Primerdesign™ Ltd

R01001

genesig™PLEX Atypical Pneumonia Real-Time PCR Multiplex Kit

Kit version: 1

Bordetella spp. (IS1002/IS481)

Legionella spp. (23S/5S Intergenic Spacer Region)

Mycoplasma pneumoniae (16S ribosomal RNA gene)

Pneumocystis spp. (rnl rRNA gene)

Chlamydia spp. (16S RNA)

genesig™PLEX kit

100 tests

GENESIG

Kits by Primerdesign

Specificity of primers and probes last reviewed on:
February 2023

For general laboratory and research use only

Introduction

Bordetella spp.

Bordetella is a Gram-negative, pleomorphic bacterium that is a significant source of morbidity and mortality around the world. The genus Bordetella consists of nine species, the most prominent being B. pertussis, B. parapertussis and B. holmseii. Bordetella has a genome of over 4.1 Mb consisting of over 4,500 coding genes.

Classically known as the causative agent of pertussis (whooping cough), Bordetella presents as a highly contagious acute respiratory illness. Morbidity and mortality are typically seen in infants too young to receive vaccinations. There are two main species of Bordetella, pertussis and parapertussis. Bordetella pertussis is the most prominent of the two and is known to excrete “pertussis toxin”.

Duration of the illness is typically 6 to 12 weeks and consists of 3 stages, catarrhal, paroxysmal and convalescent. During the catarrhal phase, pertussis is not suspected due to general symptoms seen in a common cold, eg, sore throat and mild cough. The paroxysmal phase consists of paroxysms, repetitive forceful coughs during a single exhalation; followed by a sudden inspiratory effort producing the characteristic “whoop”. The convalescent phase is characterised by the steady decrease in coughs and vomiting. Transmission is greatest during the catarrhal and early paroxysmal phase, where a patient directly infects people around them or contaminates surfaces with expelled respiratory droplets from coughing.

Legionella spp.

Legionella is a genus of Gram-negative bacterium. This genus contains over 50 species, two of which are pathogenic in humans; these being L. pneumophila and L. longbeachae. All of these bacteria can cause Legionellosis, a relatively common cause of nosocomial and community acquired pneumonia. Legionella pneumophila is a flagellated bacterium which is a non-encapsulated, aerobic bacillus of approximately 2μm in length with a single, polar flagellum. The genome of this bacterium is over 3Mbp long and encodes for around 3000 genes. There are several serotypes of L. pneumophila, of which serotype 1 is responsible for 70-90% of cases. The natural environment of L. pneumophila is hot and moist with a supply of nutrition from the sediment. These environmental features can be found in storage facilities for drinking water leading to infection of individuals by inhalation. After inhalation L. pneumophila avoids the defences of the upper respiratory tract and targets the alveolar. Here, macrophages and epithelial cells can be infected by the bacteria via phagocytosis. The generated phagosome does not fuse with the lysosome and so the bacteria avoid destruction. After several hours within the host cell, endosome enclosure allows bacterial multiplication. The bacteria express genes known to be involved in necrosis and exit the host cell and spread intracellularly.

L. pneumophila infection is known as legionellosis which can cause Legionnaires' disease or Pontiac fever. Legionnaires' disease initially presents in fever, headache, aching joints, lack of energy or tiredness that can progress into, cough, diarrhoea, nausea, abdominal pain and eventually pneumonia, which can lead to coma and death. Pontiac Fever can cause symptoms including fever, headache, tiredness, loss of appetite, muscle and joint pain, nausea, coughing but usually desists within 5 days without medical treatment. Legionella micdadei is the second most commonly isolated Legionella species and is responsible for approximately 60% of Legionella pneumonias other than those caused by L. pneumophila. The pneumonia caused is a variant of Legionnaires' disease called Pittsburgh Pneumonia and is thought to occur mainly in immunocompromised patients. The natural environment of Legionella longbeachae is in damp soil and is known to infect humans via inhalation of particles from contaminated soil or compost. This bacterium is thought to cause pneumonia in people with diseases of the immune system.

Mycoplasma pneumoniae

Mycoplasma pneumoniae is a small bacterium of the Mycoplasmataceae family that infects the mucosal membrane of the respiratory tract and causes tracheobronchitis and pneumonia. The bacterium has a small genome of around 816k nucleotides arranged in a double-stranded circular chromosome. This bacterium lacks a cell wall, the machinery for making essential organic compounds, preventing it from living outside of a host therefore making it an obligate, pathogenic species.

M. pneumoniae attaches to the epithelial cells of the host respiratory tract using adhesion proteins. It is then thought that the bacterium fuses with the membrane of the host cell, after which it produces hydrogen peroxide and other oxide radicals that cause damage to the host cells. This in turn attracts neutrophils to the infection site resulting in cytokine release and immune system activation.

The results of this bacterial infection cause symptoms that include coughing and fever. Due to the lack of a cell wall in this species, antibiotics that target the bacterial cell wall are not effective and so usual treatments include a course of erythromycin.

Pneumocystis spp.

Pneumocystis jirovecii (formerly known as Pneumocystis carinii) is a fungus of the Pneumocystidaceae family, which causes Pneumocystis Pneumonia in human hosts. Inhalation of P. jirovecii allows entry to the host lungs where it adheres to the epithelium of the alveoli. Here the cysts initially undergo meiosis and subsequently mitosis in three stages allowing an increase of the intracystic nuclei from two to eight. At the end of the third stage, excystment allows release of the trophic form of the protozoa which can be up to 4µm in diameter. The trophic form undergoes binary fission to produce many haploid trophic forms that conjugate and produce early cysts. Within the host, the protozoa only cause disease in immuno-supressed or immunocompromised individuals. In these cases, a form of

pneumonia called Pneumocystis Pneumonia (PCP) can be seen. This disease presents with symptoms including a cough, shortness of breath and fever, although the infection can spread to other organs including the liver, kidneys and spleen. A combination of antibiotics is typically used to treat the infection with a treatment course running for up to three weeks.

Chlamydia spp.

Chlamydia pneumonia and Chlamydia psittaci are a species of Chlamydia, an obligate intracellular bacterium. It is a small (0.2-1 µm), Gram-negative bacterium, that infects humans and is a major cause of pneumonia. It has a genome of 1.0-1.3 mb with 1,073 open reading frames, encoding between 900 and 1,050 proteins. As an obligate bacterium, Chlamydia requires a host cell in which to proliferate. Between host infections, Chlamydia exists as a non-biologically active elementary body which allows it to persist in the environment for a limited time. The elementary body of Chlamydia can travel from the lungs of an infected individual to an uninfected individual through expelled droplets from coughing. Upon being taken up into host cells, the elementary body transforms into a reticulate body where it replicates within endosomes. Once replication has been completed, reticulate cells can shift back into elementary bodies that are released, often resulting in host cell death. Chlamydia psittaci is predominantly an avian pathogen but can cause sporadic zoonotic disease in humans after exposure to infected birds. Transmission of Chlamydia psittaci between humans is rare but possible.

Typically, Chlamydia infection is similar to other upper respiratory tract infections, with symptoms including coughing and pharyngitis, resulting in sore throats and low-grade fevers. If left, the infection can develop into lower respiratory tract infections such as bronchitis and pneumonia.

Specificity

The genesig™PLEX Atypical Pneumonia kit is designed for the in vitro detection of *Legionella spp*., *P. jirovicii, P. carinii*, *M. pneumoniae*, *B. pertussis*, *B. parapertussis*, *B. holmesii*, *C. pneumoniae* and *C. psittaci*.

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 *Pneumocystis murina*, *Pneumocystis wakefieldiae*, *Pneumocystis oryctolagi*, and *Pneumocystis canis* which would give a signal in the Tube 1 VIC channel.

This kit is predicted to cross react with *Bordetella bronchiseptica* and *Bordetella hinzii* which would give a signal in the Tube 2 FAM channel.

This kit is predicted to cross react with *Chlamydiifrater phoenicopterid, Chlamydiifrater volucris, Candidatus Amphibiichlamydia salamandrae, Parachlamydiaceae, Neochlamydia, Neochlamydia hartmannellae, Candidatus Metachlamydia lacustris, Criblamydia sequanensis, Estrella lausannensis, Rossellomorea aquimaris, Paenibacillus, Waddlia chondrophila* and *Simkania negevensis*. This would give a signal in the Tube 2 VIC channel.

Waddlia chondrophila and *Simkania negevensis* are referred to as Chlamydia-like organisms, due to their high genetic sequence similarity. Simkania negevensis has been associated with lower respiratory tract infections in children and young adults. Waddlia chondrophila's has been associated with miscarriage and tubal infertility, it's role in respiratory disease has not been conclusively determined.

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 Tube 1 primer/probe mix (100 reactions BROWN)**
  FAM, VIC, ROX and Cy5 labelled (see table below)

Target	Fluorophore
Legionella spp.	FAM
Pneumocystis spp.	VIC
M. pneumoniae	ROX
Internal extraction control	Cy5

- **Multiplex Tube 2 primer/probe mix (100 reactions BROWN)**
  FAM, VIC and Cy5 labelled (see table below)

Target	Fluorophore
Bordetella	FAM
Chlamydia spp.	VIC
Internal extraction control	Cy5

- **Multiplex Tube 1 positive control template (RED)**
- **Multiplex Tube 2 positive control template (RED)**
- **Internal extraction control DNA (BLUE)**
- **4x Lyophilised oasig™ Master Mix (SILVER)**
- **4x oasig™ resuspension buffer (BLUE)**
- **2x Template preparation buffer (YELLOW)**
  For resuspension of the positive control template
- **2x 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.

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 two positive controls that contains templates for the 3 targets in the test Tube 1 and 2 targets in test Tube 2. The kit positive control tube 1 will give a *Legionella spp*. signal through the FAM channel, and a *Pneumocystis spp.* signal through the VIC channel and a *M.pneumoniae* signal through the ROX channel. The kit positive control tube 2 will give a *Bordetella* signal through the FAM channel, and *Chlamydia spp.* signal through the VIC channel. Each time the kit is used,

at least one positive control reaction must be included for each tube 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 each 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 Tube 1 primer/probe mix (**BROWN**)	110µl
Multiplex Tube 2 primer/probe mix (**BROWN**)	110µl

3. **Resuspend the internal control template and positive control templates 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	
Multiplex Tube 1 Positive control template (**RED**)*	500µl
Multiplex Tube 2 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 Tube 1 positive control contains templates for *Legionella spp.*, *Pneumocystis spp*., *M.pneumoniae* and Tube 2 positive control contains templates for *Bordetella* and *Chlamydia spp*. 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 and Cy5 channels.

Interpretation of results

Positive control

The Tube 1 positive control well should give an amplification plot through the FAM channel (Legionella spp.), the VIC channel (Pneumocystis spp.) and the ROX channel (M.pneumoniae). The Tube 2 positive control well should give an amplification plot through the FAM channel (Bordetella) and the VIC channel (Chlamydia spp.). 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

Multiplex Tube 1 data interpretation

Target (FAM/VIC/ROX)	Internal extraction control (Cy5)	Positive Control	Negative Control	Interpretation
FAM +	**+ / -**	**+**	**-**	**LEGIONELLA SPP. POSITIVE RESULT**
VIC +	**+ / -**	**+**	**-**	**PNEUMOCYSTIS SPP. POSITIVE RESULT**
ROX +	**+ / -**	**+**	**-**	**M. PNEUMONIAE POSITIVE RESULT**
-	**+**	**+**	**-**	**NEGATIVE RESULT**

Multiplex Tube 2 data interpretation

Target (FAM/VIC)	Internal extraction control (Cy5)	Positive Control	Negative Control	Interpretation
FAM +	**+ / -**	**+**	**-**	**BORDETELLA POSITIVE RESULT**
VIC +	**+ / -**	**+**	**-**	**CHLAMYDIA SPP. POSITIVE RESULT**
-	**+**	**+**	**-**	**NEGATIVE RESULT**

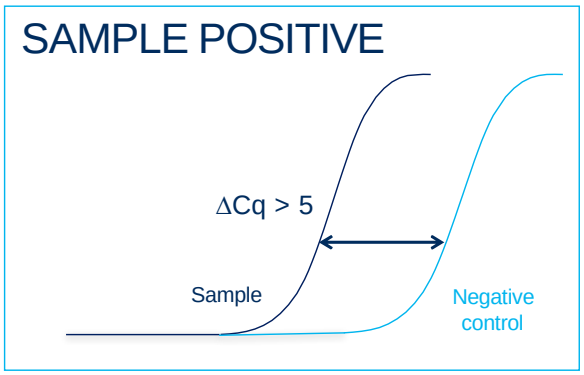
Interpretation of controls (applicable to both tubes)

Target (FAM/VIC/ROX)	Internal extraction control (Cy5)	Positive Control	Negative Control	Interpretation
+ / -	**+ / -**	**+**	**≤35**	**EXPERIMENT FAILED Due to test contamination**
+ / -	**+ / -**	**+**	**>35**	*****
-	**-**	**+**	**-**	**SAMPLE PREPARATION FAILED**
+ / -	**+ / -**	**-**	**+ / -**	**EXPERIMENT FAILED**

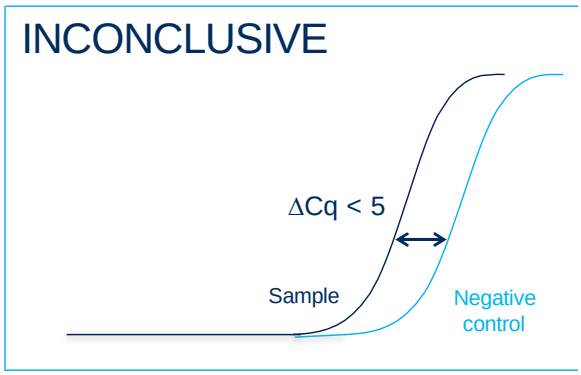
Positive control template (RED) is expected to amplify between Cq 16 and 23. Failure to satisfy this quality control criterion is a strong indication that the experiment has been compromised.

* 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.