RIDA®GENE Hospital Stool Panel

real-time RT-PCR

Art. Nr.: PG0705

100 Reactions

For *in vitro* diagnostic use.

-20 °C

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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) and 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 72h 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 CDC 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 5 genogroups with currently 29 genotypes and a multiplicity of clades. So far, human pathogens have only been described from genogroup I (GI) with 8 genotypes and from genogroup II (GII) with 17 genotypes. The remaining 4 genotypes are distributed between genogroups III to IV.
Rotaviruses belong to the Reoviridae familiy of non-enveloped icosahedral double-stranded RNA (dsRNA) viruses and are classified in seven 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 fecal-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 antibiotica-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 antibiotica-associated diarrhea and nearly all cases of pseudomembranous colitis.[16] The predisposing risk factors for CDAD are for example antibiotica exposure, advanced age as well as number and duration of hospitalization. However, *Clostridium difficile* infection is also seen in an increasing number of non-antibiotic-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 antibiotic-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) and 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, rotavirus and *Clostridium difficile* toxin A/B) are subsequently amplified by real-time PCR. The amplified targets (norovirus: ORF1/ORF2 junction region, rotavirus: NSP3 gene, *Clostridium difficile* toxin A/B: tcdA/tcdB) 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	Reagenz	Menge	Deckelfarbe
1	Reaction Mix	2x 700 µl	yellow
2	PP-Mix	1x 770 µl	green
3	Enzyme Mix	1x 80 µl	red
R	Internal Control RNA	2x 1800 µl	brown
N	PCR Water	1x 500 µl	white
P	Positive Control	1x 100 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 5 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 equipment and materials required

- The RIDA®GENE Hospital Stool Panel real-time RT-PCR Assay is suitable for use with following extraction platforms and real-time PCR instruments:
- Extraction platform:
  RIDA® Xtract (R-Biopharm)
  Maxwell® 16 (Promega)
- Real-time PCR instrument:

Roche:	LightCycler® 480II
Agilent Technologies:	Mx3005P
Applied Biosystems:	ABI 7500
Abbott:	m2000rt
Bio-Rad:	CFX96™
Cepheid:	SmartCycler®
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-techsupport@r-biopharm.de.

- RIDA®GENE Color Compensation Kit I (PG0001) for use with the LightCycler® 480
- 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

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 Material Safety Data Sheets (MSDS) at www.r-biopharm.com

8. Sample collection and Storage

8.1 DNA/RNA isolation from Stool samples

For DNA/RNA isolation of human stool samples, use a commercially available DNA isolation kit (e.g. RIDA® Xtract) or DNA extraction system (e.g. Maxwell® 16 (Promega)). Extract DNA 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 (ICR), which can either be used as PCR inhibition control or as extraction control for the sample preparation procedure and as a PCR inhibtion control.

If the ICR is used only as a PCR inhibition control, 1 µl of the ICR should be added to the Master- Mix (s. Tab. 3).

If the ICR is used as an extraction control for the sample preparation procedure and as PCR inhibition control, 20 µl of the ICR has to be added during extraction procedure. The ICR should always be added to the specimen-lysis buffer mixture and must **not** be added directly to the specimen.

9. Test procedure

9.1 Master-Mix preparation

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

We recommend to calculate an additional volume of 10 % to compensate imprecise pipetting (see Tab. 2, Tab. 3). Thaw, mix gently and centrifuge briefly the Reaction Mix, the PP-Mix, the Enzyme Mix, the Positive Control, the PCR Water and the Internal Control RNA before using. Keep reagents appropriately cold during working step (2 - 8 °C).

Tab. 2: 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	12.5 µl	137.5 µl
2	PP-Mix (Primer-Probe-Mix)	6.9 µl	75.9 µl
3	Enzyme Mix	0.7 µl	7.7 µl
	Total	**20.1 µl**	**221.1 µl**

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

Tab. 3: 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	12.5 µl	137.5 µl
2	PP-Mix (Primer-Probe-Mix)	6.9 µl	75.9 µl
3	Enzyme Mix	0.7 µl	7.7 µl
R	Internal Control RNA	1.0 µl	11 µl
	Total	**21.1 µl**	**232.1 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 PCR Water as negative control to the pre-pipetted Master Mix.

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

Sample: Add 5 µl RNA-Extract to the pre-pipetted Master Mix.

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

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

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. 4).

9.3 PCR Instrument Set-up

Tab. 4: Real-time PCR profile

Reverse Transcription	10 min, 58 °C
Initial Denaturation	1 min, 95 °C
Cycles	45 Cycles
PCR Denaturation	15 sec, 95 °C
Annealing/Extension	30 sec, 55 °C
Temperature Transition Rate / Ramp Rate	Maximum

Note: Annealing and Extension occur in the same step.

9.4 Detection Channel Set-up

Tab. 5: Selection of appropriate detection channels

Real-time PCR Instrument	Detection	Detection Channel	Note
Roche LightCycler® 480II	Norovirus	465/510	**RIDA®GENE Color Compensation Kit I (PG0001) is required**
	ICR	533/580	
	Rotavirus	533/610	
	C. difficile Toxin A/B	618/660	
Cepheid SmartCycler®	Norovirus	Channel 1	**Check that the "Manual Tres. Fluor Units" for Channel 1 is set to 10.0 and for Channel 2 to 4 is set to 5.0 ***
	ICR	Channel 2	
	Rotavirus	Channel 3	
	C. difficile Toxin A/B	Channel 4	
ABI 7500	Norovirus	FAM	**Check that passive reference option ROX is none**
	ICR	VIC	
	Rotavirus	ROX	
	C. difficile Toxin A/B	Cy5	
Abbott m2000rt	Norovirus	FAM	**-**
	ICR	VIC	
	Rotavirus	ROX	
	C. difficile Toxin A/B	Cy5	
Stratagene Mx3005P	Norovirus	FAM	**Check that passive reference option ROX is none**
	ICR	HEX	
	Rotavirus	ROX	
	C. difficile Toxin A/B	Cy5	
Qiagen Rotor-Gene Q	Norovirus	Green	**The gain settings have to be set to 5**
	ICR	Yellow	
	Rotavirus	Orange	
	C. difficile Toxin A/B	Red	
Bio-Rad CFX96™	Norovirus	FAM	**-**
	ICR	VIC	
	Rotavirus	ROX	
	C. difficile Toxin A/B	Cy5	

*** Due to variations between different cyclers, it may be required to individually adapt the "Manual Thres. Fluor Units" for channel 1.**

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 and negative controls have to show correct results (see Table 6, 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. 6: For a VALID run, the following conditions must be met:

Sample	Assay result	ICR Ct	Target Ct
PTC	Positive	NA *[1]	See Quality Assurance Certificate
NTC	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 (PTC) is not positive within the specified Ct range but the Negative Control is valid, prepare all new reactions using remaining purified nucleic acids and a new Positive Control.

If the Negative Control (NTC) is not negative but the Positive control is valid prepare all new reactions using remaining purified nucleic acids and a new Negative Control.

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 Light Cycler 480II

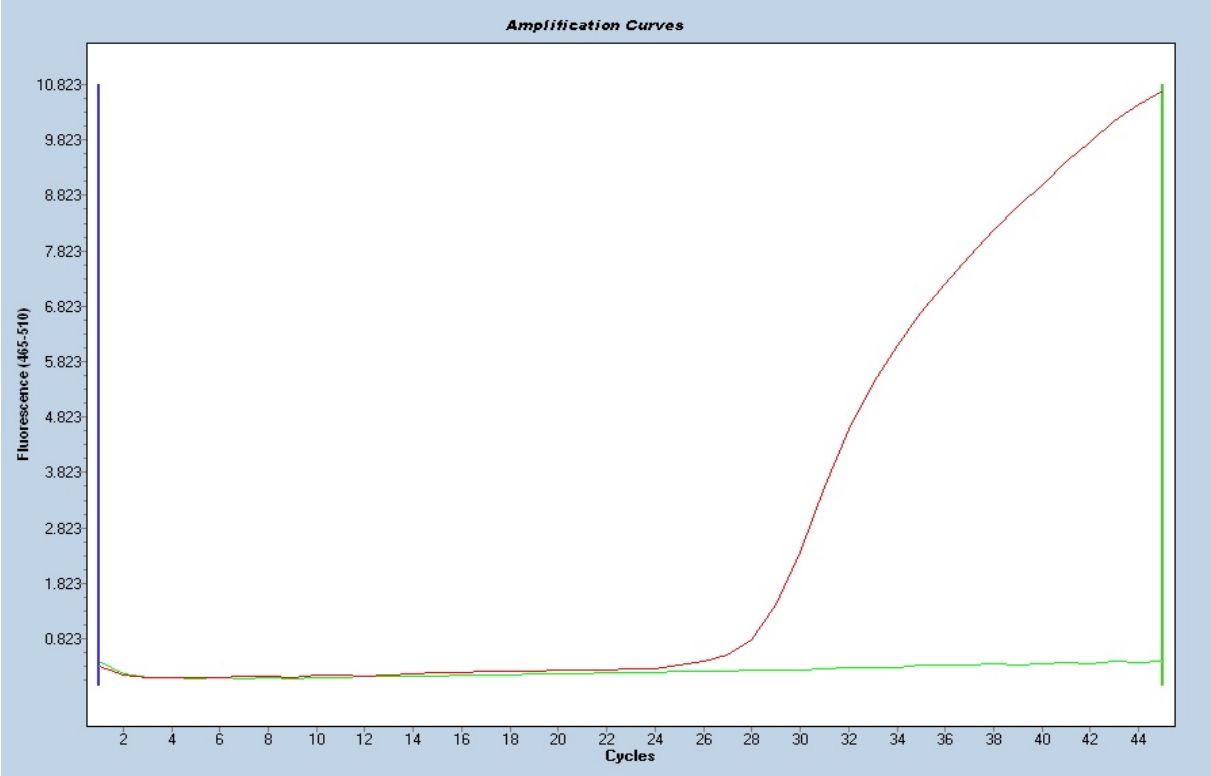


Fig. 2: Correct run of the positive and negative control (Rotavirus) on the Light Cycler 480II

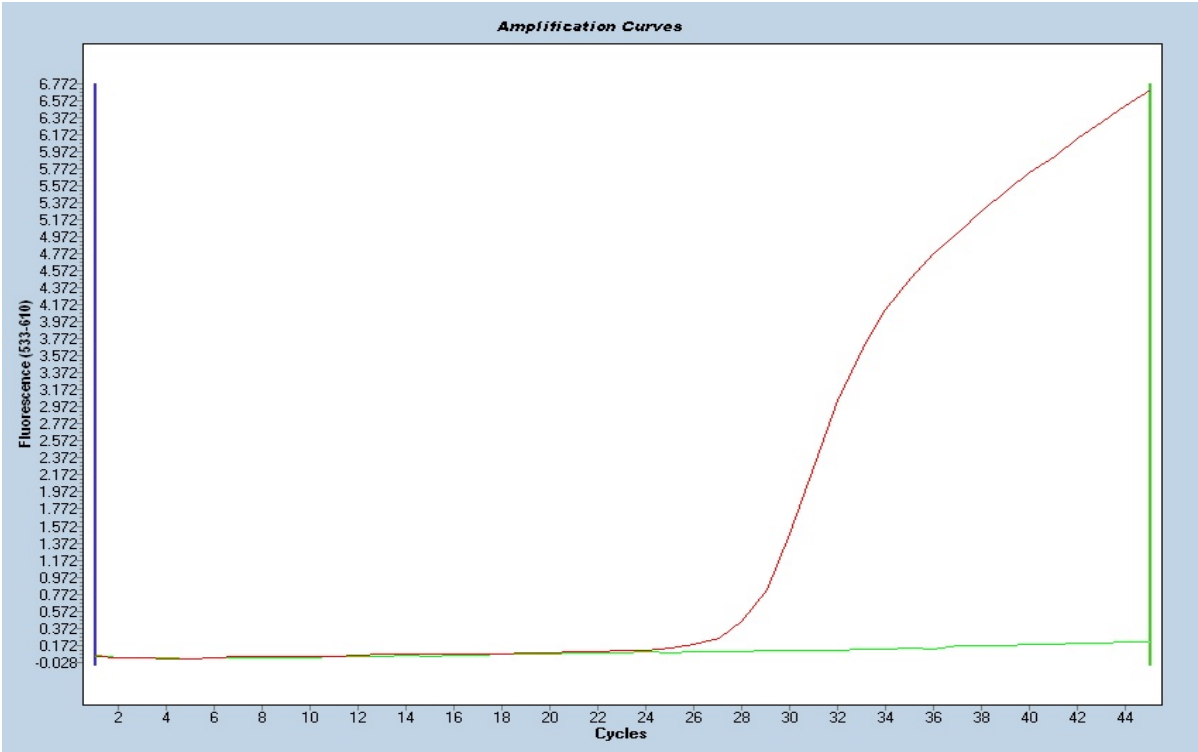
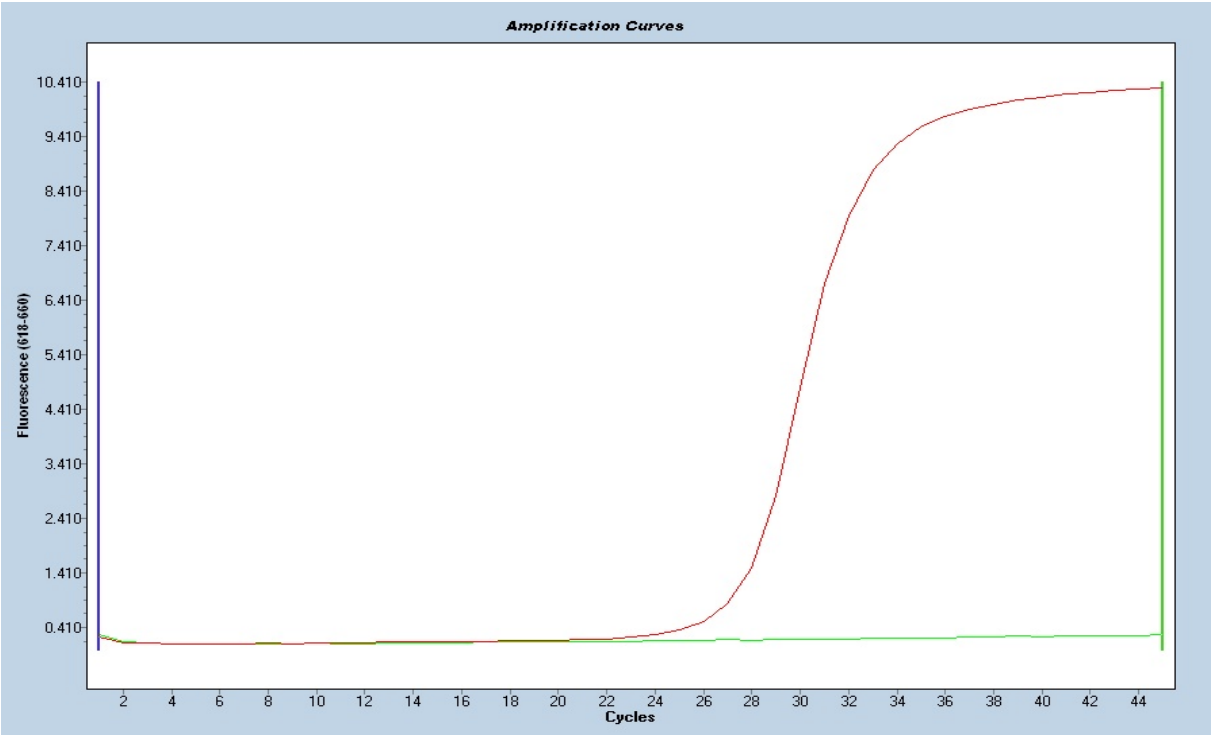


Fig. 3: Correct run of the positive and negative control (*Clostridium difficile* Toxin A/B genes) on the Light Cycler 480II



11. Result interpretation

The result interpretation is done according to Table 7.

Tab. 7: Sample interpretation

Detection				
Norovirus	**Rotavirus**	***C.difficile* Toxin A/B Gene**	**ICR**	**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, Rotavirus detected**
negative	**positive**	**positive**	**positive/negative**	**Rotavirus, *C. difficile* Toxin A/B detected**
positive	negative	**positive**	**positive/negative**	**Norovirus, *C. difficile* Toxin A/B detected**
positive	**positive**	**positive**	**positive/negative**	**Norovirus, Rotavirus, *C. difficile* Toxin A/B detected**
negative	negative	negative	**positive**	**Zielgene sind nicht detected**
negative	negative	negative	negative	**Invalid**

A sample is evaluated negative if the sample shows no amplification signal in the detection system but the Internal Control RNA (ICR) is positive. An inhibition of the PCR reaction or a failure in the extraction procedure can be excluded by the detection of the Internal Control RNA (ICR).

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

A sample is evaluated positive if the sample shows an amplification signal in the detection system but the Internal Control RNA (ICR) is negative. The detection of the internal amplification control is not necessary because high concentrations of the amplicon can cause a weak or absent signal of the internal amplification control.

A sample is evaluated invalid if both, the sample and the Internal Control RNA (ICR), show no amplification signal in the detection system. The sample contained a PCR inhibitor or a failure occurred in the extraction procedure. 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. Norovirus genogroup IV, which very rarely infect humans, will be also detected by the RIDA®GENE Hospital Stool Panel assay.
7. Rotavirus serogroup B and C will not be detected by the RIDA®GENE Hospital Stool Panel assay.
8. 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.
9. 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.

13. Performance characteristics

13.1 Analytical sensitivity

The RIDA®GENE Hospital Stool Panel multiplex real-time RT-PCR has a detection limit of ≥ 50 RNA copies per reaction for norovirus, ≥ 50 RNA copies per reaction for rotavirus and ≥ 10 DNA copies per reaction for *Clostridium difficile* A/B genes (see Fig. 4, Fig. 5, Fig. 6).

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

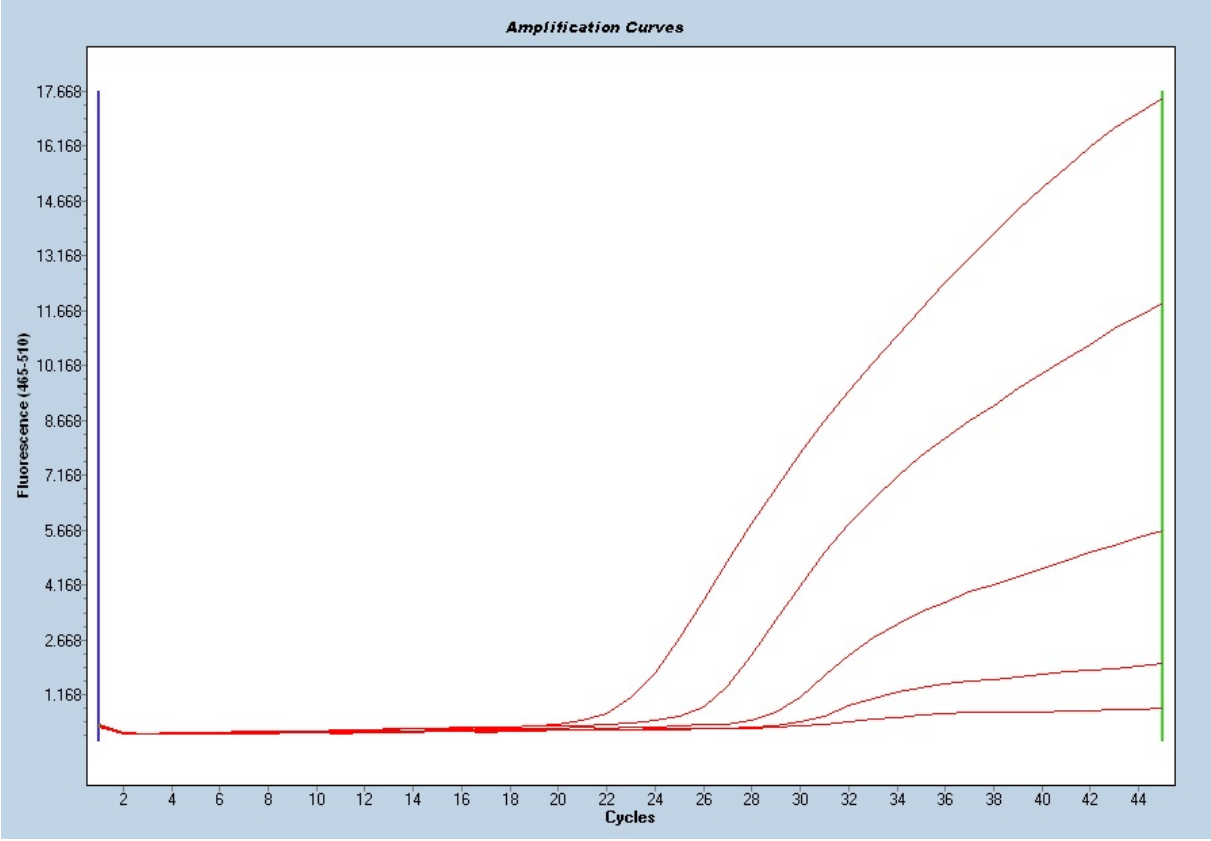


Fig. 5: Dilution series Rotavirus (10^5 – 10^1 RNA copies per µl) on the LightCycler® 480II

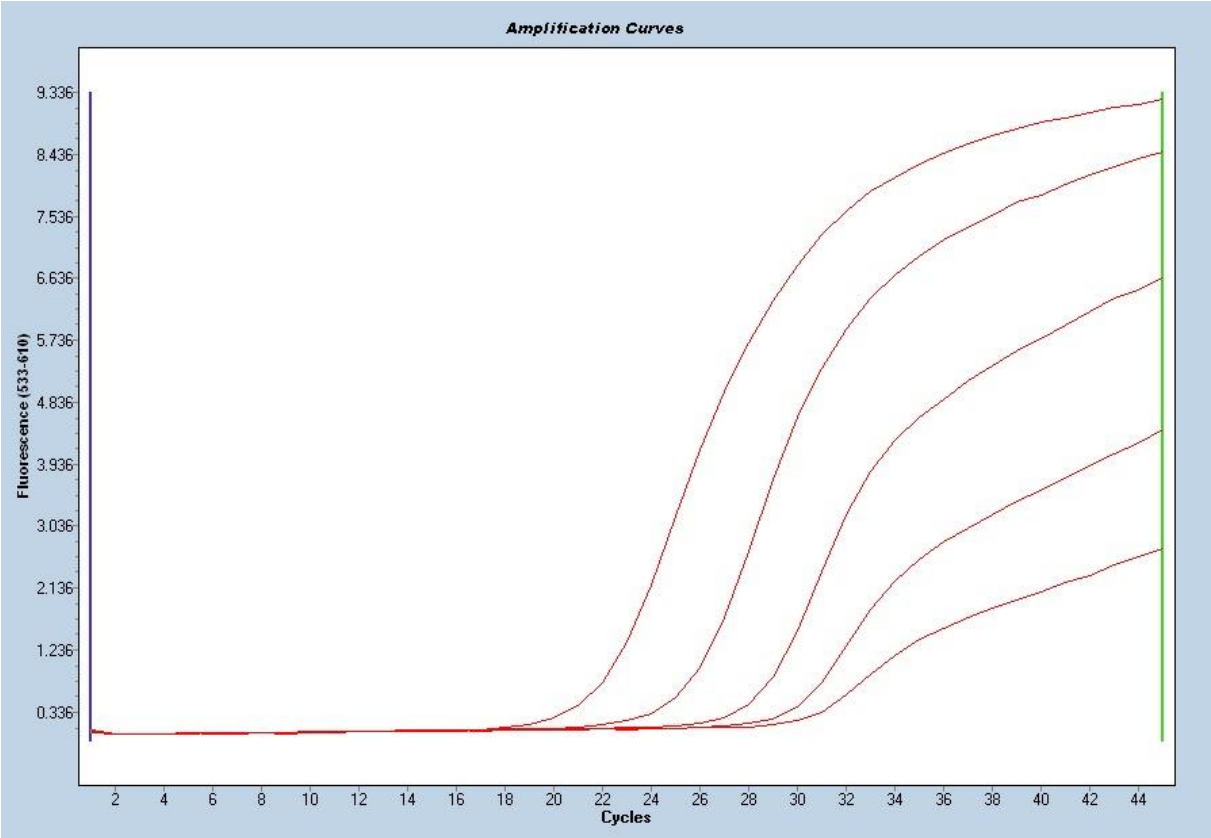
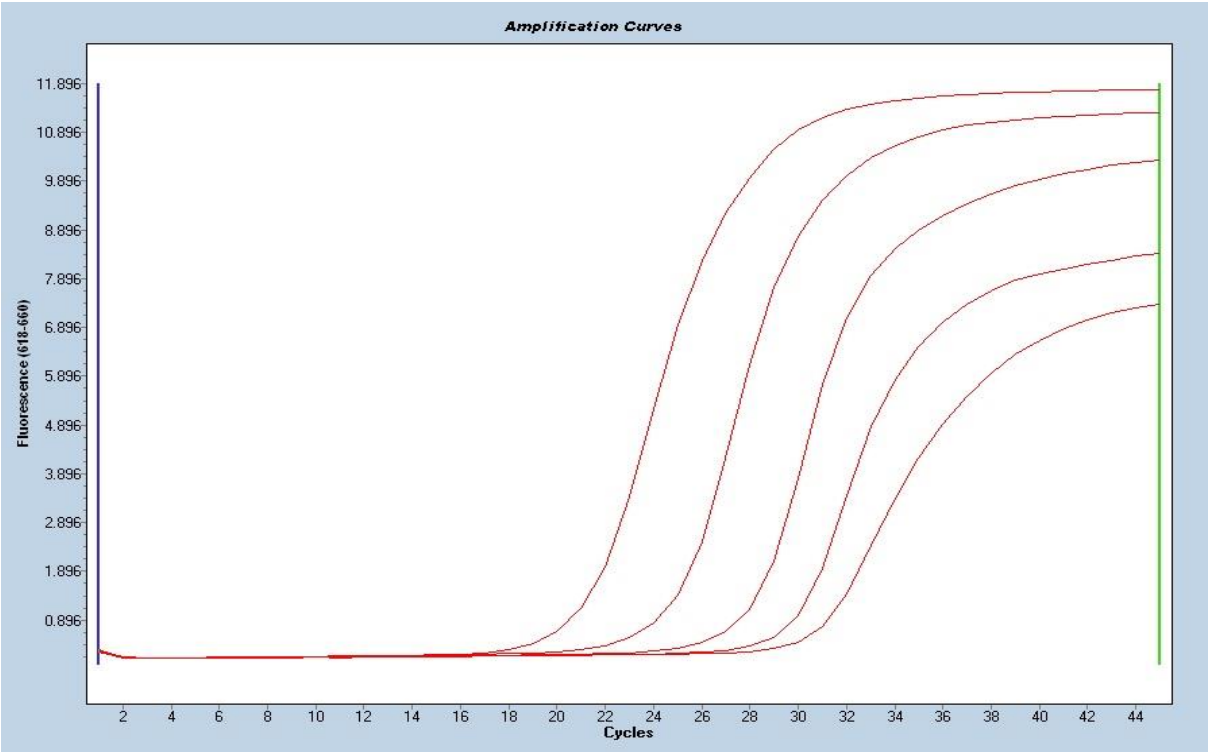


Fig. 6: Dilution series *Clostridium difficile* Toxin A/B genes (10^5 – 10^1 DNA copies per µl) on the LightCycler® 480II



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

13.2 Analytical specificity

The RIDA®GENE Hospital Stool Panel multiplex real-time RT-PCR is specific for norovirus, rotavirus and *Clostridium difficile* toxin-genes A (tcdA) and B (tcdB) in human stool samples. No cross-reaction could be detected for the following species (see Tab. 8):

Tab. 8: Cross-reactivity testing

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

13.3 Analytical reactivity

The reactivity of the RIDA®GENE Norovirus 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. 9, Tab. 10). All Norovirus genotypes and Rotavirus serotypes of the panel were detected by the RIDA®GENE Hospital Stool Panel real-time RT-PCR.

Tab. 9: Analytical reactivity testing - Norovirus

Norovirus genogroup I					
GI.1	+	GI.4	+	GI.7	+
GI.2	+	GI.5	+	GI.8	+
GI.3	+	GI.6	+		
Norovirus genogroup II					
GII.1	+	GII.4	+	GII.7	+
GII.2	+	GII.4 Sydney 2012	+	GII.10	+
GII.3	+	GII.6	+		
Norovirus genogroup IV					
GIV.1	+				

Tab.10: Analytical reactivity testing – Rotavirus

Rotavirus Serogroup A			
Serotype (samples tested)		**Serotype (samples tested)**	
G1 (4)	+	G12 (4)	+
G2 (3)	+	P[8] (14)	+
G3 (2)	+	P[4] (3)	+
G4 (3)	+	P[8] P[4] (1)	+
G9 (4)	+		

Literature

1. Polage CR, Solnick JV and Cohen SH. Nosocomial Diarrhea: Evaluation and Treatment of Causes Other Than Clostridium difficile. *Clin Infect Dis* 2012.
2. Bartel B and Gau E. Nosocomial diarrhea: a review of pathophysiology, etiology, and treatment strategies. *Hosp Pract* 2012; 40(1):130-138.
3. Weis S and Grimm M. Nosokimiale Diarrhö. *Internist* 2011; 51:167-178.
4. Pawlowski SW , Warren CA and Guerrant R. Diagnosis and Treatment of Acute or Persistent Diarrhea. Gastroenterology 2009 ; 136(6): 1874–1886.
5. Mead PS, e t al. Food- related illness and death in the United States. *Emerg Infect Dis* 1999; 5:607-625.
6. Glass RJ, *et al.* The epidemiology of enteric caliciviruses from humans: a reassessment using new diagnostics. *J Infect Dis* 2000; 181(Suppl 2):S254-261.
7. Evan HS, General outbreaks of infectious intestinal disease in England and Wales: 1995 and 1996. *Commun Dis Public Health* 1:165-171.
8. Johnston CP, *et al*. Outbreak management and implications of a nosocomial Norovirus outbreak. CID 2007; 45:534-540.
9. Corwin AL, *et al*. Shipboard impact of a probable Norwalk virus outbreak from coastal Japan. *Am J Trop Med Hyg* 1999; 61(6)898-903.
10. Kaplan JE, *et al*. An outbreak of acute nonbacterial gastroenteritis in a nursing home: demonstration of person-to-person transmission by temporal clustering of cases. *Am J Epidemiol* 1982; 116:940-948.
11. Centers for Disease Control and Prevention. Norovirus: Overwiew 2012.
12. Wilhelmi I et al. Viruses causing gastroenteritis. Clin Microbiol infect 2003, 9: 247-262.
13. Parashar UD, et al. Rotavirus and Severe Childhood Diarrhea. *Emerg Infect Dis* 2006; 12(2): 304-306.
14. Hall IC and O'Toole E. Intestinal flora in new-born infants: with a description of a new pathogenic anaerobe Bacillus difficilis. *Am J Dis Child* 1935, 49: 390–402.
15. Bartlett JG, et al. Antibiotica-associated pseudomembranous colitis due to a toxin-producing clostridia. *N Engl J Med*, 1978; 298:531-543.
16. Bartlett JG and Gerding DN. Clinical Recognition and Diagnosis of Clostridium difficile Infection. CID 2008, 46: S12-18.
17. Bartlett JG. Narrative Review: The new Epidemic of Clostridium difficile – Associated Enteric Disease. *Ann Intern Med* 2006; 145:758-764.