Primerdesign™ Ltd

R01021

# genesig™PLEX Livestock Screening Real-Time PCR Multiplex Kit

## Kit version: 1

Campylobacter fetus subsp. venerealis (flaB gene)

Arcobacter spp. (groEL region)

Tritrichomonas foetus (Beta-tubulin 1 gene)

genesig™PLEX kit

100 tests

GENESIG

Kits by Primerdesign

**Specificity of primers and probes last reviewed on:**
April 2023

For general laboratory and research use only

# Introduction

## Campylobacter foetus subsp. venerealis (Cfv)

Campylobacter fetus is a rod shaped, gram-negative bacteria within the genus Campylobacter. The species Campylobacter (C.) fetus is composed of three subspecies: C. fetus subsp. fetus (Cff), a zoonotic pathogen, C. fetus subsp. venerealis (Cfv) which causes reproductive problems in ruminants and C. fetus subsp. testudinum (Cft) which is mostly found in reptiles. Transmission of C. fetus subspecies venerealis (Cfv) occurs mainly through venereal contact, whilst subspecies fetus (Cff) occurs through ingestion of bacteria in a contaminated environment. Infertility and abortion in cattle and sheep are common outcomes of infection with Cfv. Cfv is found in bulls as an obligate coloniser of the penile and preputial mucosa and is passed on to heifers through breeding with an infected bull, resulting an inflammatory response in the uterus and oviduct which is often the main cause of embryonic mortality. Loss of embryos usually occurs in the first 15 to 21 days of conception. Late term abortions have also been reported for Cfv infections but are much less likely than early embryonic loss.

## Arcobacter

Arcobacter is a Gram negative, spiral-shaped bacterium between 0.2 – 0.5 μm in diameter and 1.0 – 3.0 μm in length. It has a genome size between 2.07 – 2.58 megabases with approximately 7474 genes. The genus consists of many species, however three (A. butzleri, A. cryaerophilus and A. skirrowii) are known to be pathogenic. In humans, the bacterium is known to cause diarrhoea, vomiting and fever. In animals, the bacterium is associated with enteritis, stillbirth and abortion of foetuses. Arcobacter has been isolated from many matrices, including environmental water sources, meat, vegetables and other food stuffs. Oral infection in animals such as pigs and chickens have shown rapid multiplication in the intestinal tract and invasion into internal organs. A. butzleri has also exhibited a level of antibiotic resistance towards β-lactam antibiotics and the glycopeptide vancomycin, making treatment extremely difficult. This bacterium can decrease productivity in cattle through reproductive loss.

## Tritrichomonas foetus

Tritrichomonas foetus (T. foetus) is a single celled flagellated protozoan parasite that reproduces by binary fission. The parasite is 5-25μm in length and is spindle shaped with an undulating membrane seen over the entire length of the body caused by a recurrent flagella. The organism has four flagella that are used to produce a jerky forward motion. This organism parasitizes both the reproductive tract of naturally bred cattle and the digestive tract of domestic cats. In cattle, the protozoa infect the female reproductive tract when transmitted from the foreskin of the bull where the parasite resides. Due to an increase in artificial insemination procedures, cases of cattle infection are decreasing. Infection in cattle may cause infertility and is thought to be linked to spontaneous abortions in the first trimester of pregnancy.

# Specificity

The genesig™PLEX Livestock Screening Real-Time PCR Multiplex kit is designed for the in vitro detection of Campylobacter fetus subsp. venerealis, Arcobacter spp. and Tritrichomonas foetus. 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 Campylobacter fetus subsp. testudinum which would give signal in the FAM channel. The detection of this species is predicted to be unlikely, as this strain has so far only been isolated from lizards and humans. This kit is not predicted to cross react with Campylobacter fetus subsp. fetus (Cff).

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 fetus subsp. venerealis | FAM |
| Arcobacter spp. | Cy5 |
| Tritrichomonas foetus | ROX |
| Internal Extraction Control | VIC |

- **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 DNA
- **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 genesig EASY DNA/RNA Extraction kit or 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.

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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 3 targets in the test. The kit positive control will give a Cfv signal through the FAM channel, an Arcobacter signal through the Cy5 channel and an T. foetus signal through the ROX 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 VIC 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**) | 55µl |

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 Cfv, Arcobacter and T.foetus. The final volume in each well is 20µl.

# qPCR amplification protocol

Amplification conditions using oasig Master Mix

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

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

# Interpretation of results

**Positive control**

The positive control well should give an amplification plot through the FAM channel (Cfv), the Cy5 channel (Arcobacter) and the ROX channel (T. foetus). There is no Internal control template within the positive control so the VIC 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, Cy5 and ROX channels may be present.

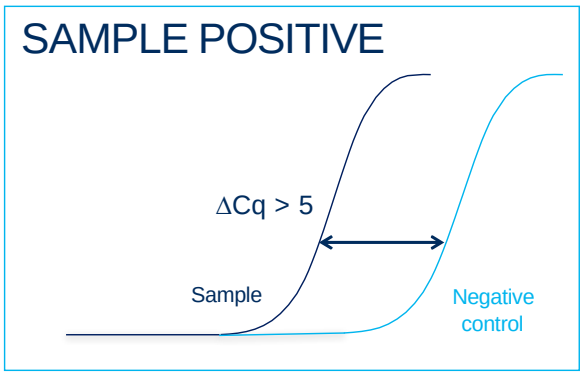
# Summary of data interpretation

| Target (FAM/Cy5/ROX) | Internal extraction control (VIC) | Positive Control | Negative Control | Interpretation |
|---|---|---|---|---|
| **FAM +** | **+ / -** | **+** | **-** | **Cfv POSITIVE RESULT** |
| **Cy5 +** | **+ / -** | **+** | **-** | **Arcobacter POSITIVE RESULT** |
| **ROX +** | **+ / -** | **+** | **-** | **T. foetus POSITIVE RESULT** |
| **-** | **+** | **+** | **-** | **NEGATIVE RESULT** |

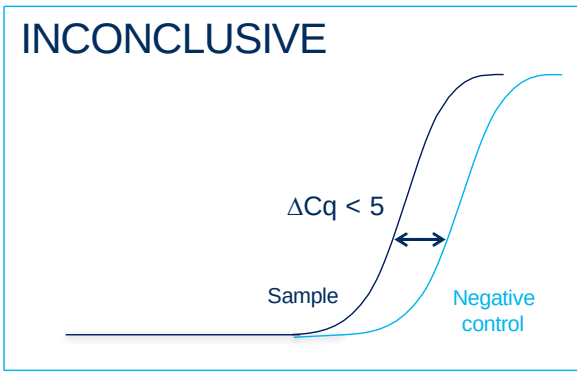
| | | | | |
|---|---|---|---|---|
| **+ / -** | **+ / -** | **+** | **≤35** | **EXPERIMENT FAILED** Due to test contamination |
| **+ / -** | **+ / -** | **+** | **>35** | ***** |
| **-** | **-** | **+** | **-** | **SAMPLE PREPARATION FAILED** |
| **+ / -** | **+ / -** | **-** | **+ / -** | **EXPERIMENT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