RIDA®UNITY EHEC/EPEC

REF UN2205

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1. Intended use

For *in vitro* diagnostic use. The RIDA®UNITY EHEC/EPEC test, performed on the RIDA®UNITY platform, is a multiplex real-time PCR for the direct qualitative detection of DNA for virulence factors of EHEC, STEC, EPEC, and EIEC/*Shigella* spp. in untreated human stool samples and culture samples from people with signs and symptoms of acute gastroenteritis.
The RIDA®UNITY EHEC/EPEC test is intended to support the differential diagnosis of *E. coli* infections (EHEC, EPEC, STEC, and EIEC/*Shigella* spp.) in patients with symptoms of gastroenteritis in conjunction with other clinical and laboratory findings. Negative results do not rule out infection with *E. coli* (EHEC, EPEC, STEC, or EIEC/*Shigella* spp.) and should not be used as the sole basis for diagnosis.

The product is intended for professional use.

2. Summary and explanation of the test

Diarrheal illnesses are a significant health problem, causing approximately 1.7 billion cases per year in children worldwide.[1] According to the World Health Organization (WHO), with approximately 525,000 deaths per year, these illnesses are the second leading cause of death in children under the age of 5, especially in developing countries.[1] One of the most common bacterial pathogens of diarrheal disease is *Escherichia coli*.[2]
E. coli is a Gram-negative, lactose-fermenting, motile bacterium and belongs to the family *Enterobacteriaceae*. It is a normal inhabitant of the gastrointestinal tract, but can also cause diarrheal diseases with high morbidity and mortality in children, especially in developing countries. The following classes of diarrheagenic *E. coli* were identified based on the bacterium's virulence characteristics, epidemiology, and clinical manifestations: enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enterohemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), and enteroaggregative *E. coli* (EAEC). All of these diarrheagenic pathotypes of *E. coli* can be transmitted via the fecal-oral route.[3]
Among the intestinal pathogenic *E. coli*, the enterohemorrhagic *E. coli* (EHEC) have gained special importance. They are a subgroup of the Shiga toxin- or verotoxin-producing *E. coli* (STEC and VTEC, respectively). The pathogenicity of STEC can be traced to their ability to colonize the intestine by adhering to the intestinal epithelial cells. After colonization, the bacteria are able to produce two cytotoxins: verotoxin 1 and 2. Owing to the similarity of the verotoxins to the Shiga toxin of *Shigella dysenteriae*, VTEC are also called STEC. Other important diagnostic EHEC pathogenicity factors are not only *stx1/stx2* (Shiga toxin genes), but also the *eae* gene (*E. coli* attaching and effacing gene), which encodes intimin, the membrane protein. This membrane protein is responsible for the adhering of the pathogen to the intestinal epithelial cells.[4] Clinical symptoms that can be caused by EHEC/STEC in humans range from bloody diarrhea to hemolytic uremic syndrome (HUS).[2, 5]
Sources of infection are primarily contaminated foods, whereas fewer than

1000 bacteria are enough to cause an EHEC/STEC infection. The worst food-related outbreak caused by STEC in Germany so far was in 2011. This outbreak resulted in 3816 identified STEC infections and 54 deaths, of which 32 were associated with HUS.[5]
Enteropathogenic *E. coli* (EPEC) are known as a cause of pediatric diarrheal diseases, especially in developing countries.[3] EPEC can be distinguished from EHEC by the absence of Shiga toxins.[3] The most common symptoms associated with an EPEC infection are watery diarrhea, abdominal pain, nausea, vomiting, and fever, whereas the infectious dose in healthy adults is about 10^8 organisms.[5]
Enteroinvasive *E. coli* (EIEC) and *Shigella* spp. likewise cause diarrheal diseases worldwide, especially in developing countries. They are both Gram-negative bacteria that are biochemically and genetically closely related to one another.[6] The pathogenicity of EIEC and *Shigella* spp. is based on the plasmid-mediated invasion of intestinal epithelial cells and their destruction. The products of the invasion plasmid antigen H gene (*ipaH*) are responsible for this process. This gene is relevant for the detection of EIEC/*Shigella* spp., making it possible to distinguish this pathotype from EHEC. Outbreaks of EIEC/*Shigella* spp. infections are considered to be mainly caused by food and manifest with diarrhea, abdominal pain, nausea, and fever.[6]

3. Test principle

The RIDA®UNITY EHEC/EPEC is a multiplex real-time PCR for the direct qualitative detection and differentiation of genes for the virulence factors of EHEC, STEC, EPEC, and EIEC/*Shigella* spp. in human stool or culture samples.
Processing is fully automated with the RIDA®UNITY system. First, the nucleic acids are extracted using the RIDA®UNITY Universal Extraction Kit and the Internal Control Kit.
The target sequence is detected always in a one-step real-time RT-PCR format (even with DNA assays), that is, reverse transcription (RT) and subsequent PCR are performed in one reaction vial. In the process, the isolated RNA (if present) is transcribed into cDNA with the help of reverse transcriptase. The specific gene fragments of the virulence factors *stx1/stx2*, *eae*, and *ipaH* are then amplified using real-time PCR.
The amplified target sequences are detected using hydrolysis probes that are labeled with a quencher at one end and a fluorescent reporter dye (fluorophore) at the other. The probes hybridize to the amplicon in the presence of a target sequence. During extension,
Taq polymerase separates the reporter from the quencher. The reporter emits a fluorescent signal that is detected by the optical unit of a real-time PCR instrument. The fluorescent signal increases with the quantity of formed amplicons. The RIDA®UNITY Internal Control Kit must be used at the same time to be able to check sample preparation and/or potential PCR inhibition.

4. Reagents provided

The reagents in the kit are sufficient for 96 determinations*.

Tab. 1: Reagents provided

REF	Reagent	Amount		Lid color
UNZ2205RM	Reaction Mix	1 ×	1935 µL	yellow, ready for use
UNZ2205EM	Enzyme Mix	1 ×	350 µL	red, ready for use
UNZ2205PC	Positive Control	1 ×	200 µL	blue, ready for use
UNZ2205NC	Negative Control	1 ×	450 µL	white, ready for use

* With repeated use and in smaller series, the number of reactions may be reduced.

5. Storage instructions

- Please follow the handling guidelines in Table 2 and store the kit directly after use according to the information specified.
- All reagents must be stored away from light at -16 °C to -28 °C and, if unopened, can be used until the expiration date printed on the label. The quality guarantee is no longer valid after the expiration date.
- All reagents should be carefully thawed prior to use (e.g., in a refrigerator at 2 - 8 °C).
- Repeated freezing and thawing up to 8 times does not affect the test properties.

Tab. 2: Storage conditions and information

	Storage temperature	Maximum storage time
unopened	-16 °C to -28 °C	Can be used until the printed expiration date
opened	-16 °C to -28 °C	8 freeze-thaw cycles

6. Reagents required but not provided

The RIDA®UNITY EHEC/EPEC multiplex real-time RT-PCR test is intended exclusively for use with RIDA®UNITY system. The following products are absolutely required for correct use:

6.1 Reagents

The following reagents are needed to perform the RIDA®UNITY EHEC/EPEC test:

Reagents	Item number
RIDA®UNITY Universal Extraction Kit (R-Biopharm AG)	UN0001
RIDA®UNITY Internal Control Kit (R-Biopharm AG)	UN0010

6.2 Laboratory equipment

The following equipment is needed to perform the RIDA®UNITY EHEC/EPEC test:

Equipment
RIDA®UNITY system; item number: ZUNITY (R-Biopharm AG)
RIDA®UNITY consumables (tips, plates, reaction vials, films). See the instructions for use for the RIDA®UNITY system, ordering information for consumables.
Vortexer
Tabletop centrifuge
Powder-free disposable gloves

External cycler (possible system enhancement)
CFX96™ Dx (Bio-Rad)

The RIDA®UNITY EHEC/EPEC kit can be used in combination with other compatible cyclers. Alternative real-time PCR instruments must be verified/validated by the user. Please contact R-Biopharm AG at pcr@r-biopharm.de to check the compatibility.

7. Warnings and precautions for the users

For *in vitro* diagnostic use.
This test must be carried out only by qualified laboratory personnel. The guidelines for working in medical laboratories must be observed.
Always adhere strictly to the operating manual when carrying out this test.
Do not pipette samples or reagents using your mouth. Avoid contact with broken skin and mucous membranes.
Wear personal protective equipment (appropriate gloves, lab coat, safety glasses) when handling reagents and samples, and wash hands after completing the test.
Do not smoke, eat, or drink in areas where samples are handled.
Avoid contaminating the samples and components of the kit with microbes and nucleases (DNase/RNase).
Clinical samples must be viewed as potentially infectious and must be disposed of appropriately, like all reagents and materials that come into contact with potentially infectious samples.
Do not exchange or mix the components (Reaction Mix, Enzyme Mix, Positive Control, Negative Control) of one kit lot with the components of another lot.
The test kit can be used for 8 weeks long after first opening (the kit can be reloaded up to 6 times). Do not use the test kit after the expiration date. These specifications are also checked by the RIDA®UNITY system.
Users are responsible for the proper disposal of all reagents and materials after use. For disposal, please adhere to national regulations.

Further details on the Safety Data Sheet (SDS) can be found under the item number at https://clinical.r-biopharm.com/search/.

For users in the European Union: Report all serious adverse events associated with the product to R-Biopharm AG and the appropriate national authorities.

The summary of safety and performance (SSP) for this product will be available at https://ec.europa.eu/tools/eudamed once the European Database on Medical Devices (EUDAMED) gets underway. In the database, search for the device using the UDI-DI located on the outer packaging of the device.

8. Collection and storage of samples

It is recommended to use fresh sample material to achieve the best possible performance of the RIDA®UNITY EHEC/EPEC assay.

Avoid repeated freezing and thawing of the sample.

Do not collect the stool samples in transport containers that contain transport media with preservatives, animal sera, metal ions, oxidizing agents, or detergents since such substances can interfere with the RIDA®UNITY tests.

It is recommended to produce aliquots of the specimens to avoid repeated thawing and freezing. Frozen specimens should be thawed immediately prior to extraction to prevent degradation of the nucleic acids.

Follow the sample storage instructions in Tables 3 to 6.

Tab. 3: Sample storage - EHEC detection

Native samples - stool		
20 - 25 °C	2 - 8 °C	-20 °C / -80 °C
≤ 7 days	≤ 7 days	≤ 6 months

Native samples - culture		
20 - 25 °C	2 - 8 °C	-20 °C / -80 °C
≤ 1 day	≤ 1 day	-

In eluate (from stool or culture)		
30 °C	2 - 8 °C	-20 °C
≤ 24 hours	≤ 36 hours	≤ 7 days

At a storage temperature of -20 °C / -80 °C, repeated freezing/thawing of the stool sample for up to 5 times does not affect the test properties.

At a storage temperature of -20 °C, repeated freezing/thawing of the eluate (from stool or culture) up to 3 times does not affect the test properties.

Do not grow cultures from previously frozen stool because freezing severely affects the growth characteristics of the pathogens and this can potentially cause false-negative results.

Tab. 4: Sample storage - STEC detection

Native samples - stool		
20 - 25 °C	2 - 8 °C	-20 °C / -80 °C
≤ 7 days	≤ 7 days	≤ 6 months

Native samples - culture		
20 - 25 °C	2 - 8 °C	-20 °C / -80 °C
≤ 1 day	≤ 1 day	-

In eluate (from stool or culture)		
30 °C	2 - 8 °C	-20 °C
≤ 24 hours	≤ 36 hours	≤ 7 days

At a storage temperature of -20 °C / -80 °C, repeated freezing/thawing of the stool sample for up to 5 times does not affect the test properties.

At a storage temperature of -20 °C, repeated freezing/thawing of the eluate (from stool or culture) up to 3 times does not affect the test properties.

Do not grow cultures from previously frozen stool because freezing severely affects the growth characteristics of the pathogens and this can potentially cause false-negative results.

Tab. 5: Sample storage - EPEC detection

Native samples - stool		
20 - 25 °C	2 - 8 °C	-20 °C / -80 °C
≤ 7 days	≤ 7 days	≤ 6 months

Native samples - culture		
20 - 25 °C	2 - 8 °C	-20 °C / -80 °C
≤ 1 day	≤ 1 day	-

In eluate (from stool or culture)		
30 °C	2 - 8 °C	-20 °C
≤ 24 hours	≤ 36 hours	≤ 7 days

At a storage temperature of -20 °C / -80 °C, repeated freezing/thawing of the stool sample for up to 5 times does not affect the test properties.

At a storage temperature of -20 °C, repeated freezing/thawing of the eluate (from stool or culture) up to 3 times does not affect the test properties.

Do not grow cultures from previously frozen stool because freezing severely affects the growth characteristics of the pathogens and this can potentially cause false-negative results.

Tab. 6: Sample storage - EIEC/*Shigella* spp. detection

Native samples - stool		
20 - 25 °C	2 - 8 °C	-20 °C / -80 °C
≤ 1 day	≤ 7 days	≤ 6 months

Native sample - culture		
20 - 25 °C	2 - 8 °C	-20 °C / -80 °C
-	≤ 1 day	-

In eluate (from stool or culture)		
30 °C	2 - 8 °C	-20 °C
≤ 24 hours	≤ 36 hours	≤ 7 days

At a storage temperature of -20 °C / -80 °C, repeated freezing/thawing of the stool sample for up to 5 times does not affect the test properties.

At a storage temperature of -20 °C, repeated freezing/thawing of the eluate (from stool or culture) up to 3 times does not affect the test properties.

Do not grow cultures from previously frozen stool because freezing severely affects the growth characteristics of the pathogens and this can potentially cause false-negative results.

8.1 DNA preparation from stool and culture samples

To isolate DNA from stool and culture samples, use the RIDA®UNITY Universal Extraction Kit. Follow the correct procedures in the instructions for use for the RIDA®UNITY Universal Extraction Kit (Section: Nucleic acid preparation from stool samples; Section: Nucleic acid preparation from culture samples).

9. Test procedure

Place both the samples and reagents of the RIDA®UNITY EHEC/EPEC on the RIDA®UNITY system at the beginning of use.
Beforehand, adequately mix the Reaction Mix, Negative Control, and Positive Control using a vortexer. Do not vortex the Enzyme Mix. Afterward, briefly centrifuge all components.
The PCR tubes for the samples to be examined must be positioned beforehand in the integrated PCR cycler.
Carriers are available for correctly loading the system with reagents and consumables. For the loading process, follow the instructions of the RIDA®UNITY system. Observe the relevant sections in the manual of the RIDA®UNITY system (Section: Performing a run).

The RIDA®UNITY EHEC/EPEC test may be used only in combination with the RIDA®UNITY Internal Control Kit. This allows for early recognition of potential PCR inhibition, verification of reagent integrity, and confirmation of successful nucleic acid extraction. The procedure is described in the instructions for use of the RIDA®UNITY Internal Control Kit (Section: Test procedure).

Automated processing is described in the RIDA®UNITY system manual (Section: Performing a run).

9.1 Device settings

9.1.1 Universal real-time PCR profile

To harmonize the RIDA®UNITY assays, the RIDA®UNITY EHEC/EPEC assay was verified exclusively in the universal profile. This makes it possible to combine DNA and RNA assays with each other. Generally speaking, reverse transcription therefore comes first in the universal profile.

Tab. 7: Universal real-time PCR profile for the RIDA®UNITY

Reverse transcription	10 min, 58 °C
Initial denaturation	1 min, 95 °C
Cycles	45 cycles
PCR Denaturation	10 sec, 95 °C
Annealing/extension	15 sec, 60 °C
Temperature transition rate/ ramp rate	Maximum

Note: Annealing and extension take place in the same step.

Tab. 8: Universal real-time PCR profile for CFX96™ Dx

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, 60 °C
Temperature transition rate/ ramp rate	Maximum

Note: Annealing and extension take place in the same step.

9.2 Detection channel setting

Tab. 9: Selection of appropriate detection channels

Real-time PCR instrument	Detection	Detection channel	Note
R-Biopharm RIDA®UNITY	*stx1/stx2*	FAM	SEEK channel stx
	Internal Control	HEX	SEEK channel ICD
	ipaH	ROX	SEEK channel ipaH
	eae	Cy5	SEEK channel eae
Bio-Rad CFX96™ Dx	*stx1/stx2*	FAM	SEEK channel stx
	Internal Control	VIC	SEEK channel ICD
	ipaH	ROX	SEEK channel ipaH
	eae	Cy5	SEEK channel eae

10. Quality control - indication of instability or expiration of reagents

The samples are evaluated using the RIDA®SEEK analytical software of the RIDA®UNITY system. The Negative Control and Positive Control must show the correct results (see Tab. 9).

The Positive Control is present in a concentration of 10^3 copies/µL. It is used in a total quantity of 5×10^3 copies in every PCR run.

The Negative Control already contains the RIDA®UNITY Internal Control. Since the controls do not contain a template, no signals are to be anticipated in the target channels. Positive signals in the IC channel with which the internal control is detected are essential (see Tab. 10).

Tab.10: A valid PCR run must meet the following conditions:

Sample	Result	IC Ct	Target gene Ct
Positive control	+	N/A*	See Certificate of Analysis
Negative control	-	Ct > 20	0

*In certain circumstances, the IC channel can have a positive signal in the positive control and therefore should not be evaluated.

If the positive control is not within the specified Ct range, but the negative control is valid, all reactions, including the controls, need to be reanalyzed in the PCR.

If the negative control is not negative, but the positive control is valid, all reactions, including the controls, need to be reanalyzed in the PCR.

If the specified values are not met, check the following items before repeating the test:

- Expiration date of the reagents used
- Functionality of the equipment being used
- Correct test procedure

If the conditions are still not fulfilled after repeating the test, please consult the manufacturer or your local R-Biopharm distributor.

11. Evaluation and interpretation

Sample evaluation and interpretation are done using the RIDA®UNITY system analytical software, RIDA®SEEK.

There is no current internationally recognized reference method or reference material for standardization. The control materials are metrologically traceable to internal R-Biopharm AG standards based on specific DNA amplicons.

For further information on metrological traceability, please contact R-Biopharm AG.

The specified values, ranges, and further details can be found in the Certificate of Analysis (CoA).

Tab.11: Result interpretation*

Detection of				
stx1/stx2	***ipaH***	***eae***	**IC**	**Result**
+	-	-	**+/-**	**STEC (EHEC) detectable**
-	**+**	-	**+/-**	**EIEC/*Shigella* spp. detectable**
-	-	**+**	**+/-**	**EPEC detectable**
+	**+**	-	**+/-**	**STEC (EHEC) and EIEC/*Shigella* spp. detectable**
+	-	**+**	**+/-**	**EHEC detectable**
-	**+**	**+**	**+/-**	**EIEC/*Shigella* spp. and EPEC detectable**
+	**+**	**+**	**+/-**	**EHEC and EIEC/*Shigella* spp. detectable**
-	-	-	**+**	**Target genes not detectable**
-	-	-	-	**Invalid**

*+= positive
- = negative

The Infection Protection Act (IfSG) describes EHEC as the Shiga toxin-producing *E. coli* (STEC) that are human pathogens. Since an exact definition of human pathogenic STEC cannot be established at this time, **every** STEC is considered a potential EHEC.(5)

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

A sample is also positive if the sample DNA shows an amplification signal, but no amplification signal can be seen for the Internal Control in the detection system.

Detecting the Internal Control is not necessary in this case because high amplicon concentrations can result in a weak or absent signal of the Internal Control.

A sample is negative if the sample DNA does not show an amplification signal, but an amplification signal is visible for the Internal Control in the detection system. Inhibition of the PCR reaction and prior extraction can be ruled out by the detection of the Internal Control.

A sample is invalid if the sample DNA and the Internal Control do not show an amplification signal in the detection system. There are inhibitors in the sample, or an error occurred during the extraction process.

12. Limitations of the method

1. The RIDA®UNITY EHEC/EPEC test detects DNA for the virulence factors of EHEC, STEC, EPEC, and EIEC/*Shigella* spp. in untreated human stool and culture samples. A connection between the level of the determined Ct value and the occurrence of severe clinical symptoms cannot be derived from this. The results obtained must always be interpreted in combination with the complete clinical symptoms.
2. The diagnosis should not be based on the result of the molecular biological analysis alone, but should always take the patient's medical history and symptoms into account.
3. This test is approved only for automated processing using the RIDA®UNITY system.
4. This test is verified only for stool and culture samples.
5. When using the culture matrix, do not transfer the agar medium into the PCR reaction because this can lead to potential interference.
6. Improper sampling, transport, storage, and handling, or a pathogen load below the test's analytical sensitivity can lead to false-negative results.
7. The presence of PCR inhibitors can lead to false-negative or invalid results.
8. As with all PCR-based *in vitro* diagnostic tests, extremely low concentrations of the target sequences, which are under the limit of detection (LoD 95 %), can be detected. The results obtained are not always reproducible.
9. Mutations or polymorphisms in the primer or probe binding sites can interfere with the detection of new or unknown variants and can lead to false-negative results using the RIDA®UNITY EHEC/EPEC.
10. A positive test result does not necessarily indicate the presence of viable organisms. A positive result indicates that the target genes (EHEC, *stx1/2, eae*; STEC, *stx1/2*; EPEC, *eae*; and EIEC/*Shigella* spp., *ipaH*) are present.
11. This assay should be performed in compliance with the regulation on good laboratory practice (GLP). Users must precisely follow the manufacturer's instructions when performing the test.

13. Performance characteristics

13.1 Clinical performance characteristics

The RIDA®UNITY EHEC/EPEC multiplex real-time PCR was compared in an external laboratory with a CE-marked reference test based on 276 stool samples from patients with symptoms of gastrointestinal infection.

The results show very high sensitivity and specificity in the detection of virulence factors of EHEC, STEC, EPEC, and EIEC/*Shigella* spp. under use of the RIDA®UNITY EHEC/EPEC kit.

Tab. 12: Detection of *stx1/2* - stool samples

		Reference PCR		
		Positive	Negative	Total
RIDA®UNITY EHEC/EPEC - *stx1/2*	Positive	122	0	122
	Negative	5	149	154
	Total	127	149	276

Relative sensitivity (95 % CI)	96.1 % (91.1 % - 98.7 %)
Relative specificity (95 % CI)	100 % (97.6 % - 100 %)

Tab. 13: Detection of *ipaH* - stool samples

		Reference PCR		
		Positive	Negative	Total
RIDA®UNITY EHEC/EPEC - *ipaH*	Positive	65	0	65
	Negative	0	211	211
	Total	65	211	276

Relative sensitivity (95 % CI)	100 % (94.5 % - 100 %)
Relative specificity (95 % CI)	100 % (98.3 % - 100 %)

Tab. 14: Detection of *eae* - stool samples

		Reference PCR		
		Positive	Negative	Total
RIDA®UNITY EHEC/EPEC - *eae*	Positive	179	0	179
	Negative	4	93	97
	Total	183	93	276

Relative sensitivity (95 % CI)	97.8 % (94.5 % - 100 %)
Relative specificity (95 % CI)	100 % (96.1 % - 100 %)

13.2 Analytical performance characteristics

13.2.1 Detection limit (LoD 95 %)

A positive control sample (negative stool samples, spiked, or using a culture sample) was measured in five dilution steps (in 0.25-log steps) for each target with 20 replicates per step in one lot to determine the LoD. This was followed by a probit analysis. Next, the calculated LoD was confirmed with 20 replicates per target for the calculated dilution step/concentration.
The following strains were used for testing:

- *stx1/stx2: Escherichia coli* D3509 (The LoD for *stx2* was determined because *stx2* is associated with hemolytic uremic syndrome.)
- *ipaH: Escherichia coli* Fr1368
- *eae: Escherichia coli* DSM8695

For the detection of EHEC, STEC, EPEC, and EIEC/*Shigella* spp.DNA using the RIDA®UNITY EHEC/EPEC assay on the UNITY system, the following limits of detection (LoD) were determined.
The results of these measurements are shown in Table 15.

Tab. 15: Results of the limit of detection of the RIDA®UNITY EHEC/EPEC test for targets *stx1/2*, *ipaH*, and *eae*.

	Matrix	*stx1/2*	*ipaH*	*eae*
LoD	Stool	476,000 CFU/mL	5300 CFU/mL	125,000 CFU/mL
	Culture	2130 CFU/mL	798 CFU/mL	2890 CFU/mL

*CFU: Colony Forming Units

The LoD for *stx1/2* in stool samples was determined at 476,000 CFU/mL.

The LoD for *ipaH* in stool samples was determined at 5300 CFU/mL.

The LoD for *eae* in stool samples was determined at 125,000 CFU/mL.

The LoD for *stx1/2* in culture samples was determined at 2130 CFU/mL.

The LoD for *ipaH* in culture samples was determined at 798 CFU/mL.

The LoD for *eae* in culture samples was determined at 2890 CFU/mL.

For the enhanced workflow using the CFX96™ Dx, these LoD values were confirmed under the assumption that we stay in a 2-3-fold LoD range.

13.2.2 Analytical specificity

Interfering substances

The presence of PCR inhibitors and interfering substances can lead to false-negative or invalid results. Therefore, the effects of various substances that may exist given their widespread use for gastrointestinal infections or widespread occurrence in the corresponding specimens were investigated.
Substances that could potentially significantly influence the test results were examined initially at high concentrations (triple the daily dose or simulation of the worst case) in an interference screen.
No interference was found for the substances listed in Table 16.

Tab. 16: Potentially interfering substances

Potentially interfering substance	Concentration
Azithromycin-ratiopharm® 500 mg film-coated tablets (azithromycin)	0.75 % [w/v]
Barium sulphate	18.5 % [v/v]
Cologran® liquid sweetener (saccharin + cyclamate)	1.3 % [w/v]
Human blood	5 % [v/v]
Charcoal tablets 250 mg (charcoal)	6 % [w/v]
Loperamid-ratiopharm® akut (loperamide)	0.02 % [v/v]
Mucins	5 % [w/v]
Stearic/palmitic acid	40 % [w/v]

Cross-reactions

Various organisms (bacteria, parasites, fungi, and viruses) that can be commonly found in the stool matrix were investigated. The microorganisms to be investigated for this assay were chosen because either they naturally occur in stool samples, or they cause corresponding symptoms as gastrointestinal pathogens. Bacterial cultures (between 10^6 and 10^9 CFU/mL), fungal or viral cultures, supernatants of viral cultures, isolates, and LGC standards of the respective organism were used for analyses.

The RIDA®UNITY EHEC/EPEC multiplex real-time PCR is specific for EHEC, STEC, EPEC, and EIEC/*Shigella* spp. No cross-reactions with the following species were detected (see Tab. 17):

Tab. 17: Potentially cross-reactive organisms.

Organism	Test result*		
	stx1/2	***ipaH***	***eae***
Arcobacter butzleri	-	-	-
Adenovirus 40	-	-	-
Adenovirus 41, human, strain Tak	-	-	-
Aeromonas hydrophila	-	-	-
Astrovirus 2	-	-	-
Bacillus cereus	-	-	-
Bacteroides fragilis	-	-	-
Campylobacter coli	-	-	-
Campylobacter fetus subsp. *fetus*	-	-	-
Campylobacter jejuni	-	-	-
Campylobacter lari subsp. *lari*	-	-	-
Campylobacter upsaliensis	-	-	-
Candida albicans	-	-	-
Citrobacter freundii	-	-	-
Clostridium bifermentans	-	-	-
Clostridium difficile	-	-	-
Clostridium novyi	-	-	-
Clostridium perfringens	-	-	-
Clostridium septicum	-	-	-
Clostridium sordellii	-	-	-
Clostridium sporogenes	-	-	-
Cryptosporidium muris	-	-	-
Cryptosporidium parvum	-	-	-

E. coli (O6)	-	-	-
Entamoeba histolytica	-	-	-
Enterobacter cloacae	-	-	-
Enterococcus faecalis	-	-	-
*Giardia intestinalis*** Portland 1	-	-	-
*Giardia intestinalis*** WB Clone C6	-	-	-
*Giardia lamblia***	-	-	-
Klebsiella oxytoca	-	-	-
Norovirus GI	-	-	-
Norovirus GII	-	-	-
Proteus vulgaris	-	-	-
Pseudomonas aeruginosa	-	-	-
Rotavirus	-	-	-
Salmonella enterica (serovar Enteritidis)	-	-	-
Salmonella enterica (serovar Typhimurium)	-	-	-
Serratia liquefaciens	-	-	-
Staphylococcus aureus	-	-	-
Staphylococcus epidermidis	-	-	-
Vibrio parahaemolyticus	-	-	-
Yersinia enterocolitica	-	-	-

* - = negative
** *Giardia intestinalis* and *Giardia lamblia* are the same organism.

13.2.3 Precision

The precision of the RIDA®UNITY EHEC/EPEC real-time PCR test was determined for the following levels of consideration.

Intra-assay precision: Determination of 5 control samples using 20 replicates each on the RIDA®UNITY under identical conditions.

Inter-assay precision: Determination of 5 control samples in 20 runs in duplicate on 10 workdays (2 runs per day) performed by different technicians under reproducible conditions.

Testing for *intra*- and *inter*-assay precision was carried out using three different lots.

The coefficients of variation obtained for each measurement using the RIDA®UNITY EHEC/EPEC real-time PCR test on the RIDA®UNITY and the CFX96™ Dx were 4.29 %.

Tab. 18: Results of the precision of the RIDA®UNITY EHEC/EPEC test for *stx1/2* from stool samples (RIDA®UNITY system).

Ct Mean value / CV		*Intra*-assay			*Inter*-assay			*Inter*-lot
		Kit lot 1	Kit lot 2	Kit lot 3	Kit lot 1	Kit lot 2	Kit lot 3	Kit lots 1-3
1	Ct	27.8	27.7	27.4	27.8	27.7	27.6	27.7
	CV (%)	1.02 %	1.19 %	1.11 %	3.25 %	3.19 %	3.18 %	3.21 %
2	Ct	29.6	29.5	29.2	30.0	29.8	29.8	29.8
	CV (%)	1.07 %	0.87 %	0.85 %	2.93 %	2.66 %	2.53 %	2.71 %
3	Ct	29.2	29.0	28.9	30.2	30.0	30.1	30.1
	CV (%)	1.72 %	1.35 %	1.46 %	3.29 %	2.85 %	3.15 %	3.10 %
4	Ct	-	-	-	32.0	31.7	31.6	31.8
	CV (%)	N/A	N/A	N/A	2.15 %	2.23 %	2.26 %	2.28 %
5	Ct	32.5	31.9	32.1	-	-	-	-
	CV (%)	2.34 %	2.05 %	2.13	N/A	N/A	N/A	N/A

Tab. 19: Results of the precision of the RIDA®UNITY EHEC/EPEC test for *stx1/2* from stool samples (CFX96™ Dx).

Ct Mean value / CV		*Intra*-assay			*Inter*-assay			*Inter*-lot
		Kit lot 1	Kit lot 2	Kit lot 3	Kit lot 1	Kit lot 2	Kit lot 3	Kit lots 1-3
1	Ct	27.2	27.1	26.9	26.1	26.0	26.0	26.0
	CV (%)	0.82 %	1.04 %	0.76 %	3.12 %	2.61 %	2.64 %	2.80 %
2	Ct	27.6	27.2	27.1	28.1	27.5	27.5	27.7
	CV (%)	1.91 %	0.95 %	0.71 %	4.03 %	3.02 %	2.86 %	3.50 %
3	Ct	27.7	27.2	27.2	28.1	27.7	27.6	27.8
	CV (%)	2.34 %	0.74 %	0.95 %	4.29 %	3.11 %	2.91 %	3.54 %
4	Ct	-	-	-	29.2	28.7	28.6	28.9
	CV (%)	N/A	N/A	N/A	2.96 %	2.90 %	2.76 %	3.04 %
5	Ct	29.6	29.5	29.6	-	-	-	-
	CV (%)	1.52 %	1.59 %	3.43 %	N/A	N/A	N/A	N/A

Tab. 20: Results of the precision of the RIDA®UNITY EHEC/EPEC test for *ipaH* from stool samples (RIDA®UNITY system).

Ct Mean value / CV		*Intra*-assay			*Inter*-assay			*Inter*-lot
		Kit lot 1	Kit lot 2	Kit lot 3	Kit lot 1	Kit lot 2	Kit lot 3	Kit lots 1-3
1	Ct	25.0	24.8	24.6	25.3	25.1	25.1	25.2
	CV (%)	1.02 %	1.11 %	1.09 %	1.84 %	1.73 %	1.83	1.80 %
2	Ct	26.2	26.1	26.0	26.3	26.1	26.2	26.2
	CV (%)	0.81 %	0.89 %	0.90 %	1.49 %	1.39 %	1.37 %	1.40 %
3	Ct	25.5	25.3	25.4	26.2	26.1	26.1	26.1
	CV (%)	0.96 %	0.99 %	0.87 %	1.72 %	1.57 %	1.53 %	1.61 %
4	Ct	-	-	-	27.1	27.0	27.0	27.0
	CV (%)	N/A	N/A	N/A	1.44 %	1.34 %	1.45 %	1.42 %
5	Ct	27.4	27.4	27.3	-	-	-	-
	CV (%)	0.88 %	0.77 %	0.69 %	N/A	N/A	N/A	N/A

Tab. 21: Results of the precision of the RIDA®UNITY EHEC/EPEC test for *ipaH* from stool samples (CFX96™ Dx).

Ct Mean value / CV		*Intra*-assay			*Inter*-assay			*Inter*-lot
		Kit lot 1	Kit lot 2	Kit lot 3	Kit lot 1	Kit lot 2	Kit lot 3	Kit lots 1-3
1	Ct	25.1	25.0	24.8	24.4	24.4	24.4	24.4
	CV (%)	1.15 %	1.25 %	1.06 %	1.49 %	1.79 %	1.70 %	1.67 %
2	Ct	25.2	25.2	25.2	25.4	25.3	25.4	25.4
	CV (%)	0.85 %	1.03 %	1.01 %	1.11 %	1.21 %	1.21 %	1.17 %
3	Ct	25.2	25.0	25.0	25.4	25.4	25.3	25.4
	CV (%)	1.04 %	1.09 %	1.14 %	1.29 %	1.46 %	1.53 %	1.43 %
4	Ct	-	-	-	26.2	26.1	26.1	26.2
	CV (%)	N/A	N/A	N/A	1.27 %	1.45 %	1.36 %	1.36 %
5	Ct	26.9	26.8	26.7	-	-	-	-
	CV (%)	0.89 %	0.53 %	0.70 %	N/A	N/A	N/A	N/A

Tab. 22: Results of the precision of the RIDA®UNITY EHEC/EPEC test for *eae* from stool samples (RIDA®UNITY system).

Ct Mean value / CV		*Intra*-assay			*Inter*-assay			*Inter*-lot
		Kit lot 1	Kit lot 2	Kit lot 3	Kit lot 1	Kit lot 2	Kit lot 3	Kit lots 1-3
1	Ct	26.2	26.3	26.0	26.4	26.5	26.5	26.5
	CV (%)	0.93 %	0.86 %	0.85 %	1.92 %	1.95 %	2.03 %	1.97 %
2	Ct	27.7	27.9	27.7	27.9	28.0	28.0	27.9
	CV (%)	0.86 %	0.92 %	0.77 %	1.85 %	1.76 %	1.75 %	1.78 %
3	Ct	27.0	27.0	27.2	27.8	27.9	27.9	27.9
	CV (%)	1.05 %	0.86 %	0.99 %	2.03 %	1.84 %	1.80 %	1.89 %
4	Ct	-	-	-	28.9	29.1	29.1	29.0
	CV (%)	N/A	N/A	N/A	1.81 %	1.78 %	1.81 %	1.80 %
5	Ct	29.1	29.4	29.4	-	-	-	-
	CV (%)	1.24 %	0.78 %	1.18 %	N/A	N/A	N/A	N/A

Tab. 23: Results of the precision of the RIDA®UNITY EHEC/EPEC test for *eae* from stool samples (CFX96™ Dx).

Ct Mean value / CV		*Intra*-assay			*Inter*-assay			*Inter*-lot
		Kit lot 1	Kit lot 2	Kit lot 3	Kit lot 1	Kit lot 2	Kit lot 3	Kit lots 1-3
1	Ct	26.5	26.6	26.5	26.3	26.4	26.4	26.4
	CV (%)	0.54 %	0.69 %	0.58 %	1.76 %	1.86 %	1.88 %	1.84 %
2	Ct	27.3	27.5	27.5	27.6	27.7	27.8	27.7
	CV (%)	0.68 %	0.68 %	0.65 %	1.49 %	1.70 %	1.51 %	1.59 %
3	Ct	27.3	27.4	27.4	27.6	27.8	27.8	27.7
	CV (%)	0.88 %	0.74 %	0.79 %	1.66 %	1.78 %	1.85 %	1.77 %
4	Ct	-	-	-	28.5	28.7	28.6	28.6
	CV (%)	N/A	N/A	N/A	1.32 %	1.58 %	1.48 %	1.47 %
5	Ct	29.1	29.3	29.2	-	-	-	-
	CV (%)	0.73 %	0.55 %	0.68 %	N/A	N/A	N/A	N/A

13.2.4 Analytical reactivity

The reactivity of the RIDA®UNITY EHEC/EPEC multiplex real-time PCR test was tested on a defined panel of *E. coli* and *Shigella* strains (see Tab. 24).

Tab. 24: Analytical reactivity testing

Strain	Result*		
	stx1/2	***ipaH***	***eae***
E. coli (stx1c, stx2b)	+	-	-
E. coli (stx1a, stx2c, eae)	+	-	+
E. coli (stx1d)	+	-	
E. coli (stx2a, eae)	+	-	+
E. coli (*stx2d*)	+	-	-
E. coli (*stx2e*)	+	-	-
E. coli (*stx2f, eae*)	+	-	+
E. coli (*stx2g*)	+	-	-
E. coli O127:H6 (*eae alpha*)	-	-	+
E. coli O157:H7 (*eae gamma*)	-	-	+
Shigella boydii (*ipaH*)	-	+	-
Shigella dysenteriae (*ipaH*)	-	+	-
Shigella flexneri (*ipaH*)	-	+	-
Shigella sonnei (*ipaH*)	-	+	-

*+ = positive (at least 2 of 3 replicates positive)
- = negative

14. Version history

Version number	Section and designation
2022-06-14	Release version

15. Explanation of symbols

General symbols

IVD	For *in vitro* diagnostic use
	Follow instructions for use
LOT	Batch number
	Use before
	Storage temperature
REF	Item number
	Number of tests
	Date of manufacture
	Manufacturer

Test-specific symbols

Reaction Mix	Reaction Mix
Enzyme Mix	Enzyme Mix
Negative Control	Negative control
Positive Control	Positive control

16. References

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