Primerdesign™ Ltd

Chlamydiaceae (all species)

16s ribosomal RNA sequence

genesig® Advanced Kit

150 tests

GENESIG

Kits by Primerdesign

For general laboratory and research use only

Introduction to Chlamydiaceae (all species)

The bacterial family, Chlamydiaceae, contains two genera of closely related, Gram-negative pathogens. They are all aerobic, non-motile bacterium with a coccoid or rod-shaped cell body. The genus Chlamydia contains three species: Chlamydia muridarum, C. suis and C. trachomatis. The latter of which infects humans while the other species infect mice and swine respectively. The second genus, Chlamydophila represents the species Chlamydophila pneumoniae, C. pecorum, C. psittaci, C. abortus, C. felis and C. caviae. All species of the Chlamydiaceae family are around 0.1µm-0.8µm in length with genomes of approximately 1Mbp length in a circular arrangement usually with one plasmid.

All species within this family are obligate pathogens, and are metabolically inert outside of a host. Upon infection of a host, the dispersal form of the bacterium, known as elementary bodies, attach to the host cells and are subsequently engulfed by phagocytosis. Once within the cell, the elementary bodies transform into reticulate bodies which undergo binary fission in the host cells' inclusion bodies to gives rise to new elementary bodies which are then released by cell lysis allowing for spread and infection of new host cells.

Each species target specific hosts and can cause a range of illnesses that vary between host species.
The most commonly known species is C. trachomatis, the causative agent of Chlamydia, the sexually transmitted disease. This bacterium can also be transmitted from mother to child during pregnancy and infect the eyes causing conjunctivitis. The genital infection causes urethritis, epididymitis and prostatitis in males and Pelvic Inflammatory Disease (PID) in females with an increased risk of contracting HIV. Transmission of the bacteria occurs via contact with infected bodily fluids which then infect mucosal membranes. The infection can be treated with a course of antibiotics.
Sexually transmitted infections in females are most often asymptomatic, but can manifest in chronic pain of the pelvic region, vaginal bleeding and painful urination. Infection of the ovaries, fallopian tubes or uterus causes Pelvic Inflammatory Disease (PID) which can lead to difficulties in conceiving, increased risk of ectopic pregnancy or infertility. Infections in males are more likely to be symptomatic, causing painful urination, discharge from the penis and swollen testicles and may eventually causes infertility if left untreated.
Eye infections that cause conjunctivitis or trachomas are thought to cause up to 15% of cases of blindness and C. trachomatis is therefore an important pathogen in this area.

Chlamydia muridarum causes pharyngitis, bronchitis, and pneumonitis in infected mice and hamsters and is most likely to be transmitted via contact with infected bodily fluids. While Chlamydia suis causes enteritis, pneumonia and conjunctivitis in infected swine and is likely to be transmitted in the same way.

Specificity

The Primerdesign genesig Kit for Chlamydiaceae (all species) (Chlamydia) genomes is designed for the in vitro quantification of Chlamydia genomes. 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.

This kit is based on a paper by Wons et. al (2017):
Wons J, Meiller R, Bergua A, Bogdan C, Geißdörfer W. Follicular Conjunctivitis due to Chlamydia felis—Case Report, Review of the Literature and Improved Molecular Diagnostics. Frontiers in Medicine. 2017;4:105. doi:10.3389/fmed.2017.00105.

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

Kit contents

- **Chlamydia specific primer/probe mix (150 reactions BROWN)**
  FAM labelled
- **Chlamydia positive control template (for Standard curve RED)**
- **Internal extraction control primer/probe mix (150 reactions BROWN)**
  VIC labelled as standard
- **Internal extraction control DNA (150 reactions BLUE)**
- **Endogenous control primer/probe mix (150 reactions BROWN)**
  FAM labelled
- **RNase/DNase free water (WHITE)**
  for resuspension of primer/probe mixes
- **Template preparation buffer (YELLOW)**
  for resuspension of internal control template, positive control template and standard curve preparation

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. However, it is designed to work well with all processes that yield high quality RNA and DNA with minimal PCR inhibitors.

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

This kit is intended for use with oasig or PrecisionPLUS 2X qPCR Master Mix.

Pipettors and Tips

Vortex and centrifuge

Thin walled 1.5 ml PCR 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 -20°C 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.

If a standard curve dilution series is prepared this can be stored frozen for an extended period. If you see any degradation in this serial dilution a fresh standard curve can be prepared from the positive control.

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 (An internal PCR control is supplied to test for non specific PCR inhibitors). 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 Chlamydia detection kits have very high priming efficiencies of >95% and can detect less than 100 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.

Trademarks

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Principles of the test

Real-time PCR

A Chlamydia specific primer and probe mix is provided and this can be detected through the FAM channel.

The primer and probe mix provided exploits the so-called TaqMan® principle. During PCR amplification, forward and reverse primers hybridize to the Chlamydia DNA. A fluorogenic probe is included in the same reaction mixture which consists of a DNA probe labeled 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 copy number determination and as a positive control for the PCR set up, the kit contains a positive control template. This can be used to generate a standard curve of Chlamydia copy number / Cq value. Alternatively the positive control can be used at a single dilution where full quantitative analysis of the samples is not required. 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 the target Chlamydia gene worked properly in that particular experimental scenario. 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 this component 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 validate any positive findings 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.

Chlamydia DNA is known to be highly prevalent within the air and environment generally and the negative control may therefore give a late positive signal due to environmental contamination. The interpretation of results section of this handbook gives guidance on how to interpret results where environmental contamination is evident.

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.

A separate primer and probe mix are supplied with this kit 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 Chlamydia 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.

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 Chlamydia primers. A poor endogenous control signal may indicate that the sample did not contain sufficient biological material.

Resuspension protocol

To minimize 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 mixes in the RNase/DNase free water supplied, according to the table below:**
   To ensure complete resuspension, vortex each tube thoroughly.

Component - resuspend in water	Volume
Pre-PCR pack	
Chlamydia primer/probe mix (BROWN)	165 µl
Internal extraction control primer/probe mix (BROWN)	165 µl
Endogenous control primer/probe mix (BROWN)	165 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 each tube thoroughly.

Component - resuspend in template preparation buffer	Volume
Pre-PCR heat-sealed foil	
Internal extraction control DNA (BLUE)	600 µl
Post-PCR heat-sealed foil	
Chlamydia Positive Control Template (RED) *	500 µl

* 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 manufacturers 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	10 µl
Chlamydia primer/probe mix (BROWN)	1 µl
Internal extraction control primer/probe mix (BROWN)	1 µl
RNase/DNase free water (WHITE)	3 µl
Final Volume	**15 µl**

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	10 µl
Endogenous control primer/probe mix (BROWN)	1 µl
RNase/DNase free water (WHITE)	4 µl
Final Volume	**15 µl**

3. **Pipette 15µl of each mix into individual wells according to your qPCR experimental plate set up.**

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

5. **Pipette 5µl of DNA template 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 a standard curve is included for quantitative analysis, prepare a reaction mix according to the table below:**

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

7. **Preparation of 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

8. **Pipette 5µl of standard template into each well for the standard curve according to your experimental plate set up.**
The final volume in each well is 20µl.

qPCR amplification protocol

Amplification conditions using oasig or PrecisionPLUS 2X qPCR Master Mix.

	Step	Time	Temp
Cycling x50	Enzyme activation	2 min	95 °C
	Denaturation	10 s	95 °C
	DATA COLLECTION *	60 s	60 °C

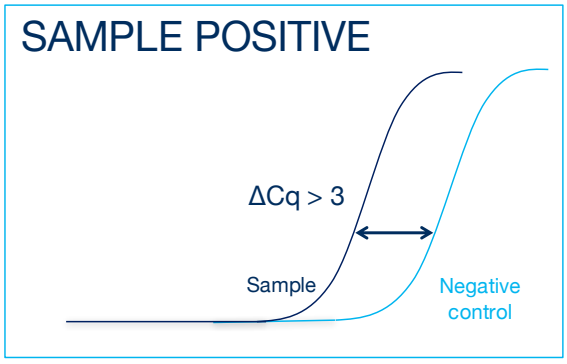
* Fluorogenic data should be collected during this step through the FAM and VIC channels

Interpretation of results

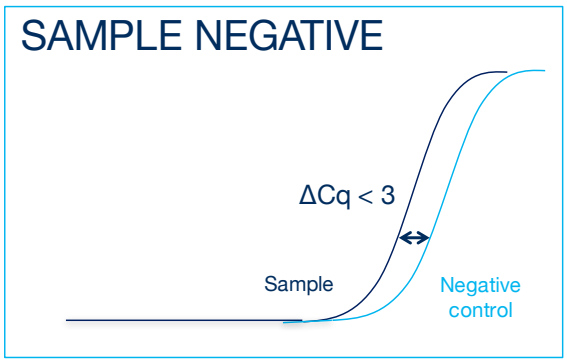
Target (FAM)	Internal control (VIC)	Positive control	Negative control	Interpretation
≤ 30	+ / -	+	-	**POSITIVE QUANTITATIVE RESULT** calculate copy number
> 30	+	+	-	**POSITIVE QUANTITATIVE RESULT** calculate copy number
> 30	-	+	-	**POSITIVE QUALITATIVE RESULT** do not report copy number as this may be due to poor sample extraction
-	+	+	-	**NEGATIVE RESULT**
+ / -	+ / -	+	≤ 30	**EXPERIMENT FAILED** due to test contamination
+ / -	+ / -	+	> 30	*
-	-	+	-	**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 > 30, the sample must be reinterpreted based on the relative signal strength of the two results:



If the sample amplifies > 3 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 < 3 Cq earlier than the negative control then the positive sample result is invalidated and a negative call is the correct result.

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 Chlamydia 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 a given 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.