ViroReal® Kit EMCV

Manual

For use with the

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

For veterinary use only

DVEV01011, DVEV01013 **100**

DVEV01051, DVEV01053 **50**

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1. Product description

ViroReal® Kit EMCV is a real-time PCR assay for detection of RNA of the encephalomyocarditis virus (EMCV) 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 kit allows the rapid and sensitive detection of RNA of EMCV from heart, liver, kidney, and spleen tissue from acutely dead animals or abortuses (e.g. with the QIAamp Viral RNA Mini Kit, Qiagen).

ViroReal® Kit EMCV detects a part of the 3´UTR RNA of EMCV. A probe-specific amplification-curve in the FAM channel indicates the amplification of EMCV specific RNA. An internal RNA positive control system for detection in VIC/HEX channel (order no. DVEV01011 or DVEV01051) or in Cy5 channel (order no. DVEV01013 or DVEV01053) allows control of RNA extraction and excludes false-negative interpretation of results caused by 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

Encephalomyocarditis virus (EMCV) is one of three virus species in the genus *Cardiovirus* in the family *Picornaviridae* and is a single stranded RNA (ssRNA) virus that causes encephalomyocarditis and reproductive disease in swine and zoologic mammals. Infected piglets suffer from encephalitis, myocarditis and sudden death. Reproductive problems include poor conception rates, embryo resorption, mummification, stillbirths, abortions and neonatal death. The disease affects the nervous, reproductive, respiratory and circulatory system of pigs. Fatal myocarditis due to EMC virus infection has been seen in primates, even-and odd-toed ungulates, elephants, carnivores and rodents. The disease can be found worldwide. The most important mode of virus transmission seems to be rodent-to-pig transmission, and rodents act as the major reservoir of the disease. Rats and other rodents probably infect pigs directly or through diseased carcasses and contaminated feeds or water supplies.

References:
Billinis, C., Paschaleri-Papadopoulou, E., Psychas, V., Vlemmas, J., Leontides, S., Koumbati, M., Kyriakis, S.C. and Papadopoulos, O. 1999. Persistence of Encephalomyocarditis virus (EMCV) infection in piglets. Vet. Microbiol. 70:171–177.

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. Contents of the Kit

4.1. ViroReal® Kit EMCV order no. DVEV01011 or DVEV01051

Labelling	Content	Amount		Storage
		DVEV01011	DVEV01051	
EMCV Assay Mix (green cap)	Primer and probe (FAM) for EMCV detection	2 x 50 µl	1 x 50 µl	-20°C
RNA IPC-1 Assay Mix (yellow cap)	Primer and probe (VIC/HEX) 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
EMCV Positive Control (red cap)	RNA positive control (approx. 1,000 target copies/µl)	1 x 300 µl	1 x 300 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

4.2. ViroReal® Kit EMCV order no. DVEV01013 or DVEV01053

Labelling	Content	Amount		Storage
		DVEV01013	DVEV01053	
EMCV Assay Mix (green cap)	Primer and probe (FAM) for EMCV 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 (red cap)	RNA internal positive control	1 x 100 µl	1 x 100 µl	-20°C
EMCV Positive Control (red cap)	RNA positive control (approx. 1,000 target copies/µl)	1 x 300 µl	1 x 300 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 EMCV are stable until the expiry date stated on the label. Repeated thawing and freezing should be avoided. Please protect kit components from light.

5. Additionally required materials and devices

- Reagents and devices for RNA-extraction
- Nuclease-free water for dilution of RNA IPC Target
- 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

6. 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 EMCV 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 RNA IPC Target is stored in RNA stabilizer that contains guanidinium thiocyanate/Triton X-100 (see MSDS, www.ingenetix.com).

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:

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 EMCV Positive Control is an *in vitro* synthesized RNA sample with a concentration of 10^3 copies/µl. It has to be stored at -20°C. Ensure a homogenous solution by gently mixing, do not vortex. To avoid sfreeze/thaw cycles, it can also be temporarily stored at 4°C if used several times on the same day.
→ As positive control please use 10 µl of the Positive Control.

7.3. Pipetting scheme

		Per sample
Preparation of Master Mix (mix well)	Nuclease-free Water*	2.0 µl
	RNA Reaction Mix	5.0 µl
	EMCV 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 EMCV
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 Cycles: 1 Analysis: None	**Program 2** Cycles: 1 Analysis: None	**Program 3** 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: 2 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) EMCV target	**Ct/Cp RNA IPC target**	**Interpretation**
Negative control	Negative	26-29*	Valid
Positive control, approx. 10,000 copies, 10 µl/PCR	<30	26-29	Valid
Extraction negative control (optional)	Negative	26-29	Valid
Negative sample	Negative	26-29	Valid
Positive sample	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 EMCV please select fluorescence display options 530 nm (FAM channel) for the EMCV target and VIC/HEX channel or 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

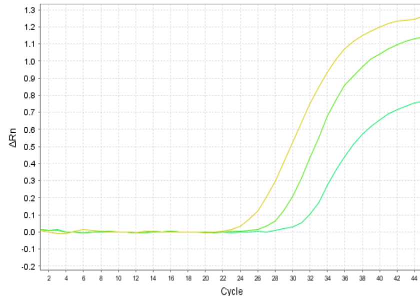
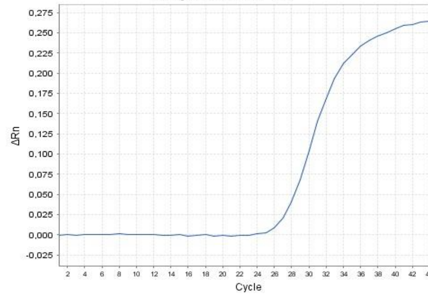
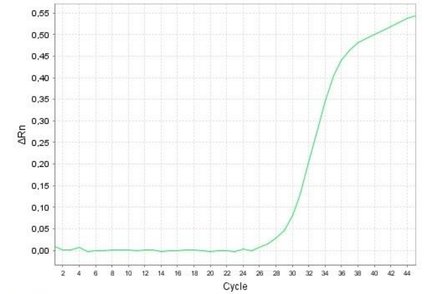
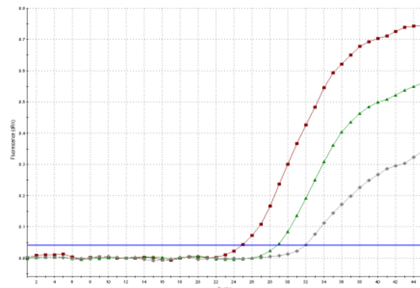
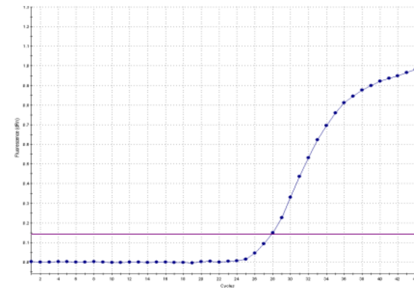
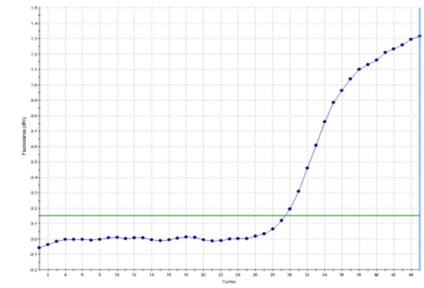
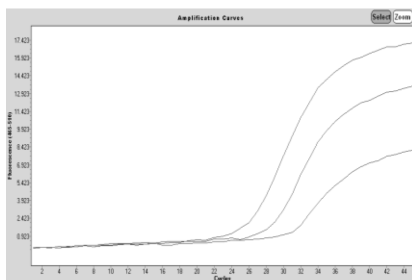
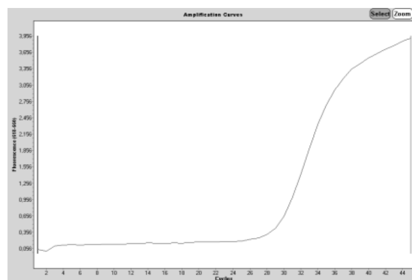
→ RNA of EMCV was amplified. The sample has to be interpreted as positive.

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

→ No RNA of EMCV 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 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 **EMCV**	Detection of internal RNA positive control **IPC-3**	Detection of internal RNA positive control **IPC-1**
 **ABI Prism® 7500:** FAM channel, 530 nm 1:10 serial dilution of an EMCV RNA positive control	 **ABI Prism® 7500:** Cy5 channel, 667 nm Detection of internal RNA positive control	 **ABI Prism® 7500:** VIC channel, 554 nm Detection of internal RNA positive control
 **Mx3005P®:** FAM channel 1:10 serial dilution of an EMCV RNA positive control	 **Mx3005P®:** CY5 channel Detection of internal RNA positive control	 **Mx3005P®:** HEX channel Detection of internal RNA positive control
 **LightCycler® 480:** FAM channel 1:10 serial dilution of an EMCV RNA positive control	 **LightCycler® 480:** Cy5 channel Detection of internal RNA positive control	

9. Troubleshooting

9.1. No EMCV 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.
- RNA might be degraded.

9.2. No signal with internal RNA positive control and no EMCV 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. EMCV 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. EMCV specific signal with negative control of RNA-extraction (optional)

- 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 EMCV 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 approx. 50-100 RNA copies/PCR.

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 EMCV strains published in the NCBI database.

11. Annex – symbols

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