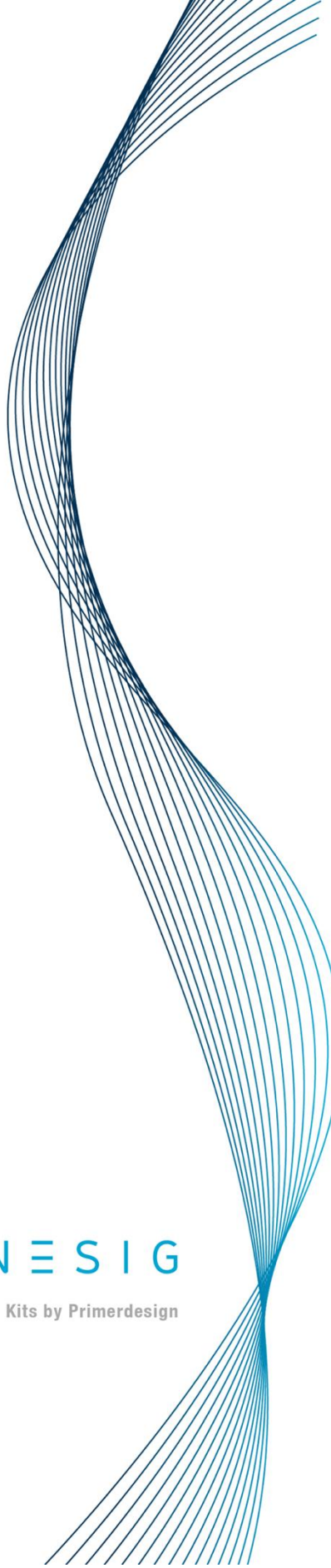
Primerdesign™ Ltd

Brucella abortus &
Brucella abortus RB51

(Multiplex kit)

genesig® kit

150 tests



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Kits by Primerdesign

For general laboratory and research use only

Introduction to *Brucella abortus*

Brucella abortus is an intracellular, blood-borne parasite. It is a Gram-negative coccobacillus that causes an infectious and contagious disease called Brucellosis. The disease primarily affects cattle, but it can also be transmitted to humans from infected animals and consuming their products. The disease can lead to great economic loss especially in the dairy and agricultural industry. The *Brucella abortus* genome contains two DNA chromosomes in a circular confirmation; the first chromosome is approximately 2.1 Mb and the second chromosome are approximately 1.2 Mb. Unusually it does not contain any plasmids or genomic islands that relate to pathogenicity and lacks many other genes that code for common virulence factors including capsules, fimbriae, exotoxins, cytolysins, resistance forms, or antigenic variation.

The most common mode of transmission to humans is through the ingestion of unpasteurized milk and cheese products as the bacteria are present in the milk glands of infected female cows. In cattle transmission can also be through ingestion but in addition, the bacteria can persist in the reproductive tracts of males, namely seminal vesicles, ampullae, testicles, and epididymides, allowing sexual transmission. In humans the bacteria enter macrophages by phagocytosis and then live in compartments of vacuolar space along the endoplasmic reticulum. They persist by inhibiting host apoptosis and go on to form chronic disease-causing lesions in the liver, spleen, bone marrow and kidneys. In cattle the bacteria additionally infect the trophoblast epithelial cells, which provide nutrition to the embryo. The trophoblast cells eventually lyse, releasing further bacteria into the blood stream of the embryo. The B.abortus cells in the blood stream go on to colonize the placenta and fetus in pregnant female cows, resulting in abortion of the fetus. Abortion can also result from insufficient anti-Brucella activity in the amniotic fluid.

In humans, the disease can be either acute or chronic and some of the symptoms include fluctuating fever, chills, sweating, headache, muscle pain and weight loss. Once a person becomes infected they are prescribed a combination of tetracycline and streptomycin for 3-6 weeks. In cattle, additional symptoms include arthritic joints and retained after-birth.

Brucella abortus strain RB51 is a licensed vaccine strain for use in cattle. *B.abortus* RB51 is a genetically stable strain; it lacks the polysaccharide O-side chains on the surface of the bacteria. The presence of the O-side chains is responsible for the initiation of an antibody-mediated response to *brucella abortus* infection. Therefore, the RB51 vaccine strain does not initiate the production of antibodies but instead produces a cell-mediated response to *brucella abortus* infections.

Cattle that have been vaccinated with the RB51 strain do not display any clinical signs of disease after vaccination and the organism is cleared from the blood stream within 3 days

of vaccination. In cases where humans may be exposed to the RB51 vaccine strain, infections of RB51 are sensitive to a range of antibiotics but are resistant to rifampicin and penicillin.

Specificity

The genesig B.abortus_MPX kit is designed for the detection of *Brucella abortus* and *Brucella abortus* RB51. The kit is designed to have a broad detection profile. Specifically, the primers represent 100% homology with over 95% of the NCBI database reference sequences available 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.

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

Kit contents

- **B.abortus_MPX primer/probe mix (150 reactions, BROWN)**
  FAM, VIC, and Cy5 labelled (see table below)

Target	Fluorophore
B.abortus	FAM
B.abortus RB51	VIC
Internal control	Cy5

- **B.abortus_MPX positive control template (RED)**
- **Internal extraction control DNA (150 reactions BLUE)**
- **Endogenous control primer/probe mix (150 reactions, BROWN)**
  FAM labelled
- **Template preparation buffer (YELLOW)**
  for resuspension of internal control template and positive control template
- **RNase/DNase free water (WHITE)**
  for resuspension of primer/probe mix

Reagents and equipment to be supplied by the user

Real-Time PCR Instrument

DNA extraction kit

This kit is recommended for use with genesig EASY DNA/RNA Extraction kit. However, it is designed to work well with all processes that yield high quality DNA with minimal PCR inhibitors.

oasig™ lyophilised or Precision®PLUS 2X qPCR Master Mix

This kit is recommended for use with oasig or PrecisionPLUS2X qPCR Master Mix.

Pipettors and tips

Vortex and centrifuge

Thin walled 1.5ml reaction 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 RNA/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 $1X10^8$ and $1X10^2$ copies of target template.

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Principles of the test

Real-time PCR

Individual primer and probes designed for B.abortus and B.abortus RB51 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 hybridise 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 two targets in the test. The positive control will give a B.abortus signal through the FAM channel and a B.abortus RB51 signal through the VIC 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 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. The supplied primer and probe mix already contains primer/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 B.abortus 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.

Endogenous control

To confirm extraction of a valid biological template, a primer and probe mix is included to detect an endogenous gene. Detection of the endogenous control is through the FAM channel and it is NOT therefore possible to perform a multiplex with the B.abortus_MPX primer/probe mix. A poor endogenous control signal may indicate that the sample did not contain sufficient biological material.

Resuspension protocol

To minimise the risk of contamination with foreign DNA, we recommend that all pipetting be 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 spilt 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	
B.abortus_MPX primer/probe mix (**BROWN**)	165µl
Endogenous control primer/probe mix (**BROWN**)	165µl

3. **Resuspend the internal extraction control DNA 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	
Pre-PCR heat-sealed foil	
Internal extraction control DNA	600µl
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.**

DNA extraction

The internal extraction control DNA can be added either to the DNA lysis/extraction buffer or to the DNA 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 protocols.**

qPCR detection protocol

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 or PrecisionPLUS 2X qPCR Master Mix	10µl
B.abortus_MPX primer/probe mix (**BROWN**)	1µl
RNase/DNase free water (**WHITE**)	4µl
Final volume	**15µl**

2. **For each DNA sample prepare an endogenous control reaction according to the table below (Optional):**
   This control reaction will provide useful information regarding the quality of the biological sample.

Component	Volume
oasig or PrecisionPLUS 2X qPCR Master Mix	10µl
Endogenous control primer/probe mix (**BROWN**)	1µl
RNase/DNase free water (**WHITE**)	4µl
Final volume	**15µl**

3. **Pipette 15µl of these mixes into each well according to your qPCR experimental plate set up.**

4. **Prepare DNA templates for each of your samples**

5. **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.

6. **If quantity estimates are required, prepare a standard curve dilution series.**

   1) Pipette 90µl of template preparation buffer into 5 tubes and label 2-6
   2) Pipette 10µl of Positive Control Template (RED) into tube 2
   3) Vortex thoroughly
   4) Change pipette tip and pipette 10µl from tube 2 into tube 3
   5) Vortex thoroughly

   Repeat steps 4 and 5 to complete the dilution series.

Standard Curve	Copy Number*
Tube 1 Positive control (RED)	2 x 10^5 per µl
Tube 2	2 x 10^4 per µl
Tube 3	2 x 10^3 per µl
Tube 4	2 x 10^2 per µl
Tube 5	20 per µl
Tube 6	2 per µl

7. **Pipette 5µl of prepared positive control dilutions into each well according to your plate set up.**
   The final volume in each well is 20µl.

   *The positive control represents equivalent quantities of B.abortus and B.abortus RB51.

qPCR amplification protocol

Amplification conditions using oasig or PrecisionPLUS 2X qPCR 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, and Cy5 channels.

Interpretation of results

Positive control

The positive control well should give an amplification plot through the FAM channel (B.abortus) and the VIC channel (B.abortus RB51). There is no endogenous 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.

Endogenous control

The signal obtained from the endogenous control reaction will vary according to the amount of biological material present in each sample. An early signal indicates the presence of a good yield of biological material. A late signal suggests that little biological material is present in the sample.

Sample data

Presence of the targets is detected in the channels indicated in the kit contents section. Positive signals indicate positive tests for those targets. Positive signal for FAM only means sample is positive for B.abortus. A positive signal through both the VIC and FAM channels indicates a positive result for the B.abortus RB51 vaccine strain.

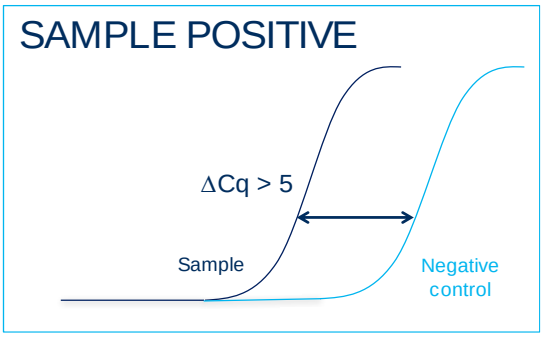
Summary of data interpretation

Target (FAM/VIC)	Internal extraction control (Cy5)	Positive control	Negative control	Interpretation
FAM +	**+ / -**	**+**	**-**	**B.abortus POSITIVE RESULT**
FAM + VIC +	**+ / -**	**+**	**-**	**B.abortus RB51 POSITIVE RESULT †**
VIC +	**+ / -**	**+**	**-**	**INVALID RESULT**
-	**+**	**+**	**-**	**NEGATIVE RESULT**
+ / -	**+ / -**	**+**	**≤ 35**	**EXPERIMENT FAILED Due to test contamination**
+ / -	**+ / -**	**+**	**> 35**	*****
-	**-**	**+**	**-**	**SAMPLE PREPARATION FAILED**
+ / -	**+ / -**	**-**	**+ / -**	**EXPERIMENT FAILED**

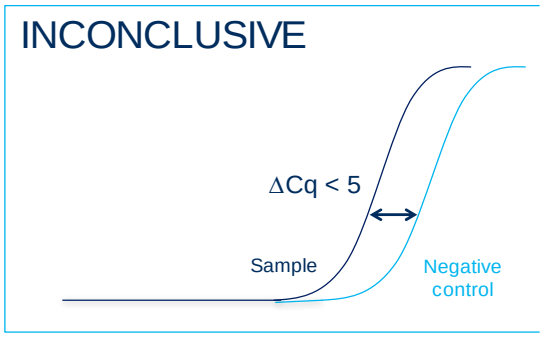
Positive control template (RED) is expected to amplify between Cq 16 and 23. Failure to satisfy this quality control criterion is a strong indication that the experiment has been compromised.

† If there is a > 3 Cq difference between VIC and FAM, this is a strong indication of co-inoculation (i.e. presence of both wild and RB51 vaccine strains).

* 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.

Internal PCR control

The Cq value obtained with the internal control will vary significantly depending on the extraction efficiency, the 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 B.abortus 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.

Endogenous control

The signal obtained from the endogenous control primer and probe set will vary according to the amount of biological material present in each sample. An early signal indicates the presence of a good yield of biological material. A late signal suggests that little biological material is present in the sample.