ViroReal® Typing Kit PCV2 a, b, d

Manual

For veterinary use only

REF **DVET002**

Σ **100**

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1. Product description

ViroReal® Typing Kit PCV2 a, b, d contains three PCR primer sets for the amplification and detection of DNA of the porcine circovirus type 2 (PCV2) a, b, d. This test is based on an intercalating dye using real-time PCR and has been developed for the Applied Biosystems® 7500 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 typing of PCV2 a, b, d from DNA samples.

ViroTyping Kit PCV2 a, b, d detects the capsid gene of porcine circovirus type 2 (PCV2) a, b, d. The test is based on phylogenetic analysis and the definition of genotype-specific marker positions for PCV2 genotypes, published by Franzo and colleagues. Amplification curves and specific melting curves for PCV2 a, b and d indicate the amplification of genotype specific DNA.

When using PCR-platforms not validated by ingenetix, an evaluation of the real-time PCR is recommended. Please be aware that some PCR-platforms have to be calibrated with the corresponding dye before performing PCR.

2. Pathogen information

Porcine circovirus type 2 (PCV2) is a member of the family *Circoviridae*, genus *Circovirus* and is an icosahedral, non-enveloped DNA virus with a single-stranded ambisense circular genome.
PCV2 infection with postweaning multisystemic wasting syndrome (PMWS) occurs all over the world. There is evidence that genetic diversity is affecting the virulence of PCV2. At the intraspecific level, two major PCV2 groups (PCV2a and PCV2b) were initially defined, followed by a third genotype named PCV2c and a fourth group, named PCV2d.
The ORF2 sequences enable differentiation of the genotypes PCV2a, PCV2b and PCV2d.

References: G. Franzo et al. Revisiting the taxonomical classification of Porcine Circovirus type 2 (PCV2): still a real challenge. Virology Journal (2015) 12:131.

3. Principle of real-time PCR

A specific DNA sequence of the pathogen genome is amplified by a set of two PCR primers that flank the target region and the generated PCR-product is detected by an intercalating nucleic acid dye with subsequent melt curve analysis.

4. General Precautions

The user should always pay attention to the following:

- Always include a negative control per PCR-run (Nuclease-free water instead of sample).
- 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. The 5x HR Master Mix can be stored up to 1 month at room temperature, routine storage is at -20°C.

5. Contents of the Kit

5.1. ViroReal® Typing Kit PCV2 a, b, d order no. DVET002

Labelling	Content	Amount DVET002
PCV2GT A Assay Mix (green cap)	Primer for detection of PCV2 a	2 x 50 µl
PCV2GT B Assay Mix (violet cap)	Primer for detection of PCV2 b	2 x 50 µl
PCV2GT D Assay Mix (amber cap)	Primer for detection of PCV2 d	2 x 50 µl
PCV2GT A Positive Control (red cap)	Control-DNA (approx. 10,000 target copies/µl)	1 x 25 µl
PCV2GT B Positive Control (red cap)	Control-DNA (approx. 10,000 target copies/µl)	1 x 25 µl
PCV2GT D Positive Control (red cap)	Control-DNA (approx. 10,000 target copies/µl)	1 x 25 µl
5x HR Master Mix (white cap)	Master Mix with internal reference (ROX)	3 x 400 µl
Nuclease-free water (blue cap)	Nuclease-free water	1 x 1000 µl

The components of ViroReal® Typing Kit PCV2 a, b, d should be stored at -15°C to -25°C, 5x HR Master Mix can be stored at room temperature up to one month. Components are stable until expiry date stated on the label.

6. Additionally required materials and devices

- Reagents and devices for DNA-extraction
- Nuclease-free 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
- 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 (Nuclease-free water, blue cap), as well as one positive control PCV2a, PCV2b and PCV2d (red cap) are included per PCR run.
Ingenetix highly recommends performing PCR analyses in duplicates, which increases the probability of pathogen detection and facilitates interpretation of results. Note that each sample has to be tested with each assay mix PCV2GT A, PCV2GT B, PCV2GT D separately (with the corresponding positive control and a negative control).

7.1. Pipetting scheme

For testing one DNA-sample prepare three reactions, one for Master Mixes PCV2a, PCV2b and PCV2d. Calculate the number of reactions required for all samples including a positive control and a negative control (NTC):

		Per sample
Preparation of Master Mix (PCV2 a) (mix well)	Nuclease-free water	10.0 µl
	HR Master Mix (5x)	4.0 µl
	PCV2GT A 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**

		Per sample
Preparation of Master Mix (PCV2 b) (mix well)	Nuclease-free water	10.0 µl
	HR Master Mix (5x)	4.0 µl
	PCV2GT B 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**

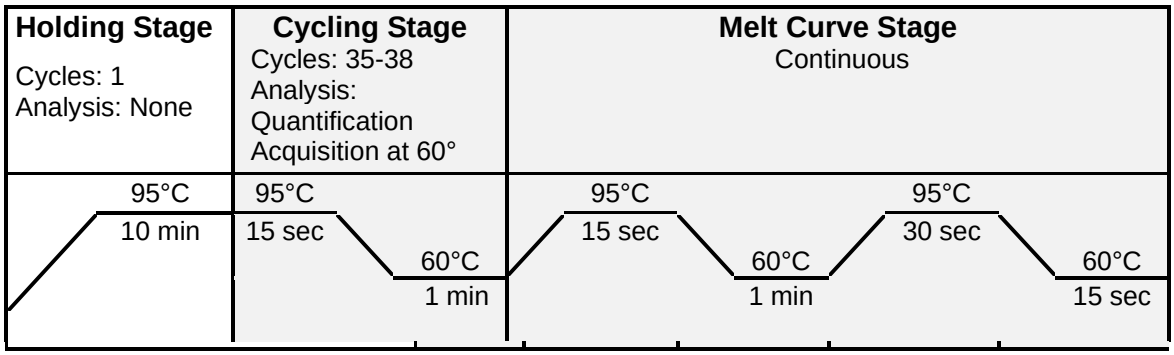
		Per sample
Preparation of Master Mix (PCV2 d) (mix well)	Nuclease-free water	10.0 µl
	HR Master Mix (5x)	4.0 µl
	PCV2GT D Assay Mix	1.0 µl
	Total volume Master Mix	**15.0 µl**
Preparation of PCR	Master Mix	15.0 µl
	Sample	5.0 µl
	Total volume	**20.0 µl**

Positive Control: Use 1 µl of PCV2 a, b or d Positive Control + 4 µl Nuclease-free water, respectively.

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 real-time PCR.

Experiment Properties	Reagents: SYBR® Green Reagents Ramp speed: Standard
Select dyes	Reporter SYBR, Quencher None
Select passive reference	ROX
Sample Volume	20 µl
Temperature Profile:	



8. Interpretation of PCR-data

For analysis of PCR results gained with ViroReal® Typing Kit PCV2 a, b, d, check amplification plots and melt curves obtained with each assay mix (PCV2GT A, PCV2GT B, PCV2GT D). The specific amplification plot and melt curve of a sample needs to be interpreted in context of the Ct/Cp and Tm [°C] values of the negative (NTC) and positive control. Be aware that the Sybr®Green method based on intercalating dyes and different primer pair sets may yield unspecific products and melting curves due to the formation of primer dimers (as shown in the Melt curve data plots and Examples of results on the following pages).

Make sure that a sample is always interpreted in context with the results of all three amplification plots (PCV2GT A, PCV2GT B and PCV2GT D) and the corresponding lowest Ct/Cp values.

Melting curve results

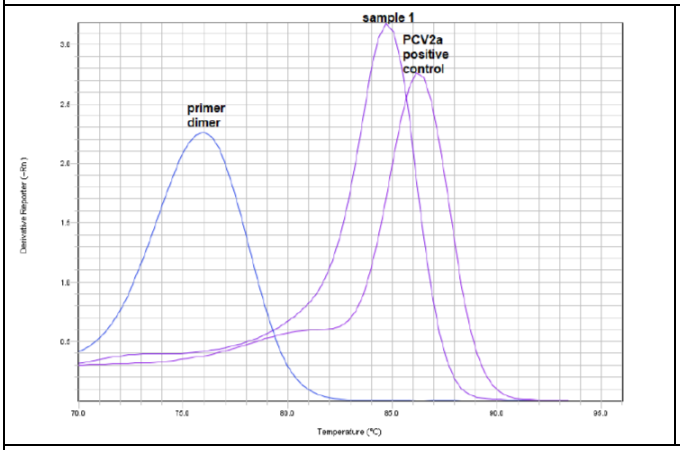
Target	Positive Control Tm [°C]	Positive Sample* Tm [°C]
PCV2a	87 (+/- 2)	85 (+/-2)
PCV2b	87 (+/- 2)	85 (+/-2)
PCV2d	87 (+/- 2)	82 (+/-2)

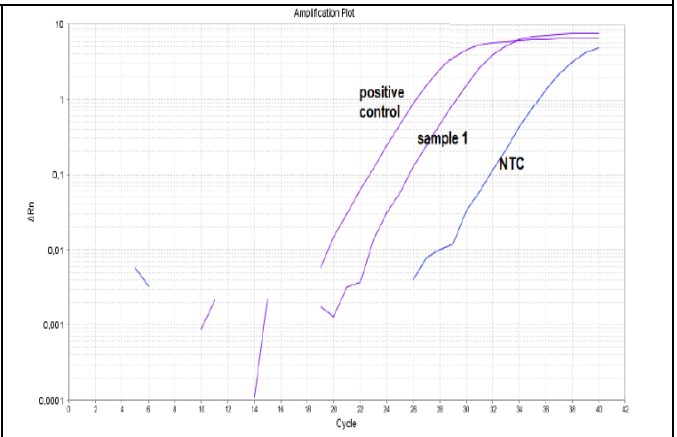
* Due to sequence variation and other factors (e.g. performance on different PCR instruments), a shift of melting point (Tm) may be observed.

Cross-reactivity of PCV2a, PCV2b and PCV2 d assays can be excluded comparing the Ct/Cp values of a sample generated in the three different assays.

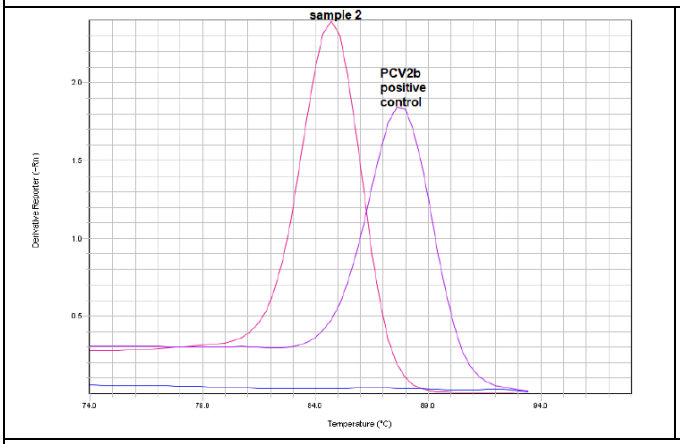
Melt curve and amplification plot data of samples with respective positive and negative controls tested with all three assays:

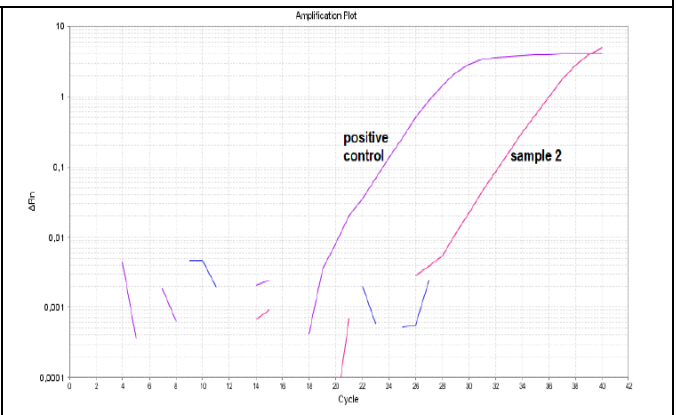
PCV2a



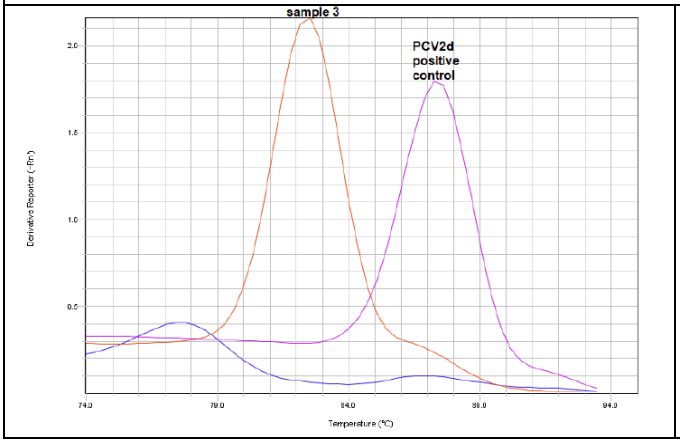


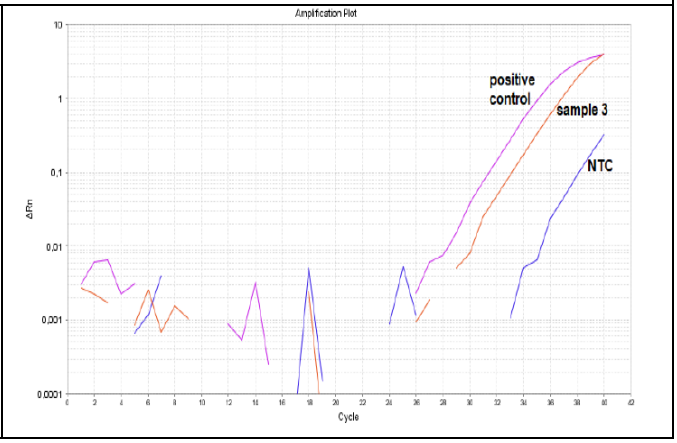
PCV2b





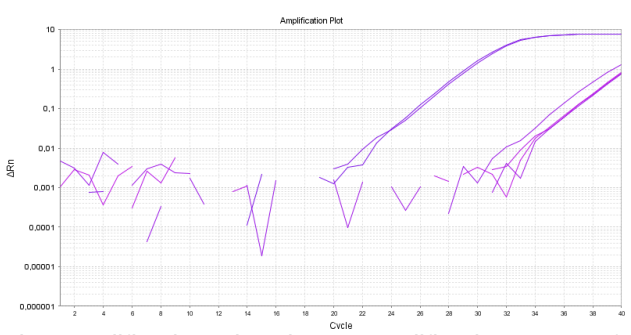
PCV2d

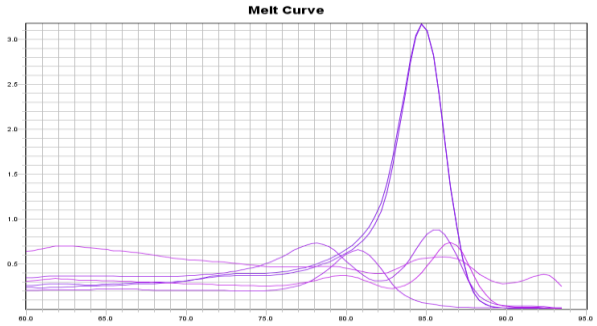




Examples of results

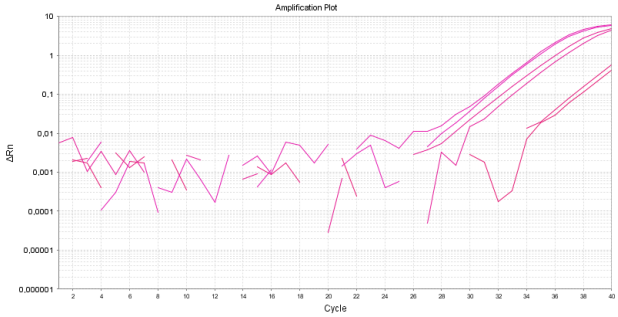
PCV2a positive:

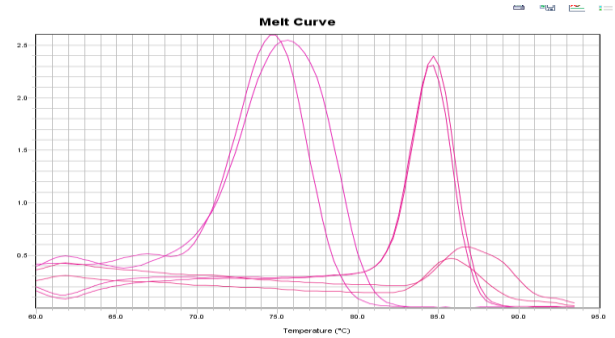




The amplification plot shows amplification curves for a PCV2a-positive sample with low Ct-values and higher Ct-values for the PCV2b and PCV2d assays run with the same sample due to unspecific amplification. The melt curve of the sample, however, is easily identifiable as a PCV2a type due to its specific peak at the expected melting point, Tm 85°C in the PCV2a reaction.

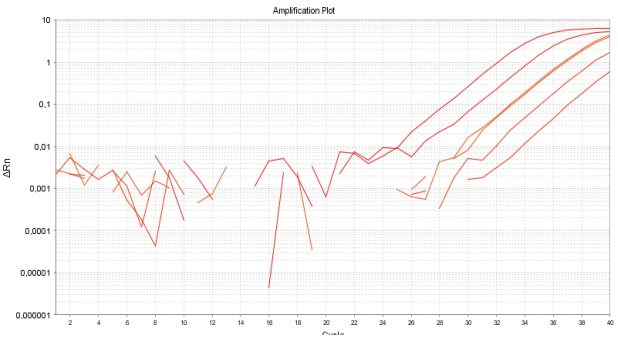
PCV2b positive:

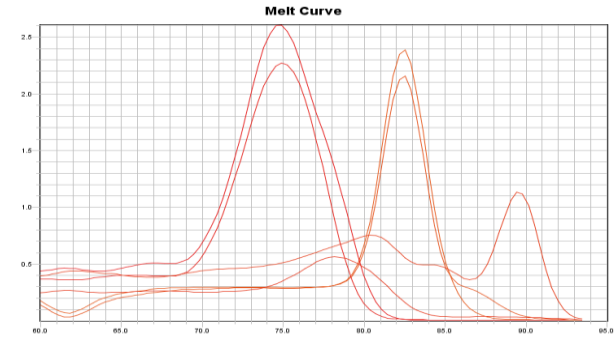




The amplification plot shows the PCV2b reaction of a low concentrated sample with Ct-values identical to nonspecific amplifications in the PCV2a and PCV2d assays of the same sample. The melt curve nonetheless is clearly identifiable as a PCV2b type in the PCV2b reaction. With the PCV2a assay of the same sample a “primer dimer” at Tm <80°C was generated and in the PCV2d assay small unspecific peaks with a higher Tm were formed.

PCV2d positive:





The amplification plot shows a sample with low concentration of PCV2d and Ct-values identical to nonspecific amplifications with PCV2a and PCV2 b assays. The melt curve of the sample in the PCV2d reaction though is specific for the PCV2d type. The same sample run with the PCV2a assay resulted in “primer dimer” formation at Tm <80°C, the PCV2b assay generated a small unspecific peak at Tm 90°C.

9. Troubleshooting

9.1. No PCV2 a, b, d 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. PCV2 a, b, d 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.

10. Specifications

ViroReal® Typing Kit PCV2 a, b, d has been evaluated with Applied Biosystems® 7500 (Fast) instrument (Thermo Fisher Scientific), LightCycler® 480 (Roche) and Mx3005P® (Agilent). For further validation data please contact ingenetix.

10.1. Analytical sensitivity.

ViroReal® Typing Kit PCV2 a, b, d is designed for genotyping purposes, thus only strong PCV2 positive samples should be subjected to testing. Analytical sensitivity of PCV2a, b, d is about 1000 target copies/PCR reaction.

10.2. Analytical specificity

The specificity is ensured by the selection of highly specific primers. The primers were checked for possible homologies to currently published sequences by sequence comparison analyses.
ViroReal® Typing Kit PCV2 a, b, d is specific for porcine circovirus type 2 a, b, d (no cross-reaction with PCV1 or PCV3). PCV2a assay tends to generate primer dimers, they are however clearly distinguishable from the target peak by means of melting temperature.

11. Annex – symbols

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

Permitted Use: The 5x HR Master Mix contains 5x HOT FIREPol® SolisGreen qPCR Mix which is a product of Solis BioDyne OÜ of 51014 Tartu, Estonia (www.sbd.ee). CYGREEN dye is a component of this qPCR Master Mix. CYGREEN is a U.S. registered trademark of Enzo Life Sciences, Inc. U.S. Patent Nos. 8,153,802 and 7,569,695 CYGREEN is a U.S. Registered trademark of Enzo Life Sciences, Inc. and this dye is the subject of the U.S. patents 12,456,502 and 7,569,695.