RIDA®GENE Enterovirus

REF PG4705

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1. Intended use

For *in vitro* diagnostic use. RIDA®GENE Enterovirus is a multiplex real-time RT-PCR for the direct, qualitative detection of enterovirus from human stool samples and cerebrospinal fluid (CSF).[1]

RIDA®GENE Enterovirus real-time RT-PCR is intended for use as an aid in diagnosis of infections caused by enteroviruses (poliovirus, echovirus, coxsackievirus, human enterovirus 70/71).

2. Summary and explanation of the test

Enteroviruses belong to the family of *Picornaviridae* and according to the current classification enteroviruses are divided into 15 species (enterovirus A-L and rhinovirus A-C), whereby the species enterovirus E-L are not described as human pathogenic.[2,3,4] The species enterovirus A includes the human enteroviruses A and the coxsackie viruses A, whereas the enteroviruses B include the human enteroviruses B, the coxsackie viruses B and also the echoviruses. Furthermore, the human enteroviruses C, the coxsackie viruses C and the polioviruses are part of the species enterovirus C. [2,3,4] Enteroviruses mainly infect infants and small children and are transmitted via the fecal-oral route but also sometimes by droplet infection and contaminated water. Most enterovirus infections are asymptomatic or present with mild cold-like symptoms. Though, due to the multitude of enterovirus species the clinical presentation in severe symptomatic diseases is of broad variety. Severe enterovirus infections are poliomyelitis, hand-foot-and-mouth disease, meningitis and myocarditis.[4]

Polioviruses are single-stranded RNA (ss-RNA) viruses and before their partial eradication they were distributed worldwide. Besides mild symptoms such as fever and cough, poliovirus can also cause poliomyelitis. Although, single outbreaks of poliomyelitis are reported consistently, the number of cases is declining worldwide. Thus, only 37 infections with poliovirus type 1 and no infections with poliovirus type 3 were reported in 2016. Poliovirus type 2 is regarded as eradicated since September 2015.[5]

In 1984, coxsackie virus A and B were first reported and named after their place of detection in Coxsackie, New York. Coxsackie viruses are present worldwide and both strains can cause the so called “summer diarrhea”. In the US, the hand-foot-and-mouth disease is mostly caused by coxsackie virus A16 whereas other severe coxsackie infections lead to conjunctivitis and myocarditis. Besides coxsackie virus, also an infection with human enterovirus 70 can lead to an acute conjuncitivitis.[6,7]

Human enterovirus 71 causes hand-foot-and-mouth disease, however infections with human enterovirus 71 are asymptomatic in most cases. This single-stranded RNA (ss-RNA) virus is distributed worldwide and most often occurs in late summer and fall.[4,9]

Echovirus is highly infectious and occurs mostly in children. Echovirus can, besides others, lead to aseptic meningitis whereby echovirus 30 is the most common meningitis causing serotype in Europe, America and Asia.[8]

3. Test principle

The RIDA®GENE Enterovirus multiplex real-time RT-PCR is a molecular diagnostic test for the direct, qualitative detection of Enterovirus RNA from human stool samples and CSF.
The detection is done in a one-step real-time RT-PCR format where the reverse transcription is followed by the PCR in the same reaction tube. The isolated RNA is transcribed into cDNA by a reverse transcriptase. Gene fragments specific for Enterovirus (5'-UTR, if present) are subsequently amplified by real-time PCR. The amplified targets are detected with hydrolysis probes, which are labeled at one end with a quencher and at the other end with a fluorescent reporter dye (fluorophore). In the presence of a target the probes hybridize to the amplicons. During the extension step the Taq-Polymerase breaks the reporter-quencher proximity. The reporter emits a fluorescent signal which is detected by the optical unit of a real-time PCR instrument. The fluorescence signal increases with the amount of formed amplicons. The RIDA®GENE Enterovirus assay contains an Internal Control RNA (ICR) as an internal control of sample preparation procedure and/or to determine possible PCR-inhibition.

4. Reagents provided

Tab. 1: Reagents provided (Reagents provided in the kit are sufficient for 100 determinations)

Kit Code	Reagent	Amount		Lid Color
1	Reaction Mix	2x	1050 µl	yellow
2	Enzyme Mix	1x	80 µl	red
R	Internal Control RNA	2x	1700 µl	brown
N	No Template Control	1x	450 µl	white
P	Positive Control	1x	200 µl	blue

5. Storage instructions

- Protect all reagents from light and store at -20 °C. All reagents can be used until. the expiration date. After expiry the quality guarantee is no longer valid.
- Carefully thaw reagents before using (e.g. in a refrigerator at 2 - 8 °C).
- Reagents can sustain up to 20 freeze/thaw cycles without influencing the assay performance (e.g. after the first thawing separate it in aliquots and freeze immediately).
- During PCR preparation all the reagents should be stored cold in an appropriate way (2 - 8 °C).

6. Additional necessary reagents and necessary equipment

The RIDA®GENE Enterovirus multiplex real-time RT-PCR assay is suitable for use with following extraction platforms and real-time PCR instruments:

Tab. 2: Necessary equipment

Extraction platform	
R-Biopharm	RIDA® Xtract
Promega	Maxwell® RSC
Real-time PCR instruments	
Roche	LightCycler® 2.0, LightCycler® 480II, LightCycler® 480z
Agilent Technologies	Mx3005P
Applied Biosystems	ABI 7500
Bio-Rad	CFX96™
QIAGEN	Rotor-Gene Q

Note: Only use 0.1 ml tubes on the Rotor-Gene Q (QIAGEN).

If you want to use other extraction platforms or real-time PCR instruments please contact R-Biopharm at mdx@r-biopharm.de.

- RIDA®GENE Color Compensation Kit II (PG0002) for use with the LightCycler® 2.0
- RIDA®GENE Color Compensation Kit IV (PG0004) for use with the LightCycler® 480II or LightCycler® 480z
- Real-time PCR consumables (plates, tubes, foil)
- Centrifuge with a rotor for the reaction vials
- Vortexer
- Pipettes (0.5 – 20 µl, 20 – 200 µl, 100 – 1000 µl)
- Filter tips
- Powder-free disposal gloves
- PCR water (nuclease-free)

7. Precautions for users

For *in-vitro* diagnostic use.
- This test must only be carried out by trained laboratory personnel. The guidelines for working in medical laboratories have to be followed.
- The instruction manual for the test procedure has to be followed.
- Do not pipet samples or reagents by mouth. Avoid contact with bruised skin or mucosal membranes.
- During handling reagents or samples, wear appropriate safety clothing (appropriate gloves, lab coat, safety goggles) and wash your hands after finishing the test procedure.
- Do not smoke, eat or drink in areas where samples or reagents are being used.
- Extraction, PCR preparation and the PCR run should be separated in different rooms to avoid cross-contaminations.
- Samples must be treated as potentially infectious as well as all reagents and materials being exposed to the samples and have to be handled according to the national safety regulations.
- Do not use the kit after the expiration date.
- All reagents and materials used have to be disposed properly after use. Please refer to the relevant national regulations for disposal.

For more details see Safety Data Sheets (SDS) at www.r-biopharm.com.

8. Collection and storage of samples

8.1 Sample preparation from stool samples

For RNA isolation of human stool samples, use a commercially available RNA extraction kit (e.g. RIDA® Xtract (R-Biopharm)) or RNA extraction system (e.g. Maxwell® RSC (Promega)). Extract viral RNA according to the manufacturer's instructions.
We recommend to dilute the stool sample before extraction 1:10 with water. Vortex intensely and centrifuge at 13,000 x g for 1 min. Use from the supernatant an appropriate volume according to the manufacturer's instruction.

The RIDA®GENE Enterovirus assay contains an Internal Control RNA that detects PCR inhibition, monitors reagent integrity and confirms that nucleic acid extraction was sufficient. The Internal Control RNA can either be used as PCR inhibition control or as extraction control for the sample preparation procedure and as PCR inhibition control.

If the Internal Control RNA is used only as a PCR inhibition control, 1 µl of the Internal Control RNA should be added to the Master- Mix (s. Tab. 4).

If the Internal Control RNA is used as a extraction control for the sample preparation procedure **and** as PCR inhibition control, 20 µl of the Internal Control RNA has to be added during extraction procedure. The Internal Control RNA should always be

added to the specimen-lysis buffer mixture and must **not** be added directly to the specimen. We also recommend to add 1 µl of the Internal Control RNA to the negative control and positive control PCR Mix.

8.2 Sample preparation from CSF

For RNA isolation of CSF samples, use a commercially available RNA extraction kit (e.g. RIDA® Xtract (R-Biopharm)) or RNA extraction system (e.g. Maxwell® RSC (Promega)). Extract viral RNA according to the manufacturer's instructions.

The RIDA®GENE Enterovirus assay contains an Internal Control RNA that detects PCR inhibition, monitors reagent integrity and confirms that nucleic acid extraction was sufficient. The Internal Control RNA can either be used as PCR inhibition control or as extraction control for the sample preparation procedure and as PCR inhibition control.

If the Internal Control RNA is used only as a PCR inhibition control, 1 µl of the Internal Control RNA should be added to the Master- Mix (s. Tab. 4).

If the Internal Control RNA is used as a extraction control for the sample preparation procedure **and** as PCR inhibition control, 20 µl of the Internal Control RNA has to be added during extraction procedure. The Internal Control RNA should always be added to the specimen-lysis buffer mixture and must **not** be added directly to the specimen. We also recommend to add 1 µl of the Internal Control RNA to the negative control and Positive Control PCR Mix.

9. Test procedure

9.1 Master-Mix preparation

Calculate the total number of PCR reactions (sample and control reactions) needed. One Positive Control and one negative control must be included in each assay run.

We recommend to calculate an additional volume of 10 % to compensate imprecise pipetting (see Tab. 3, Tab. 4). Thaw, mix gently and centrifuge briefly the Reaction Mix, the Enzyme Mix, the Positive Control, the No Template Control and the Internal Control RNA before using. Keep reagents appropriately cold during working step (2 - 8 °C).

Tab. 3: Calculation and pipetting example for 10 reactions of the Master Mix (ICR as extraction and PCR inhibition control)

Kit code	Master-Mix components	Volume per reaction	10 reactions (10 % extra)
1	Reaction Mix	19.3 µl	212.3 µl
2	Enzyme Mix	0.7 µl	7.7 µl
	Total	**20 µl**	**220 µl**

Mix the components of the Master-Mix gently and briefly spin down.

Tab. 4: Calculation and pipetting example for 10 reactions of the Master Mix (ICR only as PCR inhibition control)

Kit code	Master-Mix components	Volume per reaction	10 reactions (10 % extra)
1	Reaction Mix	19.3 µl	212.3 µl
2	Enzyme Mix	0.7 µl	7.7 µl
R	Internal Control RNA	1.0 µl	11 µl
	Total	**21.0 µl**	**231.0 µl**

Mix the components of the Master-Mix gently and briefly spin down.

9.2 Preparation of the RT-PCR-Mix

Pipette 20 µl of the Master-Mix in each reaction vial (tube or plate).

Negative control: Add 5 µl No Template Control to the pre-pipetted Master-Mix.

Note: If the Internal Control RNA is used as extraction control for the sample preparation procedure and as PCR inhibition control, we recommend to add 1 µl of the Internal Control RNA to the RT-PCR-Mix of the negative control.

Samples: Add 5 µl eluate to the pre-pipetted Master-Mix.

Positive Control: Add 5 µl Positive Control to the pre-pipetted Master-Mix.

Note: If the Internal Control RNA is used as extraction control for the sample preparation procedure and as PCR inhibition control, we recommend to add 1 µl of the Internal Control RNA to the RT-PCR Mix of the Positive Control.

Cover tubes or plate. Spin down and place in the real-time PCR instrument. The RT-PCR reaction should be started according to the PCR instrument set-up (see Tab. 5, Tab. 6).

9.3 PCR instrument set-up

9.3.1 Universal real-time RT-PCR profile

Tab. 5: Universal real-time RT-PCR profile for LightCycler® series

Reverse Transcription	10 min, 58 °C
Initial Denaturation	1 min, 95 °C
Cycles	45 Cycles
PCR Denaturation Annealing/Extension	10 sec, 95 °C 15 sec, 60 °C
Temperature Transition Rate / Ramp Rate	Maximum

Note: Annealing and extension occur in the same step.

Tab. 6: Universal real-time RT-PCR profile for Mx3005P, ABI7500, Rotor-Gene Q and CFX96™

Reverse Transcription	10 min, 58 °C
Initial Denaturation	1 min, 95 °C
Cycles	45 Cycles
PCR Denaturation Annealing/Extension	15 sec, 95 °C 30 sec, 60 °C
Temperature Transition Rate / Ramp Rate	Maximum

Note: Annealing and extension occur in the same step.

Note: The universal real-time PCR profile can also be used for DNA assays if RIDA®GENE DNA and RIDA®GENE RNA real-time PCR assays are combined in one run.

9.4 Detection channel set-up

Tab. 7: Selection of appropriate detection channels

Real-time PCR instrument	Detection	Detection channel	Note
Roche LightCycler® 2.0	Enterovirus	530	**RIDA®GENE Color Compensation Kit II (PG0002) is required**
	ICR	560	
Roche LightCycler® 480II	Enterovirus	465/510	**RIDA®GENE Color Compensation Kit IV (PG0004) is required**
	ICR	533/580	
Roche LightCycler® 480z	Enterovirus	465/510	**RIDA®GENE Color Compensation Kit IV (PG0004) is required**
	ICR	540/580	
Agilent Techn. Mx3005P	Enterovirus	FAM	**Check that reference dye is none**
	ICR	HEX	
ABI 7500	Enterovirus	FAM	**Check that passive reference option ROX is none**
	ICR	VIC	
Bio-Rad CFX96™	Enterovirus	FAM	**-**
	ICR	VIC	
Qiagen Rotor-Gene Q	Enterovirus	Green	**The gain settings have to be set to 5, according to the default settings**
	ICR	Yellow	

10. Quality control

The analysis of the samples is done by the software of the used real-time PCR instrument according to the manufacturer's instructions. Positive Control and negative control have to show correct results (see Table 8, Fig. 1) in order to determine a valid run.

The Positive Control has a concentration of 10^3 copies/µl. In each PCR run it is used in a total amount of 5 x 10^3 copies.

Tab. 8: For a valid run, the following conditions must be met:

Sample	Assay result	ICR Ct	Target Ct
Positive Control	Positive	NA *[1]	See Quality Assurance Certificate
Negative control	Negative	Ct > 20	0

[1] No Ct value is required for the ICR to make a positive call for the Positive Control.

If the positive control is not positive within the specified Ct range but the negative control is valid, prepare all new reactions including the controls.

If the negative control is not negative but the positive control is valid prepare all new reactions including the controls.

If the required criteria are not met, following items have to be checked before repeating the test:

- Expiry of the used reagents
- Functionality of the used instrumentation
- Correct performance of the test procedure

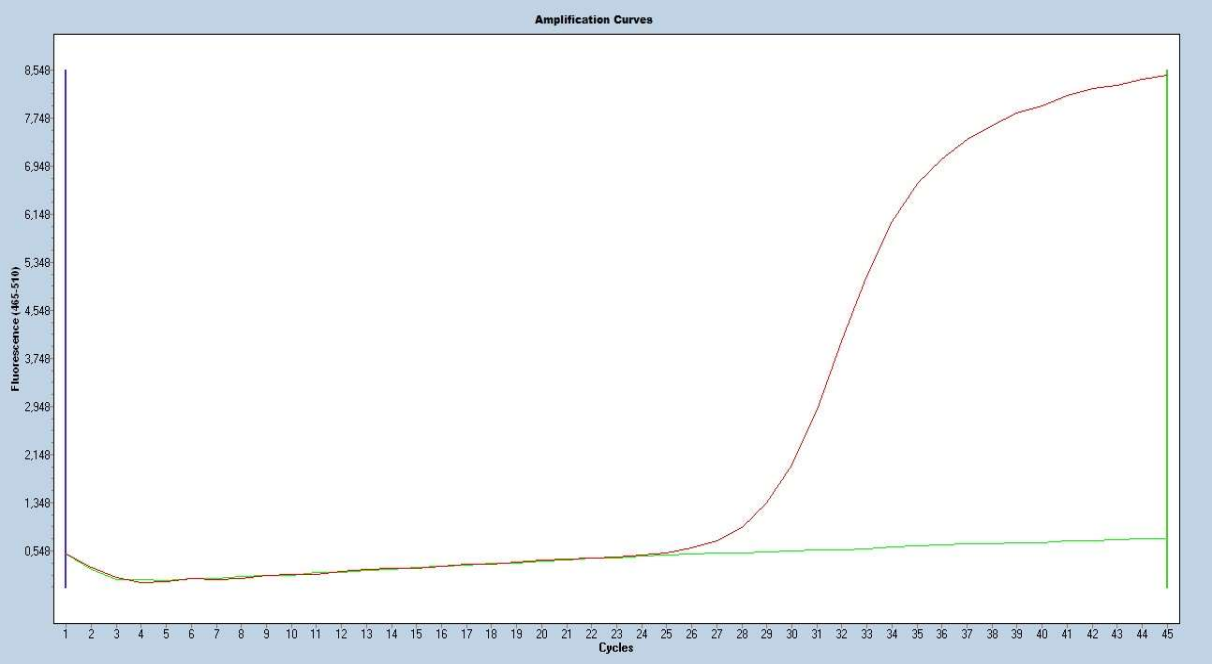

Fig. 1: Correct run of the positive control and negative control (Enterovirus) on the LightCycler® 480II

11. Result interpretation

The result interpretation is done according to Table 9.

Tab. 9: Sample interpretation

Target gene		
Enterovirus	**ICR**	**Result**
positive	**positive/negative**	**Enterovirus detected**
negative	**positive**	**Target genes not detected**
negative	negative	**Invalid**

Enterovirus is detected, if the sample RNA and the Internal Control RNA show an amplification signal in the detection system.

Enterovirus is also detected, if the sample RNA shows an amplification signal but none for the Internal Control RNA in the detection system. The detection of the Internal Control RNA is not necessary because high concentrations of the amplicon can cause a weak or absent signal of the Internal Control RNA.

Enterovirus is not detected, if the sample RNA shows no amplification signal, but an amplification signal for the Internal Control RNA in the detection system. An inhibition of the PCR reaction can be excluded by the detection of the Internal Control RNA.

A sample is invalid, if the sample RNA and Internal Control RNA show no amplification signal in the detection system. The sample contains a PCR inhibitor. The extracted sample needs to be further diluted with PCR water (1:10) and re-amplified, or the isolation and purification of the sample has to be improved.

12. Limitations of the method

1. The result of molecular analysis should not lead to the diagnosis, but always be considered in the context of medical history and symptoms of the patient.
2. This assay is only suitable for human stool samples and CSF samples.
3. Inappropriate specimen collection, transport, storage and processing or a pathogen load in the specimen below the analytical sensitivity can result in false negative results.
4. The presence of PCR inhibitors may cause invalid results.
5. Mutations or polymorphisms in primer or probe binding regions may affect detection of new variants resulting in a false negative result with the RIDA®GENE Enterovirus assay.
6. As with all PCR based *in vitro* diagnostic tests, extremely low levels of target below the limit of detection (LoD) may be detected, but results may not be reproducible.
7. A positive test result does not necessarily indicate the presence of viable organisms. However, a positive result is indicative for the presence of the target gene (5'-UTR).
8. Rhinoviruses belong to the family of *Picornaviridae*. Due to sequence similarity, it cannot be ruled out, that the RIDA®GENE Enterovirus assay shows cross reactivity to rhinoviruses.

13. Performance characteristics

13.1 Clinical performance

In a retrospective clinical validation study we analyzed 124 extracted human stool specimens with the RIDA®GENE Enterovirus assay and an in-house real-time PCR assay in an institute in Germany.

Tab.10: Correlation of the Enterovirus results with the RIDA®GENE Enterovirus multiplex real-time RT-PCR and reference in-house real-time PCR.

		In-house real-time PCR			
		Positive	Negative	Total	
RIDA®GENE Enterovirus	Positive	40	0	40	PPV: 100.0 %
	Negative	1	83	84	NPV: 98.8 %
	Total	41	83	124	

13.2 Analytical sensitivity

The RIDA®GENE Enterovirus multiplex real-time RT-PCR has a detection limit of ≥ 50 RNA copies per reaction for enterovirus.

The following figure 2 shows a dilution series of Enterovirus (10^5 – 10^1 RNA copies per µl) on the LightCycler® 480II.

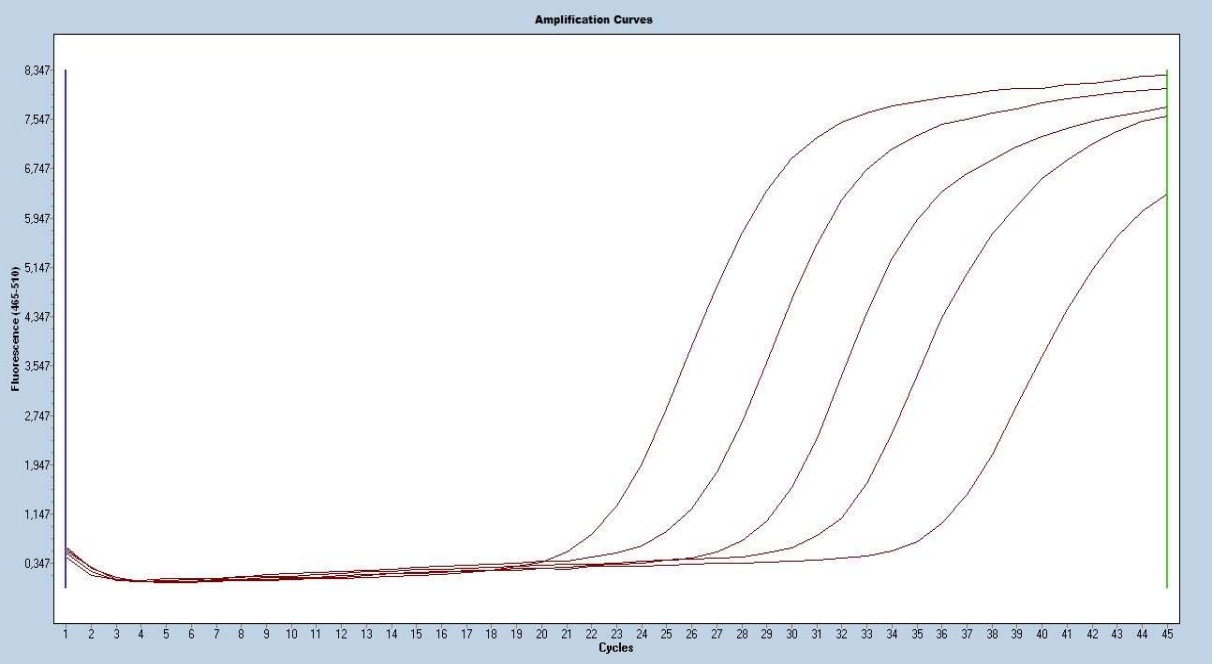

Fig. 2: Dilution series Enterovirus (10^5 – 10^1 RNA copies per µl) on the LightCycler® 480II

The detection limit of the whole procedure depends on the sample matrix, RNA extraction and RNA concentration.

13.3 Analytical specificity

The analytical specificity of the RIDA®GENE Enterovirus multiplex real-time RT-PCR is specific for Enterovirus from human stool samples. No cross-reaction could be detected for the following species (see Tab. 11):

Tab. 11: Cross-reactivity testing

Acinetobacter baumannii	-	*Clostridium bifermentans*	-	*Giardia intestinalis* Portland 1	-	*Proteus vulgaris*	-
Adenovirus 1, human, strain Adenoid 71	-	*Clostridium difficile*	-	*Giardia intestinalis* WB Clone C6	-	*Pseudomonas aeruginosa*	-
Adenovirus 7, human, strain Gomen	-	*Clostridium novyi*	-	*Haemophilus influenzae Rd*	-	Respiratory syncitial virus, human, strain Long	-
Adenovirus 40, human, strain Dugan	-	*Clostridium perfringens*	-	Herpes simplex virus 1 strain McIntyre	-	Respiratory syncitial virus, human, strain 9320	-
Adenovirus 41, Human, Strain Tak	-	*Clostridium septicum*	-	Herpes simplex virus 2 strain MS	-	Rhinovirus, human, Genogruppe A	-
Aeromonas hydrophila	-	*Clostridium sordellii*	-	Influenza virus A/PR/8/34	-	Rotavirus	-
Arcobacter butzleri	-	*Clostridium sporogenes*	-	*Klebsiella oxytoca*	-	*Salmonella enteritidis*	-
Astrovirus	-	Coronavirus 229E, human	-	*Klebsiella pneumoniae*	-	*Salmonella typhimurium*	-
Bacillus cereus	-	*Cryptosporidium muris*	-	*Legionella pneumophila*	-	*Serratia liquefaciens*	-
Bacteroides fragilis	-	*Cryptosporidium parvum*	-	Metapneumo-virus, human	-	*Shigella flexneri*	-
Bordetella parapertussis	-	Cytomegalovirus, human	-	*Mycoplasma pneumoniae*	-	*Staphylococcus aureus*	-
Bordetella pertussis	-	*E. coli* (O157:H7)	-	*Neisseria meningitidis*	-	*Staphylococcus epidermidis*	-
Campylobacter coli	-	*E. coli* (O26:H-)	-	Norovirus GG I	-	*Staphylococcus haemolyticus*	-
Campylobacter fetus subsp. *fetus*	-	*E. coli* (O6)	-	Norovirus GG II	-	*Staphylococcus hominis*	-
Campylobacter jejuni	-	*Entamoeba histolytica*	-	Parainfluenza virus 1, human, strain C35	-	*Streptococcus pneumoniae*	-
Campylobacter lari subsp. *lari*	-	*Enterobacter cloacae*	-	Parainfluenza virus 2, human, strain Greer	-	Varicella Zoster Virus (Type B)	-
Campylobacter upsaliensis	-	*Enterococcus faecalis*	-	Parainfluenza virus, serotype 3	-	*Vibrio parahaemo-lyticus*	-

Candida albicans	-	Epstein-Barr-Virus B95-8 strain	-	Parainfluenza virus 4b, human, strain CH19503	-	*Yersinia enterocolitica*	-
Citrobacter freundii	-	*Giardia lamblia*	-				

13.4 Analytical reactivity

The reactivity of the RIDA®GENE Enterovirus multiplex real-time RT-PCR was evaluated against multiple strains of Enterovirus (see Tab. 12). All strains of the panel were detected by the RIDA®GENE Enterovirus multiplex real-time RT-PCR or by sequence alignment (*).

Tab. 12: Analytical reactivity testing

Enterovirus					
Enterovirus A					
Enterovirus type 71	+	Coxsackievirus A9	+		
Enterovirus B					
Coxsackievirus B1	+	Coxsackievirus B2	+	Coxsackievirus B3	+
Coxsackievirus B4	+	Coxsackievirus B5	+	Echovirus type 6	+
Echovirus type 7	+	Echovirus type 11	+	Echovirus type 20	+
Echovirus type 25	+	Echovirus type 30	+		
Enterovirus C					
Poliovirus type 1	+	Poliovirus type 2	+	Poliovirus type 3	+
Enterovirus D					
Enterovirus type 68*	+				

14. Version history

Version number	Chapter and designation
2019-05-21	Previous version
2021-01-29	General revision 10. Quality control (Spelling mistake) 14. Version history 15. Explanation of symbols

15. Explanation of symbols

General symbols

	For *in vitro* diagnostic use
	Consult instructions for use
	Lot number
	Expiry
	Store at
	Article number
	Number of tests
	Date of manufacture
	Manufacturer

Testspecific symbols

Reaction Mix

Enzyme-Mix

Internal Control RNA

No Template Control

Positive Control

16. Literature

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