1 µl of the PCV2 Positive Control + 4 µl H_2O.
Optional: a 1:10 dilution of the positive control can be used and defined as second standard value (approx. 1000 target copies/µl).
Or: for quantitative real-time PCR the **Quantitative real-time PCR Standard PCV2** (order no. PC100) can be used. This standard contains a fragment of the PCV2 DNA at a concentration of 10^7 copies/µl. For generation of a standard curve add 1 µl of a 1:10 dilution series to 20 µl PCR reaction.

7.2. Programming of the temperature profile

Please find further information on programming the real-time PCR instrument in the respective operator's manual. Please be aware that some PCR-platforms have to be calibrated with the corresponding dye before performing multiplex-PCR.

Select dyes: FAM-TAMRA for detection of PCV2
Cy5-NONE (CR-3 Assay Mix) **or** VIC-TAMRA (CR-1 Assay Mix) for detection of IPC
Select reference dye (passive reference): ROX
Sample Volume: 20 µl
Temperature Profile:

Program 1	Program 2	Program 3
Cycles: 1 Analysis: None	Cycles: 1 Analysis: None	Cycles: 45 Analysis: Quantification Acquisition at 60°
50°C 2 min*	95°C 20 sec	95°C 5 sec 60°C 1 min

<u>For ABI PRISM® 7500:</u>
Ramp speed: Without "fast cycling" parameter

<u>For LightCycler® 480 instrument:</u>
Detection format: 2 Color Hydrolysis Probe (dyes see above)

***Note:** If viral RNA should be also detected in the same PCR run, program 1 has to be prolonged to 15 min at 50°C. This temperature profile can be used for all BactoReal®, MycoReal, ParoReal, and ViroReal® kits for the detection of DNA or RNA.

8. Interpretation of PCR-data

Examples for interpretation of positive reactions are shown in the amplification plots below.

For a valid interpretation, the following criteria must be fulfilled:

	Ct/Cp (FAM channel) PCV2 target	Ct/Cp IPC target	Interpretation
Negative control	Negative	36.0 ± 2	Valid
Positive control (undiluted, 1 µl/PCR)	28.0-31.0	36.0 ± 2	Valid
Or: positive control (1:10 diluted, 1 µl/PCR)	31.0-34.0	36.0 ± 2	Valid
Extraction negative control (optional)	Negative	36.0 ± 2	Valid
Negative sample	Negative	36.0 ± 2	Valid
Positive sample	Positive	Positive/Negative	Valid

For analysis of PCR data please proceed as follows:
For analysis of PCR results gained with ViroReal® Kit PCV2 please select fluorescence display options FAM channel for the PCV2 target and VIC/HEX channel (order no. DVEV00511, DVEV00551) or Cy5 channel (order no. DVEV00513, DVEV00553) for the internal positive control target. Samples with a positive Ct or Cp-value are considered positive. Please also check the presence of amplification-curves manually.

8.1. Signal in FAM channel:

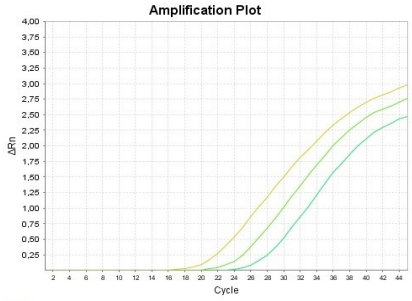
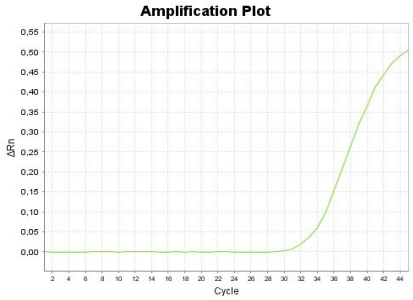
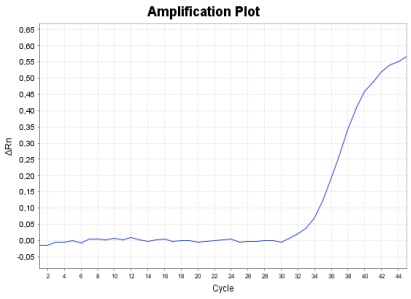
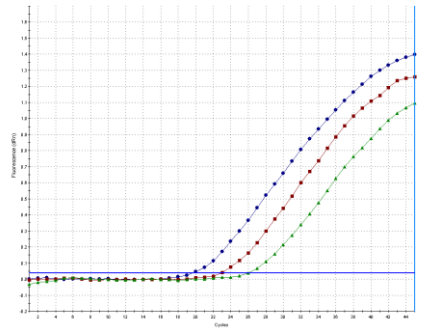
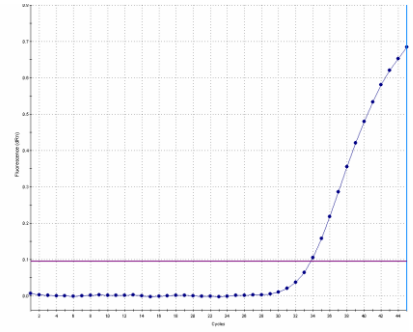
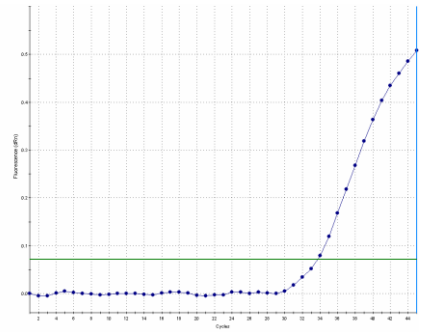
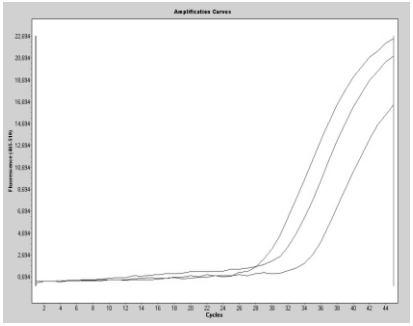
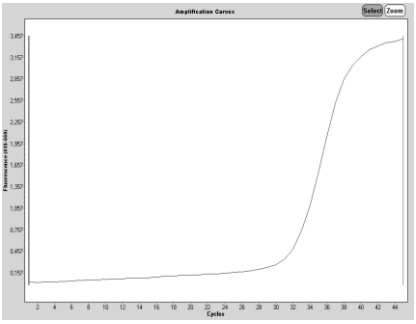
→ DNA of PCV2 was amplified. The sample has to be interpreted as positive.
PCV2 DNA can lead to a reduced or absent fluorescence signal of the internal positive control (competition of PCR).

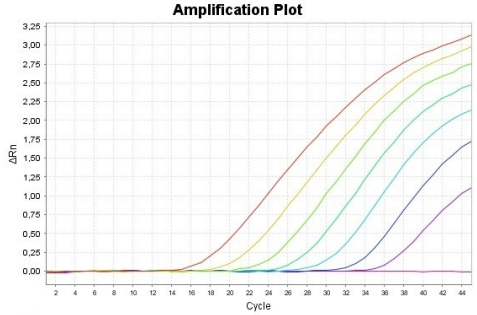
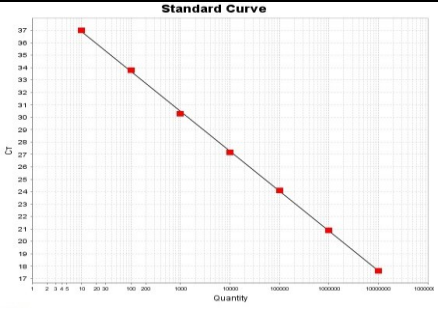
8.2. No signal in FAM channel but signal of the internal positive control:

→ No PCV2 DNA is detectable in the sample. The sample has to be interpreted as negative.
The positive signal of the internal positive control assay excludes a putative PCR inhibition.

8.3. No signals in FAM channel and no signal with internal positive control:

→ No interpretation statement can be made.
Information about possible sources of error and their solution can be found in 9. Troubleshooting.

Detection of **PCV2**	Detection of internal positive control **CR-3**	Detection of internal positive control **CR-1**
		
ABI Prism® 7500: FAM channel, 530 nm 1:10 serial dilution of PCV2 DNA	**ABI Prism® 7500:** Cy5 channel, 667 nm Internal positive control	**ABI Prism® 7500:** VIC channel, 554 nm Internal positive control
		
Mx3005P®: FAM channel 1:10 serial dilution of PCV2 DNA	**Mx3005P®:** CY5 channel Internal positive control	**Mx3005P®:** HEX channel Internal positive control
		
LightCycler® 480: FAM channel 1:10 serial dilution of PCV2 DNA	**LightCycler® 480:** Cy5 channel Internal positive control	

	
ABI Prism® 7500: 1:10 serial dilution of PCV2 Standard (order no.: PC100)	**ABI Prism® 7500:** Standard curve generated with PCV2 Standard

9. Troubleshooting

9.1. No PCV2 specific signal with positive control:

- Incorrect programming of the temperature profile of the real-time PCR instrument.
  → Compare the temperature profile with the protocol (see 7. Preparation of real-time PCR).
- Incorrect configuration of the PCR reaction.
  → Check your work steps (see 7. Preparation of real-time PCR) and repeat the PCR, if necessary.

9.2. No signal with the internal positive control and no PCV2 specific signal with sample:

- The PCR reaction was inhibited. No interpretation can be made.
  → Make sure that you use a recommended method for DNA isolation and stick closely to the manufacturer's instructions.
  → If no operating mistakes during extractions can be retraced, it is recommended to repeat the PCR with lower amounts of DNA-eluate (1/5 or 1/10 of sample volume + the adequate amount of H_2O).
- Incorrect PCR conditions.
  → Check the PCR conditions and repeat the PCR, if necessary.

9.3. PCV2 specific signal with negative control:

- A contamination occurred during preparation of the PCR.
  → Repeat the PCR with new reagents in replicates.
  → Strictly pipette the positive controls at last.
  → Make sure that work space and instruments are decontaminated at regular intervals.

9.4. PCV2 specific signal with negative control of DNA-extraction:

- A contamination occurred during extraction.
  → Repeat the extraction and PCR using new reagents.
  → Make sure that work space and instruments are decontaminated at regular intervals.

10. Specifications

ViroReal® Kit PCV2 was evaluated with the ABI PRISM® 7500 (Fast) instrument (Thermo Fisher Scientific), with the LightCycler® 480 (Roche) and the Mx3005P® (Agilent). For further validation data please contact ingenetix.

10.1. Analytical sensitivity

The analytical sensitivity is 1 template copy/PCR. The LoD95 (LoD95 = smallest number of copies of target DNA which can be detected in 95% of cases) of 4 target copies/reaction was determined by several replicates around the detection limit.

10.2. Analytical specificity

The specificity is ensured by the selection of highly specific primers and probes. The primers and probes were checked for possible homologies to currently published sequences by sequence comparison analyses. This also validated the detection of so far known PCV2 strains.
ViroReal® Kit PCV2 is specific for porcine circovirus type 2 (no cross-reaction with PCV1) and detects all PCV2 isolates (genotypes A, B and C) published in the NCBI database. 110 samples were tested and correctly analysed.

11. Annex – symbols

LOT	Batch code		Use by
REF	Catalogue number		Manufactured by
Σ	Contains sufficient for <n> tests		Store at