ViroReal® Kit PRRS Virus EU & NA 1.1

Manual

For use with the

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

For veterinary use only

REF **DVEV00113** Σ **100**

REF **DVEV00153** Σ **50**

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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 PRRS Virus EU & NA 1.1 is a real-time PCR kit for detection of RNA of both viral lineages PRRSV EU (including Lena strain) and PRRSV North America (NA) 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 RNA of PRRS virus from samples purified from blood, saliva, semen, faeces, nasal secretions, milk and tissue samples of acutely fallen ill pigs or of weak-born piglets (e.g. with the QIAamp Viral RNA Mini Kit, Qiagen).
The new version 1.1 of the kit has been adapted to currently circulating PRRSV EU-type viruses to allow improved detection of these variants.

ViroReal® Kit PRRS Virus EU & NA 1.1 detects the open-reading frame 7 including the 3'-untranslated region of PRRSV EU and NA (PRRSV type 1 and PRRSV type 2). The PRRSV EU lineage is detected in FAM channel, while the PRRSV NA is detected in 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

The porcine reproductive and respiratory syndrome virus (PRRSV) is a single strain, positive-sense RNA virus belonging to the order *Nidovirales*, family *Arteriviridae.* This virus causes the Porcine Reproductive and Respiratory Syndrome (PRRS). PRRS is one of the most economically important diseases of swines and is also known as Blue-Ear Pig Disease. The disease is characterised by reproduction defects and by multifactor respiratory problems. European (EU) lineage PRRSV isolates (type 1, prototype strain is the Lelystad Virus) are genetically divergent from North American (US) isolates (type 2, prototype strain is the VR-2332) but cause similar clinical symptoms. A great genetic heterogeneity of field isolates is observed and further complicates diagnosis.

References:
Kleiboeker SB, Lehman JR, and Fangman TF. 2002. Concurrent use of reverse transcription-polymerase chain reaction testing of oropharyngeal scrapings and paired serological testing for detection of porcine reproductive and respiratory syndrome virus infection in sows. J. Swine Health Prod. 10:251-258.

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 into 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 PRRSV 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
		DVEV00113	DVEV00153	
PRRSV EU + NA Assay Mix 1.1 (green cap)	Primer and probe (FAM + VIC/HEX) for PRRS Virus EU + NA 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
PRRS Virus EU & NA Positive Control (red cap)	RNA positive control (approx. 50,000,000 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 PRRS Virus EU & NA 1.1 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 PRRS Virus EU & NA Positive Control is an *in vitro* synthesized RNA in RNA-stabilizer. It has to be stored at -20°C. Before use it has to be freshly diluted 1:500 with nuclease-free water, which corresponds to approx. 100,000 target copies/µl.

→ As positive control please use 1 µl of the freshly 1:500 diluted PRRS Virus EU & NA Positive Control + 9 µl nuclease-free water.
Caution: The use of more than 1 µl positive control (diluted 1:500) inhibits the RT-PCR reaction.
Optional: 1:10 dilution of the 1:500 diluted positive control can be used and defined as second standard value (approx. 10,000 target copies/µl).

7.3. Pipetting scheme

		Per sample
Preparation of Master Mix (mix well)	Nuclease-free Water*	2.0 µl
	RNA Reaction Mix	5.0 µl
	PRRS Virus EU + NA Assay Mix 1.1	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 PRRS virus EU
VIC/HEX-TAMRA (554 nm) for detection of PRRS virus NA
Cy5-NONE (RNA IPC-3 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) PRRSV EU target	Ct/Cp (VIC/HEX channel) PRRSV NA target	Ct/Cp (Cy5 channel) RNA IPC target	Interpretation
Negative control	Negative	Negative	26-29*	Valid
Positive control (freshly diluted 1:500), approx. 100,000 copies, 1 µl	23-26	23-26	26-29*	Valid
Extraction negative control (optional)	Negative	Negative	26-29	Valid
Negative sample	Negative	Negative	26-29	Valid
PRRSV EU positive sample	Positive	Negative	26-29/negative	Valid
PRRSV NA positive sample	Negative	Positive	26-29/negative	Valid

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

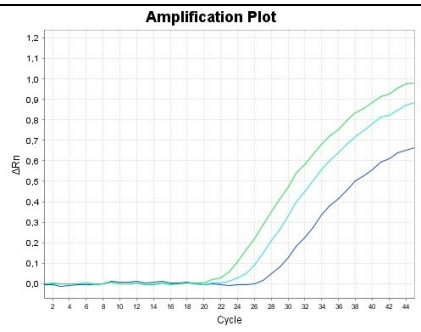
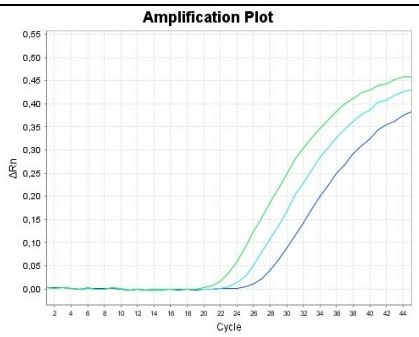
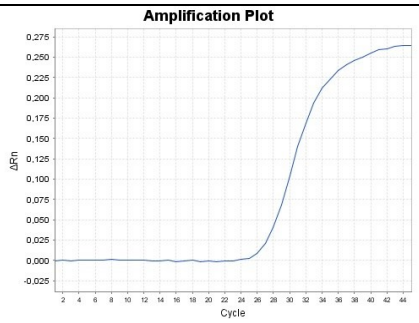
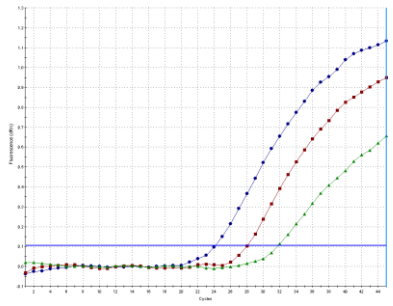
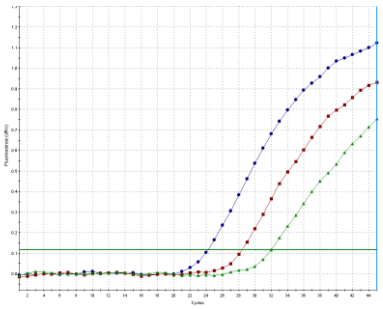
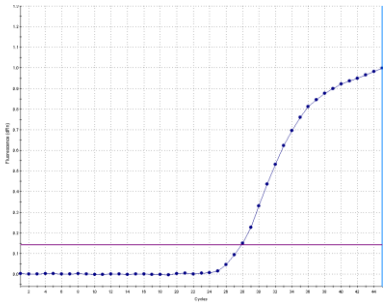
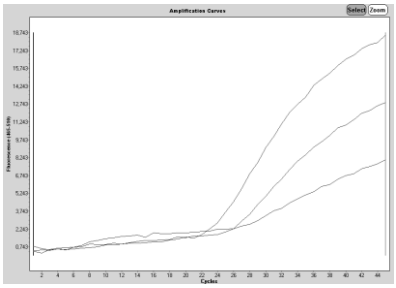
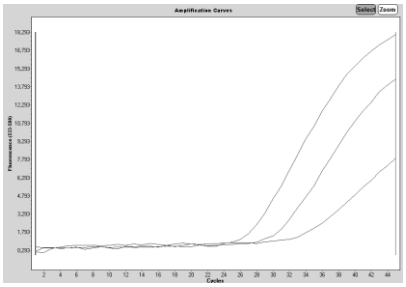
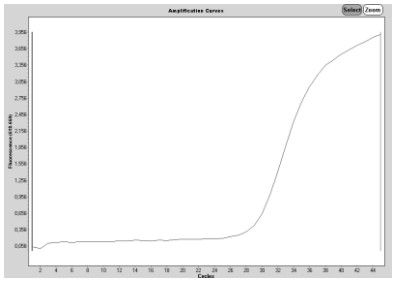
For analysis of PCR data please proceed as follows:
For analysis of PCR results gained with ViroReal® Kit PRRS Virus EU & NA 1.1 please select fluorescence display options 530 nm (FAM channel) for the PRRS virus EU target, 554 nm (VIC/HEX channel) for the PRRS virus NA target and 667 nm (Cy5 channel) 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 PRRS virus EU was amplified. The sample has to be interpreted as positive.

8.2. Signal in VIC/HEX channel:
→ RNA of PRRS virus NA 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 PRRS virus 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 PRRS virus EU FAM channel	Detection of PRRS virus NA VIC channel	Detection of internal positive control Cy5 channel
		
ABI Prism® 7500: FAM channel, 530 nm 1:10 serial dilution of the positive control	**ABI Prism® 7500:** VIC channel, 554 nm 1:10 serial dilution of the positive control	**ABI Prism® 7500:** Cy5 channel, 667 nm Detection of internal RNA positive control
		
Mx3005P®: FAM channel 1:10 serial dilution of the positive control	**Mx3005P®:** HEX channel 1:10 serial dilution of the positive control	**Mx3005P®:** CY5 channel Detection of internal RNA positive control
		
LightCycler® 480: FAM channel 1:10 serial dilution of the positive control	**LightCycler® 480:** VIC/HEX/Yellow555 channel 1:10 serial dilution of the positive control	**LightCycler® 480:** Cy5 channel, 667 nm Detection of internal RNA positive control

9. Troubleshooting

9.1. No PRRS virus EU or PRRS virus NA 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 RNA IPC no PRRS virus specific signals with 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. PRRS virus EU or PRRS virus NA specific signal with 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. PRRS virus EU or PRRS virus NA specific signal with 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 PRRS Virus EU & NA 1.1 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 12 RNA copies/PCR for PRRSV EU and 20 RNA copies/PCR for PRRSV NA. 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 56 copies/reaction for PRRSV EU and 35 copies/reaction for PRRSV NA. Sensitivity is not reduced when PCR is performed in a multiplex format. The ability to detect the PRRS virus RNA in samples is largely dependent upon the efficiency of the RNA extraction.

10.2. Analytical specificity

The primers and probes are carefully designed and are based on conserved regions. However, false-negative or less sensitive results might be obtained due to sequence heterogeneity of not yet described clinical subtypes. A great genetic heterogeneity of field isolates is observed and complicates diagnosis.
For validation, PRRS virus vaccine strains Ingelvac (US/NA) and Porcilis (EU), as well as a total of 132 confirmed PRRSV-positive field samples were tested and correctly analysed. Furthermore, the kit was tested on viral and bacterial isolates (BVDV, PCV2, PPV, *B. hyodysenteriae* and *H. parasuis*) and showed no cross-reactivity.