RIDA®GENE EHEC/EPEC

REF PG2205

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1. Intended use

For *in vitro* diagnostic use. RIDA®GENE EHEC/EPEC is a multiplex real-time PCR for the direct, qualitative detection and differentiation of genes encoding the virulence-factors of EHEC, STEC, EPEC, EIEC/*Shigella* spp. in human stool samples and cultures.[1,2]

RIDA®GENE EHEC/EPEC real-time PCR is intended for use as an aid in diagnosis of gastroenteritis caused by pathogenic *Escherichia coli* and *Shigella* spp., respectively.

2. Summary and explanation of the test

Escherichia coli (*E. coli*) are gram negative, facultatively anaerobic rod bacteria, which move by peritrichal flagellation and belong to the Enterobacteriaceae family. *E. coli* are part of the normal intestinal flora of humans and many farm animals and are generally nonpathogenic. Some *E. coli* strains are pathogenic to humans through the acquisition of certain virulence factors (e.g. genes for toxins).
The six known intestinal pathogenic *E. coli*: enterohämorrhagic *E. coli* (EHEC), enteropathogeic *E. coli* (EPEC), enterotoxic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC) and diffusely adherent *E. coli* (DAEC) can be differentiated by the virulence factors.[3]
Enterohämorrhagic *E. coli* (EHEC) are currently the most important intestinal pathogenic *E. coli*. Every year about 1000 cases of illness due to an infection with enterohaemorrhagic *E. coli* (EHEC) are reported in Germany.
EHEC are a subgroup of the Shigatoxin or Verotoxin producing *E. coli* (STEC or VTEC) and are capable to produce two cytotoxins, Verotoxin 1 and 2. Due to the similarity of the Verotoxins to the Shigatoxin of *Shigella dysenteriae*, the VTEC are also called STEC. Another important diagnostic virulence factor for EHEC is the eae gene (*E. coli* attaching and effacing gene) encoding intimin. By the detection of the ipaH gene (invasion plasmid antigen H) EHEC/STEC can be differentiated from *Shigella*/EIEC.
The clinical symptoms which are caused by EHEC range from mild diarrhoeas and severe gastroenteritis to haemorrhagic colitis which occurs in approx. 10 to 20 % of cases of infection. With 5 -10 % of infections, in babies and small children in particular as well as old patients or patients with weakened immune systems, this may also lead to a hemolytic uremic syndrome (HUS) or thrombotic thrombocytopenic purpura (TTP) as a life-threatening post-infectious complication. With HUS and TTP, mortality is particularly high among infants (approx. 10 - 15%). Acute kidney failure with a temporary need for dialysis or an irreversible loss of the kidney function resulting in a constant need for dialysis may occur. The intensity of the clinical picture depends on the predisposition of the patient, but also on the corresponding EHEC phenotype; this means that the progress of the disease also depends on the different ways in which the virulence factors are expressed. Factors, which are still unknown today also play a role. The incubation period is approximately 2 to 10 days. Because of the high environmental resistance and the infective dose for

EHEC is only at about 100 organisms. Sources of infection are contaminated foods from cattle, sheep or goats, particularly raw meat or meat products which have not been heated sufficiently, non-pasteurised raw or certified milk and contaminated fruits and vegetables. Infective chains from human to human, particularly in communal facilities such as kindergartens, homes for the elderly or hospitals, as well as direct contacts to animals are also important.[4,5]
Enteropathogenic *E. coli* (EPEC) cause particularly in infants younger than 2 years diarrhea. The virulence factor for EPEC is also the eae gene.[4]

3. Test principle

The RIDA®GENE EHEC/EPEC is a multiplex real-time PCR for the direct, qualitative detection and differentiation of genes encoding the virulence-factors of EHEC, STEC, EPEC and EIEC/*Shigella* spp.
After DNA isolation, amplification of the gene fragments specific for the virulence factors stx1/stx2, eae and ipaH (if present) occurs. 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 EHEC/EPEC assay contains an Internal Control DNA (ICD) as an internal control of sample preparation procedure and to determine possible PCR-inhibition.

4. Reagents provided

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

Kit Code	Reagent	Amount		Lid Color
1	Reaction Mix	2x	1050 µl	yellow
2	Taq-Polymerase	1x	80 µl	red
D	Internal Control DNA	2x	1700 µl	orange
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 imprinted 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 EHEC/EPEC real-time 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
bioMérieux	NucliSENS® easyMAG®
Roche	MagNA Pure
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 (BioScience grade, nuclease-free water).

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 isolation of human stool samples, use a commercially available DNA isolation kit (e.g. RIDA® Xtract (R-Biopharm)) or DNA extraction system (e.g. Maxwell® RSC (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 EHEC/EPEC assay contains an Internal Control DNA that detects PCR inhibition, monitors reagent integrity and confirms that nucleic acid extraction was sufficient. The Internal Control DNA 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 DNA is used only as a PCR inhibition control, 1 µl of the Internal Control DNA should be added to the Master-Mix (see Tab.4).

If the Internal Control DNA is used as an extraction control for the sample preparation procedure **and** as PCR inhibition control, 20 µl of the Internal Control DNA has to be added during extraction procedure. The Internal Control DNA 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 DNA to the negative control and Positive Control PCR Mix.

8.2 Sample preparation from cultures

For DNA isolation of culture samples, use a commercially available DNA isolation kit (e.g. RIDA® Xtract (R-Biopharm)) or DNA extraction system (e.g. Maxwell® RSC (Promega)). Extract DNA according to the manufacturer's instructions.
For DNA isolation from culture the following procedure is recommended: Add 1 ml PCR water into a preparation tube. Collect colonies with an inoculation loop and suspend them in the prepared PCR water. Cut or break the inoculation loop stem. Cap the preparation tube tightly and vortex strongly for 60 seconds. Heat and shake the preparation tube at 95 °C for 10 min in a heating block. Centrifuge for 1 min at 13.000 x g and apply the supernatant as sample.

Note: Repeat the centrifugation step in case of strong turbidity (if necessary).

The RIDA®GENE EHEC/EPEC assay contains an Internal Control DNA that detects PCR inhibition, monitors reagent integrity and confirms that nucleic acid extraction was sufficient. The Internal Control DNA 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 DNA is used only as a PCR inhibition control, 1 µl of the Internal Control DNA should be added to the Master-Mix (see Tab.4).

If the Internal Control DNA is used as an extraction control for the sample preparation procedure **and** as PCR inhibition control, 20 µl of the Internal Control DNA has to be added during extraction procedure. The Internal Control DNA should always be added to the specimen-PCR water mixture and must **not** be added directly to the specimen. We also recommend to add 1 µl of the Internal Control DNA 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 calculating an additional volume of 10% to compensate imprecise pipetting (see Tab. 3, Tab. 4). Thaw, mix gently and briefly centrifuge the Reaction Mix, the Taq-Polymerase, the Positive Control, the No Template Control and the Internal Control DNA 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 (ICD 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	Taq-Polymerase	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 (ICD 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	Taq-Polymerase	0.7 µl	7.7 µl
D	Internal Control DNA	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 DNA 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 DNA to the PCR-Mix of the negative control.

Sample: Add 5 µl DNA extract to the pre-pipetted Master-Mix.

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

Note: If the Internal Control DNA 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 DNA to the PCR-Mix of the positive control.

Cover tubes or plate. Spin down and place in the real-time PCR instrument. The PCR reaction should be started according to the PCR instrument set-up (Tab. 5, Tab. 6, Tab. 7, Tab. 8).

9.3 PCR instrument set-up

9.3.1 DNA real-time PCR profile

Tab. 5: DNA real-time PCR profile for LightCycler® series and Rotor-Gene Q

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: DNA real-time PCR profile for Mx3005P, ABI 7500 and CFX96™

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.

9.3.2 Universal real-time PCR profile

Note: The universal real-time PCR profile should only be used for DNA assays when combining RIDA®GENE DNA and RNA real-time PCR assays in one run.

Tab. 7: Universal real-time 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. 8: Universal real-time PCR profile for Mx3005P, ABI 7500, 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.

9.4 Detection channel set-up

Tab. 9: Selection of appropriate detection channels

Real-time PCR instrument	Detection	Detection channel	Note
Roche LightCycler® 480II	stx1/stx2	465/510	**RIDA®GENE Color Compensation Kit IV (PG0004) is required**
	ICD	533/580	
	ipaH	533/610	
	eae	618/660	
Roche LightCycler® 480 z	stx1/stx2	465/510	**RIDA®GENE Color Compensation Kit IV (PG0004) is required**
	ICD	540/580	
	ipaH	540/610	
	eae	610/670	
Agilent Techn. Mx3005P	stx1/stx2	FAM	**Check that reference dye is none**
	ICD	HEX	
	ipaH	ROX	
	eae	Cy5	
ABI 7500	stx1/stx2	FAM	**Check that passive reference option ROX is none**
	ICD	VIC	
	ipaH	ROX	
	eae	Cy5	
Bio-Rad CFX96™	stx1/stx2	FAM	**-**
	ICD	VIC	
	ipaH	ROX	
	eae	Cy5	
Qiagen Rotor-GENE Q	stx1/stx2	Green	**The gain settings have to be set to 5, according to the default settings**
	ICD	Yellow	
	ipaH	Orange	
	eae	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. Positive control and negative control have to show correct results (see Table 10, 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. 10: 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

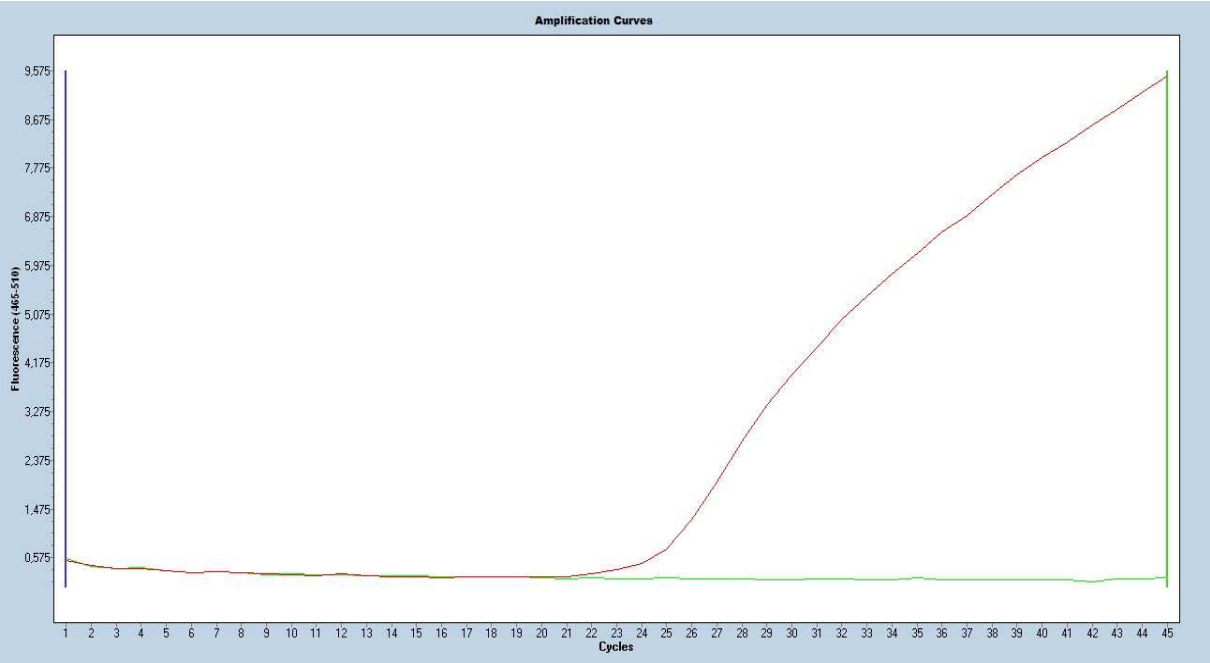

Fig. 1: Correct run of the positive and negative control (stx1/stx2) on the LightCycler® 480II

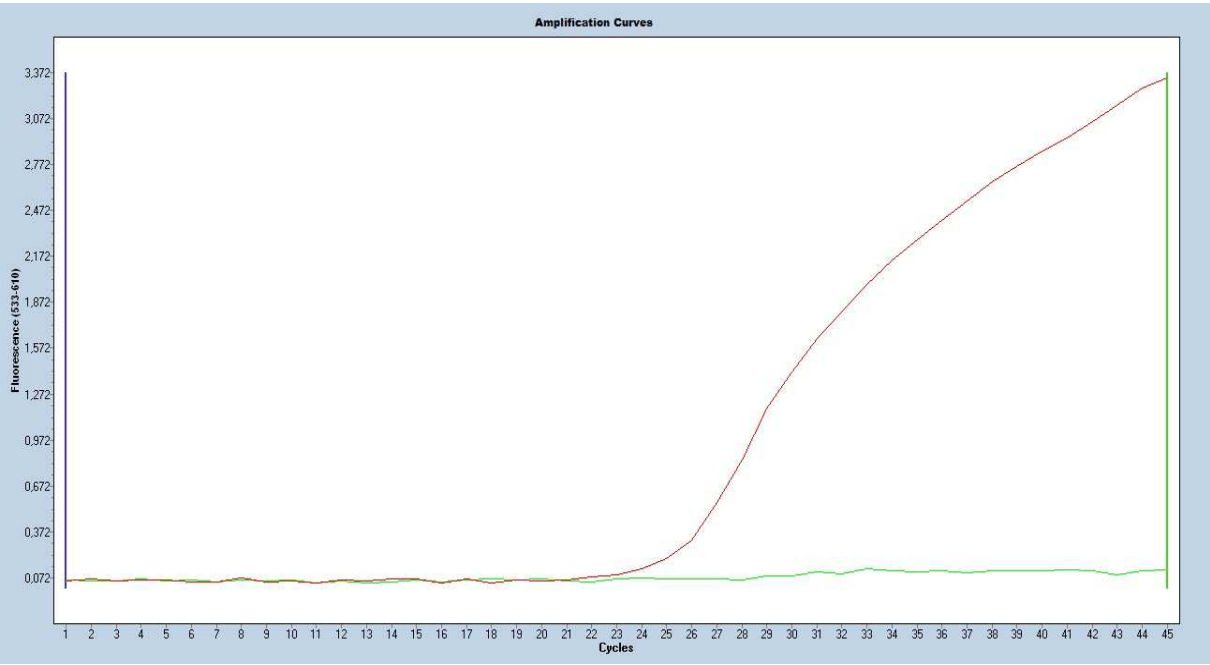

Fig. 2: Correct run of the positive and negative control (ipaH) on the LightCycler® 480II

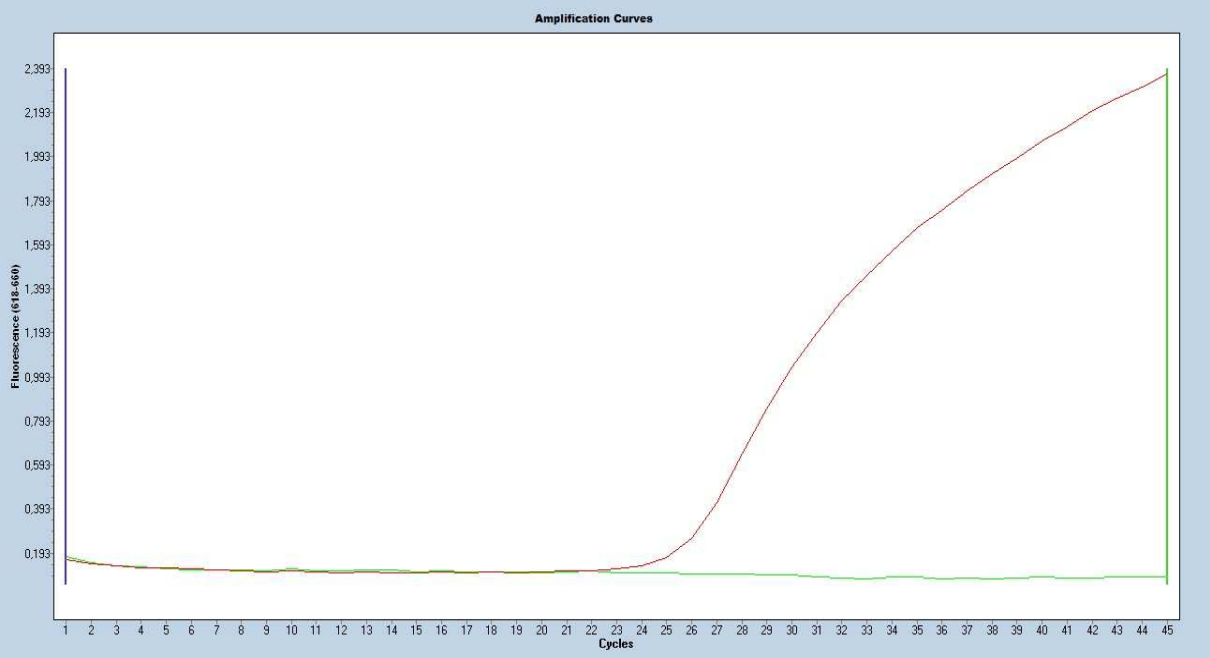

Fig. 3: Correct run of the positive and negative control (eae) on the LightCycler® 480II

11. Result interpretation

The result interpretation is done according to table 11.

Tab.11: Sample interpretation

Virulence factor genes				
stx1/stx2	**ipaH**	**eae**	**ICD**	**Result**
positive	negative	negative	**positive/ negative**	**STEC (EHEC) detected**
negative	**positive**	negative	**positive/ negative**	**EIEC/*Shigella* spp. detected**
negative	negative	**positive**	**positive/ negative**	**EPEC detected**
positive	**positive**	negative	**positive/ negative**	**STEC (EHEC) and EIEC/*Shigella* spp. detected**
positive	negative	**positive**	**positive/ negative**	**EHEC detected**
negative	**positive**	**positive**	**positive/ negative**	**EIEC/*Shigella* spp. and EPEC detected**
positive	**positive**	**positive**	**positive/ negative**	**EHEC and EIEC/*Shigella* spp. detected**
negative	negative	negative	**positive**	**Target genes not detected**
negative	negative	negative	negative	**Invalid**

According to the German Infection Protection Act (IfSG) EHEC are those STEC (Shigatoxin-producing *E. coli*), which are pathogenic for humans. As there is no specific definition for human pathogenic STEC, each STEC has to be regarded as potential EHEC.[5]

A sample is evaluated negative, if the sample shows no amplification signal in the detection system, but the Internal Control DNA 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 DNA.

A sample is evaluated positive, if both, the sample and the Internal Control DNA, 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 DNA 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 DNA 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 assay is only validated for stool and culture 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 EHEC/EPEC 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 genes (stx1/stx2, ipaH, eae).
8. Mucin can show interfering characteristics already in small quantities.

13. Performance characteristics

13.1 Analytical sensitivity

The RIDA®GENE EHEC/EPEC multiplex real-time PCR has a detection limit of ≥ 10 DNA copies per reaction for stx1/stx2, ipaH and eae, respectively.

The following figures 4, 5 and 6 show a dilution series of stx1/stx2, ipaH and eae (each 10^5 – 10^1 DNA copies per µl) on the LightCycler® 480II.

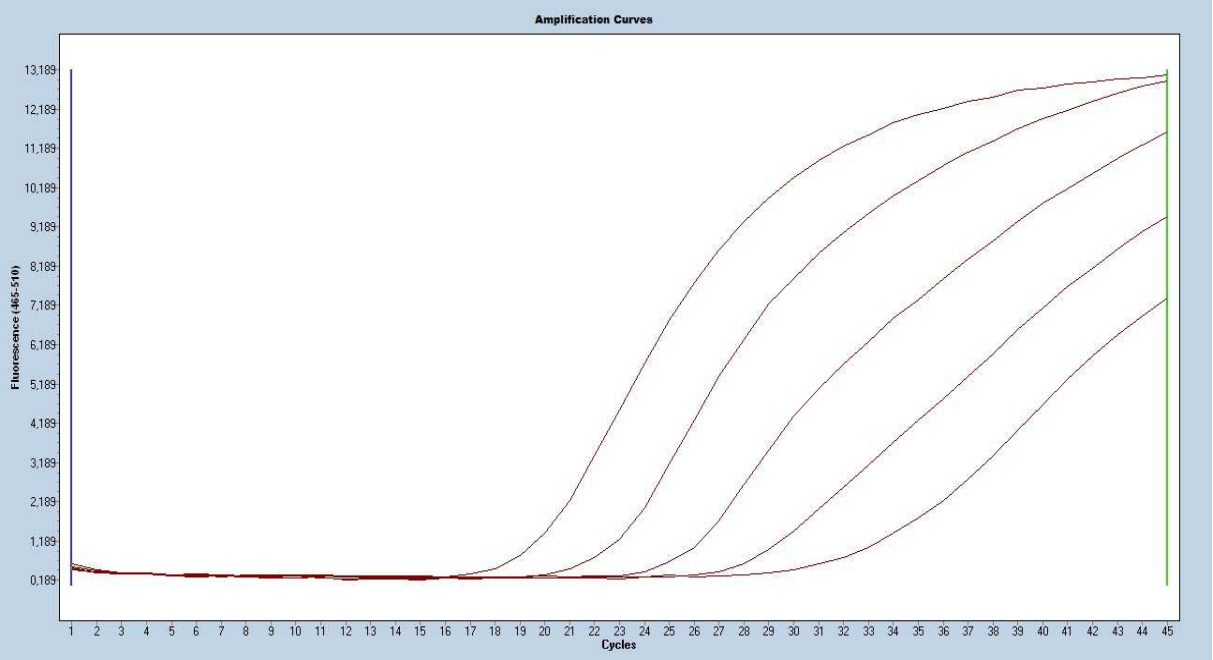

Fig. 4: Dilution series Shigatoxin genes stx1/stx2 (10^5 – 10^1 DNA copies/µl) on the LightCycler® 480II

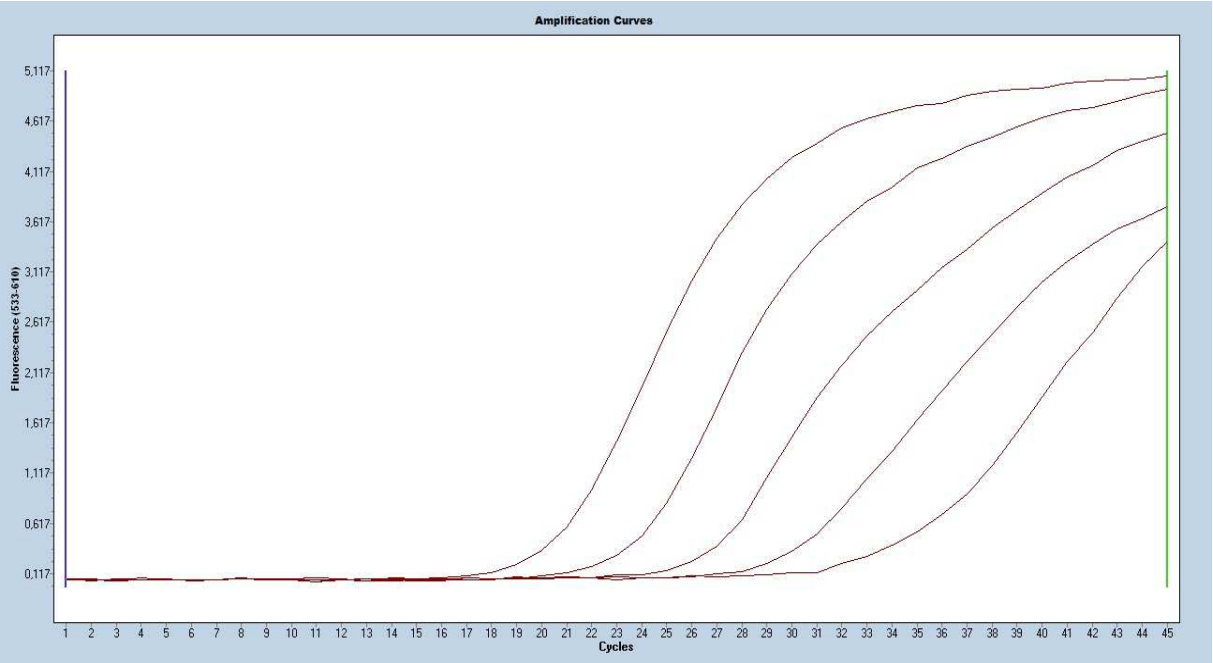

Fig. 5: Dilution series ipaH gene (10^5 – 10^1 DNA copies/µl) on the LightCycler® 480II

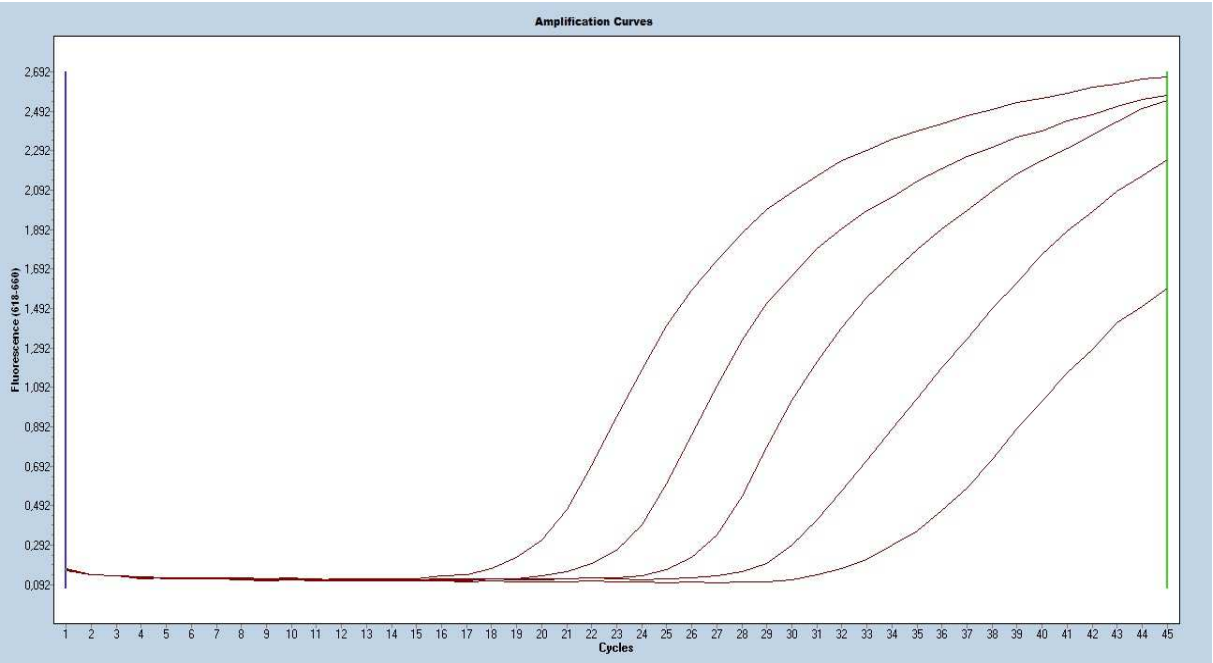

Fig. 6: Dilution series eae gene (10^5 – 10^1 DNA copies/µl) on the LightCycler® 480II

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

13.2 Analytical specificity

The RIDA®GENE EHEC/EPEC multiplex real-time PCR is specific for stx1/stx2, ipaH and eae. No cross-reaction could be detected for the following species (see Tab. 12).

Tab. 12: Cross-reactivity testing

Adenovirus 40, human, strain Dugan	-	*Clostridium difficile*	-	*Giardia lamblia*	-
Adenovirus 41, human, strain Tak	-	*Clostridium novyi*	-	*Klebsiella oxytoca*	-
Aeromonas hydrophila	-	*Clostridium perfringens*	-	Norovirus GI	-
Arcobacter butzleri	-	*Clostridium septicum*	-	Norovirus GII	-
Astrovirus	-	*Clostridium sordelli*	-	*Proteus vulgaris*	-
Bacillus cereus	-	*Clostridium sporogenes*	-	*Pseudomonas aeruginosa*	-
Bacteroides fragilis	-	*Cryptosporidium muris*	-	Rotavirus	-
Campylobacter coli	-	*Cryptosporidium parvum*	-	*Salmonella enteritidis*	-
Campylobacter fetus subsp. *fetus*	-	*E.coli* (O6)	-	*Salmonella typhimurium*	-
Campylobacter jejuni	-	*Entamoeba histolytica*	-	*Serratia liquefaciens*	-
Campylobacter lari subsp. *lari*	-	*Enterobacter cloacae*	-	*Staphylococcus aureus*	
Campylobacter upsaliensis	-	*Enterococcus faecalis*	-	*Staphylococcus epidermidis*	-
Candida albicans	-	*Giardia intestinalis* Portland1	-	*Vibrio parahaemolyticus*	-
Citrobacter freundii	-	*Giardia intestinalis* WB Clone 6	-	*Yersinia enterocolitica*	-
Clostridium bifermentas	-				

13.3 Analytical reactivity

The reactivity of the RIDA®GENE EHEC/EPEC real-time PCR was evaluated against multiple subtypes of the stx1 and stx2 gene as well as subtypes of the ipaH and eae gene (see Tab. 13). Below subtypes of stx1 and stx2 were detected by the RIDA®GENE EHEC/EPEC multiplex real-time PCR:

Tab.13: Analytical reactivity testing

stx1 subtypes					
stx1a	+	stx1c	+	stx1d	+
stx2 subtypes					
stx2a	+	stx2d	+	stx2g	+
stx2b	+	stx2e	+		
stx2c	+	stx2f	+		
ipaH subtypes					
Shigella boydii	+	*Shigella flexneri*	+	*Shigella sonnei*	+
Shigella dysenteriae	+				
eae subtypes					
eae alpha	+	eae gamma	+		

14. Version history

Version number	Chapter and designation
2014-08-14	Release version
2018-08-24	General revision
2018-08-24	4. Reagents provided 6. Additional necessary reagents and necessary equipment 8. Collection and storage of samples 9. Test procedure 10. Quality control 11. Result interpretation 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

1. Müller D, *et al.* Identification of Unconventional Intestinal Pathogenic *Escherichia coli* Isolates Expressing Intermediate Virulence Factor Profiles by Using a Novel Single-Step Multiplex PCR. Applied and Environmental Microbiology 2007; 73 (10): 3380–3390.
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