Instruction for Use

virellaPRRSV

real time RT-PCR Kit

For the qualitative *in-vitro* detection of RNA from Porcine Reproductive and Respiratory Syndrome Virus.

REF	G01102-32	G01102-96
Σ	32	96

- 18°C

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1 Intended Use

The virellaPRRSV real time RT-PCR Kit is an assay for the simultaneous detection of the RNA of the North American (NA), European (EU), and the highly pathogenic (HP) strain of the NA genotype of Porcine Reproductive and Respiratory Syndrome Virus (PRRSV). Extracted RNA from swine blood, serum, tissue, bronchial swabs, saliva, bronchial lavage, semen, and cell culture supernatant samples can be used for analysis.

2 Pathogen Information

PRRSV infections are highly prevalent in swine and lead to high economical losses. The panzootic disease causes reproductive failure in breeding stock and respiratory tract illness in young pigs. PRRSV is a member of the genus *Arterivirus*, family Arteriviridae, order Nidovirales. The two prototype strains of PRRSV are the North American strain, VR-2332, and the European strain, the Lelystad virus (LV). The European and North American PRRSV strains cause similar clinical symptoms, but represent two distinct viral genotypes whose genomes diverge by approximately 40%. In the early 2000s a highly pathogenic strain of the North American genotype emerged in China. This strain, HP-PRRSV, is more virulent than all other strains, and causes great losses in Asian countries.

3 Principle of the Test

The virellaPRRSV real time RT-PCR contains specific primers and hydrolysis probes for the detection of RNA of the NA, EU, and HP strains of PRRSV. Extracted RNA from swine blood, serum, tissue, bronchial swabs, saliva, bronchial lavage, semen, and cell culture supernatant samples can be used for analysis. The reverse transcription (RT) of viral RNA to cDNA and the subsequent amplification of virus specific fragments are performed in a one-step RT-PCR. The amplification can be detected when specific probes are hydrolysed by the Polymerase. The emitted fluorescence is measured in the FAM (NA), Cy5 (EU) and ROX (HP) channel.

Furthermore, the virellaPRRSV real time RT-PCR contains a Control RNA, which is detected simultaneously. Added during the extraction, the Control RNA allows not only monitoring of RT-PCR inhibition but also detects possible mistakes during extraction. This greatly reduces the risk of false-negative results. The Control RNA can also be used solely as Internal Control by adding it directly to the Mastermix. The fluorescence of the Control RNA is measured in the VIC®/HEX/JOE™/TET channel.

4 Package Contents

The reagents supplied are sufficient for 32 or 96 reactions respectively.

Table 1: Components of the virellaPRRSV real time RT-PCR Kit.

Label	Lid Colour	Content 32	Content 96
Reaction Mix	yellow	1 x 506 µl	2 x 759 µl
Enzyme	blue	1 x 6.4 µl	1 x 19.2 µl
Positive Control (NA, HP, EU)	red	1 x 50 µl	1 x 100 µl
Negative Control	green	1 x 50 µl	1 x 100 µl
Control RNA	colourless	1 x 160 µl	2 x 240 µl

5 Equipment and Reagents to be Supplied by User

- DNA/RNA isolation kit (e.g. NukEx Pure RNA/DNA, gerbion Cat. No. G05004 or NukEx Mag RNA/DNA, gerbion Cat. No. G05012)
- PCR grade Water
- Sterile microtubes
- Pipets (adjustable volume)
- Sterile pipet tips with filter
- Table centrifuge
- Vortexer
- Real time PCR instrument
- Optical PCR reaction tubes with lid
- Optional: Liquid handling system for automation
- Optional: Colour Compensation Kit for use of virellaPRRSV real time RT-PCR with LightCycler 480II, available from gerbion on request

6 Transport, Storage and Stability

The virellaPRRSV real time RT-PCR Kit is shipped on dry ice or cool packs.
All components must be stored at maximum -18°C in the dark immediately after receipt. Do not use reagents after the date of expiry printed on the package. Up to 20 freeze and thaw cycles are possible.
For convenience, opened reagents can be stored at +2-8°C for up to 6 months.
Protect kit components from direct sunlight during the complete test run.

7 Important Notes

- The virellaPRRSV real time RT-PCR must be performed by qualified personnel only.
- Good Laboratory Practice (GLP) has to be applied.
- Samples must always be regarded as potentially infectious material and all equipment used has to be treated as potentially contaminated.

8 General Precautions

- Stick to the protocol described in the Instruction for Use.
- Set up different laboratory areas for the preparation of samples and for the set up of the RT-PCR in order to avoid contaminations.
- Pipettes, tubes and other materials must not circulate between those different laboratory areas.
- Always use filter tips.
- Regularly decontaminate equipment and benches with ethanol-free decontaminant.
- Do not combine virellaPRRSV real time RT-PCR Kit components of different lot numbers.

9 Sample Material

Starting material for the virellaPRRSV real time RT-PCR is the nucleic acid isolated from clinical specimens (e.g. swine blood, serum, tissue, bronchial swabs, saliva, bronchial lavage, semen) and cell culture supernatant samples.

10 Sample Preparation

Commercial kits for RNA isolation such as the following are recommended:

- NukEx Pure RNA/DNA, gerbion Cat. No. G05004
- NukEx Mag RNA/DNA, gerbion Cat. No. G05012

Important: In addition to the samples always run a "water control" in your extraction. Treat this water control analogous to a sample.
Comparing the amplification of the Control RNA in the sample to the amplification of the Control RNA in the water control will give insights on possible inhibitions of the real time RT-PCR. Furthermore, possible contaminations during RNA extraction will be detectable.

Please note chapter ‚Control RNA'.

If the real time RT-PCR is not performed immediately, store extracted RNA according to the instructions given by the nucleic acid extraction extraction kit's manufacturer.

11 Control RNA

A Control RNA is supplied and can be used as extraction control or only as inhibition control. This allows the user to control the RNA isolation procedure and to check for possible real time RT-PCR inhibition.

a) Control RNA used as Extraction Control:
virellaPRRSV real time RT-PCR Control RNA is added to the extraction.
Add 5 µl Control RNA per extraction (5 µl x (N+1)). Mix well. Perform the RNA isolation according to the manufacturer's instructions. Please follow protocol A.
The Control RNA must be added to the Lysis Buffer of the extraction kit.

b) Control RNA used as Internal Control of the real time PCR:
If only inhibitions of the real time RT-PCR will be checked please follow protocol B.

12 Real time RT-PCR

12.1 Important Points Before Starting:

- Please pay attention to chapter 7 ‚Important Notes'.
- Before setting up the real time RT-PCR familiarise yourself with the real time PCR instrument and read the user manual supplied with the instrument.
- The programming of the thermal profile should take place before the PCR set up.
- In every RT-PCR run one Positive Control and one Negative Control should be included.

- Before each use, all reagents - except the Enzyme - should be thawed completely at room temperature, thouroughly mixed, and centrifuged very briefly.

12.2 Procedure

If the Control RNA is used to control both, the real time RT-PCR and the RNA isolation procedure, please follow protocol A. If the Control RNA is solely used to detect possible inhibition of the real time RT-PCR, please follow protocol B.

Protocol A

The Control RNA was added during RNA extraction (see chapter ‚Control RNA'). In this case, prepare the Master Mix according to Table 2.

The Master Mix contains all of the components needed for RT-PCR except the sample. Prepare a volume of Master Mix for at least one sample more than required, in order to compensate for pipetting inaccuracy.

Table 2: Preparation of the Master Mix

Volume per Reaction	Volume Master Mix
15.8 µl Reaction Mix	15.8 µl x (N+1)
0.2 µl Enzyme	0.2 µl x (N+1)

Protocol B

The Control RNA is used for the control of the real time RT-PCR only (see chapter ‚Control RNA'). In this case, prepare the Master Mix according to Table 3.

The Master Mix contains all of the components needed for real RT-PCR except the sample. Prepare a volume of Master Mix for at least one sample more than required, in order to compensate for pipetting inaccuracy.

Important: Dilute the Control RNA 1:10 in PCR-grade dH_2O (e.g. 1 µl Control RNA + 9 µl PCR grade Water before adding it to the Master Mix.

Table 3: Preparation of the Master Mix

Volume per Reaction	Volume Master Mix
15.8 µl Reaction Mix	15.8 µl x (N+1)
0.2 µl Enzyme	0.2 µl x (N+1)
0.2 µl Control RNA* **(diluted 1:10)**	0.2 µl x (N+1)*

*The increase in volume caused by adding the Control RNA is not taken into account when preparing the PCR assay. The sensitivity of the detection system is not impaired.

Protocol A and B: real time RT-PCR set up

- Place the number of optical PCR reaction tubes needed into the respective tray of the real time PCR instrument.
- Pipet **16 µl** of the Master Mix into optical PCR reaction tubes.
- Add **4 µl** of the eluates from the RNA isolation (including the eluate of the water control), the Positive Control, and the Negative Control to the corresponding optical PCR reaction tubes (Table 4).
- Close the optical PCR reaction tubes immediately after filling in order to reduce the risk of contamination.

Table 4: Preparation of the real time PCR

Component	Volume
Master Mix	16.0 µl
Sample	4.0 µl
Total Volume	20.0 µl

12.3 Instrument Settings

For the real time RT-PCR use the thermal profile shown in Table 5.

Table 5: real time RT-PCR thermal profile

Description	Time	Temperature	Number of Cycles
Reverse Transcription	10 min	50°C	1
Initial Denaturation	1 min	95°C	1
Amplification of cDNA			
Denaturation	10 sec	95°C	45
Annealing and Extension	40 sec Aquisition at the end of this step	60°C	

Dependent on the real time instrument used, further instrument settings have to be adjusted according to Table 6.

Alternatively, the thermal profile shown below can be used.

Description	**Time**	**Temperature**	**Number of Cycles**
Reverse Transcription	20 min	50°C	1
Initial Denaturation	15 min	95°C	1
Amplification of cDNA			
Denaturation	15 sec	95°C	45
Annealing	30 sec Aquisition at the end of this step	57°C	
Extension	30 sec	72°C	

Table 6: Overview of the instrument settings required for the virellaPRRSV real time RT-PCR Kit.

Real time PCR Instrument	Parameter Reaction Mix	Detection Channel	Notes		
LightCycler 480II			Colour Compensation required		
			Melt Factor	Quant Factor	Max Integration Time (sec)
	PRRSV NA	465-510	1	10	1
	PRRSV HP	533-610	1	10	2
	Control RNA	533-580	1	10	2
	PRRSV EU	618-660	1	10	3
Stratagene Mx3000P / Mx3005P	PRRSV NA	FAM	Gain 8		Reference Dye: None
	PRRSV HP	ROX	Gain 1		
	Control RNA	HEX	Gain 1		
	PRRSV EU	Cy5	Gain 4		
ABI 7500	PRRSV NA	FAM	Option Reference Dye ROX: NO		
	PRRSV HP	ROX			
	Control RNA	JOE			
	PRRSV EU	Cy5			
Rotor-Gene Q, Rotor-Gene 3000 Rotor-Gene 6000	PRRSV NA	Green	Gain 5		
	PRRSV HP	Orange	Gain 5		
	Control RNA	Yellow	Gain 5		
	PRRSV EU	Red	Gain 5		
mic qPCR Cycler	PRRSV NA	Green	Gain 8		
	PRRSV HP	Orange	Gain 10		
	Control RNA	Yellow	Gain 10		
	PRRSV EU	Red	Gain 10		

13 Data Analysis

The virus specific amplifications are measured in the FAM, ROX, and Cy5 channel. The amplification of the Control RNA is measured in the VIC®/HEX/JOE™/TET channel. The Positive Control contains in vitro transcripts of the respective nucleic acid sequences of PRRSV NA, PRRSV HP, and PRRSV EU strains. For the Positive Control, signals in the FAM, ROX, and Cy5 channels must be detected. The interpretation of the test results is described in table 7.

Table 7: Interpretation of results

Signal/Ct Values				Interpretation
FAM Channel PRRSV NA	ROX Channel PRRSV HP	Cy5 Channel PRRSV EU	HEX Channel Control RNA	
positive	negative	negative	positive or negative*	Positive result, the sample contains PRRSV NA-RNA.
positive	positive	negative	positive or negative*	Positive result, the sample contains PRRSV HP-RNA.
negative	negative	positive	positive or negative*	Positive result, the sample contains PRRSV EU-RNA.
negative	negative	negative	≤ 34**	Negative result, the sample contains no PRRSV NA-RNA, PRRSV HP-RNA and PRRSV EU-RNA.
negative	negative	negative	negative or > 34**	No diagnostic statement can be made. The real time RT-PCR is either inhibited or errors occurred while RNA extraction.

* A strong positive signal in the FAM, Cy5 and/or ROX can inhibit the IC. In such cases the result for the Control RNA can be neglegted.

** Depending on the PCR instrument and/or the chosen extraction method, the Ct values might be shifted. The water control can be used as reference. If the HEX Ct value of a sample differs a lot from the water control, partial inhibition has occurred, leading to false negative results in case of weak positive samples.

Figure 1 and Figure 2 show examples for positive and negative real time RT-PCR results.

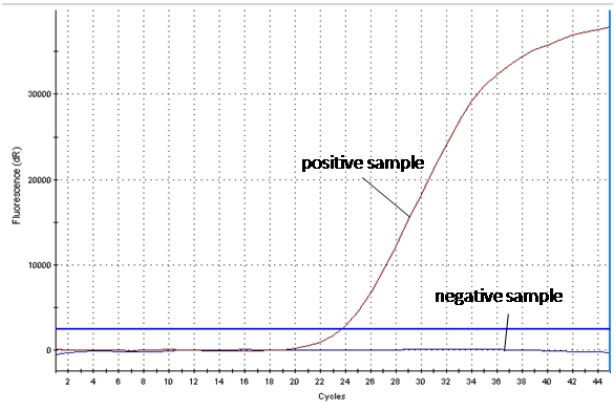

Figure 1: The positive sample shows virus-specific amplification, whereas no fluorescence signal is detected in the negative sample.

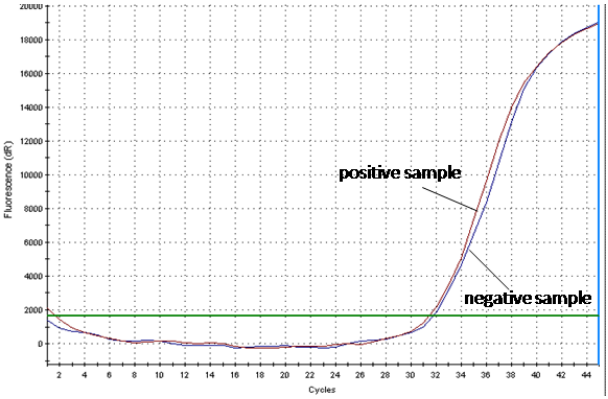

Figure 2: The positive sample as well as the negative sample show a signal in the Control RNA specific VIC®/HEX/JOE/TET channel. The amplification signal of the Control RNA in the negative sample shows, that the missing signal in the virus specific channels is not due to RT-PCR inhibition or failure of RNA isolation, but that the sample is a true negative.

14 Assay Validation

Set a threshold as follows:

Negative Controls

All negative controls should be below the threshold. If there is a potential contamination (appearance of a curve in the negative control or a cluster of curves in specimens at high C_T – for example above 36), results obtained are not interpretable and the whole run (including extraction) has to be repeated.

Positive Controls

All the positive controls must show a positive (i.e. exponential) amplification curve. The positive controls must fall below a C_T of 30.

Internal Controls

All internal controls must show a positive (i.e. exponential) amplification curve. The internal control must fall below a C_T of 34. If the internal control is above C_T 34, this points to a purification problem or a strong positive sample that can inhibit the IC. In the latter case, the assay is valid. If a water control run is performed, the IC must fall below a C_T of 34.

15 Troubleshooting

The following troubleshooting guide is included to help you with possible problems that may arise when performing a real time RT-PCR. If you have further questions, please do not hesitate to contact our scientists on info@gerbion.com.

No fluorescence signal in the FAM, ROX, Cy 5 channel of the Positive Controls	
The selected channel for analysis does not comply with the protocol	Select the FAM channel for analysis of the PRRSV NA specific amplification, the ROX channel for analysis of the PRRSV HP specific amplification and the Cy5 channel for analysis of the PRRSV EU specific amplification. Select the VIC®/HEX/JOE™/TET channel for the amplification of the Control RNA. Due to amplification in the specific channels, amplification of the Internal Control can be inhibited in the Positive Control.
Incorrect configuration of the real time RT-PCR	Check your work steps and compare with ‚Procedure' on page 7.
The programming of the thermal profile is incorrect	Compare the thermal profile with the protocol (Table 5, page 10).

Incorrect storage conditions for one or more kit components or kit expired	Check the storage conditions and the date of expiry printed on the kit label. If necessary, use a new kit and make sure kit components are stored as described in ‚Transport, Storage and Stability', page 5.
Weak or no signal of the Control RNA and simultaneous absence of a signal in the virus specific FAM channel, ROX channel or Cy5 channel.	
real time RT-PCR conditions do not comply with the protocol	Check the real time RT-PCR conditions (page 7 ff).
real time RT-PCR inhibited	Make sure that you use an appropriate isolation method (see ‚Sample Preparation', page 5) and follow the manufacturer's instructions. Make sure that the ethanol-containing washing buffer of the isolation kit has been completely removed. An additional centrifugation step at high speed is recommended before elution of the RNA.
RNA loss during isolation process	In case the Control RNA was added before extraction, the lack of an amplification signal can indicate that the RNA isolation was not successful. Make sure that you use an appropriate isolation method (commercial kits are recommended) and stick to the manufacturer's protocol.
Incorrect storage conditions for one or more components or kit expired	Check the storage conditions and the date of expiry printed on the kit label. If necessary, use a new kit and make sure kit components are stored as described in ‚Transport, Storage and Stability', page 5.
Detection of a fluorescence signal in the FAM channel, ROX channel or Cy5 channel of the Negative Control.	
Contamination during preparation of the RT-PCR	Repeat the real time RT-PCR in replicates. If the result is negative in the repetition, the contamination occured when the samples were pipetted into the optical PCR reaction tubes. Make sure to pipet the Positive Control last and close the optical PCR reaction tube immediately after adding the sample. If the same result occurs, one or more of the kit components might be contaminated. Make sure that work space and instruments are decontaminated regularly. Use a new kit and repeat the real time RT-PCR.

16 Kit Performance

16.1 Analytical Sensitivity

The limit of detection (LoD) of virellaPRRSV real time RT-PCR was determined using serial dilutions of in vitro transcripts (PRRSV NA, PRRSV HP, PRRSV EU) in a Stratagene Mx3005 real time PCR instrument.

Table 9: Samples tested for the validation of the sensitivity of virellaPRRSV real time RT-PCR.

PRRSV NA	Copies per Reaction	Expected Result	virellaPRRSV
	1.000.000	positive	positive
	100.000	positive	positive
	10.000	positive	positive
	1.000	positive	positive
	100	positive	positive
	10	positive	positive

PRRSV HP	Copies per Reaction	Expected Result	virellaPRRSV
	1.000.000	positive	positive
	100.000	positive	positive
	10.000	positive	positive
	1.000	positive	positive
	100	positive	positive
	10	positive	positive

PRRSV EU	Copies per Reaction	Expected Result	virellaPRRSV
	1.000.000	positive	positive
	100.000	positive	positive
	10.000	positive	positive
	1.000	positive	positive
	100	positive	positive
	10	positive	positive

16.2 Analytical Specificity

The specificity of the virellaPRRSV real time RT-PCR was evaluated by in silico analysis and by amplification of RNA and DNA of other relevant viruses and bacteria found in clinical samples.

The virellaPRRSV real time RT-PCR showed positive results for the samples containing RNA from PRRSV NA, PRRSV HP, and PRRSV EU, whereas samples containing other pathogens were reliably tested negative. The results are shown in table 10.

Table 8: Bacterial and viral pathogens tested for the determination of the analytical specificity of virellaPRRSV real time RT-PCR.

Sample	Status	virellaPRRSV real time RT-PCR		
		FAM PRRSV (NA)	ROX PRRSV (HP)	Cy5 PRRSV (EU)
11/ ASFV Niederlande DNA	ASFV pos.	negative	negative	negative
12/ ASFV"Estland" DNA	ASFV pos.	negative	negative	negative
13/ ASFV"Kenia" DNA	ASFV pos.	negative	negative	negative
14/ ASFV"Sardinien" DNA	ASFV pos.	negative	negative	negative
20/ CSFV"Litauen", RNA	CSFV pos.	negative	negative	negative
CSF0751 (RV213/18)	CSFV pos.	negative	negative	negative
CSF0679 (RV213/18)	CSFV pos.	negative	negative	negative
CSF0383 (RV213/18)	CSFV pos.	negative	negative	negative
CSF0932 (RV213/18)	CSFV pos.	negative	negative	negative
CSF0306 (RV213/18)	CSFV pos.	negative	negative	negative
CSF0653 (RV213/18)	CSFV pos.	negative	negative	negative
QCMD ADVDNA14-06 (RV134/14)	*Adenovirus*	negative	negative	negative
QCMD BPDNA 14-08 (RV127/14)	*Bordetella pertussis*	negative	negative	negative
Instand RV 370_15 Intern 141/15 Probe 370056	*Influenza A*	negative	negative	negative
Instand RV 370_15 Intern 141/15 Probe 370055	*Influenza B*	negative	negative	negative
QCMD MTBDNA14-01 (RV 124/14)	*Mycobacterium tuberculosis*	negative	negative	negative
Instand RV 541/2015 (RV 151/15) Probe 1515413	*Mycoplasma pneumoniae*	negative	negative	negative
ATCC-VR-1360	*Chlamydia pneumoniae*	negative	negative	negative
QCMD CVRNA 14-02	*Coronavirus*	negative	negative	negative
ATCC 15597	*Escherichia coli*	negative	negative	negative
PRRSV (EU) Lelystad Virus	*PRRSV EU*	negative	negative	**positive**
PRRSV (NA) ATCC VR 2332	*PRRSV NA*	**positive**	negative	negative
PRRSV HP PRRS-Virus 2, Stamm China FLI RVB-0581	*PRRSV HP*	negative	**positive**	negative

16.3 Diagnostic Sensitivity and Specificity

During the validation study of the virellaPRRSV real time RT PCR positive and negative clinical samples, previously characterized using other real time RT-PCR, were tested. The diagnostic sensitivity was found to be 100% and the diagnostic specificity 100%.

Table 9: Overview of the amount of samples tested and the diagnostic sensitivity and specificity

		positive samples	negative samples
PRRSV positive			
	PRRSV EU	22	0
	PRRSV HP	1	0
	PRRSV NA	11	0
PRRSV negative			
	PRRSV EU	0	18
	PRRSV HP	0	39
	PRRSV NA	0	29
Sensitivity		100%	
Specificity		100%	

17 Abbreviations and Symbols

cDNA	complementary Deoxyribonucleid Acid
RNA	Ribonucleid Acid
PCR	Polymerase Chain Reaction
RT	Reverse Transcription
ASFV	African Swine Fever Virus
CSFV	Classical Swine Fever Virus
REACTION MIX	Reaction Mix
ENZYME	Enzyme
CONTROL +	Positive Control
CONTROL −	Negative Control
CONTROL RNA IC	Control RNA

REF	Catalog number
Σ	Contains sufficient for <n> test
-18°C	Upper limit of temperature
	Manufacturer
	Use by YYYY-MM-DD
LOT	Batch code
CONT	Content
	Consult instructions for use

18 Literature

[1] Moh A. Alkhamis, Andreia G. Arruda, Robert B. Morrison, Andres M. Perez. Novel approaches for Spatial and Molecular Surveillance of Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) in the United States, Nature.com., Scientific Reports volume 7, Article number: 4343 (2017)

[2] Pileri, E; Mateu, E (28 October 2016). "Review on the transmission porcine reproductive and respiratory syndrome virus between pigs and farms and impact on vaccination". Veterinary research. 47 (1): 108