Primerdesign™ Ltd

R01004

genesig™ PLEX Gastrointestinal Bacterial Multiplex qPCR Kit

version: 1

Campylobacter coli/jejuni (rplD)

E. coli (Stx1, Stx2, and Stx2f)

Shigella species and EIEC (ipaH)

Salmonella enterica subspecies enterica (invA)

genesig™ PLEX kit

100 tests

GENESIG

Kits by Primerdesign

Specificity of primers and probes last reviewed on:
September 2022

For general laboratory and research use only

Introduction

Campylobacter coli and Campylobacter jejuni

The genus Campylobacter are Gram-negative, spiral, microaerophilic bacteria. Motile, with either uni- or bi-polar flagella, the organisms have a somewhat curved, rod-like appearance, and are oxidase-positive. Campylobacter jejuni is now recognised as one of the main causes of bacterial foodborne disease in many developed countries. At least a dozen species of Campylobacter have been implicated in human disease, with C. jejuni and C. coli the most common.

Campylobacteriosis is an infection by campylobacter. The common routes of transmission are fecal-oral, person-to-person sexual contact, ingestion of contaminated food or water. It produces an inflammatory, sometimes bloody, diarrhoea, periodontitis or dysentery syndrome, mostly including cramps, fever and pain. The infection is usually self-limiting and in most cases, symptomatic treatment by reposition of liquid and electrolyte replacement is enough in human infections. The use of antibiotics, on the other hand, is controversial.

This is most commonly caused by C. jejuni, a spiral and comma shaped bacterium normally found in cattle, swine, and birds, where it is non-pathogenic. But the illness can also be caused by C. coli (also found in cattle, swine, and birds).

One cause of the effects of campylobacteriosis is tissue injury in the gut. The sites of tissue injury include the jejunum, the ileum, and the colon. C jejuni appears to achieve this by invading and destroying epithelial cells.

Escherichia coli (Stx1, Stx2, and Stx2f)

Escherichia coli are one of many species of bacteria living in the lower intestines of mammals, known as gut flora. When located in the large intestine, it assists with waste processing, vitamin K production, and food absorption. Discovered in 1885 by Theodor Escherich, a German paediatrician and bacteriologist, E. coli are abundant: the number of individual E. coli bacteria in the faeces that a human defecates in one day averages between 100 billion and 10 trillion. However, the bacteria are not confined to the environment, and specimens have also been located, for example, on the edge of hot springs. The bacteria are Gram-negative, rod-shaped, flagellated and non-spore forming. Most strains are non-pathogenic but some cause food poisoning in humans with transmission largely being through the faecal-oral route. E. coli have a circular, DNA genome of approximately 4.6 Mb but also carry plasmids.

Shiga toxin-producing E. coli (STEC) are a form of enterohaemorrhagic E. coli that cause illness ranging from mild intestinal disease to severe kidney disease. The shiga toxin can cause haemorrhagic colitis, the source of the bloody diarrhoea associated with E. coli O157:H7 infections, as well as being responsible for haemolytic uremic syndrome (HUS). When the

Shiga toxin is released, it can translocate to organs other than the digestive tract such as the kidneys and central nervous system. The ability of the shiga toxins to pass through cell barriers is possibly due to the increased permeability of the intestinal epithelial cells resulting from effects of the body's own immune system. The body increases permeability of cell barriers so that important cells of the immune system (neutrophils/PMN's) can reach the E. coli infection. Shiga toxin may use this opportunity to break through the walls of the digestive tract, enter the blood stream, and bind white blood cells for transport to locations such as the kidney or brain. Transmission is predominantly through consumption of contaminated foods.

Enterohaemorrhagic E. coli are found in humans, cattle, and goats. There are a number of E. coli serogroups that produce shiga toxin such as O157:H7, O26, O111, and O103. The Shiga toxins of STEC can be divided into Shiga toxin 1 (Stx1) and Shiga toxin 2 (Stx2), each with several sub-variants. Variant stx2f is one of the latest described in literature, found in E. coli strains from pigeons, it has been rarely associated with symptomatic human infections. Recently however, studies have found that human stx2f STEC infections are more common than anticipated in the Netherlands, with an estimated 20% of all STEC infections constituting the stx2f gene. Although Stx2f STEC infections appear to be relatively mild compared to other STEC infections, new data points to stx2f STEC emerging.

Shigella (sonnei/flexneri/boydii/dysenteriae)

Shigella is a genus of four species of Gram-negative bacterium that is split into four serogroups. Shigella species have genomes ranging in size from 4.4Mbp to 4.8Mbp in length in a circular formation and are the causative agents of Shigellosis.

Shigella species infect the M cells of the intestinal epithelium and then releases proteins into the host cell via the type-three secretion system that allows bacterial invasion. The spread of bacteria between cells results in degradation of the epithelial lining of the intestine resulting in leakage.

Infection with Shigella species results in symptoms such as fever, vomiting, abdominal pain and diarrhoea, resulting from inflammation of the colon and intestinal lumen. Severe symptoms can be treated with antibiotics and a course of rehydration therapy.

S. dysenteriae is the species of serogroup A and causes dysentery in many developing countries. Serogroup B consists of serotypes of S. flexneri which is found in developing countries and Serotype C contains S. boydi which is limited to the Indian subcontinent. These 3 species are physiologically similar whereas S. sonnei of Serogroup D, found in developed countries, is more genetically divergent.

Enteroinvasive E.coli (EIEC) is closely related to Shigella and also causes dysentery by the same invasion mechanism, the ipaH gene.

Salmonella enterica subspecies enterica (typhimurium/enteritidis/newport/javiana/heidelberg)

Salmonella spp. are members of the family Enterobacteriaceae. They are Gram-negative, facultatively anaerobic, flagellated, rod-shaped organisms. They are approximately 0.7 to 1.5 µm in diameter and 2 to 5 µm in length and responsible for a large number of cases of foodborne illness throughout the world. Salmonella have circular DNA genomes with a mean length of approximately 4530 kb, although this can vary by up 1000 kb. Salmonella classification is extremely complex, however, the genus is divided into two species: S. enterica and S. bongori. S. enterica is then itself divided into 6 biochemically distinct subspecies and the Salmonella genus is further classified into serovars (serotypes) based on the lipopolysaccharide (O), flagella protein (H), and sometimes the capsular (VI) antigens. There are more than 2500 known serovars and within a serovar there may be strains that differ in virulence.

Salmonella are mainly transmitted by the faecal-oral route. They are carried asymptomatically in the intestines or gall bladder of many animals, being continuously or intermittently shed in the faeces. Humans can become infected if they do not wash their hands after contact with infected animals or animal faeces. In such instances the bacteria adhere to and enter the cells of the intestinal epithelium. The toxins produced by the bacteria can damage and kill the cells that line the intestines, which results in intestinal fluid loss. The bacteria can survive for weeks in a dry environment and far longer in water thus they are frequently present in polluted waters. Salmonella can also be carried latently in the mesenteric lymph nodes or tonsils; these bacteria are not shed but can become reactivated after stress or immunosuppression. In addition, fomites and vectors can spread Salmonella and vertical transmission occurs in birds, with contamination of the vitalize membrane, albumen and possibly the yolk of eggs. Salmonella spp. can also be transmitted in utero in mammals.

There are two different disease conditions that are distinct to salmonellosis; gastroenteritis and enteric typhoid fever. The gastroenteritis is a non-systemic infection of the intestinal tract and regional lymph nodes that gives rise to headache, muscle aches, diarrhoea, vomiting, abdominal cramping, chills, fever, nausea and dehydration. In contrast, the enteric typhoid fever is a systemic disease in which the microorganism replicates within the cells of the reticuloendothelial system. The symptoms usually appear 6 to 72 hours after ingesting contaminated food although individuals can be infected with the bacteria without having symptoms. Those with and without symptoms shed the bacteria in their stool and it is important that personal hygiene be maintained at all times.

Specificity

The genesig™PLEX kit is designed for the in vitro detection of Cambylobacter coli, Campylobacter jejuni, Escherichia coli Shiga toxin 1 & 2, Salmonella enterica, Shigella spp. and enteroinvasive Escherichia coli (EIEC).

The assays within this kit are predicted to detect over 95% of sequences available on the NCBI database at the time of design.

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

This kit is predicted to cross react with *Escherichia marmotae* which would give signal in the ROX channel, Acinobacter haemolyticus and Citrobacter freundii which would give a signal in the HEX channel and Salmonella bongori which would give a signal in the Cy5.5 channel.

Stx genes (E. coli target) are present in a subset of sequences from the following species: Bacteriophages, Shigella dysenteriae, Shigella sonnei, Acinetobacter haemolyticus, Citrobacter freundii, Enterobacter cloacae and Escherichia albertii, suggesting that these species of enterobacteria are susceptible to infection by phages and genetic exchange can occur between the phage and the host. Due to the high similarity of the stx genes between these species, cross reactivity is unavoidable.

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 and Cy5 labelled (see table below)

Target	Fluorophore
Campylobacter	FAM
E. coli	VIC
Shigella & EIEC	ROX
Internal extraction control	Cy5
Salmonella	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
- **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 exig®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

For a positive control, the kit contains a single positive control that contains templates for the 4 targets in the test. The kit positive control will give a Campylobacter signal through the FAM channel, an E. coli signal through the VIC channel, a Shigella signal through the ROX channel, and a Salmonella 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 and probe mix 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 Campylobacter, E. coli, Shigella, and Salmonella. 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 (Campylobacter), the VIC channel (E. coli), the ROX channel (Shigella & EIEC), and the Cy5.5 channel (Salmonella). 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.

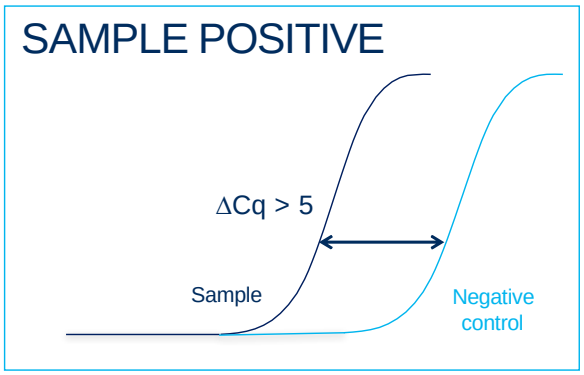
Summary of data interpretation

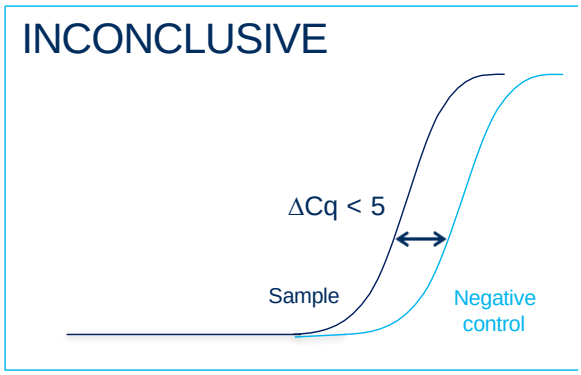
Target (FAM/VIC/ROX/ Cy5.5)	Internal extraction control (Cy5)	Positive Control	Negative Control	Interpretation
FAM +	**+ / -**	**+**	**-**	**CAMPYLOBACTER POSITIVE RESULT**
VIC +	**+ / -**	**+**	**-**	**E. COLI POSITIVE RESULT**
ROX +	**+ / -**	**+**	**-**	**SHIGELLA/EIEC POSITIVE RESULT**
Cy5.5 +	**+ / -**	**+**	**-**	**SALMONELLA 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.

* 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.