Vibrio cholerae Real-TM
Handbook

Real Time PCR Kit for the qualitative detection and identification of pathogen Vibrio cholerae strains in the biological materials

for use with RotorGene™ 3000/6000/Q (Corbett Research, Qiagen)

REF B53-50FRT

REF TB53-50FRT

Σ 50

NAME

Vibrio cholerae Real-TM

INTRODUCTION

Vibrio cholerae (also *Kommabacillus*) is a gram negative comma-shaped bacterium with a polar flagellum that causes cholera in humans. *V. cholerae* and other species of the genus *Vibrio* belong to the gamma subdivision of the Proteobacteria. There are two major biotypes of *V. cholerae* identified by hemaggluttination testing, classical and El Tor, and numerous serogroups.

V. cholerae pathogenicity genes code for proteins directly or indirectly involved in the virulence of the bacteria. They are interesting targets to detect and to study *V. cholerae* infections. Because of their same transcriptional regulation and their implication in the same pathway, pathogenicity genes are generally organized in operons and/or gene clusters. In *V. cholerae*, most of virulence genes are located in two pathogenicity plasmids, which are organized as prophages:

- CTX (Cholera ToXins) plasmid;
- and TCP (Toxin-Coregulated Pilus) plasmid, also named VPI (*V. cholerae* Pathogenicity Island).

Virulent and epidemic strains of *V. cholerae* require these two genetic elements to cause infections.

Cholera is an infection of the small intestine that is caused by the bacterium *Vibrio cholerae.* The primary symptoms of cholera are profuse painless diarrhea and vomiting of clear fluid. These symptoms usually start suddenly, one to five days after ingestion of the bacteria. The diarrhea is frequently described as "rice water" in nature and may have a fishy odor. An untreated person with cholera may produce 10-20 liters of diarrhea a day with fatal results. Cholera is caused by eating contaminated food. Transmission is primarily due to the fecal contamination of food and water due to poor sanitation. This bacterium can, however, live naturally in any environment. Antibiotic treatments for one to three days shorten the course of the disease and reduce the severity of the symptoms.

INTENDED USE

Kit **Vibrio cholerae Real-TM** PCR kit is an *in vitro* nucleic acid amplification test for qualitative detection and identification of *Vibrio cholerae* DNA in biological material and environmental compartments by using real-time hybridization-fluorescence detection.

PRINCIPLE OF ASSAY

The detection of *Vibrio cholerae* DNA (Hly sequence is present) and identification of pathogen *Vibrio cholerae* strains (main virulence factors – CtxA, tcpA – are present), belonging to serogroups O1 (target wbeT present) or O139 (target wbf present) by the polymerase chain reaction (PCR) is based on the amplification of pathogen genome specific region using special *Vibrio cholerae* strains primers. In real-time PCR, the amplified product is detected using fluorescent dyes. These dyes are usually linked to oligonucleotide probes which bind specifically to the amplified product during thermocycling. The monitoring of the fluorescence intensities during the real-time PCR allows the detection of accumulating product without re-opening the reaction tubes after the PCR run.

Vibrio cholerae 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 isolation process of each individual sample and to identify possible reaction inhibition.

PCR analysis is carried out in multiplex format in two tubes:

- "Screen" – the amplification of CtxA target (FAM/Green), tcpA target (ROX/Orange) and IC target (JOE/Yellow/HEX),
- "Type" - the amplification of Hly target (JOE/Yellow/HEX) - cholera germs of all groups, wbeT (FAM/Green) - belonging to serogroup O1, wbf (ROX/Orange) - belonging to serogroup O139.

It is necessary to carry out both "Screen" and "Type" reactions for results interpretation.

MATERIALS PROVIDED

Module No.1: Real Time PCR kit (B53-50FRT)

Part N° 2 –" **Vibrio cholerae Real-TM"**

- **PCR-mix-1-FRT Screen**, 55 tubes;
- **PCR-mix-1-FRT Type**, 55 tubes;
- **PCR-mix-2-FRT**, 0,77 ml;
- **Pos C+ Screen**, 0,1 ml;
- **Pos C+ Type**, 0,1 ml;
- **Pos IC**, 0,1 ml;
- **Negative Control C-***, 2 x 1,2 ml;
- **Internal Control IC****, 0,5 ml;
- **DNA-buffer**, 0,5 ml;

Contains reagents for 55 tests.

Module No.2: Complete Real Time PCR test with DNA purification kit (TB53-50FRT)

Part N° 1 – **"DNA/RNA Prep":** sample preparation;

- **Lysis Sol**, 15 ml;
- **Prec Sol**, 20 ml;
- **Washing Sol 3**, 25,0 ml;
- **Washing Sol 4**, 10,0 ml
- **RE-buffer**, 4 x 1,2 ml;

Contains reagents for 50 extractions

Part N° 2 –" **Vibrio cholerae Real-TM"**

- **PCR-mix-1-FRT Screen**, 55 tubes;
- **PCR-mix-1-FRT Type**, 55 tubes;
- **PCR-mix-2-FRT**, 0,77 ml;
- **Pos C+ Screen**, 0,1 ml;
- **Pos C+ Type**, 0,1 ml;
- **Pos IC**, 0,1 ml;
- **Negative Control C-***, 2 x 1,2 ml;
- **Internal Control IC****, 0,5 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*

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:

- Real Time Thermalcycler
- Tubes or PCR plate
- Workstation
- Pipettes with sterile, RNase-free filters tips
- Tube racks

STORAGE INSTRUCTIONS

Vibrio cholerae Real-TM and **DNA-RNA Prep** must be stored at +2-8°C. The kits can be shipped at room temperature but should be stored at 2-8°C immediately on receipt.

STABILITY

Vibrio cholerae 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

1.   Xn

Component Lysis Sol 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: 20/21/22-36/38; S: 36/37/39). **Risk Phrases:**R 20/21/22 Harmful by inhalation, in contact with the skin and if swallowed, R 22 Harmful if swallowed, R 36/38 Irritating to eyes and skin. **Safety Phrases:** S 13 Keep away from food, drink and animal feedstuffs

Component Prec Sol contains 2-propanol*: flammable. Irritant. (R10-36-67, S7-16-24/25-26). **Risk Phrases:** R10: Flammable, R36/37/38: Irritating to eyes, respiratory system and skin, R67: Vapors may cause drowsiness and dizziness. **Safety Phrases:** S7: Keep container tightly closed, S16: Keep away from sources of ignition - No smoking, S24/25: Avoid contact with skin and eyes, S26: In case of contact with eyes, rinse immediately with plenty of water and seek medical advice;

1. Wear disposable gloves, laboratory coats and eye protection when handling specimens and reagents. Thoroughly wash hands afterward.
2. Do not pipette by mouth.
3. Do not eat, drink, smoke, apply cosmetics, or handle contact lenses in laboratory work areas.
4. Do not use a kit after its expiration date.
5. Dispose all specimens and unused reagents in accordance with local regulations
6. Avoid contact of specimens and reagents with the skin, eyes and mucous membranes. If these solutions come into contact, rinse immediately with water and seek medical advice immediately.
7. Material Safety Data Sheets (MSDS) are available on request.
8. Use of this product should be limited to personnel trained in the techniques of DNA amplification.
9. Workflow in the laboratory must proceed in a uni-directional manner, beginning in the Extraction Area and moving to the Amplification and Detection Area. Do not return samples, equipment and reagents in the area where you performed previous step.

** 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, PREPARATION, STORAGE AND TRANSPORT

Vibrio cholerae Real-TM kit is intended for the analysis of DNA extracted by using DNA isolation kit from biological material and environmental compartments.

The following material is used for analysis:

Clinical material samples:

- 1.0 –2.0 g (or 1-2 ml in case of diarrhea) of feces, native or transferred into tube with 5 ml of 1 % peptone water are used after preliminary preparation;
- 1–2 ml of vomit masses, native or transferred into 5 ml of peptone water are used after preliminary preparation;
- rectum wall scraping from depth of 5-6 cm, taken by dry sterile probe (probe's working part with tampon is to be placed into 1.5 ml tube with 0.5 ml of 1 % peptone water, the rest of the probe is to be broken and deleted). 50 µl of solution is used for analysis.

Autopsy material samples:

- upper, medial and lower sections small intestine content is transferred into tube with 5 ml of 1 % peptone water are used after preliminary preparation.

Environmental samples (for monitoring purpose):

- water (from water body, wastewater, drinking water) is sampled and treated in compliance with local authorities requirements. First peptone water (after preliminary preparation) is used for analysis;
- silt and hydrobionts are sampled and treated in compliance with local authorities requirements. First peptone water (after preliminary preparation) is used for analysis.

Environmental samples (nidus of infection):

- water (from water body, wastewater, drinking water) is sampled and treated in compliance with local authorities requirements. Then it is preliminary filtered out through the filters with pore diameter – 8 µm (or paper filters) and finally filtered out by using of filters with pore diameter – 0.45 µm. These filters are placed into the tubes with 3 ml of physiological solution and boiled during 10 min. 50 µl of solution is used for analysis. In case of negative analysis result it's needed to make the inoculation of washouts from filters in compliance with local authorities requirements and to test the first peptone water (after preliminary preparation).
- washouts from the surfaces of object (10 x 10 cm area), sampled by sterile probe, which was wetted by physiological solution (probe's working part with tampon is to be placed into 1.5-ml tube with 0.5 ml of 1 % peptone water, the rest of the probe is to be broken and deleted). 50 µl of solution without preliminary preparation is used for analysis.

Food products: are sampled ant treated in compliance with local authorities requirements. First peptone water (after preliminary preparation) is used for analysis.

Germ cultures, suspected in *Vibrio cholerae* presence:

- colony is to be resuspended in 0,5 ml of physiological solution or phosphate buffer. 50 µl of suspension is used for analysis.

Studied material's transportation is strictly carried out in compliance with local authorities requirements.

Material's preliminary preparation

Any operation with studied material transportation is carried out in compliance with local authorities requirements.

All manipulations, connected with probes preparation, are carried out by varying volume pipettors with using of disposable polypropylene microtubes of 1.5 ml or 10.0 ml volume and tips with aerosol barriers. Disposable plastic dishes (tubes, tips) are to be thrown into the special container with suitable disinfectant. They are to be utilized in compliance with local authorities requirements.

Native faeces:

A. 10-20 % faeces suspension preparation (watery faeces are used without suspension preparation).
   1. 4 ml of saline or phosphate buffer is to be transferred into 5 ml volume tubes with tightly closed cap.
   2. 0.5 – 1.0 g (near 1-2 ml) faeces are transferred into tubes. Use an individual tip with aerosol barriers (or disposable spatula) for each tube. The content of the tube is to be stirred carefully to form the homogeneous suspension.

B. Faeces bacterial fraction preparation:
   From tubes with faecal suspension 1 ml is to be transferred to 1.5 ml tube with tightly closed cap then it's to be centrifuged during 5 min at 12000 rpm. For DNA isolation 50 µl of light fraction from the board of transparent liquid and solid faecal fractions is to be used.

Feces or vomit masses, placed into 1 % peptone water:

A. The content of the tube is to be stirred carefully to form the homogeneous suspension.
B. Bacterial fraction preparation:
   1 ml of suspension is to be transferred to 1.5 ml tube with tightly closed cap then it's to be centrifuged during 5 min at 12000 rpm. For DNA isolation 50 µl of light fraction from the board of transparent liquid and solid fecal fractions is to be used.

Autopsy material samples (small intestine content):

The content of the tube is to be stirred carefully to form the homogeneous suspension. For DNA isolation 50 µl of suspension is to be used.

Liquid culture (peptone water after bacterial inoculation):

– from the surface of peptone water 1.2-1.4 ml is sampled into the tube of 1.5 ml volume then it is to be centrifuged during 10 min at maximum revolutions (10-12 thousand rpm). Supernatant is to be deleted. Precipitate is to be resuspended in 300 µl of saline or phosphate buffer. 50 µl of solution is used for analyses.

Specimens can be stored at +2-8°C for no longer than 48 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

Sacace Biotechnologies recommends to use the following kit:

- ⇨ **DNA/RNA Prep** (Sacace, REF K-2-9);
- ⇨ **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. Prepare required number of 1.5 ml disposable polypropylene micro centrifuge tubes including one tube for Negative Control of Extraction (**Negative Control**, **C-**).
2. Add to each tube **10 µl** of **IC** (Internal Control) and **300 µl** of **Lysis Sol**
3. Add **100 µl** of samples to the appropriate tubes using pipette tips with aerosol barriers.
4. Prepare Controls as follows:
    - add **100 µl** of **Negative Control C–** to the tube labeled C*neg*
5. Vortex the tubes and incubate for 5 min at 65°C. Centrifuge for 7-10 sec.
6. Add **400 µl** of **Prec Sol** and mix by vortex. Centrifuge all tubes at 13,000 r/min for 5 min 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 the tubes.
7. Add **500 µl of Wash Sol 3** into each tube. Vortex vigorously to ensure pellet washing. Centrifuge all tubes at 13,000 r/min for 60 sec 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 the tubes.
8. Add **200 µl of Wash Sol 4** into each tube. Vortex vigorously to ensure pellet washing. Centrifuge all tubes at 13,000 r/min for 60 sec 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 the tubes.
9. Incubate all tubes with open caps at **65 °C for 5 min**.
10. Resuspend the pellet in **50 µl of RE-buffer** (elution volume can be increased up to 90 µl). Incubate for 5 min at 65°C and vortex periodically.
11. Centrifuge the tubes at 13000g for 60 sec.

The supernatant contains RNA/DNA ready for amplification. If amplification is not performed 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.

PROTOCOL

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

Preparing tubes for PCR.

1. Prepare the required number of the tubes with **PCR-mix-1 Screen** and **PCR-mix-1 Type** for amplification of DNA from clinical and control samples. Mark tubes by «S» and «T»
2. Add **7 µl** of **PCR-mix-2** to the surface of the wax layer of each tube ensuring that it does not fall under the wax and mix with **PCR-mix-1**
3. Using tips with aerosol barrier add **10 µl** of **DNA samples** obtained from clinical or control samples at the DNA extraction stage into prepared tubes.
4. Carry out the control amplification reactions:

NCA - Add **10 µl** of **DNA-buffer** to the tube labeled NCA (Negative Control of Amplification).

C+$_{screen}$ - Add **10 µl** of **Positive Control DNA *Vibrio cholerae* screen (Pos C+$_{screen}$)** to the tube with **PCR-mix-1-FRT Screen** labeled **C+$_{screen}$** (Positive Control of Amplification).

C+$_{type}$ - Add **10 µl** of **Positive Control DNA *Vibrio cholerae* type (Pos C+ $_{type}$)** to the tube with **PCR-mix-1-FRT Type** labeled **C+$_{type}$** (Positive Control of Amplification).

IC+ - Add **10 µl** of **Positive Control IC (Pos IC)** to the tube with **PCR-mix-1-FRT Screen** labeled **IC+** (Positive Control of Amplification).

Amplification

1. Program the Rotor-Gene according to manufacturer's manual.
2. Create a temperature profile on your Rotor-Gene instrument as follows:

RG program

Step	*Temperature, °C*	*Time*	*Fluorescence detection*	*Cycle repeats*
Hold	95	5 min	–	1
Cycling	95	10 sec	–	10
	60	25 sec	–	
	72	10 sec	–	
Cycling 2	95	10 sec	–	35
	56	25 sec	FAM/Green, JOE/Yellow, ROX/Orange	
	72	10 sec	–	

3. Fluorescence detection is on the 2-nd pass **(56 °C)** in FAM/Green, JOE/Yellow and ROX/Orange fluorometer channels.
4. Make the adjustment of the fluorescence channel sensitivity:

Channel	Threshold	More Settings/Outlier Removal	Slope Correct
FAM/Green	0.05	5 %	off
JOE/Yellow	0.05	5 %	off
ROX/Orange	0.1	5 %	off

DATA ANALYSIS

The signal is considered to be positive, if the corresponding fluorescence accumulation curve crosses threshold line. Results are accepted as relevant if both positive and negative controls of amplification along with negative control of extraction are passed.

Results interpretation

Results for controls for test with PCR-mix-1-FRT Screen

Control	Controlled stage	Results			Interpretation
		FAM/ Green (CtxA)	JOE/Yellow (IC)	ROX/Orange (tcpA)	
C-	DNA isolation	Neg	Pos (< 33)	Neg	OK
NCA	Amplification	Neg	Neg	Neg	OK
$C+_{screen}$	Amplification	Pos (< 33)	Neg	Pos (< 33)	OK
IC+	Amplification	Neg	Pos (< 33)	Neg	OK

Results for controls for test with PCR-mix-1-FRT Type

Control	Controlled stage	Results			Interpretation
		FAM/ Green (O1)	JOE/Yellow (V.cholerae)	ROX/Orange (O139)	
C-	DNA isolation	Neg	Neg	Neg	OK
NCA	Amplification	Neg	Neg	Neg	OK
$C+_{type}$	Amplification	Pos (< 33)	Pos (< 33)	Pos (< 33)	OK

Interpretation of results for PCR-analysis

Variants	PCR-mix-1-FRT Screen			PCR-mix-1-FRT Type		
	Ct value on channel					
	FAM/Green (CtxA)	JOE/Yellow (IC)	ROX/Orange (tcpA)	FAM/Green (O1)	JOE/Yellow (*V.cholerae*)	ROX/Orange (O139)
V.cholerae O1 toxigenic	Pos (< 33)	Any value or its absence	Pos (< 33)	Pos (< 33)	Pos (< 33)	Neg
V.cholerae O139 toxigenic	Pos (< 33)	Any value or its absence	Pos (< 33)	Neg	Pos (< 33)	Pos (< 33)
V.cholerae O1 NON toxigenic, but contained the sequence **tcpA**	Neg	Pos (< 33)	Pos (< 33)	Pos (< 33)	Pos (< 33)	Neg
V.cholerae O139 NON toxigenic, but contained the sequence **tcpA**	Neg	Pos (< 33)	Pos (< 33)	Neg	Pos (< 33)	Pos (< 33)
V.cholerae O1 NON toxigenic	Neg	Pos (< 33)	Neg	Pos (< 33)	Pos (< 33)	Neg
V.cholerae O139 NON toxigenic	Neg	Pos (< 33)	Neg	Neg	Pos (< 33)	Pos (< 33)
V.cholerae not O1 and not O139	Neg	Pos (< 33)	Neg	Neg	Pos (< 33)	Neg
Comma bacillus are not detected	Neg	Pos (< 33)	Neg	Neg	Neg	Neg

SPECIFICATIONS

Analytical specificity

The absence of nonspecific reactions of components of the PCR kit was demonstrated for DNA of closely related microorganisms, microorganisms representative of normal microflora, and some other pathogens causing intestinal infections, namely: *Vibrio parahaemolyticus, V.alginolyticus, V.anguillarum, V.mimicus, V.splendidus, V.fluvialis, and V.proteolyticus; Escherichia coli; Salmonella enteritidis* and *S.typhi; Shigella flexneri* and *Sh.sonnei; Campylobacter fetus* and *C.jejuni; Klebsiella pneumonia; Listeria monocytogenes; Proteus vulgaris; Pseudomonas aeruginosa; Staphylococcus aureus; Morganella morganii, Enterobacter faecalis; Aeromonas; Plesiomonas shidel; Commomonas; and* human cDNA/DNA.

False-positive results were not detected in the study of 100 fecal samples without enteritis and 50 fecal samples with enteritis of bacterial and viral etiology.

Analytical sensitivity.

The analytical sensitivity of the kit **Vibrio cholerae Real-TM** was not less than 1000 copies/ml

TROUBLESHOOTING

1. If the Ct values are absent on FAM/Green and ROX/Orange channels and the Ct value on JOE/Yellow channel is also absent or exceeds “33” when using of PCR-mix-1-FRT Screen then the PCR analysis and DNA isolation should be repeated.
2. If the positive signal on any target except IC (negative result on JOE/Yellow channel when using of PCR-mix-1-FRT Type and the Ct value does not exceed “33” on JOE/Yellow channel when using of PCR-mix-1-FRT Screen) is obtained then the result sample is considered to be invalid. The sampling and the analysis should be repeated.
3. If the Ct value on JOE/Yellow channel is absent when using of PCR-mix-1-FRT Type and the conditions of item 1 are satisfied then the analysis should be repeated from the stage of DNA extraction.
4. The absence of positive signal in a sample with positive control could indicate that the amplification program is chosen in correctly or it could indicate of other mistakes made during PCR run. In this case the PCR analysis should be repeated.
5. Positive signal for negative control (C-) on FAM/Green and/or ROX/Orange and for Negative Control of Amplification (NCA, DNA-buffer) on any channel indicates the reagent or sample contamination. In such case the analysis results are considered to be invalid. The analyses should be repeated and measures to detect and eliminate the contamination source are to be taken.

KEY TO SYMBOLS USED

REF	List Number		Caution!
LOT	Lot Number		Contains sufficient for <n> tests
	Consult instructions for use	VER	Version
	Store at		Expiration Date
	Manufacturer		Flammable
			Harmful

* Rotor-Gene™ Technology is a registered trademark of Corbett Research

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