ViroReal® Kit Enterovirus

Kit version 1.1

Instructions for Use

CE

IVD **For *in vitro* diagnostic use**

REF **DHUV00353**

Σ **50 reactions**

ingenetix GmbH
Haidingergasse 1
1030 Vienna, Austria
T +43(0)1 36 198 01
office@ingenetix.com
www.ingenetix.com

Index

1 Intended purpose 3
2 Product description 3
3 Pathogen information 3
4 Principle of one-step reverse transcription real-time PCR 4
5 Contents of the kit, stability, and storage 4
6 Additionally required materials and devices 5
7 Precautions and safety information 5
7.1 General information 5
7.2 Specific information 5
8 Limitations 6
9 Preparation of samples 7
9.1 Sample collection and storage 7
9.2 Recommended extraction methods 7
9.3 Quality control for RNA extraction and RT PCR inhibition with IPC 7
9.3.1 Application of IPC as control of extraction and RT real-time PCR 7
9.3.2 Application of IPC as quality control for RT PCR reaction 7
10 Preparation of real-time PCR 8
10.1 Pipetting scheme 8
10.2 Programming of temperature profile 9
11 Interpretation of data 10
11.1 Controls 10
11.2 Clinical samples 11
12 Troubleshooting 11
12.1 No virus specific signal with positive control and with IPC 11
12.2 Virus specific signal with negative control 11
12.3 Virus specific signal with negative control of extraction 11
12.4 IPC specific signal with PCR negative control and positive control 11
12.5 No signal with IPC and no virus specific signal with sample 12
13 Specifications and performance evaluation 13
13.1 Test performance on different real-time PCR instruments 13
13.2 Limit of detection, LoD95% 14
13.3 Linearity and dynamic measuring range 14
13.4 Precision 14
13.5 Analytical specificity 14
13.6 Diagnostic evaluation 15
14 References 15
15 Revision history 15

Explanation of symbols

LOT	Batch code		Use by date
REF	Catalogue number		Manufacturer
Σ	Contains sufficient for <n> tests		Temperature limit (Store at)
CE	This product fulfils the requirements of the European Directive 98/79 EC for *in vitro* diagnostic medical devices	IVD	In vitro diagnostic medical device
	Consult instructions for use	UDI	Unique device identifier
	Keep away from sunlight	QTY	Contents

1 Intended purpose

ViroReal® Kit Enterovirus is a non-automated IVD test, based on one-step reverse transcription real-time PCR (RT-PCR), for the qualitative detection of RNA (5´UTR region) of the genus *Enterovirus*. The detection of following serotypes has been confirmed: Coxsackie-Virus A1, coxsackie-virus A5, coxsackie-virus A6, coxsackie-virus A13, coxsackie-virus A21, coxsackie-virus B2, coxsackie-virus B3, coxsackie-virus B5, echovirus 1, echovirus 6, echovirus 11, echovirus 14, echovirus 30, enterovirus 71, enterovirus 90, enterovirus 99, enterovirus D68, poliovirus type 1, poliovirus type 2 and poliovirus type 3. Some rhinoviruses can also be detected, but with expected lower sensitivity.

Proper specimens are RNA extracts isolated from samples of the human respiratory tract (nasopharyngeal swabs) or stool.

This test is suitable for patients of all ages with suspected enterovirus infection and is intended as an aid in the diagnosis of enterovirus infection in combination with patient history and additional clinical information.

The test is intended for professional use and is limited to qualified personnel instructed in the procedures of real-time PCR and *in vitro* diagnostic procedures.

2 Product description

ViroReal® Kit Enterovirus is a one-step reverse transcription real-time PCR test and detects RNA of the 5´UTR region of the genus *Enterovirus.* The detection of following serotypes has been confirmed with ViroReal® Kit Enterovirus: Coxsackie-Virus A1, coxsackie-virus A5, coxsackie-virus A6, coxsackie-virus A13, coxsackie-virus A21, coxsackie-virus B2, coxsackie-virus B3, coxsackie-virus B5, echovirus 1, echovirus 6, echovirus 11, echovirus 14, echovirus 30, enterovirus 71, enterovirus 90, enterovirus 99, enterovirus D68, poliovirus type 1, poliovirus type 2 and poliovirus type 3. Some rhinoviruses can also be detected, but with expected lower sensitivity.

A probe-specific amplification-curve in the FAM channel indicates the amplification of enterovirus specific RNA. The internal RNA positive control (IPC) is detected in Cy5 channel and serves as a control for RNA extraction and possible RT-PCR inhibition. The target for the RNA IPC (artificial target RNA) is added during sample extraction.

This test has been validated with the ABI® 7500 Fast instrument (fast cycle parameters are not supported, Thermo Fisher Scientific) and was also tested with QuantStudio™ 7 Pro (Thermo Fisher Scientific), Mx3005P® (Agilent), qTOWER^{3}G (Analytik Jena), MIC instrument (bio molecular systems) and LightCycler® 480 II (Roche Diagnostics). It is also compatible with other real-time PCR instruments which detect and differentiate fluorescence in FAM and Cy5 channel (e.g., QuantStudio™ 5, cobas z 480 Analyzer (Roche)).

When using PCR-platforms not tested by ingenetix, an evaluation of the multiplex-PCR shall be done. Keep in mind that some PCR-platforms first have to be calibrated with the corresponding dye before performing multiplex-PCR.

Ingenetix ViroReal®, BactoReal®, MycoReal®, PanReal and ParoReal® Kits are optimized to run under the same thermal cycling conditions. RNA and DNA can be analysed in one run.

3 Pathogen information

Enteroviruses are non-enveloped, positive single-stranded RNA viruses (ss(+)RNA) of the family *Picornaviridae*. The genus *Enterovirus* has a worldwide distribution and includes numerous viral groups infecting humans, bovines, equines, simians, porcines and rodents.
Enteroviruses infecting humans are poliovirus, coxsackievirus, echovirus, human enteroviruses and human rhinoviruses. Enteroviruses are the causative agents e.g., for infection of the respiratory tract (summer flu, pharyngitis, pneumonia) and infection of inner organs (myocarditis, encephalitis, pleurodynia), aseptic meningitis, herpangina, hand-foot-mouth disease, haemorrhagic conjunctivitis and poliomyelitis.

4 Principle of one-step reverse transcription real-time PCR

The test is based on multiplex one-step reverse transcription real-time PCR (RT-PCR) by 5´-nuclease-assay technology. In a first step, a specific RNA sequence is transcribed into cDNA and subsequently the DNA is amplified. The generated PCR-products are detected by an oligonucleotide-probe labelled with a fluorescent dye. This allows the sequence-specific detection of PCR amplificates.

During PCR, primers are extended by *Taq* polymerase and probes hybridized to the target are cleaved by the 5'-exonuclease activity of *Taq* polymerase. According to the accumulation of PCR product, the fluorescence of the probe increases with each PCR cycle. The change in fluorescence of the different dyes is recorded cycle by cycle in the real-time PCR instrument in the closed reaction tube at different fluorescence wavelengths.
The Cq value (Cq = Quantification cycle, Ct = Cycle threshold, Cp = Crossing point) describes the cycle at which the fluorescence rises significantly above the background fluorescence.

5 Contents of the kit, stability, and storage

Table 1

Labelling	Content	Amount	Storage
Enterovirus + IPC3 Assay Mix (green cap)	Primer + probes for detection of - Enterovirus (FAM) - RNA IPC (Cy5)	1 x 50 µl	-25 to -15 °C
IPC-Target (RNA) (orange cap)	Target for RNA IPC (internal RNA positive control system)	1 x 200 µl	-25 to -15 °C
Enterovirus Positive Control (red cap)	RNA positive control (approx. 1000 target copies/µl)	1 x 300 µl	-25 to -15 °C
RNA Reaction Mix (white cap)	PCR reaction mix for RNA amplification	1 x 250 µl	-25 to -15 °C
Nuclease-free water (blue cap)	Nuclease-free water	1 x 1000 µl	-25 to -15 °C

RNA Reaction Mix: The Master Mix provided with the kit has been designed for reliable, high-sensitivity one-step reverse transcription real-time PCR even in the presence of common reaction inhibitors. The Master Mix contains a thermostable MMLV Reverse Transcriptase, an RNase inhibitor, a highly purified Taq Polymerase for rapid hot-start PCR, dNTPs, ROX™ dye (passive reference) and buffer components – additives optimized to handle RT-PCR inhibitors.

Delivery and Storage

Shipment is with dry ice. When stored properly, the kit components are stable until the specified expiration date. This also applies after opening. Store kit protected from light.

Quality Control Release Testing

In accordance with the ISO 13485 certified Quality Management System of ingenetix, each lot is tested against predetermined specifications to ensure consistent product quality.
Quality control is performed with an artificial RNA representing parts of the pathogen RNA. The RNA concentration was determined using a standard curve of a plasmid containing parts of the pathogen sequence.

6 Additionally required materials and devices

- Reagents and devices for RNA-extraction which are appropriate for the listed sample material (see 9. Preparation of the samples)
- Nuclease-free water
- Powder-free disposable gloves
- Pipettes (adjustable)
- Filter pipette tips
- Vortex-Mixer
- Benchtop centrifuge with rotor for 2 ml reaction tubes
- Real-time PCR instrument detecting and differentiating fluorescence in FAM and Cy5 channel
- Appropriate 96 well optical reaction plates or reaction tubes with corresponding (optical) closing material
- Optional: Laminar flow box
- Optional: PCR workstation

7 Precautions and safety information

7.1 General information

- For *in vitro* diagnostic use. The use of this kit is limited to qualified personnel instructed in real-time PCR and *in vitro* diagnostic procedures.
- Transportation of clinical specimens must comply with local regulations for the transport of biological materials.
- Specimens should be handled as if infectious in accordance with safe laboratory procedures. Wear protective powder-free disposable gloves when handling kit reagents and specimens.
- Discard specimens, reagents, and waste according to your local safety regulations.
- Improper collection, transport or storage of specimens may hinder the ability of the assay to detect the target sequences.
- Quality of RNA has a profound impact on the test performance. Ensure that the used RNA extraction system is compatible with real-time PCR technology.
- The real-time PCR instrument should be calibrated, serviced, and cleaned regularly.
- Protect kit components from light.
- Avoid mixing reagents of different kits and lots and check expiry date of the kit.

7.2 Specific information

A workflow must be followed to avoid false positive results due to detection of contaminating RNA.

<u>Recommended measures to avoid RNA contamination:</u>

- Use separated workspaces for specimen preparation, set-up of real-time PCR and amplification. Supplies and equipment must be dedicated to each of these separate workspaces to ensure workflow in the laboratory from pre- to post-PCR.
- Laboratory benches and devices must be cleaned regularly.
- Sample preparation should be performed in a laminar flow box. Clean laminar flow box regularly in all areas.
- If possible, leave consumables and pipettes in the laminar flow sterile bench and PCR workstation.
- Preparation of real-time PCR should be done in a PCR workstation.
- The use of sterile aerosol-resistant pipette tips is required.
- Use only RNA-free consumables.
- Wear lab coat.
- Work only with powder-free disposable gloves, do not touch the outer surface of the palm and fingers of the gloves when putting them on. Change gloves frequently. To avoid skin contact, wear gloves over the sleeves of the lab coat. Use disposable sleeve protectors if possible.
- Do not touch the rim or threads of open vials.
- Caution is advised when handling specimens and positive control to avoid cross-contamination.
- Store positive and potentially positive material separately from all other reagents.
- For a valid interpretation of results, a negative control shall be included during RNA-extraction (e.g., extraction of water instead of sample material), to exclude false-positive results due to contamination with RNA of pathogen during extraction.
- Optional: include a negative control of PCR per PCR-run (nuclease-free water instead of sample, NTC).

8 Limitations

- Reliable results with this test are only achieved by appropriate specimen collection, transport, and storage, as well as an appropriate RNA extraction procedure.
- RNA extraction and enterovirus detection have been validated for nasopharyngeal swabs and stool samples with this kit. Test performance with other specimen types has not yet been assessed.
- A negative test result does not exclude the possibility of enterovirus infection, because test results may be affected by improper specimen collection, technical error, specimen mix-up or viral quantities below the assay sensitivity. PCR inhibitors may lead to invalid results.
- For this kit highly specific primers and probes have been selected. However, false-negative, or less sensitive results might be obtained due to sequence heterogeneity within the target region of yet unknown clinical subtypes.
- Results should be interpreted in context of other clinical and laboratory findings.

9 Preparation of samples

ViroReal® Kit Enterovirus is suitable for analysis of RNA extracts of samples from the human respiratory tract (nasopharyngeal swabs) and stool.

Sample preparation should be performed with the recommended measures to avoid RNA contamination (see precautions and safety information). Always include an extraction negative control during RNA-extraction (e.g., extraction of water instead of sample material).

Purified RNA should be stored at -25 to -15 °C or at -80 to -60 °C.

9.1 Sample collection and storage

Samples from the respiratory tract and stool can be stored in microcentrifuge tubes. Swabs are stored either dry or in isotonic saline solution (NaCl 0.9%, not provided). Swabs can be collected with swab material proper for PCR (e.g., sterile polyester or rayon swabs with aluminium or plastic shaft).
It is recommended to process samples immediately after collection. Store samples at 2-8 °C for no longer than 48 hours. Do not freeze native samples prior to extraction. Samples in lysis buffer can be frozen.

9.2 Recommended extraction methods

Ensure that the extraction system used is not contaminated with RNA of pathogens detected with ViroReal® Kit Enterovirus. Extract samples with an RNA extraction system compatible with RT real-time PCR technology and appropriate for the sample material. Extract RNA from 140-200 µl sample (depending on the extraction method) and elute in approx. 50 µl.

For manual extraction recommended
- QIAamp Viral RNA Mini Kit (Qiagen)

When using extraction methods not recommended by ingenetix, an evaluation of the extraction method must be performed.

9.3 Quality control for RNA extraction and RT PCR inhibition with IPC

The RNA IPC system (internal RNA positive control) is used as a control for RNA extraction, identifies possible RT PCR inhibition and confirms the integrity of kit reagents.
For this, an artificial RNA (IPC-Target (RNA), approx. $5x10^5$ copies/µl) is added during extraction.

Note: The Cq values of the IPC depend on the extraction method and on the type of sample material. Negative samples should show Cq values of the IPC between 27-30. The extraction method used must be validated accordingly with sample material. Use the IPC-Target (RNA) freshly diluted (1:10 with nuclease-free water) in the extraction, if Cq values < 27 are determined during validation with samples.

9.3.1 Application of IPC as control of extraction and RT real-time PCR

The IPC-Target (RNA) must be added during extraction.

→ Per sample spike 1 µl IPC-Target (RNA) (orange cap) directly into the appropriate volume of lysis buffer (or spike it to the sample after the lysis buffer has been added to the sample), then continue the extraction procedure.

Caution: The IPC-Target (RNA) shall not be added to sample material in the absence of lysis buffer, as degradation may occur. It must be added to the lysis buffer.

9.3.2 Application of IPC as quality control for RT PCR reaction

If the IPC-Target (RNA) has not been added during extraction, it can be added at a later stage to the PCR master mix.

→ Freshly dilute the IPC-Target (RNA) 1:100 with nuclease-free water and add 1 µl of the dilution/PCR reaction.

Caution: The IPC-Target (RNA) shall not be added undiluted to the master mix (inhibition of PCR).

10 Preparation of real-time PCR

- Include one positive control (red cap), one extraction negative control and optionally one PCR negative control (NTC, e.g., nuclease-free water) per PCR run.
- It is generally recommended to analyse samples in duplicates, which increases the probability of pathogen detection and facilitates interpretation of results.
- Best use RNA immediately after extraction and always keep it on ice. Alternatively, use RNA stored at -80 °C to -15 °C. Thaw RNA on ice, avoid prolonged exposure to room temperature and immediately refreeze the RNA after use.
- Just before use, thaw the RNA Reaction Mix, and invert 2 to 3 times to ensure a homogenous solution. The RNA Reaction Mix does not freeze at -20 °C, but gelling may occur. The RNA Reaction Mix should not be warmed to room temperature. Thaw the other kit components completely at room temperature. When thawed, mix components carefully, centrifuge briefly with low speed and subsequently put on ice. Prepare Master Mix for RT-PCR on ice.
- **Positive Control**
  → As positive control, use 10 µl of the Positive Control (red cap). Always, pipette positive control at last. Enterovirus Positive Control is an *in vitro* synthesized RNA with a concentration of approx. 10^3 copies/µl. It must be stored at -20 °C. Before use, gently mix Positive Control, do not vortex. To avoid frequent freeze/thaw cycles, it can also be temporarily stored at 4 °C if used several times on the same day.

10.1 Pipetting scheme

		Per sample
Preparation of Master Mix (mix well)	Nuclease-free Water*	4.0 µl
	RNA Reaction Mix	5.0 µl
	Enterovirus + IPC3 Assay Mix	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**

*10 µl sample can be used. When using a volume other than 10 µl, the volume of nuclease free water has to be adjusted accordingly.

→ **If IPC-Target (RNA) was not added during extraction:** Freshly dilute the IPC-Target (RNA) (orange cap) 1:100 with nuclease-free water and add 1 µl per sample directly to the master mix. In this case, the IPC-Target (RNA) is used as a quality control for the RT PCR reaction.

Caution: The use of more than 1 µl diluted IPC-Target (RNA) per reaction might cause inhibition of the real-time PCR reaction.

- Prepare the Master Mix according to the number of samples, calculating an additional volume of approx. 10% for pipetting loss.
- Pipette 10 µl of the prepared Master Mix per sample into the well of the optical reaction plate.
- Then add 10 µl of the extracted sample or controls. Pipette the positive control at last.
- Seal the plate with a suitable optical sealing material.
- Vortex the sealed plate for 1-2 seconds and briefly centrifuge the plate.

10.2 Programming of temperature profile

Please find further information on programming of the real-time PCR instrument in the respective operator's manual. Keep in mind that some PCR-platforms must be calibrated with the corresponding dye before performing a multiplex-PCR.

Sample Volume: 20 µl
Ramp speed: "Standard" without "fast cycling" parameter for ABI® 7500 Fast Instrument, QuantStudio™ (Thermo Fisher Scientific)

Program 1 Cycles: 1 Analysis: None	**Program 2** Cycles: 1 Analysis: None	**Program 3** Cycles: 45 Analysis: Quantification Acquisition at 60 °C
50 °C 15 min	95 °C 2 min	95 °C 5 sec 60 °C 1 min

Ad program 1: This temperature profile can be used for all ingenetix ViroReal®, BactoReal®, MycoReal®, PanReal and ParoReal® kits for the detection of DNA or RNA.
Ad program 2: the previous temperature profile with 20 sec in program 2 can still be used.

<u>Select detection channels</u>

FAM channel: Detection of enterovirus
Cy5 channel: Detection of IPC

For ABI® 7500 Instrument, QuantStudio™ 5/6/7 (Thermo Fisher Scientific), Mx3005P® (Agilent)
FAM-TAMRA
Cy5-NONE
Passive reference dye: ROX

For MIC Instrument (bio molecular systems)
FAM: Green
Cy5: Red
Passive reference dye: no ROX needed

For cobas z 480 Analyzer / LightCycler® 480 II (Roche)
Detection format: 2 Color Hydrolysis Probe
FAM: Excitation at 465 nm, Emission at 510 nm
Cy5: Excitation at 610 / 618 nm, Emission at 670 / 660 nm
Passive reference dye: no ROX needed

11 Interpretation of data

For the analysis of the PCR results, select the fluorescence display options FAM channel for the pathogen target and Cy5 channel for the IPC.
Please note that some PCR platforms require Color Compensation when using a multiplex PCR.

IMPORTANT: Samples should be checked in both logarithmic (Roche instrument: Abs Quant/Fit Points) and linear view and compared to the negative control. In addition to the Cq values, also check the amplification curves and adjust the threshold (noise band) if necessary.
After you have saved the new settings, export the data. For the cobas z 480 Analyzer, export tables per dye.

Assessment of clinical specimen test results should be performed after data of the positive and negative controls and of the IPC have been examined and determined to be valid. If results of controls are not valid, no interpretation of results with clinical samples is possible.

Negative samples:
Samples without amplification curves (no Cq values, undetermined) are regarded as negative. No RNA was detected in these samples because there is no infection with the corresponding pathogen, or the pathogen RNA concentration is below the detection limit of the test.

Positive samples:
Samples with Cq values < 45 in the fluorescence channel for the pathogen target are regarded as positive.

Table 2 shows the criteria for valid positive and negative controls. Table 3 shows interpretation of data with clinical samples.

11.1 Controls

Table 2 Criteria for valid positive and negative controls, IPC-Target (RNA) was added during extraction

	Cq FAM channel Enterovirus target	Cq Cy5 channel IPC[1]	Interpretation	Action
Positive control	< 30	Negative	Valid	-
Positive control	Negative	Negative	Invalid	See 12.1
Extraction negative control	Negative	27-30[2]	Valid	-
Extraction negative control	Negative	Negative	Invalid	See 12.1
Extraction negative control	Positive	27-30[2]	Invalid	See 12.3
NTC PCR (optional)	Negative	Negative	Valid	-
NTC PCR (optional)	Positive	Negative	Invalid	See 12.2

[1] If the IPC-Target (RNA) was added directly to the master mix, all samples in the Cy5 channel must be positive.
[2] Cq values of the IPC are dependent on the extraction method and should show Cq values of 27-30 (see 9. preparation of samples).

11.2 Clinical samples

Samples with positive Cq-values are considered positive, see Table 3.

Table 3 Interpretation of data with clinical samples

	Cq FAM channel Enterovirus target	Cq Cy5 channel IPC[1]	Interpretation	Action
Clinical sample	Negative	27-30[2]	Negative	-
Clinical sample	Positive	Positive/Negative[2]	Positive	-
Clinical sample	Negative	Negative	Invalid	See 12.5

[1] A positive signal of the IPC excludes potential PCR inhibition. However, IPC Cq-values should show comparable results among samples. A shift of Cq- values can indicate a partial inhibition of PCR. Cq values of the IPC are dependent on the extraction method and should show Cq values of 27-30 (see 9. preparation of samples).
[2] High pathogen load in the sample can lead to a reduced or absent fluorescence signal of the IPC.

In case of invalid data, analysis has to be repeated with the remaining or newly extracted RNA sample (see 12. Troubleshooting).

12 Troubleshooting

12.1 No virus specific signal with positive control and with IPC

- Incorrect programming of the temperature profile or incorrect setting of detection channels on the real-time PCR instrument.
  → Compare temperature profile and setting of detection channels with details specified in the protocol.
- Incorrect configuration of the PCR reaction.
  - → Check your pipetting steps with the pipetting scheme and repeat PCR, if necessary.
  - The RNA may have been degraded.
- The IPC-Target (RNA) was added undiluted directly to the mastermix and not freshly diluted 1:100. The PCR reaction is therefore inhibited.
  → Freshly dilute IPC-Target (RNA) 1:100 and repeat PCR.
- No Positive Control was added.
  → Repeat PCR in case all clinical samples are negative.
- For control of the reverse transcription real-time PCR only, 1 µl of freshly 1:100 diluted IPC-Target (RNA) must be added to the mastermix. If the IPC-Target (RNA) has been forgotten to be added:
  → Freshly dilute IPC-Target (RNA) and repeat PCR.
- For control of RNA extraction and PCR inhibition, the undiluted IPC-Target (RNA) must be added during extraction to the lysis buffer. If the IPC-Target (RNA) has been forgotten to be added:
  → Repeat RNA extraction.

12.2 Virus specific signal with negative control

- A contamination occurred during preparation of the RT-PCR.
  → Repeat the RT-PCR with new reagents in replicates.
  → Strictly pipette positive control at last.
  → Make sure that workspace and instruments are cleaned at regular intervals.

12.3 Virus specific signal with negative control of extraction

- A contamination occurred during extraction.
  → Repeat RNA extraction and RT-PCR using new reagents.
  → Make sure that workspace and instruments are cleaned at regular intervals.

12.4 IPC specific signal with PCR negative control and positive control

- The IPC-Target (RNA) has been added to lysis buffer during extraction, but there is IPC specific signal with PCR negative control and positive control: Contamination with the IPC-Target (RNA).
  → Make sure that workspace and instruments are cleaned at regular intervals.

12.5 No signal with IPC and no virus specific signal with sample

- Incorrect assignment of detection channels in sample.
  → Please verify the correct assignment of detection channels.
- Incorrect RT-PCR conditions.
  → Check the RT-PCR conditions and repeat the RT-PCR, if necessary.
- The RNA might be degraded.
- If IPC-Target (RNA) was added during extraction:
  - Inhibition of RT-PCR may have occurred.
  - RNA extraction was unsuccessful.
  - The IPC-Target (RNA) was not added to the lysis buffer of the sample.
  - The extracted sample was not added to the RT-PCR reaction.

  → No statement is possible. Verify you use a recommended method for RNA isolation and re-examine the single steps of the RNA extraction.
- → If no operating mistakes during RNA extractions can be retraced, it is recommended to repeat the RT-PCR with lower amounts of RNA-eluate (1/5 or 1/10 of sample volume + the adequate volume of nuclease-free water).

13 Specifications and performance evaluation

13.1 Test performance on different real-time PCR instruments

Performance of ViroReal® Kit Enterovirus with an ABI® 7500 instrument (Thermo Fisher Scientific) is shown in Figure 1.

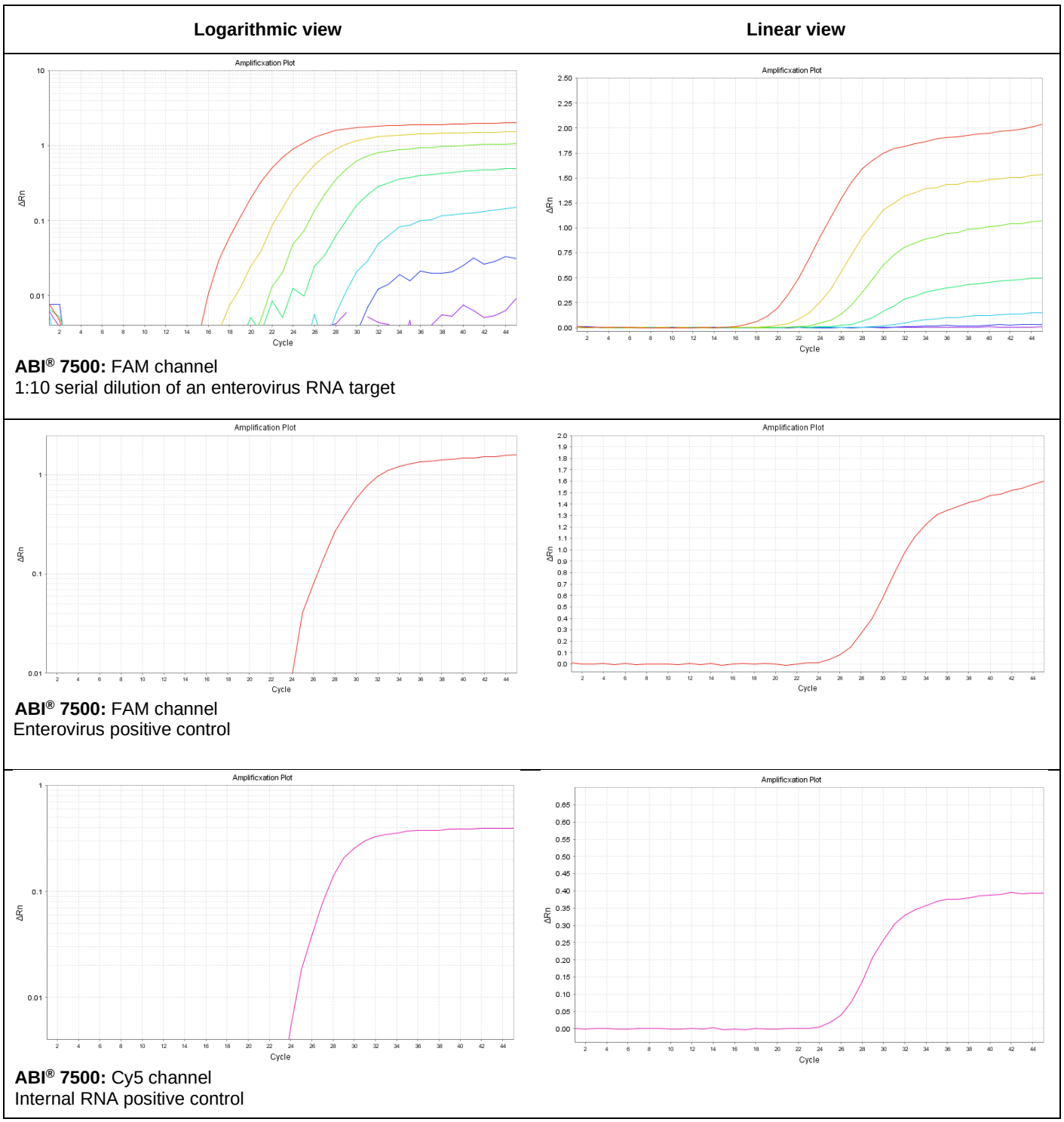

Figure 1 Performance of ViroReal® Kit Enterovirus with ABI® 7500

This test has been validated with the ABI® 7500 Real-time PCR System (Thermo Fisher Scientific) and tested with a LightCycler® 480 Instrument II (Roche), Mx3005P® QPCR System (Agilent), MIC instrument (bio molecular systems), QuantStudio™ 7 Pro (Thermo Fisher Scientific) and qTOWER^{3}G (Analytik Jena), but is also compatible with other real-time PCR instruments which detect and differentiate fluorescence in FAM and Cy5 channel.

13.2 Limit of detection, LoD95%

Method: ViroReal® Kit Enterovirus was tested with a 10-fold dilution series of a synthetic RNA representing a fragment of enterovirus RNA.
The limit of detection (LoD95% = smallest number of copies of target RNA which can be detected in 95% of cases) was determined with dilutions of the RNA around the analytical sensitivity using a non-linear curve fitting with the Graph Pad Prism Software.

Result: At least 10 target copies/reaction could be detected. The LoD95% is 30.6 target copies/reaction.

13.3 Linearity and dynamic measuring range

Method: Linearity was determined using a 10-fold dilution series (10^6 – 10^1 target copies/reaction) of the synthetic RNA. The number of determinations (n) per dilution was nine.

Result: The assay shows linearity over the range of 100 to 1,000,000 target copies/reaction with a slope of - 3.35 ± 0.05 and an R^2 of > 0.99 as shown in Figure 2.

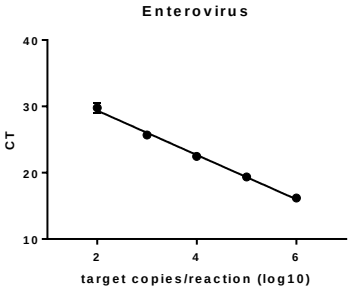

Figure 2 Ten-fold dilution series of enterovirus RNA plotted against CT

13.4 Precision

Method: Precision within a run (intra-assay), between multiple runs (inter-assay) and between two lots (inter-lot) was determined.

Result: The mean values of the coefficients of variation (CV%) are 1.2% for intra-assay precision, 1.7% for inter-assay precision and 1.1% for inter-lot precision.

13.5 Analytical specificity

Method BLAST analysis: The selection of highly specific primers and probes ensures analytical specificity. The specificity of primer and probes was validated *in silico* using the Basic Local Alignment Search Tool (BLAST) against the NCBI database. Primers and probes have been checked for possible homologies to currently published sequences. This analysis validates the detection of so far known enterovirus strains.

Result: Based on BLAST analysis, no relevant sequence variabilities were observed in the primer and probe region of those enterovirus strains detected by the test. Some rhinoviruses are detected as well, but with expected lower sensitivity.

Method testing inclusivity: RNA of defined enterovirus isolates has been tested with ViroReal® Kit Enterovirus, namely coxsackie-virus A1, coxsackie-virus A5, coxsackie-virus A6, coxsackie-virus A13, coxsackie-virus A21, coxsackie-virus B2, coxsackie-virus B3, coxsackie-virus B5, echovirus 1, echovirus 6, echovirus 11, echovirus 14, echovirus 30, enterovirus 71, enterovirus 90, enterovirus 99, enterovirus D68, poliovirus type 1, poliovirus type 2 and poliovirus type 3.

Result: All enterovirus isolates were detected.

Method testing exclusivity: Analytical specificity has been further evaluated by testing genomic DNA or RNA of viruses (adenovirus, parainfluenza virus, metapneumovirus, respiratory syncytial virus, influenza A, influenza B) and of bacteria (*Bordetella pertussis, Bordetella parapertussis, Streptoccocus pneumoniae, Mycoplasma pneumoniae, Haemophilus influenzae*).

Result: No cross-reactions have been observed.

13.6 Diagnostic evaluation

Method:
For diagnostic evaluation, 114 clinical samples, previously tested for the presence of enterovirus, have been analysed with ViroReal® Kit Enterovirus. The analyses included 76 samples positively tested for enterovirus (26 RNA samples isolated from nasopharyngeal swabs and 50 RNA samples isolated from stool) and 38 stool samples negatively tested for enterovirus.

Result:
ViroReal® Kit Enterovirus confirmed the presence of enterovirus in all 76 positive samples. Selected samples have been additionally subjected to sequencing, which confirmed the presence of enterovirus. In all 38 samples negatively tested for enterovirus, ViroReal® Kit Enterovirus confirmed the absence of enterovirus (Table 4, Table 5).

The diagnostic evaluation of ViroReal® Kit Enterovirus is shown in Table 4 and Table 5.

Table 4 Overall results obtained with 88 tested stool samples, 2x2 contingency table

	Reference			
		pos	**neg**	**Total**
ViroReal® Kit Enterovirus	**pos**	**50**	**0**	**50**
	neg	**0**	**38**	**38**
	Total	**50**	**38**	**88**

Table 5 Statistical evaluation of the diagnostic validation with stool samples

	Value	95% CI
Sensitivity	100%	92.89% to 100.00%
Specificity	100%	90.75% to 100.00%
NPV	100%	90.75% to 100.00%
PPV	100%	92.89% to 100.00%
Prevalence	57%	

14 References

Verstrepen WA, Kuhn S, Kockx MM, Van De Vyvere ME, Mertens AH. Rapid detection of enterovirus RNA in cerebrospinal fluid specimens with a novel single-tube real-time reverse transcription-PCR assay. J Clin Microbiol 2001; 39:4093–6.

15 Revision history

Revision	Date	Description
1.3en	03 March 2020	4. Information about RNA Reaction Mix was added 8.Additional information about sample preparation was added 8.2 CAUTION! Positive Control was changed from storage in 15 µl RNA stabilizer (contained Guanidinium thiocyanate/Triton X-100) to storage in 150 µl citrate buffer. It must not be diluted 1:500 anymore. 9. Table 1 and 2 were inserted 11.1. qTOWER3G (Analytik Jena) and MIC instrument (bio molecular systems) were added as validated real-time PCR devices 11.3 Added information about precision 11.5 Table 3 and 4 were inserted

1.4en	01 May 2023	**Change of the company address** **Explanation of symbols** Update **1. Intended purpose** Intended use specified in more detail **5. Contents, stability and storage** Kit version 1.1: Changed fill volume of IPC-Target (RNA) and Positive Control. The RNA IPC-Target is not stored in RNA/DNA Stabilizer anymore, concentration was changed from approx. $6x10^5$ copies/µl to approx. $6x10^4$ copies/µl and was renamed to IPC-Target (RNA). RNA IPC-3 Assay Mix has been removed. The former RNA IPC-3 Assay Mix is now included in the Enterovirus Assay Mix. **6. Additionally required materials and equipment** Update **7. Precautions and safety instructions** Update of this section Inclusion of additional information on specimen collection and storage, processing of specimens after collection, storage conditions of specimens, transport and storage conditions of purified RNA. Recommended measures to avoid RNA contamination added. **9. Preparation of samples** Update Note that for Cq values <27, the IPC-Target (RNA) must be diluted prior to extraction. **10. Preparation of real-time PCR** The pipetting scheme has changed. Dilution of IPC-Target (RNA) was changed from 1:500 to 1:100 The temperature profile has changed - the activation step in program 2 has been extended from 20 sec to 2 min. **11. Interpretation of PCR data** Update of tables for results with positive and negative controls and clinical samples. Inclusion of an explanation of how to evaluate clinical specimen test results in relation to controls. **12. Troubleshooting** Update regarding references in the new results tables. **13. Specifications and performance evaluation** Update.

Note:
Any serious incident that has occurred in relation to the product shall be reported to the manufacturer and the competent authority of the Member State in which the user and/or the patient is established.

Technical support
ingenetix GmbH, Haidingergasse 1, 1030 Vienna, Austria
Telephone: +43 (0)1 36 198 01; **E-Mail:** office@ingenetix.com