Primer Design Ltd

R01046

genesig®PLEX Gastrointestinal Bacterial II Multiplex Kit

Kit Version: 1

Vibrio spp. (rpoB gene)

Escherichia coli O157 (Z3276 ORF region)

Yersinia enterocolitica (ail gene)

Clostridium difficile (toxb gene)

genesig®PLEX kit

100 tests

GENESIG

Kits by Primerdesign

Specificity of primers and probes last reviewed on:
21st December 2022

For general laboratory and research use only

Introduction

Vibrio spp.

Vibrio encompasses a group of gram-negative, rod-shaped bacteria, known for their distinctive curved or comma-shaped morphology. They exhibit facultative anaerobic behaviour, thriving in both oxygen-rich and oxygen-depleted environments. The rpoB gene encodes a vital subunit of bacterial RNA polymerase, an essential enzyme governing DNA-to-RNA transcription during gene expression. This gene is highly conserved across bacterial species due to its pivotal role in transcriptional processes.

These Vibrio species are commonly encountered in aquatic settings, yet some can pose a pathogenic threat, leading to ailments in humans and other animals. Notably, Vibrio cholerae, a prominent pathogenic species in this genus, is the causative agent of the lethal diarrheal disease cholera. Additionally, Vibrio parahaemolyticus can induce gastroenteritis. In immunocompromised individuals, Vibrio infections may escalate into severe skin and soft tissue infections or bloodstream infections.

Gastrointestinal Vibrio infections manifest in a spectrum of symptoms, varying in intensity from mild to severe. Watery diarrhoea, dehydration, nausea, abdominal cramps, fever, and vomiting are common indicators of such infections. Supportive care typically suffices for mild cases of Vibrio gastroenteritis, involving adequate hydration and rest until symptoms abate. In instances of pronounced dehydration, oral rehydration solutions or intravenous (IV) fluids may be warranted. Certain Vibrio species may necessitate antibiotic treatment, such as doxycycline and azithromycin.

E. coli O157

Escherichia coli O157:H7, commonly known as E. coli O157, is a specific strain of the bacterium Escherichia coli, characterized by unique surface antigens. These antigens play a crucial role in distinguishing and classifying various E. coli strains. E. coli O157 is particularly noteworthy for its association with gastrointestinal infections and foodborne diseases.

Transmission of E. coli O157 typically occurs through the consumption of contaminated food or water. While it is frequently linked to undercooked ground beef, it can also be found in other sources, including unpasteurized milk, fresh produce, and contaminated water. Preventing E. coli O157 infections necessitates adherence to stringent food hygiene and safety practices.

Infection with this strain can result in a range of symptoms, such as severe and often bloody diarrhoea, abdominal cramps, nausea, vomiting, and low-grade fever. Moreover, E. coli O157 infections have the potential to lead to serious complications, including Haemolytic Uremic Syndrome (HUS) and kidney failure. In most cases, the primary treatment for E. coli O157 infections involves supportive care, including maintaining adequate hydration. The use of antibiotics is generally discouraged due to the potential to increase the risk of HUS. However, in severe cases, especially when complications like HUS arise, hospitalization and specialized medical attention become necessary.

Given its capacity to cause severe illnesses and complications, public health agencies closely monitor E. coli O157, investigating outbreaks to pinpoint their sources and prevent further infections. It is imperative for individuals to be well-informed about safe food handling practices to reduce the risk of E. coli O157 infections.

Y. enterocolitica

Yersinia enterocolitica, a gram-negative, rod-shaped bacterium within the Enterobacteriaceae family, shares close genetic ties with other Yersinia species, including Yersinia pseudotuberculosis and Yersinia pestis, responsible for bubonic plague. Yersinia enterocolitica frequently surfaces as a culprit behind foodborne illnesses in humans, with varying prevalence across regions, and it is notably associated with a condition known as yersiniosis, a gastrointestinal infection showcasing a diverse array of symptoms.

Transmission of Yersinia enterocolitica predominantly occurs through the consumption of contaminated food or water. Common sources of infection encompass undercooked or raw pork products, unpasteurized milk, and vegetables tainted with the bacterium. Preventing Yersinia enterocolitica infection usually involved adopting sound food handling practices and ensuring thorough cooking.

Symptoms of yersiniosis, though diverse, often encompass watery or bloody diarrhoea, abdominal pain and cramping (which may mimic appendicitis in some instances), fever, and nausea with vomiting. In many instances, yersiniosis spontaneously resolves without specific treatment. Nevertheless, in severe or prolonged cases or when complications arise, healthcare providers may prescribe antibiotics such as ciprofloxacin or doxycycline as a therapeutic intervention.

C. difficile

Clostridium difficile, often abbreviated as C. difficile or C. diff, is a bacterium recognized for its potential to trigger gastrointestinal infections, particularly among individuals who have recently taken antibiotics or possess compromised immune systems. This gram-positive, spore-forming bacterium can be encountered in various environments, with a notable presence in healthcare establishments like hospitals and long-term care facilities, where it is causes healthcare-associated infections. Toxin B, often referred to as ToxB, stands out as one of the key toxins produced by C. difficile and plays a pivotal role in the disease process. This bacterium produces two main toxins, the other being toxin A. Toxin B, a potent cytotoxin, inflicts damage to the cells lining the colon, subsequently inciting inflammation and manifesting the characteristic symptoms of C. difficile infection.

C. difficile spores are highly resilient and capable of persisting in the environment for prolonged durations. In healthcare settings, transmission of C. difficile infections (CDIs) frequently occurs through contact with contaminated surfaces or via the hands of healthcare personnel, who may inadvertently spread these tenacious spores. CDIs arise from the overgrowth of C. difficile within the colon, giving rise to a spectrum of gastrointestinal symptoms predominantly attributed to the bacterium's toxin production, including toxin B. These symptoms can encompass watery or blood-tinged diarrhoea, abdominal pain and cramps, fever, loss of appetite, and dehydration. In severe instances, C. difficile infection may lead to life-threatening complications such as pseudomembranous colitis, toxic megacolon, and colon perforation.

Treatment of C. difficile infection typically involves discontinuing the triggering antibiotics (if feasible) and initiating targeted antibiotics to combat C. difficile. Commonly employed antibiotics include metronidazole and vancomycin. For severe cases or those with complications, supplementary medical interventions may be required. Prevention of C. difficile infection entails adherence to proper hand hygiene practices, judicious use of antibiotics to minimize disruption of the gut microbiota, and rigorous cleaning and disinfection of healthcare environments.

Specificity

The genesig®PLEX kit is designed for the in vitro detection of Vibrio spp, E. coli O157, Y. enterocolitica and C. difficile (ToxB positive). The assays within this kit are predicted to detect over 95% of sequences available on the NCBI database since the date of last review.

The dynamics of genetic variation means that new sequence information may become available after the initial design. Primer Design Ltd periodically reviews the detection profiles of our kits and when required releases new versions.

The E. coli O157 design is predicted to cross react with some deposited sequence for Shigella dysenteriae found in wastewater samples, which would give signal in the Cy5.5 channel.

If you require further information or have a specific question about the detection profile of this kit, then please send an email to techsupport@primerdesign.co.uk and our team will answer your question.

Kit contents

- **Multiplex primer/probe mix (2x 50 reactions BROWN)**
  FAM, VIC, ROX, Cy5, and Cy5.5 labelled (see table below)

Target	Fluorophore
Vibrio spp.	FAM
Y. enterolitica	HEX
C. difficile	ROX
Internal control	Cy5
E. coli O157	Cy5.5

- **Multiplex positive control template (RED)**
- **Internal extraction control DNA (BLUE)**
- **2x Lyophilised oasig™ Master Mix (SILVER)**
- **2x oasig™ resuspension buffer (BLUE)**
- **Template preparation buffer (YELLOW)**
  For resuspension of the positive control template and internal extraction control
- **RNase/DNase free water (WHITE)**
  For resuspension of the primer/probe mix

Reagents and equipment to be supplied by the user

Real-time PCR Instrument

Extraction kit

This kit is recommended for use with exsig®Mag however, it is designed to work well with all processes that yield high quality RNA and DNA with minimal PCR inhibitors.

Pipettors and filter tips

Vortex and centrifuge

1.5ml tubes

qPCR plates or tubes

Kit storage and stability

This kit is stable at room temperature but should be stored at -20ºC on arrival. Once the lyophilised components have been resuspended, they should not be exposed to temperatures above -20ºC for longer than 30 minutes at a time and unnecessary repeated freeze/thawing should be avoided. The kit is stable for six months from the date of resuspension under these circumstances. Primerdesign does not recommend using the kit after the expiry date stated on the pack.

Suitable sample material

All kinds of sample material suited for PCR amplification can be used. Please ensure the samples are suitable in terms of purity, concentration, and DNA integrity. Always run at least one negative control with the samples. To prepare a negative control, replace the template DNA sample with RNase/DNase free water.

Dynamic range of test

Under optimal PCR conditions genesig kits have very high priming efficiencies of >90% and can detect between $1X10^6$and $1X10^2$ copies of target template.

Notices and disclaimers

This product is developed, designed and sold for research purposes only. It is not intended for human diagnostic or drug purposes or to be administered to humans unless clearly expressed for that purpose by the Food and Drug Administration in the USA or the appropriate regulatory authorities in the country of use. During the warranty period Primerdesign genesig detection kits allow precise and reproducible data recovery combined with excellent sensitivity. For data obtained by violation to the general GLP guidelines and the manufacturer's recommendations the right to claim under guarantee is expired. PCR is a proprietary technology covered by several US and foreign patents. These patents are owned by Roche Molecular Systems Inc. and have been sub-licensed by PE Corporation in certain fields. Depending on your specific application you may need a license from Roche or PE to practice PCR. Additional information on purchasing licenses to practice the PCR process may be obtained by contacting the Director of Licensing at Roche Molecular Systems, 1145 Atlantic Avenue, Alameda, CA 94501 or Applied Biosystems business group of the Applera Corporation, 850 Lincoln Centre Drive, Foster City, CA 94404. In addition, the 5' nuclease assay and other homogeneous amplification methods used in connection with the PCR process may be covered by U.S. Patents 5,210,015 and 5,487,972, owned by Roche Molecular Systems, Inc, and by U.S. Patent 5,538,848, owned by The Perkin-Elmer Corporation.

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Principles of the test

Real-time PCR

Individual primer and probes designed for each pathogen have been combined into a single reaction and these can be detected through the different fluorescent channels as described in the kit contents.

The primer and probe mix provided exploits the so-called TaqMan® principle. During PCR amplification, forward and reverse primers hybridize to the target DNA. Fluorogenic probes are included in the same reaction mixture which consists of a DNA probe labelled with a 5`-dye and a 3`-quencher. During PCR amplification, the probe is cleaved, and the reporter dye and quencher are separated. The resulting increase in fluorescence can be detected on a range of qPCR platforms.

Positive control

The kit contains a single positive control that contains templates for the 4 targets in the test. This will give a Vibrio spp. signal through the FAM channel, a Y. enterolitica signal through the VIC channel, a C. difficile signal through the ROX channel, and an E. coli O157 signal through the Cy5.5 channel. Each time the kit is used, at least one positive control reaction must be included in the run. A positive result indicates that the primers and probes for detecting each target are working properly in that particular run. If a negative result is obtained the test results are invalid and must be repeated. Care should be taken to ensure that the positive control does not contaminate any other kit component which would lead to false positive results. This can be achieved by handling these components in a post PCR environment. Care should also be taken to avoid cross-contamination of other samples when adding the positive control to the run. This can be avoided by sealing all other samples and negative controls before pipetting the positive control into the positive control well.

Negative control

To confirm the absence of contamination, a negative control, or No Template Control (NTC) reaction should be included every time the kit is used. For this reaction, the RNase/DNase free water should be used instead of template. A negative result indicates that the reagents have not become contaminated while setting up the run.

Internal DNA Extraction Control

When performing DNA extraction, it is often advantageous to have an exogenous source of DNA template that is spiked into the lysis buffer. This control DNA is then co-purified with the sample DNA and can be detected as a positive control for the extraction process. Successful co-purification and qPCR for the control DNA also indicates that PCR inhibitors are not present at a high concentration. Within the Multiplex primer/probe mix are primers and probes to detect the exogenous DNA using qPCR. The primers are present at PCR limiting concentrations which allows multiplexing with the target sequence primers. Amplification of the control DNA does not interfere with detection of the target DNA even when present at low copy number. The Internal control is detected through the Cy5 channel and gives a Cq value of 28+/-3.

Resuspension protocol

To minimise the risk of contamination with foreign DNA, we recommend that all pipetting is performed in a PCR clean environment. Ideally this would be a designated PCR lab or PCR cabinet. Filter tips are recommended for all pipetting steps.

1. **Pulse-spin each tube in a centrifuge before opening.**
   This will ensure lyophilised primer probe mix or template is in the base of the tube and is not lost upon opening the tube.

2. **Resuspend the primer/probe mix in the RNase/DNase free water supplied, according to the table below:**
   To ensure complete resuspension, vortex the tube thoroughly.

Component – resuspend in water	Volume
Pre-PCR pack	
Multiplex primer/probe mix (**BROWN**)	55 µl

3. **Resuspend the internal control template and positive control template in the template preparation buffer supplied, according to the table below:**
   To ensure complete resuspension, vortex the tube thoroughly.

Component – resuspend in template preparation buffer	Volume
Pre-PCR heat-sealed foil	
Internal extraction control DNA (**BLUE**)	500µl
Post-PCR heat-sealed foil	
Positive control template (**RED**)*	500µl

*** This component contains high copy number template and is a VERY significant contamination risk. It must be opened and handled in a separate laboratory environment, away from the other components.**

4. **Resuspend the lyophilised Master Mix in oasig resuspension buffer, according to the table below:**

Component – resuspend in oasig resuspension buffer	Volume
Lyophilised Master Mix (SILVER)	525µl

DNA extraction

The internal extraction control DNA can be added to either lysis/extraction buffer or to the sample once it has been resuspended in lysis buffer.

DO NOT add the internal extraction control DNA directly to the unprocessed biological sample as this will lead to degradation and a loss in signal.

1. **Add 4µl of the Internal extraction control DNA (BLUE) to each sample in DNA lysis/extraction buffer per sample.**

2. **Complete DNA extraction according to the manufacturer's recommended protocols.**

qPCR detection protocol

For optimum performance and sensitivity
All pipetting steps and experimental plate set up should be performed on ice. After the plate is prepared, proceed immediately to the amplification protocol. Prolonged incubation of reaction mixes at room temperature can lead to PCR artifacts that reduce the sensitivity of detection.

1. **For each DNA sample prepare a reaction mix according to the table below:**
   Include sufficient reactions for positive and negative controls.

Component	Volume
oasig Master Mix (SILVER)	10µl
Multiplex primer/probe mix (**BROWN**)	1µl
RNase/DNase free water (**WHITE**)	4µl
Final volume	**15µl**

2. **Pipette 15µl of this mix into each well according to your qPCR experimental plate set up.**

3. **Pipette 5µl of DNA sample into each well according to your experimental plate set up.**
   For negative control wells use 5µl of RNase/DNase free water. The final volume in each well is 20µl.

4. **Pipette 5µl of positive control template into each well according to your plate set up.**
   The positive control contains templates for Vibrio spp., Y. enterolitica, C. difficile, and E. coli O157. The final volume in each well is 20µl.

qPCR amplification protocol

Amplification conditions using oasig Master Mix

	Step	Time	Temp
	Enzyme activation	2 mins	95°C
Cycling x 50	Denaturation	10 secs	95°C
	DATA COLLECTION*	60 secs	60°C

* Fluorogenic data should be collected during this step through the FAM, VIC, ROX, Cy5 and Cy5.5 channels.

Interpretation of results

Positive control

The positive control well should give an amplification plot through the FAM channel (Vibrio spp.), the VIC channel (Y. enterolitica), the ROX channel (C. difficile), and the Cy5.5 channel (E. coli O157). There is no Internal control template within the positive control so the Cy5 channel should give no signal (flat amplification plot). The positive control signals indicate that the kit is working correctly to detect each target.

No template control (NTC)

The NTC should give a flat line (flat amplification plots) through all channels. Signals in the NTC indicate cross contamination during plate set up.

Internal DNA extraction control

The Cq value obtained with the internal control will vary significantly depending on the extraction efficiency, the quality and quantity of DNA added to the PCR reaction and the individual machine settings. Cq values of 28±3 are within the normal range. When amplifying a target sample with a high genome copy number, the internal extraction control may not produce an amplification plot. This does not invalidate the test and should be interpreted as a positive experimental result.

Sample data

Presence of the targets are detected in the channels indicated in the kit contents section. Positive signals indicate positive tests for those targets. It may be possible for samples to contain multiple targets, therefore positive results in the FAM, VIC and ROX channels may be present.

Summary of data interpretation

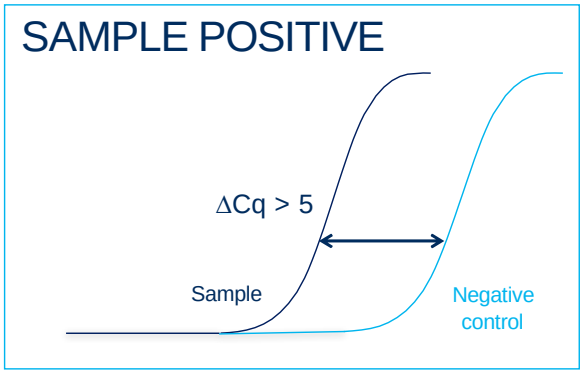
Target (FAM/VIC/ROX/ Cy5.5)	Internal extraction control (Cy5)	Positive Control	Negative Control	Interpretation
FAM +	**+ / -**	**+**	**-**	**VIBRIO SPP. POSITIVE RESULT**
VIC +	**+ / -**	**+**	**-**	**Y. ENTEROLITICA POSITIVE RESULT**
ROX +	**+ / -**	**+**	**-**	**C. DIFFICILE POSITIVE RESULT**
Cy5.5 +	**+ / -**	**+**	**-**	**E. COLI O157 POSITIVE RESULT**
-	**+**	**+**	**-**	**NEGATIVE RESULT**

+ / -	**+ / -**	**+**	**≤35**	**EXPERIMENT FAILED** Due to test contamination
+ / -	**+ / -**	**+**	**>35**	*****
-	**-**	**+**	**-**	**SAMPLE PREPARATION FAILED**
+ / -	**+ / -**	**-**	**+ / -**	**EXPERIMENT FAILED**

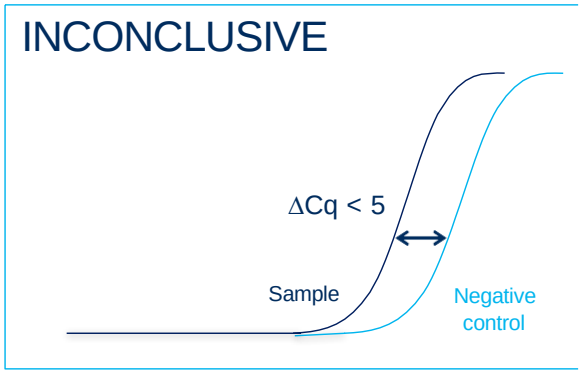
Positive control template (RED) is expected to amplify between Cq 16 and 23. Failure to satisfy this quality control criterion is a strong indication that the experiment has been compromised.

Please note that high concentrations of the Vibrio target could prevent detection of other assay targets within the same sample. Although coinfection is unlikely, if suspected, alternative testing measures should be considered.

* Where the test sample is positive, and the negative control is positive with a Cq >35, the sample must be reinterpreted based on the relative signal strength of the two results:



If the sample amplifies > 5 Cq earlier than the negative control then the sample should be reinterpreted (via the table above) with the negative control verified as negative.



If the sample amplifies < 5 Cq earlier than the negative control then the positive sample result is invalidated and the result should be determined inconclusive due to test contamination. The test for this sample should be repeated.