IVD For *in Vitro* Diagnostic Use

CE

Yersinia enterocolitica / Y.pseudotuberculosis Real-TM

Handbook

Real Time PCR kit for qualitative detection and differentiation of virulent and avirulent *Yersinia enterocolitica* strains and *Yersinia pseudotuberculosis* strains in environmental sample and clinical materials

REF B64-50FRT

REF TB64-50FRT

Σ 50

NAME

Yersinia enterocolitica/Y.pseudotuberculosis Real-TM

INTRODUCTION

Yersinia genus presently consists of 11 species, three of which can cause disease in humans and animals: Y. enterocolitica, Y. pseudotuberculosis and Y. pestis.

Yersinia enterocolitica is an enteric pathogen causing a variety of gastrointestinal and systemic syndromes, including enterocolitis, terminal ileitis, mesenteric lymphadenitis and septicemia. Human yersiniosis is attributed to contaminated pork, milk, water, and tofu consumption, as well as blood transfusion. Infected individuals may shed *Y enterocolitica* in stools for 90 days after the symptom resolution, suggesting that early detection of *Y enterocolitica* from diarrheal stool samples is critical in preventing its transmission and an eventual out break.

Yersinia enterocolitica is differenziated in avirulent and virulent strains (virulence is assessed by genes encoding enterotoxin (*Yst*), attachment invasion locus (*ail*), and plasmid pYV adhesion (*yadA*), which could potentially promote adherence to and invasion of antigen-sampling intestinal epithelial cells known as M cells.

INTENDED USE

Kit **Yersinia enterocolitica/Y.pseudotuberculosis Real-TM** is a Real-Time test for the qualitative detection and differentiation of avirulent and virulent *Yersinia enterocolitica* strains (virulence is assessed by genes encoding enterotoxin (*Yst*), attachment invasion locus (*ail*), and plasmid pYV adhesion (*yadA*)) and *Yersinia pseudotuberculosis* strains in environmental samples and clinical material.

PRINCIPLE OF ASSAY

Yersinia enterocolitica/Y.pseudotuberculosis Real-TM test is based on two major processes: isolation of DNA from specimens, and Real Time amplification. *Yersinia enterocolitica and Y.pseudotuberculosis* detection by the polymerase chain reaction (PCR) is based on the amplification of pathogen genome specific region using specific primers and detection via fluorescent dyes. These dyes are linked with probes of oligonucleotides which bind specifically to the amplified product. The real-time PCR monitoring of fluorescence intensities allows the accumulating product detection without reopening of reaction tubes after the PCR run. **Yersinia enterocolitica/Y.pseudotuberculosis Real-TM** PCR kit is a qualitative test which contain the Internal Control (IC). It must be used in the isolation procedure in order to control the process of each individual sample extraction and serves also to identify possible reaction inhibition.

MATERIALS PROVIDED

Module No.1: Real Time PCR kit (B64-50FRT)

Part N° 2– **"Y. enterocolitica/Y.pseudotuberculosis Real-TM":** Real Time amplification kit

- **PCR-mix-1 Y.enterocolitica/Y.pseudotuberculosis**, 0,6 ml;
- **PCR-mix-1 Y.enterocolitica typing**, 0,6 ml;
- **PCR-mix-2**, 2 x 0,3 ml;
- **Hot Start Taq Polymerase**, 2 x 0,03 ml;
- **Pos DNA Y.enterocolitica/Y.pseudotuberculosis/IC C+,** 0,1 ml;
- **DNA-buffer**, 0,5 ml;
- **Negative Control C-**, 1,2 ml;*
- **Internal Control (IC)**, 1,0 ml.**

Contains reagents for 55 tests.

Module No.2: Complete Real Time PCR test with DNA purification kit (TB64-50FRT)

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**, 5,0 ml.

Contains reagents for 50 extractions

Part N° 2– **"Y. enterocolitica/Y.pseudotuberculosis Real-TM":** Real Time amplification kit

- **PCR-mix-1 Y.enterocolitica/Y.pseudotuberculosis**, 0,6 ml;
- **PCR-mix-1 Y.enterocolitica typing**, 0,6 ml;
- **PCR-mix-2**, 2 x 0,3 ml;
- **Hot Start Taq Polymerase**, 2 x 0,03 ml;
- **Pos DNA Y.enterocolitica/Y.pseudotuberculosis/IC C+,** 0,1 ml;
- **DNA-buffer**, 0,5 ml;
- **Negative Control C-**, 1,2 ml;*
- **Internal Control (IC)**, 1,0 ml.**

Contains reagents for 55 tests.

* *must be used during the sample preparation procedure: add 100 µl of C– (Negative Control) to labeled Cneg*
** *add 10 µl of Internal Control to all samples during the DNA isolation procedure directly to the sample/lysis mixture*

MATERIALS REQUIRED BUT NOT PROVIDED

- DNA extraction kit (module No. 1)
- Real Time Thermalcycler
- PCR tubes or PCR plates
- Workstation
- Pipettes with sterile, RNase-free filters tips
- Tube racks

STORAGE INSTRUCTIONS

Store kit **Yersinia enterocolitica/Y.pseudotuberculosis Real-TM** at -20°C. **DNA-Sorb-B** must be stored at 2-8°C. The kits can be shipped at 2-8°C but should be stored at -20°C and 2-8°C immediately on receipt.

STABILITY

Yersinia enterocolitica/Y.pseudotuberculosis Real-TM test 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

Yersinia enterocolitica/Y.pseudotuberculosis Real-TM can analyze DNA extracted from:

- *water:* centrifuge 10-20 ml for 10 min at maximum speed. Discard the supernatant and leave about 100 µl of solution for DNA extraction;
- *feces:*

  prepare 10-20% feces suspension, for instance adding 4ml of Saline Solution and 1,0 gr (approx. 1,0 ml) of feces in 5 ml tube (the same can be done in 2,0 ml tube). The DNA/RNA purification must be done immediately, if it is not possible add 20% glycerine and store at -20°C.

  Vortex to get an homogeneous suspension and centrifuge for 5 min to 7000-12000g. the bacterial fraction (white-yellowish line between the sediment and the supernatant) for the extraction of bacterial DNA.
- *Parenchymal organs (or biopsy) of animals.*

Specimens can be stored at +2-8°C for no longer than 12 hours, or frozen at -20°C to -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/RNA Prep** (Sacace, REF K-2-9);
- ⇨ **DNA-Sorb-B** (Sacace, REF K-1-1/B);
- ⇨ **SaMag Bacterial DNA Extraction kit** (Sacace, REF SM006).

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°C until disappearance of ice crystals.
2. Prepare required quantity of 1.5 ml polypropylene tubes.
3. Add to each tube **300 µl** of **Lysis Solution** and **10 µl** of **IC**.
4. Add **100 µl** of **Samples** to the appropriate tube.
5. Prepare Controls as follows:
   - add **100 µl** of **C–** (**Negative Control**) to labeled C*neg*.
6. Vortex the tubes, incubate 5 min at 65°C and centrifuge for 5 sec.
7. Vortex vigorously **Sorbent** and add **25 µl** to each tube.
8. Vortex for 5-7 sec and incubate all tubes for 10 min at room temperature. Vortex periodically.
9. Centrifuge all tubes for 1 min at 5000g and using a micropipette with a plugged aerosol barrier tip, carefully remove and discard supernatant from each tube without disturbing the pellet. Change tips between tubes.
10. Add **300 µl** of **Washing Solution 1** to each tube. Vortex vigorously and centrifuge for 1 min at 5000g and using a micropipette with a plugged aerosol barrier tip, carefully remove and discard supernatant from each tube without disturbing the pellet. Change tips between tubes.
11. Add **500 µl** of **Washing Solution 2** to each tube. Vortex vigorously and centrifuge for 1 min at 10000g and using a micropipette with a plugged aerosol barrier tip, carefully remove and discard supernatant from each tube without disturbing the pellet. Change tips between tubes.
12. Repeat step 11.
13. Incubate all tubes with open cap for 5 min at 65°C.
16. Resuspend the pellet in **50 µl** of **DNA-eluent.** Incubate for 5 min at 65°C and vortex periodically.
17. Centrifuge the tubes for 2 min at maximum speed (12000-16000 g). The supernatant contains DNA ready for amplification. The amplification can be performed on the same day of extraction.

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

REAL TIME AMPLIFICATION

Total reaction volume is **25 µl**, the volume of DNA sample is **10 µl**.

1 Prepare required quantity of reaction tubes (2 tubes for each sample + Controls)

2 Prepare the reaction mix for required number of samples.

3 For N reactions mix for each PCR-Mix-1 in a new tube:

10*(N+1) µl of **RT-PCR-mix-1 Y. enterocolitica / Y. pseudotuberculosis (or Y. enterocolitica typing)**

5.0*(N+1) µl of **PCR-mix-2**

0.5*(N+1) µl of **Polymerase**

4 Vortex the tube, then centrifuge shortly. Add **15 µl** of prepared reaction mix into each appropriate tube.

5 Using tips with aerosol filter add **10 µl** of DNA samples obtained at the stage of DNA isolation and mix carefully by pipetting**.**

N.B. If the DNA-Sorb isolation kit is used as a DNA extraction kit, re-centrifuge all the tubes with extracted DNA for 2 min at maximum speed (12000-16000 g) and take carefully supernatant. N.B. don't disturb the pellet, sorbent inhibit reaction

6 Prepare for each mix 2 controls:

- add **10 µl** of **DNA-buffer** to the tube labeled PCR Negative Control;
- add **10 µl** of **Pos DNA Y.enterocolitica/Y.pseudotuberculosis/IC C+** to the tube labeled Pos PCR control.

Amplification:

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

	Rotor-type Instruments[1]			**Plate-type Instruments**[2]		
Step	***Temp, °C***	***Time***	**Repeats**	***Temp, °C***	***Time***	**Repeats**
Hold	**95**	15 min	1	**95**	15 min	1
Cycling	**95**	10 s	45	**95**	10 s	45
	60	25 s *fluorescent signal detection*		**60**	25 s *fluorescent signal detection*	
	72	10 s		**72**	10 s	

[1] *For example Rotor-Gene™ 3000/6000 (Corbett Research, Australia)*

[2] *For example SaCycler-96 (Sacace),CFX96/ iQ5™/iQ iCycler™ (BioRad, USA); Mx3000P/Mx3005P™ (Stratagene, USA), Applied Biosystems® 7300/7500 Real Time PCR (Applera), SmartCycler® (Cepheid)*

Fluorescent signal is detected in the channels designed for the FAM/Green, JOE/Yellow/HEX/Cy3, and ROX/Orange/TexasRed fluorophores on the 2nd step of stage Cycling.

INSTRUMENT SETTINGS

Rotor-type instruments (Rotor-Gene 3000, Rotor-Gene 6000, Rotor-Gene Q, etc.)

Channel	Threshold	More Settings/ Outlier Removal	Slope Correct	Auto Gain Calibration Setup
FAM/Green	*0.05*	*10 %*	*On*	*5-10 FL*
JOE/Yellow	*0.05*	*10 %*	*On*	*5-10 FL*
ROX/Orange	*0.05*	*10 %*	*On*	*5-10 FL*

Plate-type instruments (SaCycler-96, CFX96, iCycler iQ, iQ5, SC etc.)

Channel	Threshold
FAM	The threshold line should be set in the result analysis window (***Log View***) at the minimum level where it does not cross the fluorescence curve of negative samples.
HEX/Joe/Cy3	
ROX/TexasRed	

Ct boundary values

Sample	Channel	Ct
C+	FAM/Green	*40*
	JOE/Yellow	*40*
	ROX/Orange	*40*
C-	FAM/Green	*40*
Test samples	FAM/Green	*40*
	JOE/Yellow	*40*
	ROX/Orange	*40*

DATA ANALYSIS

The results are interpreted by the device software through the presence of crossing of fluorescence curve with the threshold line.

Detection channels and the pathogens correspondence:

Detection channel	PCR-mix-1 *Y.enterocolitica / Y.pseudotuberculosis*	PCR-mix-1 *Y. enterocolitica* typing
FAM/Green	Internal Control DNA	*Y.enterocolitica* DNA (*Ail*-positive)
JOE/Yellow/HEX/Cy3	*Y.pseudotuberculosis* DNA (all strains)	*Y.enterocolitica* DNA (*Yst*-positive)
ROX/Orange/TexasRed	*Y. enterocolitica* DNA (all strains)	*Y.enterocolitica* DNA (*yadA*-positive)

Interpretation of results:

PCR-mix-1	Ct value in the channel			Result
	FAM/Green	JOE/Yellow/ HEX/Cy3	ROX/Orange/ TexasRed	
PCR-mix-1 Y.enterocolitica / Y.pseudotuberculosis	**Pos (< 40)**	Neg (or > 40)	Neg (or > 40)	*Yersinia enterocolitica* and *Yersinia pseudotuberculosis* DNA **are not detected**
	Pos (< 40)	**Pos (< 40)**	Neg (or > 40)	*Y. pseudotuberculosis* DNA **is detected**
	Pos (< 40)	Neg (or > 40)	**Pos (< 40)**	*Y. enterocolitica* DNA **is detected**[1]
	Neg (or > 40)	Neg (or > 40)	Neg (or > 40)	**Invalid result** Repeat extraction and PCR
***PCR-mix-1 Y.enterocolitica* typing**	**Pos (< 40)**	Neg (or > 40)	Neg (or > 40)	*Y. enterocolitica* virulence factor (attachment invasion locus *Ail*) **is detected**
	Neg (or > 40)	**Pos (< 40)**	Neg (or > 40)	*Y. enterocolitica* virulence factor (*Yst* enterotoxin) **is detected**
	Neg (or > 40)	Neg (or > 40)	**Pos (< 40)**	*Y. enterocolitica* virulence factor (plasmid pYV adhesin *yadA*) **is detected**
	Neg (or > 40)	Neg (or > 40)	Neg (or > 40)	*Y. enterocolitica* virulence factors **are not detected**

[1]The analysis of samples with PCR-mix-1 *Yersinia enterocolitica* type is required for assessment of the *Yersinia enterocolitica* strain virulence.

SPECIFICATIONS

Sensitivity

The analytical sensitivity of **Yersinia enterocolitica/Y.pseudotuberculosis Real-TM** PCR kit is specified in the table below.

Pathogen	Test material	DNA extraction kit	Analytical sensitivity, GE/ml*
Y.enterocolitica	Feces	DNA/RNA Prep	1×10^3
Y.pseudotuberculosis	Feces	DNA/RNA Prep	1×10^3

* Genome equivalents (GE) of the microorganism per 1 ml of a sample.

11.2. Specificity

The analytical specificity of **Yersinia enterocolitica/Y.pseudotuberculosis Real-TM** PCR kit is ensured by selection of specific primers and probes as well as stringent reaction conditions. The primers and probes were checked for possible homologies to all sequences deposited in gene banks by sequence comparison analysis. Specificity was confirmed using the following microorganism strains:

- Strains from the VGNKI collection: *Salmonella enteritidis* S-6, *S.choleraesuis* 370, *S.typhimurium* 371, *S.dublin* 373, *S.typhi* C1, *S.abortusovis* 372, and *S.gallinarum-pullorum; Shigella flexneri* 851b; *Campylobacter fetus* ssp*. fetus* 25936 and *C.jejuni* ssp*. jejuni* 43435; *Klebsiella K* 65 SW4; *Listeria monocytogenes* USKHCH 19 and *L.monocytogenes* USKHCH 52; *Proteus vulgaris* 115/98; *Pseudomonas aeruginosa* DN c1; *Staphylococcus aureus* 653 and *S.aureus* 29112; *Morganella morganii* 619 c 01; and *Enterococcus faecalis* 356.
- Strains of *Yersinia enterocolitica* and *Y.pseudotuberculosis*.
- Vaccination strains of *Yersinia pestis*.

There were no nonspecific responses in tests with human DNA and a DNA panel of the above-mentioned microorganisms.

The clinical specificity of **Yersinia enterocolitica/Y.pseudotuberculosis Real-TM** PCR kit was confirmed in laboratory clinical trials.

TROUBLESHOOTING

Results of analysis are not taken into account in the following cases:

- If Ct value of the Positive Controls of PCR (C+) is greater than the boundary value (> 38) in the FAM/Green, JOE/Yellow/HEX/Cy3 or ROX/Orange channel, the PCR and detection should be repeated for all samples in which Ct value is greater than the boundary value (> 40) in the FAM/Green, JOE/Yellow/HEX, or ROX/Orange channel with appropriate PCR-mix-1.
- If no signal is detected for the positive controls of amplification, it may suggest that the programming of the temperature profile of the used instrument was incorrect, or that the configuration of the PCR reaction was incorrect, or that the storage conditions for kit components did not comply with the manufacturer's instruction, or that the reagent kit expired. Programming of the used instrument, storage conditions, and the expiration date of the reagents should be checked, and then PCR should be repeated.
- If a positive result (the fluorescence curve crosses the threshold line) is detected for a sample that has a fluorescence curve without the typical exponential growth phase (the curve is linear), this may suggest incorrect setting of the threshold line or incorrect calculation of baseline parameters. Such a result should not be considered as positive. Once the threshold line has been set correctly, PCR analysis of the sample should be repeated (if iCycler iQ or iQ5 instruments are used).

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	**C–**	Negative control of Extraction
	Consult instructions for use	**C+**	Positive Control of Amplification
	Expiration Date	**IC**	Internal Control

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