IVD For *in Vitro* Diagnostic Use

CE

Helicobacter pylori Real-TM

Handbook

Real Time PCR kit for qualitative detection of *Helicobacter pylori*

REF B9-50FRT

REF TB9-50FRT

Σ 50

NAME

H. pylori Real-TM

INTRODUCTION

Helicobacter pylori is a Gram-negative, microaerophilic bacterium that can inhabit various areas of the stomach and duodenum. It causes a chronic low-level inflammation of the stomach lining and is strongly linked to the development of duodenal and gastric ulcers and stomach cancer. Over 80% of individuals infected with the bacterium are asymptomatic. More than 50% of the world's population harbour *H. pylori* in their upper gastrointestinal tract. Approximately 10-20% of those colonized by *H. pylori* will ultimately develop gastric and duodenal ulcers. *H. pylori* infection is also associated with a 1-2% lifetime risk of stomach cancer and a less than 1% risk of gastric MALT lymphoma.

INTENDED USE

Kit **H. pylori Real-TM** is a test for the qualitative detection of *Helicobacter pylori* in the biopsy sample of stomach mucous membrane, sputum, feces and other biological materials.

PRINCIPLE OF ASSAY

Kit **H.pylori Real-TM** is based on two major processes: isolation of DNA from specimens and Real Time amplification. *H.pylori* DNA is extracted from the specimens, amplified using Real-Time amplification and detected fluorescent reporter dye probes specific for *H.pylori* DNA and Internal Control. Internal Control (IC) serves as an amplification control for each individually processed specimen and to identify possible reaction inhibition. IC is detected in a channel other than the *H.pylori* .

MATERIALS PROVIDED

Module No.1: Real Time PCR kit (B9-50FRT)

Part N° 2 – **"H.pylori TM":** Real Time amplification kit

- **PCR-mix-1-FRT**, 0,6 ml;
- **PCR-Buffer-FRT**, 0,3 ml;
- **TaqF Polymerase**, 0,03 ml;
- **H.pylori C+**, 0,1 ml;
- **IC C+**, 0,1 ml;
- **Negative Control C-***, 1,2 ml;
- **Internal Control IC****, 1,0 ml;
- **DNA-buffer**, 0,5 ml;

Contains reagents for 55 tests.

Module No.2: Complete Real Time PCR test with DNA purification kit(TB9-50FRT)

Part N° 1 – **"DNA-sorb-B":** Sample preparation kit

- **Lysis Solution**, 15 ml;
- **Washing Solution 1**, 15 ml;
- **Washing Solution 2**, 50 ml;
- **Sorbent**, 1,25 ml;
- **DNA-eluent**, 5 ml.

Contains reagents for 50 tests.

Part N° 2 – **"H.pylori TM":** Real Time amplification kit

- **PCR-mix-1-FRT**, 0,6 ml;
- **PCR-Buffer-FRT**, 0,3 ml;
- **TaqF Polymerase**, 0,03 ml;
- **H.pylori C+**, 0,1 ml;
- **IC C+**, 0,1 ml;
- **Negative Control C-***, 1,2 ml;
- **Internal Control IC****, 1,0 ml;
- **DNA-buffer**, 0,5 ml;

Contains reagents for 55 tests.

**must be used in the isolation procedure as Negative Control of Extraction.*
***add 10 µl of Internal Control during the DNA isolation directly to the sample/lysis mixture (see DNA-Sorb-B REF K-1-1/B protocol).*

MATERIALS REQUIRED BUT NOT PROVIDED

Zone 1: sample preparation:

- DNA extraction kit (module No. 1)
- Biological cabinet
- Vortex
- 65°C ± 2°C dry heat block
- Desktop microcentrifuge for “eppendorf” type tubes (RCF max. 16,000 x g)
- Tube racks
- Microcentrifuge tubes, 1,5 - 2,0 ml
- Pipettes with sterile, RNase-free filters tips
- Biohazard waste container
- Disposable gloves, powderless
- Refrigerator, Freezer

Zone 2: Real Time amplification:

- Disposable gloves, powderless
- Biohazard waste container
- Refrigerator, Freezer
- Real Time Thermal cycler
- Workstation
- Pipettes (adjustable)
- Sterile pipette tips with filters

STORAGE INSTRUCTIONS

H.pylori Real-TM must be stored at 2-8°C. **TaqF Polymerase, PCR-mix-1-FRT, PCR-Buffer-FRT** must be stored at -20°C. **DNA-sorb-B** kit must be stored at 2-8°C. The kits can be shipped at 2-8°C but should be stored at 2-8°C and -20°C immediately on receipt.

STABILITY

H.pylori Real-TM is stable up to the expiration date indicated on the kit label. The product will maintain performance through the control date printed on the label. Exposure to light, heat or humidity may affect the shelf life of some of the kit components and should be avoided. Repeated thawing and freezing of these reagents should be avoided, as this may reduce the sensitivity.

QUALITY CONTROL

In accordance with Sacace’s ISO 13485-Certified Quality Management System, each lot is tested against predetermined specifications to ensure consistent product quality.

WARNINGS AND PRECAUTIONS

IVD

***In Vitro* Diagnostic Medical Device**

For *In Vitro* Diagnostic Use Only

The user should always pay attention to the following:

- ⚠ Lysis Solution contains guanidine thiocyanate*. Guanidine thiocyanate is harmful if inhaled, or comes into contact with skin or if swallowed. Contact with acid releases toxic gas. (Xn; R: 20/21/22-36/37/38; S: 36/37/39).
- Use sterile pipette tips with aerosol barriers and use new tip for every procedure.
- Store extracted positive material (samples, controls and amplicons) away from all other reagents and add it to the reaction mix in a separate area.
- Thaw all components thoroughly at room temperature before starting an assay.
- When thawed, mix the components and centrifuge briefly.
- Use disposable gloves, laboratory coats and eye protection when handling specimens and reagents. Thoroughly wash hands afterwards.
- Do not eat, drink, smoke, apply cosmetics, or handle contact lenses in laboratory work areas.
- Do not use a kit after its expiration date.
- Dispose of all specimens and unused reagents in accordance with local authorities' regulations.
- Specimens should be considered potentially infectious and handled in a biological cabinet in accordance with appropriate biosafety practices.
- Clean and disinfect all sample or reagent spills using a disinfectant such as 0.5% sodium hypochlorite, or other suitable disinfectant.
- Avoid sample or reagent contact with the skin, eyes, and mucous membranes. If skin, eyes, or mucous membranes come into contact, rinse immediately with water and seek medical advice immediately.
- Material Safety Data Sheets (MSDS) are available on request.
- Use of this product should be limited to personnel trained in the techniques of DNA amplification.
- The laboratory process must be one-directional, it should begin in the Extraction Area and then move to the Amplification and Detection Areas. Do not return samples, equipment and reagents to the area in which the previous step was performed.

Some components of this kit contain sodium azide as a preservative. Do not use metal tubing for reagent transfer.

**** Only for Module No.2***

PRODUCT USE LIMITATIONS

All reagents may exclusively be used in in vitro diagnostics. Use of this product should be limited to personnel trained in the techniques of DNA amplification (UNI EN ISO 18113-2:2012). Strict compliance with the user manual is required for optimal PCR results. Attention should be paid to expiration dates printed on the box and labels of all components. Do not use a kit after its expiration date.

SAMPLE COLLECTION, STORAGE AND TRANSPORT

H.pylori Real-TM can analyze DNA extracted from:

- *tissue* (≈1,0 gr) homogenized with mechanical homogenizer or scalpel, glass sticks, teflon pestles and dissolved in 1,0 ml of saline water or PBS sterile. Vortex vigorously and incubate 30 min at room temperature. Transfer the supernatant into a new 1,5 ml tube
- *gastric juice- 0,5-1,0 ml;*
- *dental bacterial plaque* dissolved in 0,1 ml of sterile saline solution*;*
- *sputum:* add 1 volume of sputum to 1 volumes of β-mercaptoethanol 0,1M (reagent not provided. β-mercaptoethanol is a toxic reagent, please follow the supplier MSDS). Vortex well and incubate at room temperature for 30 min, mix batchly. Transfer 1,0 ml of clinical material to a sterile polypropylene tube (1,5 ml) and centrifuge at 10000g/min for 10 min. Discard the supernatant and leave about 100 µl of solution for DNA extraction;
- *feces:*
  - Prepare required quantity of 1,5 ml polypropylene tubes with 0,8 ml of Saline Solution. Add to each tube 0,1 g of feces. Vortex to get the homogeneous suspension and centrifuge for 5 min to 7000-12000g and using a micropipette with a plugged aerosol barrier tip transfer in a new tube 0,1 ml of the bacterial fraction (white-yellowish line between the sediment and the supernatant). Add 0,8 ml of sterile Saline Solution.
  - Vortex vigorously and centrifuge for 5 min at 7000-12000 g. Remove and discard the supernatant.
  - Resuspend the pellet in 0,3 ml of Saline Solution

It is recommended to process samples immediately after collection. Store samples at 2–8 °C for no longer than 24 hours, or freeze at –20/80°C. Transportation of clinical specimens must comply with country, federal, state and local regulations for the transport of etiologic agents.

DNA ISOLATION

Any commercial RNA/DNA isolation kit, if IVD-CE validated for the specimen types indicated herein at the “SAMPLE COLLECTION, STORAGE AND TRANSPORT” paragraph, could be used.

Sacace Biotechnologies recommends to use the following kits:

- ⇨ **DNA-Sorb-B** (Sacace, REF K-1-1/B);
- ⇨ **DNA/RNA Prep** (Sacace, REF K-2-9);
- ⇨ **SaMag Bacterial DNA Extraction kit** (Sacace, REF SM006) for sputum, feces.

Please carry out DNA extraction according to the manufacture's instruction. Add 10 µl of Internal Control during DNA isolation procedure directly to the sample/lysis mixture.

SPECIMEN AND REAGENT PREPARATION*

1. **Lysis Solution** and **Washing Solution** (in case of their storage at +2-8°C) should be warmed up to 60–65°C until disappearance of ice crystals. Prepare required quantity of 1.5 ml polypropylene tubes including one tube for **Negative Control of Extraction**.
2. Add to each tube **300 µl** of **Lysis Solution** and **10 µl** of **Internal Control.**
3. Add **100 µl** of **Samples** to the appropriate tube.
4. Prepare Controls as follows:
   - add **100 µl** of **C–** (**Negative Control** provided with the amplification kit) to the tube labeled C*neg*.
5. Vortex the tubes and incubate for 5 min at 65°C. Centrifuge for 7-10 sec. If the sample is not completely dissolved it is recommended to re-centrifuge the tube for 5 min at a maximum speed (12000-16000 g.) and transfer the supernatant into a new tube for DNA extraction.
6. Vortex vigorously **Sorbent** and add **25 µl** to each tube.
7. Vortex for 5-7 sec and incubate all tubes for 3 min at room temperature. Repeat this step.
8. Centrifuge all tubes for 30 sec at 8000g and using a micropipette with a plugged aerosol barrier tip, carefully remove and discard supernatant from each tube without disturbing the pellet.
9. Add **300 µl** of **Washing Solution 1** to each tube. Vortex vigorously and centrifuge for 30 sec at 8000g. Remove and discard supernatant from each tube.
10. Add **500 µl** of **Washing Solution 2** to each tube. Vortex vigorously and centrifuge for 30 sec at 8000g. Remove and discard supernatant from each tube.
11. Repeat step 10 and incubate all tubes with open cap for 5 min at 65°C.
12. Resuspend the pellet in **50 µl of DNA-eluent.** Incubate for 5 min at 65°C and vortex periodically.
13. Centrifuge the tubes for 1 min at maximum speed (12000-16000 g). The supernatant contains DNA ready for amplification. If amplification is not performed in the same day of extraction, the processed samples can be stored at 2-8°C for at maximum period of 5 days or frozen at –20°/-80°C.

**** Only for Module No.2***

PCR PROTOCOL:

1. Prepare required quantity of reaction tubes for samples (N) and controls (+2).
2. Prepare in the new sterile tube for each sample **10*(N+1) µl** of **PCR-mix-1-FRT**, **5,0*(N+1) µl** of **PCR-Buffer-FRT** and **0,5*(N+1) µl** of **TaqF Polymerase.** Vortex and centrifuge for 2-3 sec.

Table 1. Mix preparation x No Samples

Volume for 1 reaction	Volume of reagents		
	10.00	5.00	0.50
No reactions	**PCR-mix-1-FRT, µl**	**PCR-Buffer-FRT, µl**	**TaqF Polymerase, µl**
8	80	40	4.0
10	100	50	5.0
12	120	60	6.0
14	140	70	7.0
16	160	80	8.0
18	180	90	9.0
20	200	100	10.0
22	220	110	11.0
24	240	120	12.0
26	260	130	13.0
28	280	140	14.0
30	300	150	15.0
32	320	160	16.0

3. Add to each tube **15 µl** of **Reaction Mix** and **10 µl** of **extracted DNA** sample to appropriate tube. Mix by pipetting.
4. Prepare for each panel 3 controls:
   - add **10 µl** of **DNA-buffer** to the tube labeled Amplification Negative Control;
   - add **10 µl** of **H.pylori C+** to the tube labeled Amplification Positive Control;
   - add **10 µl** of **IC C+** to the tube labeled Amplification Internal Control;
5. Insert the tubes in the thermalcycler.

Amplification

Create a temperature profile on your Real-time instrument as follows:

	Rotor type instruments[1]				*Plate type or modular instruments*[2]			
Stage	***Temp, °C***	***Time***	***Fluorescence detection***	***Cycle repeats***	***Temp,°C***	***Time***	***Fluorescence detection***	***Cycle repeats***
Hold	95	15 min	–	1	95	15 min	–	1
Cycling	95	10 s	–	45	95	10 s	–	45
	60	25 s	Fam (Green) Joe(Yellow)		60	30 s	Fam, Joe/HEX/Cy3	
	72	10 s	–		72	10 s	–	

[1] *For example Rotor-Gene™ 3000/6000/Q (Corbett Research, Qiagen)*
[2] *For example, SaCycler-96™ (Sacace), CFX/iQ5™ (BioRad); Mx3005P™ (Agilent), ABI® 7300/7500/StepOne Real Time PCR (Applied Biosystems), SmartCycler® (Cepheid), LineGeneK® (Bioer)*

Fluorescence is detected at the 2nd step of Cycling 2 stage (60 °C) in FAM/Green and JOE/Yellow/Hex/Cy3 fluorescence channels.

H.pylori is detected on the JOE(Yellow)/HEX/Cy3 channel, *IC DNA* on the FAM (Green) channel

INSTRUMENT SETTINGS

Rotor-type instruments

Channel	Calibrate/Gain Optimisation…	Threshold	More Settings/ Outlier Removal	Slope Correct
FAM/Green	*from 5 Fl to 10 Fl*	*0.05*	*10 %*	*On*
JOE/Yellow	*from 4 Fl to 8 Fl*	*0.05*	*10 %*	*On*

Plate-type instruments

The threshold line should cross only sigmoid curves of signal accumulation of positive samples and should not cross the baseline; otherwise, the threshold level should be raised. Set the threshold at a level where fluorescence curves are linear and do not cross curves of the negative samples.

Ct boundary values

Sample	Channel	Ct
C+	FAM/Green	40
	JOE/Yellow/HEX	40
C–	FAM/Green	40
Samples	FAM/Green	40

Interpretation of amplification's results

Ct value in channel		Interpretation
FAM/Green	**JOE/Yellow/HEX**	
Ct value is definied	Pos (< 40)	*Helicobacter pylori* DNA is **detected**
Pos (< 40)	Neg (> 40) or undefined	*Helicobacter pylori* DNA is **not detected**
Neg (> 40) or undefined	Neg (> 40) or undefined	**Invalid result**

SPECIFICATIONS

Analytical specificity

Analytical specificity of the primers and probes was validated with 100 negative samples. They did not generate any signal with the specific for H.pylori primers and probes. The potential cross-reactivity of the kit **H.pylori Real-TM** was tested also against the group control listed in the following table. The results of H.pylori were interpreted on the Joe channels while IC results (recombinant structure inserted in DNA of fago λ) were interpreted on the Fam channel . The kit did not give any cross-reactivities with these pathogens.

Testing the specificity of the kit with other pathogens: *Campylobacter jejuni subsp. jejuni* 43435, *Campylobacter fetus subsp. fetus* 25936, 20 strains *Campylobacter jejuni*, 20 strains *Campylobacter coli,* 5 strains *Campylobacter lari*, 5 strains *Campylobacter hyointestinalis*, 9 strains *Campylobacter fetus*, *Salmonella enteritidis S*-6, *Salmonella choleraesuis* 370, *Salmonella typhimurium* 371, *Salmonella dublin* 373, *Salmonella typhi* C1, *Salmonella abortusovis* 372, *Salmonella gallinarum-pullorum, Shigella flexneri* 851b, *Klebsiella* K 65 SW4, *Listeria monocitogenes* 19, *Listeria monocitogenes* 52, *Proteus vulgaris* 115/98, *Pseudomonas aeruginosa*, *Staphilococcus aureus* 653, *Staphilococcus aureus* 29112, *Morganella morganii* 619 01, *Enterobacter faecalis* 356

Analytical sensitivity and reproducibility.

The analytical sensitivity of the **H.pylori Real-TM** kit was determined using the Standard DNA of the H.Pylori. This Standard was serially diluted in the DNA-buffer.

The analytical sensitivity of the kit **H.pylori Real-TM** was not less than 500 copies/ml

TROUBLESHOOTING

1. Weak or no signal of the IC for the Negative Control of extraction.
    - The PCR was inhibited.
        - ⇒ Make sure that you use a recommended DNA extraction method and follow to the manufacturer's instructions.
        - ⇒ Re-centrifuge all the tubes before pipetting of the extracted DNA for 2 min at maximum speed (12000-16000 g) and take carefully supernatant. Don't disturb the pellet, sorbent inhibit reaction.
    - The reagents storage conditions didn't comply with the instructions.
        - ⇒ Check the storage conditions
    - The PCR conditions didn't comply with the instructions.
        - ⇒ Check the PCR conditions and select for the IC detection the fluorescence channel reported in the protocol.
    - The IC was not added to the sample during the pipetting of reagents.
        - ⇒ Make attention during the DNA extraction procedure.
2. Weak or no signal of the Positive Control.
    - The PCR conditions didn't comply with the instructions.
        - ⇒ Check the amplification protocol and select the fluorescence channel reported in the manual.
3. Joe(Yellow)/Hex/Cy3 signal with Negative Control of extraction.
    - Contamination during DNA extraction procedure. All samples results are invalid.
        - ⇒ Decontaminate all surfaces and instruments with sodium hypochlorite and ethanol.
        - ⇒ Use only filter tips during the extraction procedure. Change tips between tubes.
        - ⇒ Repeat the DNA extraction with the new set of reagents.
4. Any signal with Negative Control of PCR (DNA-buffer).
    - Contamination during PCR preparation procedure. All samples results are invalid.
        - ⇒ Decontaminate all surfaces and instruments with sodium hypochlorite and ethanol or special DNA decontamination reagents.
        - ⇒ Pipette the Positive control at last.
        - ⇒ Repeat the PCR preparation with the new set of reagents.

KEY TO SYMBOLS USED

REF	List Number		Caution!
LOT	Lot Number		Contains sufficient for <n> tests
IVD	For *in Vitro* Diagnostic Use	VER	Version
	Store at	**NCA**	Negative Control of Amplification
	Manufacturer	**NCE**	Negative control of Extraction
	Consult instructions for use	**C+**	Positive Control of Amplification
	Expiration Date	**IC**	Internal Control

Sacace Biotechnologies Srl
via Scalabrini, 44 – 22100 – Como – Italy Tel +390314892927 Fax +390314892926
mail: ***info@sacace.com*** *web:* ***www.sacace.com***