H. pylori ClariRes Assay

Instructions for Use

For use with the
LightCycler® 1.1/1.2/1.5/2.0 instrument

CE

IVD **For *in vitro* diagnostic use**

REF **RTGM100**

Σ **50 reactions**

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Explanation of symbols

LOT	Batch code		Use by date
REF	Catalogue number		Manufacturer
Σ	Contains sufficient for <n> tests		Temperature limit (Store at)
CE	This product fulfils the requirements of the European Directive 98/79 EC for *in vitro* diagnostic medical devices	IVD	In vitro diagnostic medical device
	Consult instructions for use	UDI	Unique device identifier
	Keep away from sunlight	QTY	Contents

1. Intended purpose

H. pylori ClariRes Assay is a non-automatic, qualitative *in vitro* diagnostic test, based on real-time PCR, for the detection of *H. pylori* in combination with clarithromycin susceptibility testing. The test allows detection of a *H. pylori* infection with simultaneous detection of the wild-type (susceptible to clarithromycin) as well as of three most common point mutations (A2142C, A2142G, and A2143G) in the 23S rRNA gene of *H. pylori* responsible for clarithromycin resistance. Mixed infections with clarithromycin resistant and susceptible stains can be interpreted up to a ratio of 1:10. The *H. pylori* ClariRes Assay allows the examination of DNA extracted from fresh or frozen stool or gastric tissue biopsies without pre-enrichment. This test is suitable for patients of all ages with suspected infection with *Helicobacter pylori*.

H. pylori ClariRes Assay is compatible with the real-time PCR instruments LightCycler® 1.1/1.2/1.5 or 2.0 instrument (Roche).
The use of this kit is limited to qualified personnel instructed in the procedures of real-time PCR and *in vitro* diagnostics.

2. Product description

A non-invasive *H. pylori* detection in combination with clarithromycin susceptibility testing from stool samples provides a convenient monitoring of a *H. pylori* infection for the patient.

The *H. pylori* ClariRes Assay is a test for the simultaneous detection of *H. pylori* and of the three major point mutations in the 23S rRNA gene associated with clarithromycin resistance in *H. pylori* isolates. The three point mutations and the wild-type-sequence of the 23S rRNA gene of *H. pylori* are amplified in one PCR reaction and are subsequently differentiated by melting curve analyses in a LightCycler® 1.1/1.2/1.5 or 2.0 instrument (Roche) (see 11. interpretation of the LightCycler® PCR-data). The presence of mixed infections results in two melting temperatures and can be interpreted up to a ratio of 1:10.

The *H. pylori* ClariRes Assay contains primers and a fluorescence-labeled probe for the detection of the 23S rRNA gene of *H. pylori*. Furthermore, it contains an internal positive control to verify the absence of PCR-inhibitors for reliable negative results, two positive control samples for *H. pylori* (wild-type and one mutant), a negative control and an operating manual.

3. Pathogen information

Helicobacter pylori colonizes the human stomach and is associated with gastritis and gastroduodenal ulcer and gastric cancer. At present, several diagnostic tests for *H. pylori* detection are available. Invasive methods requiring gastric endoscopy include rapid urease testing, culture, histology and molecular diagnostics. Non-invasive approaches include faecal antigen detection, serologic testing, urea breath testing and molecular diagnostics. Infection with *H. pylori* can be effectively treated with proton pump inhibitors and various antibiotics. Clarithromycin is an integral part of first line therapies to treat *H. pylori* infection. Since clarithromycin is a widely used antimicrobial drug, the prevalence of clarithromycin resistant *H. pylori* strains is increasing continuously. Resistance to clarithromycin is mainly due to three major point mutations at two positions (A2142C, A2142G, and A2143G) within the peptidyltransferase region of the 23 S rRNA of *H. pylori*.

References:
Claudia Schabereiter-Gurtner, Alexander M. Hirschl, Brigitte Dragosics, Peter Hufnagl, Sonja Puz, Zsuzsanna Kovách, Manfred Rotter and Athanasios Makristathis. 2004. Novel real-time PCR assay for detection of *Helicobacter pylori* infection and simultaneous clarithromycin susceptibility testing in stool and biopsy specimens. J. Clin. Microbiol. 42:4512-8.

4. Principle of real-time PCR

A specific DNA sequence of the pathogen´s genome is amplified and the generated PCR-product is detected by an oligonucleotide-probe labelled with a fluorescent dye.
Using the *H. pylori* ClariRes Assay, PCR amplification is followed by melting curve analysis on the LightCycler® instrument. In case of a wild-type target sequence the probe dissociates at its melting temperature (Tm). In case of a mutation in the target sequence, it dissociates earlier (at a lower temperature). This technology allows the sequence specific detection of the 23S rRNA gene of *H. pylori* and the determination of the three major point mutations in the PCR-product.

5. Contents of the kit, stability and storage

Labelling	Content	Amount
H. pylori ClariRes Assay Mix (green cap)	Primers and probe for *H. pylori* and IC detection	1 x 25 µl
H. pylori ClariRes Internal Positive Control (orange cap)	Internal positive control (please dilute freshly 1:100 before use)	1 x 25 µl
Nuclease-Free Water (blue cap)	Water as negative control	1 x 1000 µl
H. pylori Wild-type Positive Control (red cap)	Control-DNA of a *H. pylori* 23S rRNA wild-type (clarithromycin susceptible)	1 x 25 µl
H. pylori Resistant Positive Control (red cap)	Control-DNA of a *H. pylori* 23S rRNA mutant (A2142G) (clarithromycin resistant)	1 x 25 µl

The components of the *H. pylori* ClariRes Assay should be stored at -25 to -15 °C and are stable until the expiry date stated on the label. Repeated thawing and freezing should be avoided.

Quality Control Release Testing
In accordance with ingenetix's ISO 13485-certified Quality Management System, each lot is tested against predetermined specifications to ensure consistent product quality.

6. Additionally required materials and devices

- Reagents and devices for DNA-extraction appropriate for the biological sample material
- Disposable powder-free gloves
- Pipettes (adjustable) and sterile pipette tips with filters
- Vortex Mixer
- Desktop centrifuge with rotor for 2 ml reaction tubes
- LightCycler® FastStart DNA Master SYBR Green I (Roche Diagnostics order no. 12239264001; kit for 480 reactions of 20 µl final reaction volume, *or* order no. 03003230001; kit for 96 reactions of 20 µl final reaction volume)
- LightCycler® Capillaries (20 µl) (Roche Diagnostics order no. 04929292001: 1 pack containing 5 boxes, each with 96 capillaries and stoppers)
- LightCycler® Cooling Block
- LightCycler® Capping Tool
- LightCycler® 1.1/1.2/1.5 oder 2.0 Instrument
- LightCycler® Carousel Centrifuge

7. Precautions and safety information

- For *in vitro* diagnostic use. The use of this assay is limited to persons instructed in the procedures of real-time PCR and *in vitro* diagnostics.
- Transportation of clinical specimens must comply with local regulations for the transport of etiologic agents.
- Improper collection, transport or storage of specimens may hinder the ability of the assay to detect the target sequences.
- Clean benches and devices periodically.
- Use sterile filter pipette tips.
- Specimens should be handled as if infectious in accordance with safe laboratory procedures. Wear protective disposable powder-free gloves when handling assay reagents and specimens.
- Use separated working areas for specimen preparation, reagent preparation and amplification. Supplies and equipment must be dedicated to each of these separate working areas and ensure workflow in the laboratory from pre- to post-PCR.
- Be careful when handling samples and positive control to avoid cross contamination. Change gloves after handling of samples or positive control. Store positive or potentially positive material separately from reagents
- Prevent contamination of work equipment and reagents with DNA/RNA, nuclease or amplification products by good laboratory practice.
- Quality of DNA has a profound impact on the test performance. Ensure that the used DNA extraction system is compatible with real-time PCR technology.
- Always include a negative control per PCR-run (nuclease-free water instead of sample).
- For a valid interpretation of results, a negative control should be included during DNA-extraction (e.g. extraction of water instead of sample material), in order to exclude false-positive results due to contamination with DNA of pathogen during extraction.
- Please note the expiry date of the assay.
- Do not interchange or mix reagents from assays with different lot numbers.
- Repeated thawing and freezing of assay components should be avoided. Protect assay components from light.
- Use established laboratory practices according to your local safety regulations for discarding specimens, reagents and waste.

8. Limitations

- Optimal performance of this test requires appropriate specimen collection, transport and storage, as well as an appropriate DNA extraction procedure.
- DNA extraction and *H. pylori* detection have been validated for stool samples and biopsies with this assay.
- A negative test result does not exclude the possibility of *H. pylori* infection, because test results may be affected by improper specimen collection, technical error, specimen mix-up or pathogen quantities below the assay sensitivity. The presence of PCR inhibitors may lead to invalid results.
- For this assay highly specific primers and probes have been selected. However, false-negative or less sensitive results might be obtained due to sequence heterogeneity within the target region of not yet described clinical subtypes.
- For clarithromycin resistance testing, the three major point mutations at two positions (A2142C, A2142G, and A2143G) within the 23S rRNA most commonly associated with clarithromycin resistance in *H. pylori* isolates are detected.
- Mixed infections with clarithromycin resistant and susceptible strains result in multiple melting curves and can be interpreted up to a 1:10 quantitative ratio. The mutant (resistant) cannot be detected in the presence of approximately 10-fold higher concentration of the wild type (sensitive) compared to the mutant (resistant).
- Stool samples must be fresh or frozen.
- Results should be interpreted in consideration of clinical and laboratory findings.

9. Preparation of samples

Extract samples with a DNA extraction system compatible with real-time PCR technology and appropriate for the biological sample material (e.g. QIAamp Fast DNA Stool Mini Kit for stool samples and QIAamp DNA Mini Kit, Qiagen for biopsies).
When using extraction methods not recommended by ingenetix, an evaluation of the extraction method must be performed.
Kits for DNA-extraction are provided by different manufacturers. Please use the sample volume as recommended by the respective manufacturer and follow the respective manual. It has to be assured that all reagents are free of *H. pylori* DNA.
Analysing of too small stool volumes can have an impact on the sensitivity of the test. If possible, 500 mg stool should be examined. If too much biopsy material is used, the test may be inhibited.

Sample collection and storage:

- Stool samples: It is recommended to process samples immediately after collection. Store samples at 2-8°C for no longer than 48 hours or freeze at -20/-80°C in microcentrifuge tubes.
- Gastric tissue biopsies can be stored in microcentrifuge tubes containing sterile physiological saline (approx. 100 µl) at 2-8°C for up to 48 h. If it is not guaranteed that the sample will be processed within 48 h, the sample shall be immediately frozen dry without additives at -20/-80°C.

Purified DNA should be stored at -15°C to -25°C.

10. Preparation of real-time PCR

Please make sure that at least one negative control (water, blue cap), as well as one positive control (red cap) and one extraction negative control (optional, recommended) are included per PCR run.
Ingenetix highly recommends performing PCR analyses of stool samples in duplicates, which increases the probability of detection of *H. pylori* in stool samples.
Please calculate the number of required PCR reactions including positive and negative controls. Calculate a surplus (5-10%) for the loss of volume during pipetting.

Quality control for PCR inhibition
The DNA IPC system (internal DNA positive control system) consists of a separate target (*H. pylori* ClariRes Internal Positive Control), which must be freshly diluted and added to the master mix. The DNA IPC identifies possible PCR inhibition and confirms the integrity of the kit reagents.

Dilution of the internal positive control:
Prepare a fresh 1:100 dilution of the internal positive control (orange cap) (e.g. 1 µl IC + 99 µl water, mix well).

Please use the following pipetting scheme (mix the master mix well):

		Per sample
Preparation of master mix	H_2O	12.1 µl
	$MgCl_2$ stock solution [25 mM]	2.4 µl
	LC-SYBR*	2.0 µl
	H. pylori ClariRes Assay Mix	0.5 µl
	H. pylori ClariRes Internal Positive Control; freshly diluted 1:100	1.0 µl
	Total volume	**18.0 µl**
Preparation of PCR assay	Master mix	18.0 µl
	Sample	2.0 µl
	Total volume	**20.0 µl**

*LC-SYBR (LC™-FastStart DNA Master SYBR® Green I. For preparation of the LC-SYBR master mix, please follow instructions of the manufacturer.

Before use, all reagents need to be thawed completely and gently mixed.

Positive/Negative Controls: As positive control please use 1 µl of the *H. pylori* ClariRes Wild-type Positive Control or 1 µl of the *H. pylori* ClariRes Resistant-type Positive Control (A2142G) + 1 µl H_2O. As negative control please use 2 µl of the negative control (water, blue cap).

Pipette 18 µl of the master mix into the reservoir of each capillary. Then add 2 µl of the eluted sample DNA. Correspondingly, 1 µl of the *H. pylori* ClariRes Wild-type Positive Control or 1 µl of the *H. pylori* ClariRes Resistant Positive Control + 1 µl H_2O or 2 µl of the negative control have to be used.
Close the capillaries. To transfer the mixture from the reservoir into the capillary, centrifuge the capillaries.

Programming of the temperature profile in the LightCycler® instrument:
Please find further information on programming the LightCycler® instruments in the respective LightCycler® Operator's Manuals. For programming the correct temperature profile for the *H. pylori* ClariRes Assay, open the LightCycler® software, activate in the menu strip the option *NEW* and choose *LightCycler® Experiment*.
Please create a temperature profile according to the following four steps i) Initial activation of the Hot Start-Enzyme ii) Amplification of the DNA iii) Melting curve analysis and iv) Cooling.

Denaturing 1 Cycle

Target Temp.	Incubation time	Temp. Trans. Rate	Sec.Target Temp.	Step Size	Step Delay	Acquisition Mode
95	00:10:00	20.00	0	0.0	0	NONE

Amplification 70 Cycles

Target Temp.	Incubation time	Temp. Trans. Rate	Sec.Target Temp.	Step Size	Step Delay	Acquisition Mode
95	00:00:05	20.00	0	0.0	0	NONE
65	00:00:10	20.00	0	0.0	0	SINGLE
72	00:00:06	20.00	0	0.0	0	NONE

Melting 1 Cycle

Target Temp.	Incubation time	Temp. Trans. Rate	Sec.Target Temp.	Step Size	Step Delay	Acquisition Mode
95	00:00:00	20.00	0	0.0	0	NONE
37	00:01:00	20.00	0	0.0	0	NONE
95	00:00:00	0.2	0	0.0	0	CONT

Cooling 1 Cycle

Target Temp.	Incubation time	Temp. Trans. Rate	Sec.Target Temp.	Step Size	Step Delay	Acquisition Mode
40	00:00:10	20.00	0	0.0	0	NONE

11. Interpretation of LightCycler® PCR-data

Examples for interpretation of melting curves are shown in Figures 1-6.

For analysis of PCR data collected with the LightCycler® instrument please use the LightCycler® software. Please consider the instructions given in the LightCycler® Instrument Operator's Manual.

Assessment of clinical specimen test results should be performed after the positive and negative controls and the IPC have been determined to be valid. If results of controls are not valid, no interpretation is possible.

Table 1: Interpretation of controls

	Melting temperature Tm	**Interpretation**
Negative control of PCR (NTC)	47°C	Valid
H. pylori Wild-type Positive Control	63°C (+/-1°C)	Valid
H. pylori Resistant Positive Control	54°C (+/-1°C)	Valid

For the analysis of PCR data please proceed as follows:

- **IMPORTANT:** When using the *H. pylori* ClariRes Assays please do not use a Colour Compensation. Activate the function *Analysis* in the menu strip and select the option *Tm Calling*.
- For analysing the PCR-results please choose fluorescence display option 670 nm (LightCycler® 2.0) or F3 (LightCycler® 1.1/1.2/1.5). Please always check the presence of melting curves also manually. For this, click with the left mouse button on the respective sample positions.

Once analysis is completed, the following results are possible:

Table 1: Interpretation of clinical samples

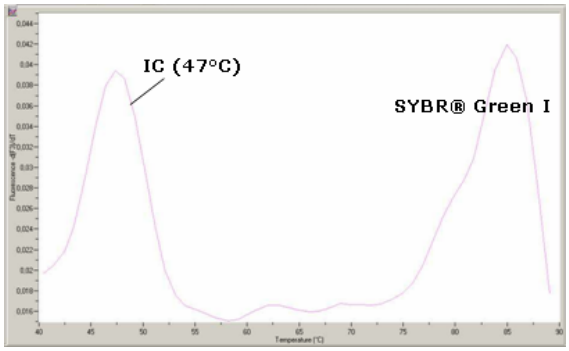
Melting temperature Tm	**Detection of**	**Interpretation**
47°C	Internal positive control (IC)	No inhibition of PCR-reaction
63°C (+/-1°C)	Wild-type	Sample contains *H. pylori* (Clarithromycin susceptible strain)
58°C (+/-1°C)	Mutant A2142C	Sample contains *H. pylori* (Clarithromycin resistant strain)
54°C (+/-1°C)	Mutant A2142G or A2143G	Sample contains *H. pylori* (Clarithromycin resistant strain)
63°C (+/-1°C) + 58°C (+/-1°C)[1]	Wild-type + Mutant A2142C	Sample contains *H. pylori* (Clarithromycin susceptible + resistant strain)
63°C (+/-1°C) + 54°C (+/-1°C)	Wild-type + Mutant A2142G or A2143G	Sample contains *H. pylori* (Clarithromycin susceptible + resistant strain)
54°C (+/-1°C) + 58°C (+/-1°C)	Mutant A2142C + Mutant A2142G or A2143G	Sample contains *H. pylori* (2 different clarithromycin resistant strains)
75°C - 85°C	SYBR® Green I signal due to specific or unspecific PCR-products or primer-dimers	No interpretation of melting curves >70°C
Tm >70°C or no melting curve	-	**No diagnostic statement can be made** Information regarding error sources and their solution can be found in 12. Troubleshooting.

In case of detection of *H. pylori*, the detection of the IC at 47°C is not essential, since high concentrations of *H. pylori* DNA can lead to a reduced or absent fluorescence signal of the internal positive control (competition of PCR).

Always perform analyses of stool samples in duplicates. In cases where only one out of two reactions is positive for *H. pylori,* the sample should be considered as positive.

In case of invalid data, analysis has to be repeated with the remaining or newly extracted DNA sample. Information about possible sources of error and their solution can be found in 12. Troubleshooting.

Examples of melting curves of *H. pylori* clarithromycin resistant (mutant) or clarithromycin susceptible (wild-type) strains and of the internal positive control (IC) in clinical material:



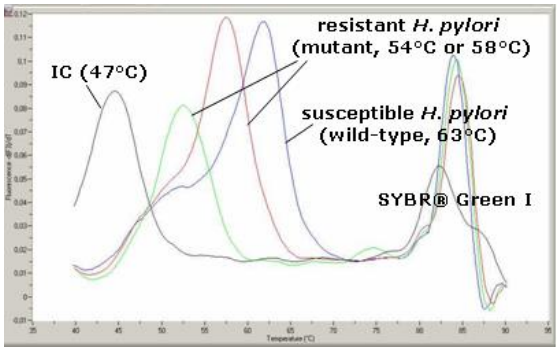
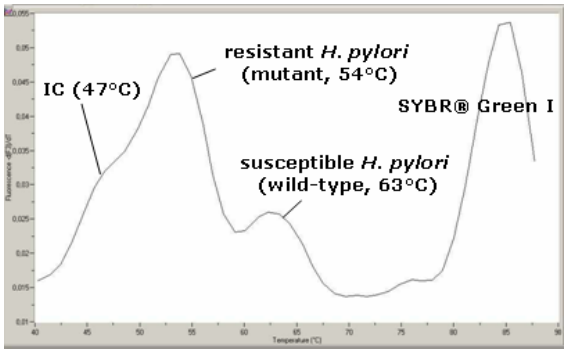


Figure 2



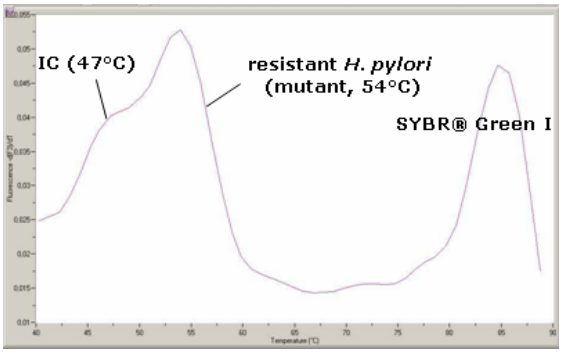


Figure 4

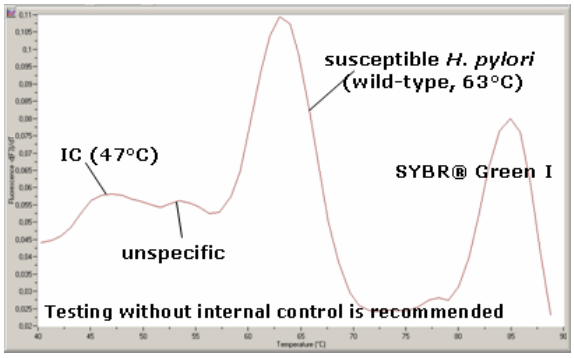


Figure 5

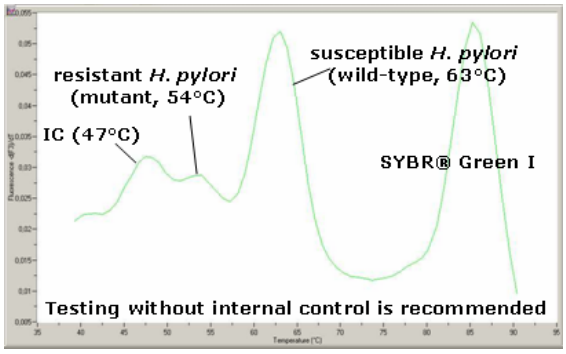


Figure 6

12. Troubleshooting

12.1. No *H. pylori* specific signal with positive controls

- Incorrect programming of the temperature profile of the LightCycler® instrument.
  → Compare the temperature profile with the protocol (see programming of the LightCycler® instrument).
- Incorrect configuration of the PCR reaction.
  → Check your work steps by means of the pipetting scheme (see preparation of real-time PCR) and repeat the PCR, if necessary.

12.2. No signal with IC and no *H. pylori* specific signal with clinical sample

- The PCR reaction was inhibited. No diagnostic statement can be made.
  → Make sure that you use a recommended method for DNA isolation and stick closely to the manufacturer's instructions.
  → If no operating mistakes during extractions can be retraced, it is recommended to repeat the PCR with lower amounts of DNA-eluate (1/5 or 1/10 of sample volume + the adequate amount of H_2O).
- Incorrect PCR conditions.
  → Check the PCR conditions and repeat the PCR, if necessary.

12.3. Questionable, weak signal of low fluorescence at 54°C in clinical sample (Fig. 5 and 6)

- A low amount of DNA of a *H. pylori* mutant A2142G or A2143G might be suppressed by the IC.
  → Repeat PCR without internal positive control.

12.4. Signal with negative control

- A contamination occurred during preparation of the PCR.
  → Repeat the PCR with new reagents in replicates.
  → Strictly pipette the positive controls at last.
  → Make sure that work space and instruments are decontaminated at regular intervals.

12.5. Signal with negative control of DNA-extraction (optional)

A contamination occurred during extraction.
→ Repeat the extraction and PCR using new reagents.
→ Make sure that work space and instruments are decontaminated at regular intervals.

13. Specification

13.1. Analytical sensitivity – Limit of detection (LoD)

The analytical sensitivity (defined as the smallest amount of DNA that can be detected) is 0.5 fg *H. pylori* DNA/PCR (equal to 0.3 CFU).
The LoD 95% (defined as the concentration where 95% of 20 PCR repeats were positive) is 5 fg *H. pylori* DNA/PCR (equal to 3 CFU). This means, that 5 fg/PCR-reaction can be detected with a probability of 95%.

13.2. Analytical specificity

The specificity is ensured by the selection of specific primers and probes. The primers and probes were checked for possible homologies to currently in gene banks published sequences by sequence comparison analyses. This also ensured the detection of all so far known *H. pylori* strains.
The test reveals 100% cross-reactivity with *H. fennelliae, H. heilmanii, H. acinonychis* and *H. cetorum. Helicobacter fennelliae* can cause gastroenteritis, proctitis and bacteremia, while *H. heilmanii* can cause chronic gastritis in humans. *Helicobacter acinonychis* and *H. cetorum* have no clinical relevance for humans.
The poor cross-reactivity with other non-pylori *Helicobacter* species is assumed to have no diagnostic relevance.
The *H. pylori* ClariRes Assay was tested with DNA of *H. hepaticus* (ATCC 51448), *H. bilis* (ATCC 51630), *H. pullorum* (clinical isolate), *H. rappini* (ATCC 43879) and *Campylobacter jejuni* (clinical isolate). The test revealed cross-reactivity with *H. hepaticus, H. bilis* and *H. rappini*. The analytical sensitivity for these species was 40 fg (equal to 20 CFU) and the amplification was delayed for 10 cycles. When 1 g stool was spiked with 3,000 pg DNA (1.6×10^6 CFU) of these species, no amplification was observed.
Due to the poor amplification of the above species, it is assumed that their cross-reactivity has no diagnostic relevance.
A clinical evaluation was performed in the study of Schabereiter-Gurtner et al., 2007. In this study 92 gastic biopsy samples and 92 stool samples of the same patients were analyzed. Regarding the detection of the clarythromycin resistance, the specificity is 100% both for stool and biopsy samples, the sensitivity is 73% for stool and 82% for biopsies. Regarding the detection of *H. pylori*, the test has a specificity of 98% (both for stool and biopsy samples) and a sensitivity of 98% for stool and 100% for biopsies.

14. Revision history

Revision	Date	Description
V2.5e	18 November 2021	Ad 1. More detailed information about intended use. Ad 5. Information about quality control release tests Ad 7. Additional information on safety for sample transport and storage Ad 8. Additional information on limitations of the method regarding detection of mutations and mixed infections Ad 9. Information on sample preparation added Ad 11. Table 1 added Ad 14 Added
V2.6en	01 May 2023	**Change of the company address** **Explanation of symbols** Update
V2.7en	10. July 2023	**Contents of the kit, stability and storage** Update

Note:
Any serious incident that has occurred in relation to the device shall be reported to the manufacturer and the competent authority of the Member State in which the user and/or the patient is established.

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