ViroReal® Kit PEDV & SDCV

Manual

For use with the

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

For veterinary use only

REF	**DVEV01913**	Σ	**100**
REF	**DVEV01953**	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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Explanation of symbols

LOT	Batch code		Use by
REF	Catalogue number		Manufactured by
Σ	Contains sufficient for <n> tests		Store at
	Corrosion, GHS05		Exclamation mark, GHS07

1. Product description

ViroReal® Kit PEDV & SDCV is a real-time PCR kit for detection of RNA of both porcine epidemic diarrhea virus (PEDV) and swine delta coronavirus (SDCV, also called porcine delta coronavirus PDCoV) using one-step reverse transcription real-time PCR. 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 PEDV RNA and SDCV RNA isolated from feces of acutely affected pigs (e.g. with the QIAamp Viral RNA Mini Kit, Qiagen).

ViroReal® Kit PEDV & SDCV detects the nucleocapsid protein gene (N gene) of PEDV as well as the 3'UTR of SDCV. The PEDV is detected in the FAM channel, while the SDCV is detected in the VIC/HEX channel. An internal RNA positive control system (detection in Cy5 channel) allows control of RNA extraction and excludes false-negative interpretation of results due to inhibition of reverse transcription 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

Porcine epidemic diarrhea virus (PEDV), a member of the genus *Coronavirus* (Alphacoronavirus), family *Coronaviridae*, is a positive-sense, enveloped, single-stranded RNA virus. Severity of disease is variable and dependent on the epidemiological status of the herd. When epidemic, PEDV causes acute watery diarrhea and vomiting in a large proportion at all ages of swine. If endemic, then diarrhea is observed with lower morbidity in suckling and recently weaned pigs. The PED virus is similar to, but antigenically distinct from transmissible gastroenteritis virus (TGEV).
Swine delta coronavirus (SDCV, PDCoV), a member of the genus *Coronavirus* (Deltacoronavirus), family *Coronaviridae*, is a positive-sense, enveloped, single-stranded RNA virus. SDCV is a new virus first found in pigs in Hong Kong in 2012 and 2014 in the United States. SDCV causes acute watery diarrhea and vomiting in a large proportion at all ages of swine. SDCV infection is clinically similar to, but genetically distinct from porcine epidemic diarrhea virus (PEDV) and transmissible gastroenteritis virus (TGEV).

References:

- Kim O et al. 2002. Comparison of reverse transcription polymerase chain reaction. immunohistochemistry, and in situ hybridization for the detection of porcine epidemic diarrhea virus. Can J Vet Res. 66:112-116.
- Patrick C. Y. Wooa, Susanna K. P. Laua, Carol S. F. Lama, Kwok-Yung Yuena. 2012. Discovery of Seven Novel Mammalian and Avian Coronaviruses in the Genus Deltacoronavirus Supports Bat Coronaviruses as the Gene Source of Alphacoronavirus and Betacoronavirus and Avian Coronaviruses as the Gene Source of Gammacoronavirus and Deltacoronavirus. J. Virology 86:7.

3. Principle of real-time PCR

When detecting pathogens by reverse transcription real-time PCR, a specific RNA sequence of the pathogen genome is transcribed to cDNA and amplified. 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 (Nuclease-free water instead of sample).
- Optional: for valid interpretation of results, a negative control should be included during RNA-extraction (for example extraction of water instead of sample material), in order to exclude false-positive results due to contamination with porcine coronavirus RNA 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.
- Always keep the RNA Reaction Mix on ice.
- Use the RNA immediately after extraction and store at -20°C to -80°C as soon as possible.
- **Caution:** the Positive Control and the RNA IPC Target are stored in RNA stabilizer that contains guanidinium thiocyanate/Triton X-100 (see MSDS, www.ingenetix.com).

5. Contents of the Kit

Labelling	Content	Amount		Storage
		DVEV01913	DVEV01953	
PEDV Assay Mix (green cap)	Primer and probe (FAM) for PEDV detection	2 x 50 µl	1 x 50 µl	-20°C
SDCV Assay Mix (purple cap)	Primer and probe (VIC/HEX) for SDCV detection	2 x 50 µl	1 x 50 µl	-20°C
RNA IPC-3 Assay Mix (yellow cap)	Primer and probe (Cy5) for RNA IPC detection	2 x 50 µl	1 x 50 µl	-20°C
RNA IPC Target (orange cap)	RNA internal positive control	1 x 100 µl	1 x 100 µl	-20°C
PEDV Positive Control (red cap)	RNA positive controls (approx. 5,000,000 target copies/µl)	1 x 15 µl	1 x 15 µl	-20°C
SDCV Positive Control (red cap)	RNA positive controls (approx. 5,000,000 target copies/µl)	1 x 15 µl	1 x 15 µl	-20°C
RNA Reaction Mix (white cap)	4 x Reaction Mix	2 x 250 µl	1 x 250 µl	-20°C
Nuclease-free water (blue cap)	Nuclease-free water	2 x 1000 µl	1 x 1000 µl	-20°C

The components of ViroReal® Kit PDEV & SDCV 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 RNA-extraction
- Nuclease-free water for dilution of RNA IPC Target and positive control
- 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, VIC/HEX and 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.

- Prepare master mix on ice.
- Thaw RNA Reaction Mix on ice, and invert 2 to 3 times to ensure homogenous solution. Do not let it warm to room temperature.
- Use RNA immediately after extraction and store at -20 to -80°C as soon as possible.

7.1. Internal RNA positive control (RNA IPC):

An internal RNA positive control system containing the RNA IPC assay and the RNA IPC Target excludes false-negative interpretation of results due to inhibition of reverse transcription real-time PCR.

→ Dilute RNA IPC Target freshly 1:500 with nuclease-free water and add to the master mix (use 1 µl/reaction).

→ Alternatively, for control of RNA extraction and PCR inhibition the RNA IPC Target can be added during extraction. Spike 1 µl of undiluted RNA IPC Target into the sample material after the lysis buffer was added. Caution: Do not add the RNA IPC Target directly to the sample material.

7.2. Positive Control:

The PEDV & SDCV Positive Controls are *in vitro* synthesized RNAs in RNA-stabilizer. They have to be stored at -20°C. Before use they have to be freshly diluted 1:500 with nuclease-free water, which corresponds to approx. 10,000 target copies/µl. Optional: 1:10 dilution of the 1:500 diluted positive control can be used and defined as second standard value (approx. 1,000 target copies/µl).

→ As positive controls please use 1 µl of the freshly 1:500 diluted PEDV Positive Control + 1 µl of the freshly 1:500 diluted SDCV Positive Control + 8 µl nuclease-free water.

Caution: The use of more than 1 µl positive controls (diluted 1:500) inhibits the RT-PCR reaction.

7.3. Pipetting scheme

		Per sample
Preparation of Master Mix (mix well)	Nuclease-free Water*	1.0 µl
	RNA Reaction Mix	5.0 µl
	PEDV Assay Mix	1.0 µl
	SDCV Assay Mix	1.0 µl
	RNA IPC Assay Mix	1.0 µl
	RNA IPC Target# (freshly diluted 1:500)	1.0 µl
	Total volume Master Mix	**10.0 µl**
Preparation of PCR	Master Mix	10.0 µl
	RNA-Sample*	10.0 µl
	Total volume	**20.0 µl**

*1-10 µl of the sample can be used. When using an amount < 10 µl of the sample, the amount of H_2O has to be changed accordingly.
#If RNA IPC Target not already added during extraction.

7.4. 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.

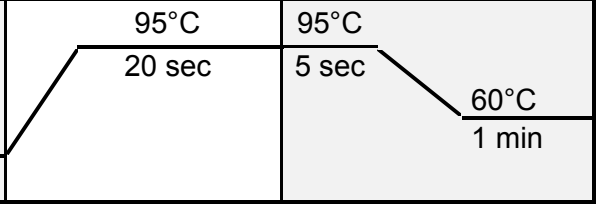
Select dyes: FAM-TAMRA (530 nm) for detection of PEDV
VIC/HEX-TAMRA (554 nm) for detection of SDCV
Cy5-NONE (RNA IPC-3 Assay Mix) **or** VIC/HEX-TAMRA (RNA IPC-1 Assay Mix) for detection of RNA IPC

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 15 min	95°C 20 sec	95°C 5 sec 60°C 1 min



For ABI PRISM® 7500:
Ramp speed: Without "fast cycling" parameter

For LightCycler® 480 instrument:
Detection format: 3 Color Hydrolysis Probe (dyes see above)

Note: These instrument parameters can be used for all BactoReal®, MycoReal, ParoReal and ViroReal® kits on all PCR instruments.

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) PEDV target	Ct/Cp (VIC/HEX channel) SDCV target	Ct/Cp (Cy5 channel) RNA IPC target	Interpretation
Negative control	Negative	Negative	26-29*	Valid
Positive control (freshly diluted 1:500), approx. 10,000 copies, 1 µl/PCR	26-29	26-29	26-29*	Valid
Extraction negative control (optional)	Negative	Negative	26-29	Valid
Negative sample	Negative	Negative	26-29	Valid
PEDV positive sample	Positive	Negative	26-29/negative	Valid
SDCV positive sample	Negative	Positive	26-29/negative	Valid

*In the case that the RNA IPC target has been added to the master mix

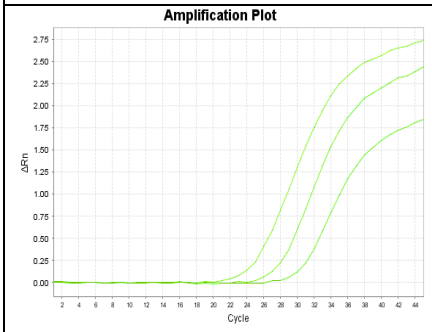
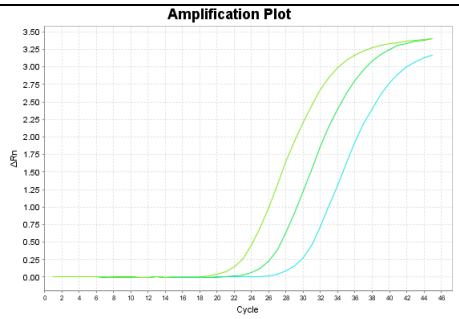
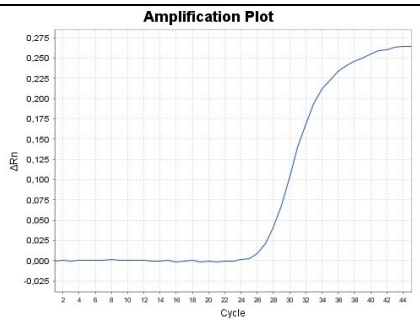
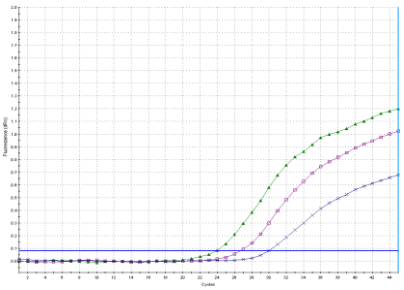
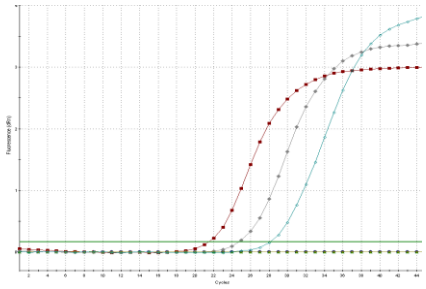
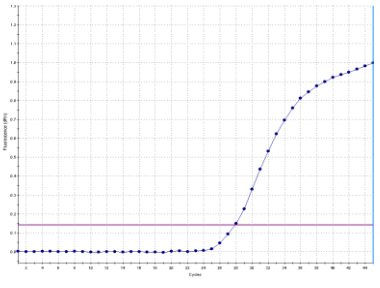
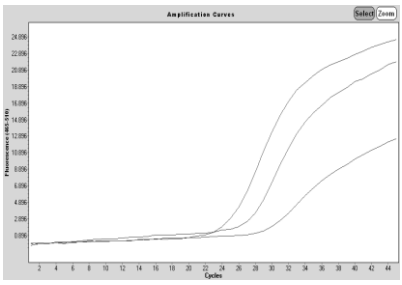
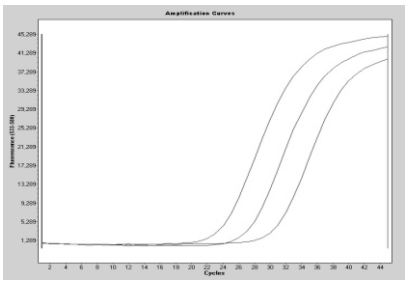
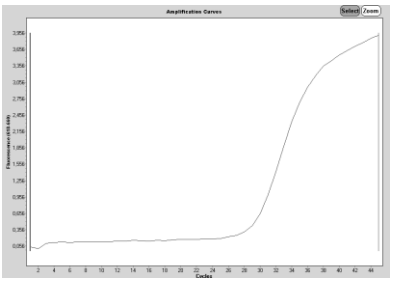
For analysis of PCR data please proceed as follows:
For analysis of PCR results gained with ViroReal® Kit PEDV & SDCV please select fluorescence display options 530 nm (FAM channel) for the PEDV target, 554 nm (VIC/HEX channel) for the SDCV target and 667 nm (Cy5 channel) for the RNA IPC target. Samples 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:
→ RNA of PEDV was amplified. The sample has to be interpreted as positive.

8.2. Signal in VIC/HEX channel:
→ RNA of SDCV was amplified. The sample has to be interpreted as positive.

8.3. No signal in FAM channel and VIC/HEX channel but signal of the RNA IPC:
→ No RNA of porcine coronaviruses PEDV and SDCV 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.

8.4. No signal in FAM, VIC/HEX or Cy5 channel:
→ No interpretation statement can be made.
Information about possible sources of error and their solution can be found in 9. Troubleshooting.

Detection of PEDV FAM channel	Detection of SDCV VIC/HEX channel	Detection of internal positive control Cy5 channel
		
ABI Prism® 7500 FAM channel, 530 nm 1:10 serial dilution of a PEDV positive control	**ABI Prism® 7500:** VIC channel, 554 nm 1:10 serial dilution of a SDCV positive control	**ABI Prism® 7500:** Cy5 channel, 667 nm Detection of internal RNA positive control
		
Mx3005P®: FAM channel 1:10 serial dilution of a PEDV positive control	**Mx3005P®:** HEX channel 1:10 serial dilution of a SDCV positive control	**Mx3005P®:** CY5 channel Detection of internal RNA positive control
		
LightCycler® 480: FAM channel 1:10 serial dilution of a PEDV positive control	**LightCycler® 480:** VIC/HEX/Yellow555 channel 1:10 serial dilution of a SDCV positive control	**LightCycler® 480:** Cy5 channel, 667 nm Detection of internal RNA positive control

9. Troubleshooting

9.1. No PEDV or SDCV virus 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. Check the correct fluorescence display options FAM, VIC/HEX and Cy5 channel).
- RNA might be degraded.
  → Prepare a fresh 1:500 dilution of the positive controls and repeat the PCR.

9.2. No signal with the RNA IPC and no porcine coronavirus specific signals with the sample:

- The PCR reaction was inhibited. No interpretation can be made.
  → Make sure that you use a recommended method for RNA 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 RNA-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. PEDV or SDCV specific signal with the negative control:

- A contamination occurred during preparation of the PCR.
  → Repeat 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. PEDV or SDCV specific signal with the negative control of RNA-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. Specification

ViroReal® Kit PEDV & SDCV 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 10 RNA copies/PCR for PEDV and 2 RNA copies/PCR for SDCV. The limit of detection (LoD95 = smallest number of copies of target RNA which can be detected in 95% of cases) was determined by several replicates around the detection limit. The LoD95 is 37 target copies/reaction for PEDV and 18 target copies/reaction for SDCV.

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 PEDV and SDCV strains published in the NCBI database. The kit was tested with one TGEV, one PHEV, three PEDV, eight PRRSV EU, four PPV and seven PCV2 strains. It was positive with PEDV and showed no cross-reaction with the others. ViroReal® Kit PEDV & SDCV is specific for PEDV and SDCV and detects all PEDV strains, and SDCV and porcine coronavirus HKU15 strains published in the NCBI database.