RIDA®GENE Hospital Stool Panel

REF PG0705

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1. Intended use

For *in vitro* diagnostic use. RIDA®GENE Hospital Stool Panel is a multiplex real-time RT-PCR for the direct, qualitative detection and differentiation of norovirus (genogroup I and II), rotavirus and *Clostridium difficile* toxin genes A (tcdA) / B (tcdB) in human stool samples.
RIDA®GENE Hospital Stool Panel real-time RT-PCR is intended for use as an aid in diagnosis of a hospital acquired gastroenteritis.

2. Summary and explanation of the test

Hospital acquired or nosocomial diarrhea is defined as an acute episode of diarrhea after 72 hours of hospitalization (3-day rule). The causes of hospital acquired diarrhea may be infectious or non-infectious (e.g. medication or chemotherapy) and affects up to one third of hospitalized patients. The most common infective cause of hospital acquired diarrhea is *Clostridium difficile*. Apart from *Clostridium difficile* other predominant infectious causes are norovirus and rotavirus. Hospital acquired diarrhea can cause serious complications in patients and increases length and costs of hospital stay.[1,2,3,4]
Noroviruses cause by far the most cases of non-bacterial gastroenteritis outbreaks.[5,6,7] A gastroenteritis caused by norovirus is manifested by severe nausea, vomiting and diarrhea. Noroviruses are egested by stool and with the vomit. An airborne transmission through aerosols containing the virus is often the cause of a very rapid spreading in shared facilities.[8,9,10] The Center for Disease Control (CDC, Atlanta, USA) estimates that more than 21 million cases of acute gastroenteritis, 70.000 hospitalisations and 800 deaths are caused by norovirus infections each year in the United States.[11] Noroviruses belong to the family of Caliciviridae and are small, non-enveloped viruses with a single-stranded RNA (ssRNA). They can be grouped in 7 genogroups with currently over 30 genotypes and a multiplicity of clades. So far, human pathogens have only been described from genogroup I (GI) with 9 genotypes, from genogroup II (GII) with 22 genotypes and from genogroup IV.[18,19]
Rotaviruses belong to the Reoviridae family of non-enveloped, icosahedral, double-stranded RNA (dsRNA) viruses and are classified in 7 serogroups (A - G). Human infections are only caused by serogroup A, B and C, although rotavirus serogroup A accounts for more than 90 % of the infections.[12] Symptoms of rotavirus infection are usually vomiting, watery diarrhoea and abdominal pain. The virus is transmitted by the faecal-oral route through contaminated hands and objects. Rotavirus is the main cause of diarrhoea in children aged under five and is responsible for the death of an estimated 611,000 children worldwide each year.[13]
Clostridium difficile, a gram-positive, spore-forming anaerobic bacterium was first described in 1935 by Hall and O'Toole as a component of the intestinal microflora in healthy neonates.[14] In the late 1970s, however, *Clostridium difficile* was identified as the cause of antibiotics-associated diarrhea and pseudomembranous colitis.[15] Today *Clostridium difficile* is one of the most common causes of nosocomial diarrhea. *Clostridium difficile* is responsible for 15 - 25% of antibiotics-associated diarrhea and

nearly all cases of pseudomembranous colitis.[16] The predisposing risk factors for CDAD are for example antibiotics exposure, advanced age as well as number and duration of hospitalization. However, *Clostridium difficile* infection is also seen in an increasing number of non-antibiotics-treated and non-hospitalized individuals. The symptoms range from mild diarrhea to intestinal infections of variable severity, including pseudomembranous colitis, the most severe form of antibiotics-induced inflammatory bowel disease. Clinically symptomatic cases are caused by toxigenic *Clostridium difficile* strains that produce toxin A and toxin B. In recent years the incidence and severity of *Clostridium difficile* infections increased worldwide.[17]
Real-time RT-PCR permits a rapid, highly sensitive and specific detection of the infectious cause of diarrhea. An early and reliable diagnosis of the diarrhea causing pathogen makes it possible to administer specific treatment of hospitalized patients and also to initiate hygiene measures to prevent nosocomial transmission.

3. Test principle

The RIDA®GENE Hospital Stool Panel multiplex real-time RT-PCR is a molecular diagnostic test for the direct, qualitative detection and differentiation of norovirus (genogroup I and II), rotavirus and *Clostridium difficile* toxin genes A (tcdA) / B (tcdB) in human stool samples.
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 (norovirus, rotavirus) is transcribed into cDNA by a reverse transcriptase. Gene fragments specific for norovirus (ORF1/ORF2 junction region), rotavirus (NSP3) and *Clostridium difficile* toxin A/B (tcdA/tcdB) 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 Hospital Stool Panel 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 Hospital Stool Panel multiplex real-time RT-PCR assay is suitable for use with following extraction platforms and real-time PCR instruments:

Tab. 2: Necessary equipment

Extraction platforms	
R-Biopharm	RIDA® Xtract
Promega	Maxwell® RSC
Real-time PCR instruments	
Roche	LightCycler® 480II, LightCycler® 480 z
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 IV (PG0004) for use with the LightCycler® 480II and the LightCycler® 480 z
- 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 DNA/RNA isolation of human stool samples, use a commercially available nucleic acid extraction kit (e.g. RIDA® Xtract (R-Biopharm)) or nucleic acid extraction system (e.g. Maxwell® RSC (Promega)). Extract viral nucleic acid according to the manufacturer's instructions.
We recommend to dilute the stool samples before extraction 1:3 with water. Vortex the diluted stool sample intensely and centrifuge at 1000 x g for 30 sec. Use from the supernatant the appropriate volume according to the manufacturer's instruction.

The RIDA®GENE Hospital Stool Panel 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 (see Tab.4).

If the Internal Control RNA is used as an 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 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.

Sample: 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® 480II	Norovirus	465/510	**RIDA®GENE Color Compensation Kit IV (PG0004) is required**
	ICR	533/580	
	Rotavirus	533/610	
	C. difficile Toxin A/B	618/660	
Roche LightCycler® 480 z	Norovirus	465/510	**RIDA®GENE Color Compensation Kit IV (PG0004) is required**
	ICR	540/580	
	Rotavirus	540/610	
	C. difficile Toxin A/B	610/670	
Agilent Techn. Mx3005P	Norovirus	FAM	**Check that the reference dye is none**
	ICR	HEX	
	Rotavirus	ROX	
	C. difficile Toxin A/B	Cy5	
ABI 7500	Norovirus	FAM	**Check that passive reference option ROX is none**
	ICR	VIC	
	Rotavirus	ROX	
	C. difficile Toxin A/B	Cy5	
Bio-Rad CFX96™	Norovirus	FAM	**-**
	ICR	VIC	
	Rotavirus	ROX	
	C. difficile Toxin A/B	Cy5	
Qiagen Rotor-Gene Q	Norovirus	Green	**The gain settings have to be set to 5, according to the default settings**
	ICR	Yellow	
	Rotavirus	Orange	
	C. difficile Toxin A/B	Red	

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. Negative control and positive control have to show correct results (see Table 8, Fig. 1, Fig. 2, Fig. 3) 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	ICD 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

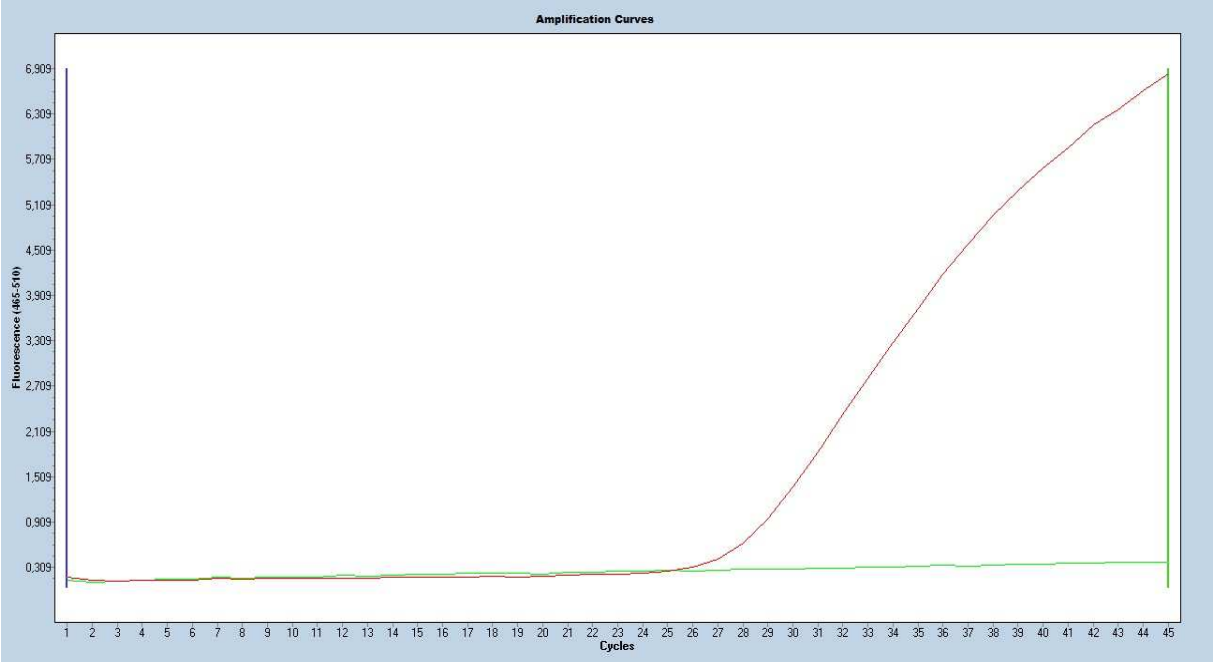

Fig. 1: Correct run of the positive and negative control (norovirus) on the LightCycler® 480II

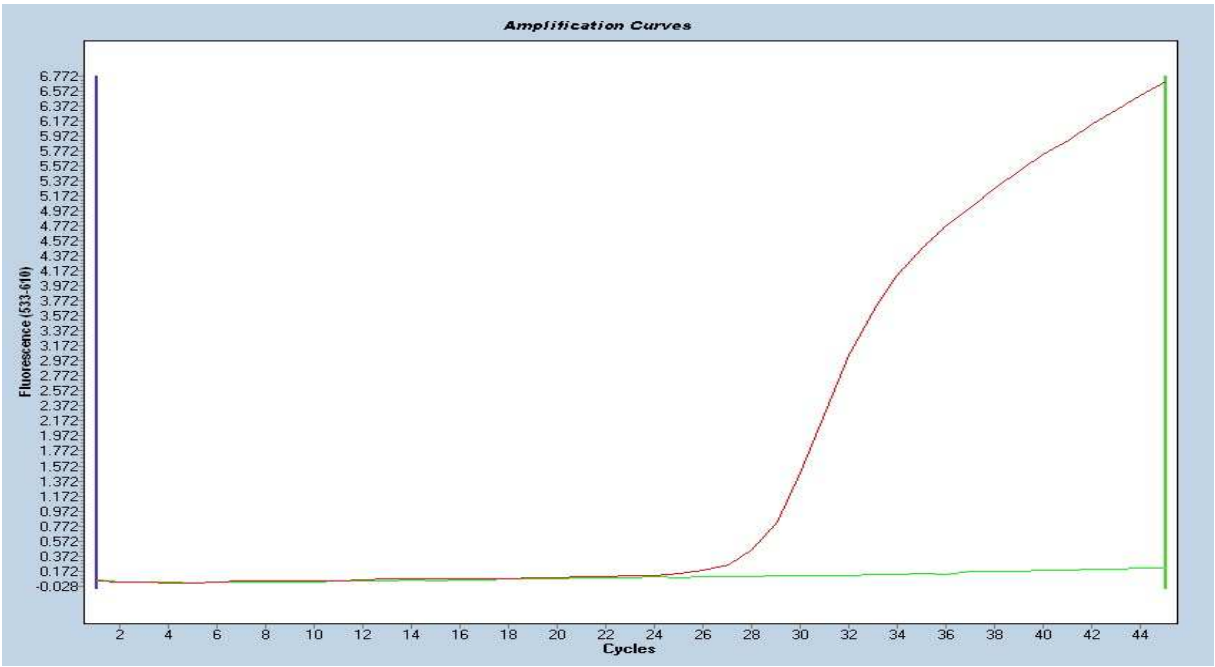

Fig. 2: Correct run of the positive and negative control (rotavirus) on the LightCycler® 480II

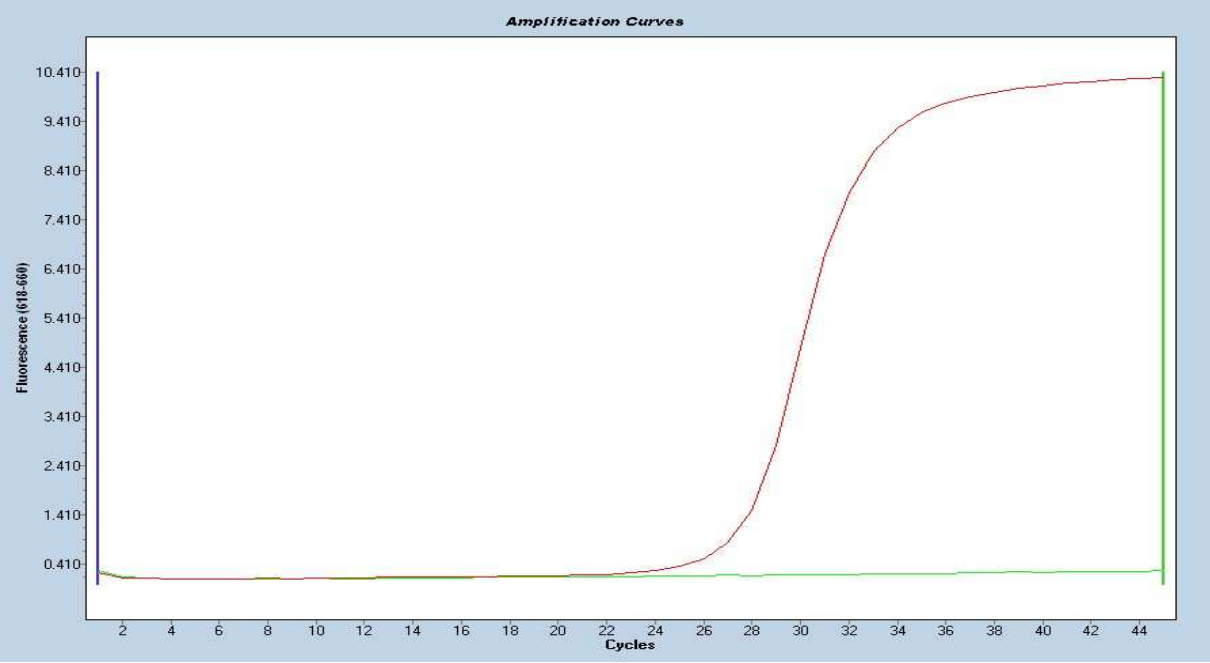

Fig. 3: Correct run of the positive and negative control (*C. difficile* toxin genes A/B) on the LightCycler® 480II

11. Result interpretation

The result interpretation is done according to Table 9.

Tab. 9: Sample interpretation

Detection				
Norovirus	**Rotavirus**	***C.difficile* toxin gene A/B**	**ICR/ICD**	**Result**
positive	negative	negative	**positive/negative**	**Norovirus detected**
negative	**positive**	negative	**positive/negative**	**Rotavirus detected**
negative	negative	**positive**	**positive/negative**	***C. difficile* toxin A/B detected**
positive	**positive**	negative	**positive/negative**	**Norovirus and Rotavirus detected**
negative	**positive**	**positive**	**positive/negative**	**Rotavirus and *C. difficile* Toxin A/B detected**
positive	negative	**positive**	**positive/negative**	**Norovirus and *C. difficile* Toxin A/B detected**
positive	**positive**	**positive**	**positive/negative**	**Norovirus, Rotavirus and *C. difficile* Toxin A/B detected**
negative	negative	negative	**positive**	**Target genes not detected**
negative	negative	negative	negative	**Invalid**

A sample is evaluated positive, if the sample RNA and the Internal Control RNA show an amplification signal in the detection system.

A sample is also evaluated positive, 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.

A sample is evaluated negative, 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 evaluated 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 test is only validated for stool samples.
3. Inappropriate specimen collection, transport, storage and processing or a viral 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 norovirus genotypes resulting in a false negative result with the RIDA®GENE Hospital Stool Panel 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 respective target genes (Norovirus: ORF1/ORF2 junction region, Rotavirus: NSP3 gene, *Clostridium difficile* toxin A/B: tcdA/tcdB).
8. Norovirus genogroup IV, which very rarely infect humans, will be also detected by the RIDA®GENE Hospital Stool Panel assay.
9. Rotavirus serogroup B and C will not be detected by the RIDA®GENE Hospital Stool Panel assay.

13. Performance characteristics

13.1 Analytical sensitivity

The RIDA®GENE Hospital Stool Panel multiplex real-time RT-PCR has a detection limit of ≥ 50 copies per reaction for norovirus, rotavirus and *Clostridium difficile* toxin genes A/B.

The following figures 4, 5 and 6 show dilution series of norovirus, rotavirus (each 10^5 - 10^1 RNA copies per µl) and *Clostridium difficile* toxin genes A/B (10^5 - 10^1 DNA copies per µl) on the LightCycler® 480II.

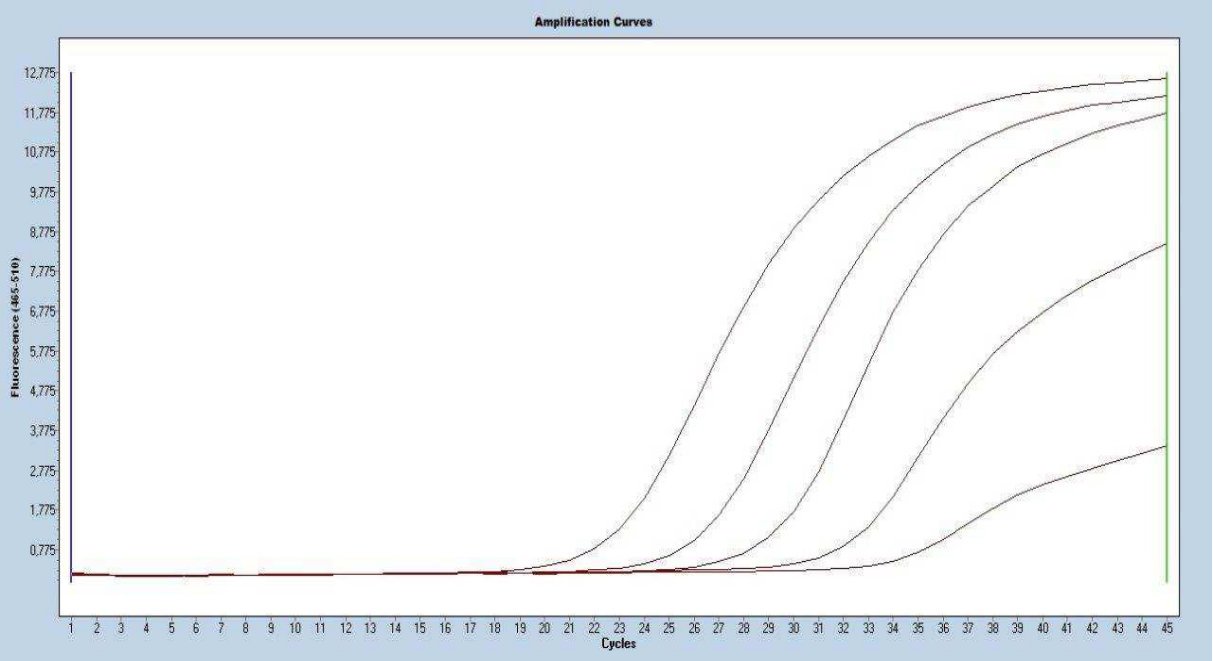

Fig. 4: Dilution series norovirus (10^5 – 10^1 RNA copies per µl) on the LightCycler® 480II

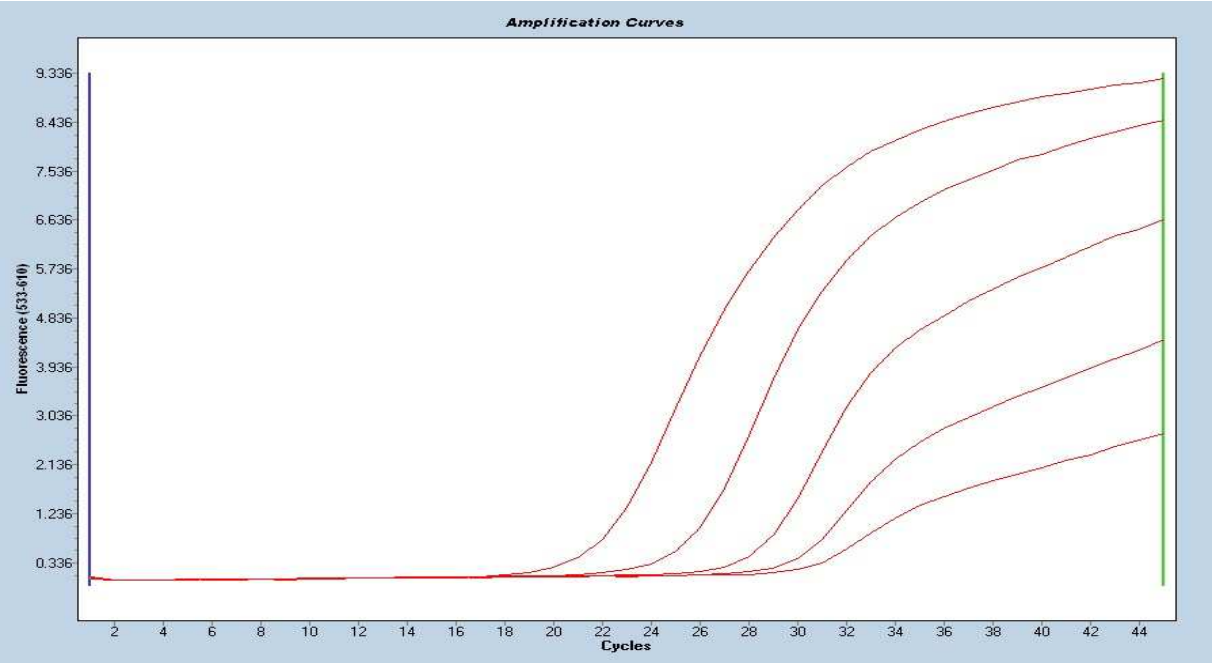

Fig. 5: Dilution series rotavirus (10^5 – 10^1 RNA copies per µl) on the LightCycler® 480II

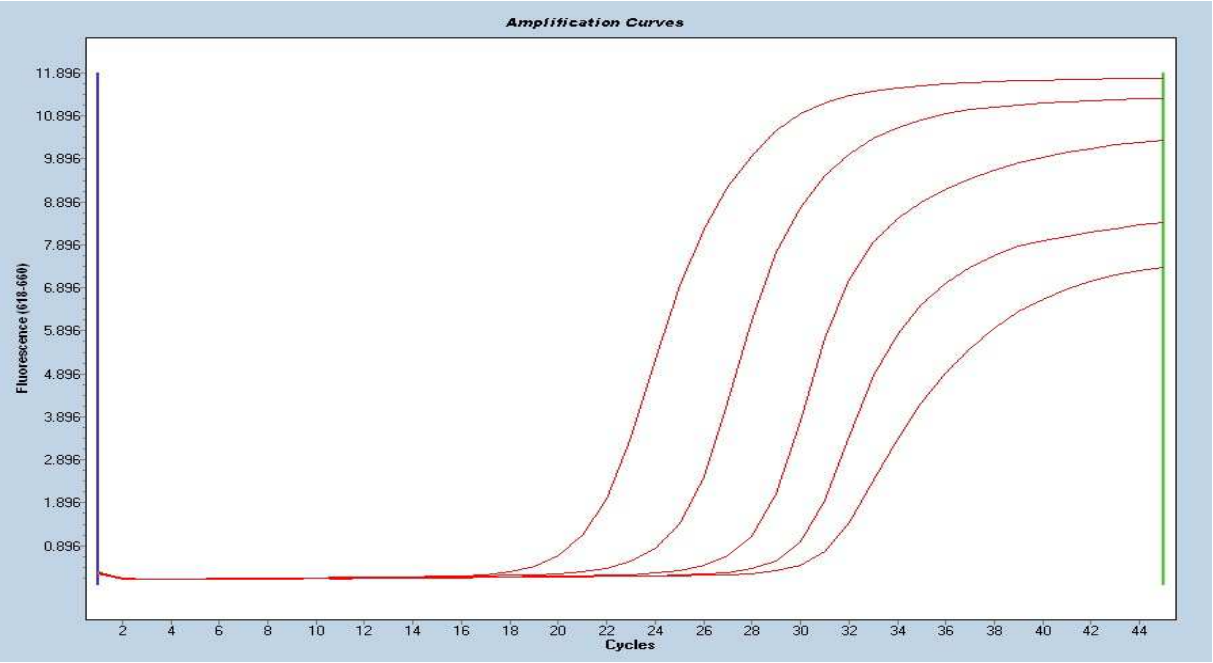

Fig. 6: Dilution series *Clostridium difficile* toxin genes A/B (10^5 – 10^1 RNA copies per µl) on the LightCycler® 480II

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

13.2 Analytical specificity

The RIDA®GENE Hospital Stool Panel multiplex real-time RT-PCR is specific for norovirus (genogroups I, II and IV), rotavirus (serogroup A) and *Clostridium difficile* toxin genes A/B. No cross-reaction could be detected for the following species (see Tab. 10):

Tab. 10: Cross-reactivity testing

Adenovirus 1, human, strain Adenoid 71	-	*Campylobacter fetus* subsp. *fetus*	-	*Cryptosporidium muris*	-	*Klebsiella oxytoca*	-
Adenovirus 7, human, strain Gomen	-	*Campylobacter lari* subsp. *lari*	-	*Cryptosporidium parvum*	-	*Proteus vulgaris*	-
Adenovirus 40, human, strain Dugan	-	*Campylobacter upsaliensis*	-	*E. coli* (O157:H7)	-	*Pseudomonas aeruginosa*	-
Adenovirus 41, human, strain Tak	-	*Candida albicans*	-	*E. coli* (O26:H-)	-	*Salmonella enteritidis*	-
Aeromonas hydrophila	-	*Citrobacter freundii*	-	*E. coli* (O6)	-	*Salmonella typhimurium*	-
Arcobacter butzleri	-	*Clostridium bifermentans*	-	*Entamoeba histolytica*	-	*Serratia liquefaciens*	-
Astrovirus	-	*Clostridium novyi*	-	*Enterobacter cloacae*	-	*Shigella flexneri*	-
Bacillus cereus	-	*Clostridium perfringens*	-	*Enterococcus faecalis*	-	*Staphylococcus aureus*	-
Bacteroides fragilis	-	*Clostridium septicum*	-	*Giardia intestinalis* Portland 1	-	*Staphylococcus epidermidis*	-
Campylobacter coli	-	*Clostridium sordellii*	-	*Giardia intestinalis* WB Clone C6	-	*Vibrio parahaemolyticus*	-
Campylobacter jejuni	-	*Clostridium sporogenes*	-	*Giardia lamblia*	-	*Yersinia enterocolitica*	-

13.3 Analytical reactivity

The reactivity of the RIDA®GENE Hospital Stool Panel multiplex real-time RT-PCR was evaluated against multiple genotypes of the norovirus genogroup I, II and IV and different serotypes of the rotavirus species A (see Tab. 11). All Norovirus genotypes and Rotavirus serotypes of the panel were detected by the RIDA®GENE Hospital Stool Panel multiplex real-time RT-PCR.

Tab. 11: Analytical reactivity testing

Norovirus					
Genogroup I					
GI.1 – Norwalk	+	GI.3 – Desert Shield, Birmingham	+	GI.6 – Hesse	+
GI.2 – Southhampton, Whiterose	+	GI.4 – Chiba, Malta	+	GI.7 – Winchester	+
GI.2 – Southhampton, Southhampton	+	GI.5 – Musgrove	+	GI.8 – Boxer	+
Genogroup II					
GII.1 – Hawaii	+	GII.4 – Sydney 2012	+	GII.17 – Kawasaki	+
GII.2 – Melksham	+	GII.6 – Seacroft	+	GII.b – Hilversum	+
GII.3 – Toronto	+	GII.7 – Leeds	+	GII.c – Den Haag	+
GII.4 – Bristol, Grimsby 2004	+	GII.10 – Erfurt	+		
Genogroup IV					
GIV.1 – Alphatron	+				
Rotavirus					
Serogroup A					
Serotype G1	+	Serotype G2	+	Serotype G3	+
Serotype G4	+	Serotype G9	+	Serotype G12	+

14. Version history

Version number	Chapter and designation
2018-09-20	General revision 2. Summary and explanation of the test 4. Reagents provided 5. Storage instructions 6. Additional necessary reagents and necessary equipment 9. Test procedure 10. Quality control 12. Limitations of the method 13. Performance characteristics 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

Not applicable

16. Literature

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