Primerdesign™ Ltd

R01002

genesig™PLEX Bacterial Meningitis Real-Time PCR Multiplex Kit

Kit version: 1

N. meningitidis (ctrA gene)

S. pneumoniae (glycoside hydrolase family 1 protein)

H. influenzae (hpd3 gene)

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

N. meningitidis

Neisseria meningitidis is a non-motile, aerobic, Gram-negative bacterium of the *Neisseriaceae* family and is a causative agent of meningitis and other meningococcal diseases. There are 13 serotypes of this species and the genomes that have been wholly sequenced are around 2.2Mbp in length, arranged as a single, circular chromosome.

This bacterium resides on mucosal membranes such as in the respiratory tract and can be transmitted between hosts by infected saliva as aerosols. Pili facilitate attachment of the bacterium to the host cell membrane, allowing the bacterium to grow by utilising host provided salts, amino acids and iron. The iron is gained from heme in the blood and due to this, infection sites are often seen in combination with an aggregation of blood cells. Once the bacterium has entered the host blood stream, it can gain access to the meninges of the brain resulting in inflammation. A polysaccharide capsule containing variable surface protein allows the bacterium to evade phagocytosis by the host immune system. Another virulence factor is the production of a lipopolysaccharide endotoxin which is upregulated during the bacterial growth phase.

Infection usually begins with symptoms including fatigue, fever and headaches but rapidly progresses to coma and in around 10% of cases, death. As soon as infection with this bacterium is suspected, a course of antibiotics can be prescribed.

S. pneumoniae

Streptococcus pneumoniae, or *pneumococcus*, is a Gram-positive, alpha-hemolytic diplococcus bacterium and a member of the genus *Streptococcus*. A significant human pathogen, *S. pneumoniae* was recognized as a major cause of pneumonia in the late 19th century and is the subject of many humoral immunity studies.

Despite the name, the organism causes many types of infection other than pneumonia, including acute sinusitis, otitis media, meningitis, osteomyelitis, septic arthritis, endocarditis, peritonitis, pericarditis, cellulitis, and brain abscess. *S. pneumoniae* is the most common cause of bacterial meningitis in adults and children, and is one of the top two isolates found in otitis media. *Pneumococcal pneumonia* is more common in the very young and the very old.

S. pneumoniae can be differentiated from *Streptococcus viridans*, which is also alpha hemolytic, using an optochin test, as *S. pneumoniae* is optochin sensitive. The encapsulated, gram-positive coccoid bacteria have a distinctive morphology on gram stain, the so-called, "lancet shape." It has a polysaccharide capsule that acts as a virulence factor for the organism; 91 different capsular types are known, and these types differ in virulence, prevalence, and extent of drug resistance.

S. pneumoniae has several virulence factors, including the polysaccharide capsule mentioned earlier, that help it evade a host's immune system. It has pneumococcal surface proteins that inhibit complement-mediated opsonization, and it secretes IgA1 protease that will destroy secretory IgA produced by the body.

The risk of pneumococcal infection is much increased in persons with impaired IgG synthesis, impaired phagocytosis, or defective clearance of pneumococci. In particular, the absence of a functional spleen, through congenital asplenia, splenectomy, or sickle-cell disease predisposes individuals to a more severe course of infection, most commonly seen in post-splenectomy patients.

H. influenzae

Haemophilus influenzae is a Gram-negative bacterium of the *Pasteurellaceae* family. This non-motile coccobacillus has a genome of around 1.8Mbp arranged in a single circular conformation coding for 1740 genes and is around 1µm in length.

H. influenzae resides in the mucosal membrane of the nasopharynx and can be transmitted via respiratory droplets. The bacteria colonise the nasopharynx via interactions between outer membrane proteins and host mucin. Other bacterial proteins are then expressed that impair ciliary activity, minimising the effect of the mucociliary escalator. With bacteria within the mucus, a combination of pili and outer membrane proteins mediate attachment to the host epithelial cells allowing colonisation. Once the bacteria have colonised the nasopharynx, individuals invade the host cells and migrate between the epithelial cells by disrupting tight junctions. The bacteria evade the host immune response by expressing proteases that act on IgA present within the respiratory tract.

H. influenzae species can be classified into 2 types, encapsulated on nonencapsulated. Nonencapsulated species often cause non-invasive infections. Of the encapsulated strains, type b (Hib) is thought to be the most pathogenic although is only found in around 7% of the population whereas other strains can be found in around 75% of people. Infection with Hib is most commonly seen in children and can cause bacteremia and acute bacterial meningitis and less frequently epiglottitis, cellulitis and ear infections as well as being involved in respiratory tract infections. Nonencapsulated strains are more frequently seen in adults and can cause pneumonia. In 1988 a Hib vaccine was introduced which has dramatically lowered the number of cases of Hib infections.

Specificity

The genesig™PLEX Bacterial Meningitis kit is designed for the in vitro detection of S. pneumoniae, H. influenzae and N. meningitidis.

The assays within this kit are predicted to detect over 95% of sequences available on the NCBI database at the time of design. The target of the N. menigitidis assay is the ctrA gene as recommended by the CDC. At least 16% of N. menigitidis isolates lack the ctrA gene, however these are non-groupable (not encapsulated) and rarely cause disease as infections are opportunistic.

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.

The N. meningitidis assay is predicted to cross react with Neisseria brasiliensis, Neisseria weixii and Neisseria animalis which would give signal in the ROX channel.

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
S. pneumoniae	FAM
H. influenzae	VIC
N. meningitidis	ROX
Internal extraction control	Cy5

- **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 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 an *S. pneumoniae* signal through the FAM channel, an *H. influenzae* signal through the VIC channel and an *N. meningitidis* 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 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**)	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 N.meningitidis, S.pneumoniae, H.influenzae. 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 (S.pneumoniae), the VIC channel (H.influenzae) and the ROX channel (N.meningitidis). 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.

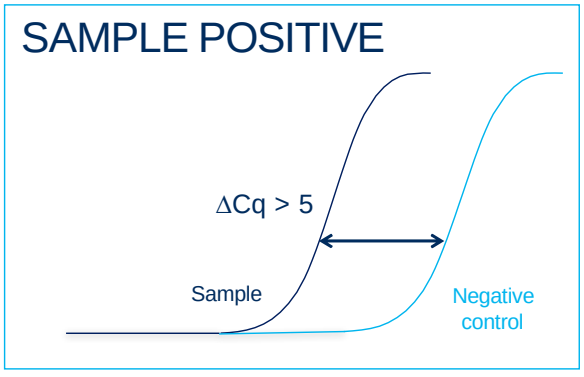
Summary of data interpretation

Target (FAM/VIC/ROX)	Internal extraction control (Cy5)	Positive Control	Negative Control	Interpretation
FAM +	**+ / -**	**+**	**-**	**S. PNEUMONIAE POSITIVE RESULT**
VIC +	**+ / -**	**+**	**-**	**H. INFLUENZAE POSITIVE RESULT**
ROX +	**+ / -**	**+**	**-**	**N. MENINGITIDIS POSITIVE RESULT**
-	**+**	**+**	**-**	**NEGATIVE RESULT**

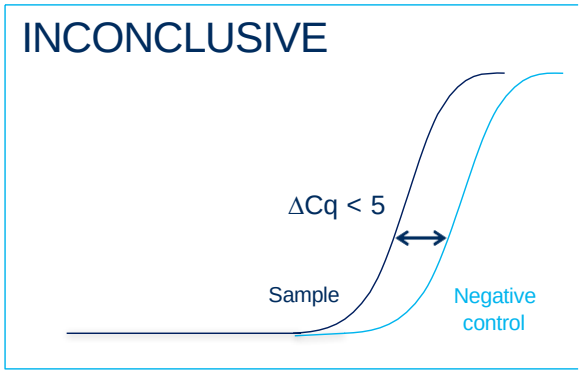
+ / -	**+ / -**	**+**	**≤35**	**EXPERIMENT FAILED** Due to test contamination
+ / -	**+ / -**	**+**	**>35**	*****
-	**-**	**+**	**-**	**SAMPLE PREPARATION FAILED**
+ / -	**+ / -**	**-**	**+ / -**	**EXPERIMENT FAILED**

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.