Primerdesign

R01207

Pneumocystis jirovecii

Kit version: 1

Target region:

Kexin-like serine protease (kex1) gene

genesig® Complete DNA Kit

150 tests

GENESIG

Kits by Primerdesign

For general laboratory and research use only

Product Description

This genesig® Complete qPCR detection kit targets kexin-like serine protease (kex1) gene from Pneumocystis jirovecii (P. jirovecii). Pneumocystis jirovecii is a fungi of the Pneumocystidaceae family, the genome of which has not been fully sequenced. This species of fungus causes Pneumocystis Pneumonia in human hosts.

Inhalation of P. jirovecii allows entry to the host lungs here 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 2 to 8. 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 immune-compromised 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 3 weeks.

Specificity

The kit is designed for the in vitro quantification of Pneumocystis jirovecii genomes within human samples, and to have a broad detection profile.

Please note that this species has been reclassified in the past and was previously known as P.carinii f. sp. Hominis. The target sequence within the KEX1 gene has previously been shown to be a good genetic marker for P. jirovecii in other clinical real-time PCR based studies (Rohner P, et.al 2009). This gene has no known homology with other species in the P. carinii at the primer binding sites. The primers and probe sequences in this kit have 100% homology with a broad range of clinically relevant reference sequences based on a comprehensive bioinformatics analysis.

The dynamics of genetic variation means that new sequence information may become available after the initial design. If you require further information or have a specific question about the detection profile of this kit then please send an e-mail to techsupport@primerdesign.co.uk and our team will answer your question.

Kit contents

Quantity	Component	Tube	Cap Colour
1	**P.jirovecii primer/probe mix (150 reactions)** FAM labelled		BROWN
1	**P.jirovecii positive control template**		RED
1	**Internal extraction control primer/probe mix (150 reactions)** VIC labelled as standard		BROWN
1	**Internal extraction control DNA (150 reactions)**		BLUE
1	**Endogenous control primer/probe mix (150 reactions)** FAM labelled, Target: human ACTB as standard		BROWN
3	**oasig® lyophilised 2X qPCR Master Mix (50 reactions per glass vial)**		SILVER
4	**oasig® resuspension buffer** For resuspension of the lyophilised Master Mix (and lyophilised ROX, if required)		BLUE
1	**oasig® lyophilised ROX** ROX passive reference dye that (if required) can be added to oasig® lyophilised 2X qPCR Master Mix.		BROWN
1	**RNase/DNase free water** for resuspension of the primer/probe mixes		WHITE
3	**Template preparation buffer** for resuspension of internal control template, positive control template and standard curve preparation		YELLOW

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 nucleic acid with minimal PCR inhibitors.

Pipettors and filter tips

Vortex and centrifuge

1.5 ml microtubes

qPCR plates or reaction tubes

Kit storage and stability

This kit is stable for shipping at ambient temperature but should be stored at -20°C upon 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.

Primer Design Ltd does not recommend using the kit after the expiry date stated on the pack.

Suitable sample material

This kit can be used with all types of samples from various origins. Please ensure that the extracted nucleic acid samples are suitable in terms of purity, concentration, and DNA integrity. An internal extraction control is provided to test for non-specific PCR inhibitors.

Dynamic range of test

Under optimal PCR conditions the kit can achieve priming efficiencies between 90-110% and detect less than 100 copies of target template. If running a positive control standard curve for a quantitative result, and an efficiency of between 90% to 110% is not achieved, then the run should be repeated with a freshly prepared standard curve.

Principles of the test

Real-time PCR

A target 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 target DNA. A fluorogenic probe is 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 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 target 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 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. It is also known as a No Template Control or NTC.

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.

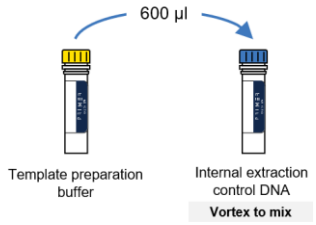
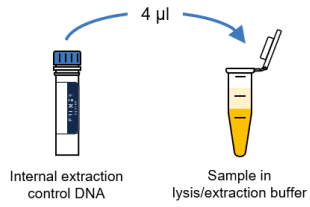
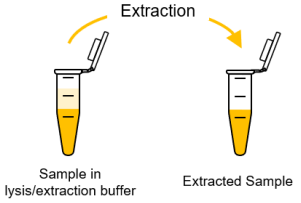
A separate primer and probe mix is supplied with this kit to detect the exogenous DNA using qPCR. The primers are present at PCR limiting concentrations which allows multiplexing with the target sequence primers. Amplification of the control DNA does not interfere with detection of the target DNA even when present at low copy number. The Internal control is detected through the VIC channel and gives a Cq value of 28+/-3 depending on the level of sample dilution.

Endogenous control

A primer/probe mix for detection of the endogenous control gene is included in the kit, which allows confirmation of a valid biological sample from the host. Detection of the endogenous control is through the FAM channel, and it is therefore NOT possible to perform a multiplex reaction with the target specific primer/probe mix. Amplification of the endogenous control may depend on the sample type used. Please note that if samples from a different species are used, the endogenous control may not be appropriate, but the internal extraction control is advised to be used.

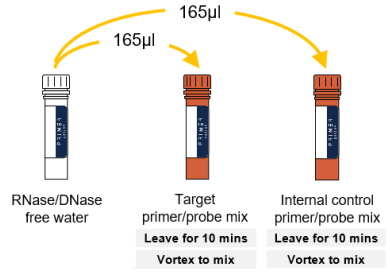
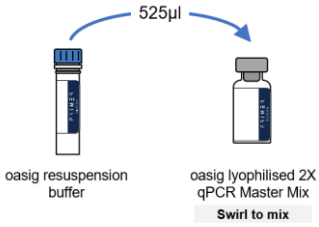
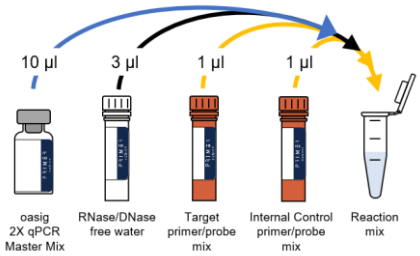
genesig® Complete DNA Workflow

1. Sample Preparation

A) Resuspend the internal control	**B)** Add to the sample in lysis/extraction buffer *	**C)** Extract sample(s) with chosen protocol
		

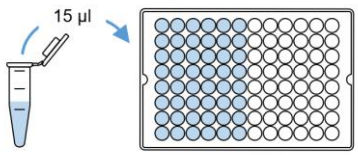
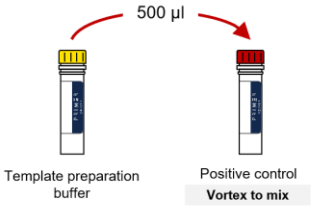
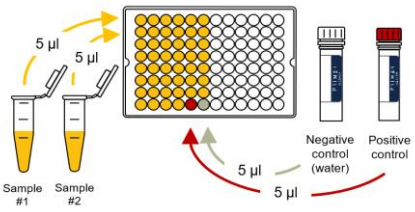
*NOTE – DO NOT add internal extraction control DNA directly to the unprocessed biological sample as this will lead to degradation and a loss in signal.

2. Reaction Mix Preparation

A) Resuspend the primer/probe mix	**B)** Resuspend the oasig® Master Mix	**C)** Combine to make the reaction mix **
		

**NOTE – Displayed amounts are required for each reaction. Multiply each volume by the number of reactions required including overage.

3. Plate Setup

A) Add 15 µl of reaction mix to each well	**B)** Resuspend the Positive Control	**C)** Add 5 µl of the sample(s) to the well(s).
		

Resuspension protocol

To minimize 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 mixes in the RNase/DNase free water supplied, according to the table below:**
   To ensure complete resuspension, allow primer/probe mix to rehydrate for 10 minutes at room temperature. Vortex each tube thoroughly, followed by pipetting up and down 10 times. Failure to mix well can produce poor kit performance.

Component - resuspend in water	Volume
Pre-PCR pack	
P.jirovecii 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 oasig® lyophilised 2X qPCR Master Mix in oasig® resuspension buffer, according to the table below:**

Component - resuspend in oasig® resuspension buffer	Volume
oasig® lyophilised 2X qPCR Master Mix (**SILVER**)	525 µl

Please note: If the kit is intended to be used with platforms that use ROX as a passive reference dye, please proceed to the **ROX Passive Reference Dye Handling Protocol** at the end of this handbook.

4. **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	
P.jirovecii 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 4 µl of the Internal extraction control DNA (BLUE) to each sample in DNA lysis/extraction buffer.**

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® lyophilised 2X qPCR Master Mix (SILVER)	10 µl
P.jirovecii 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. **(Optional – if you wish to run an endogenous control reaction for each sample, you will need to purchase additional Master Mix for this). For each DNA sample prepare an endogenous control reaction according to the table below.**
   This control reaction will provide useful information regarding the quality of the biological sample.

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

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

4. **Pipette 5 µl of DNA template into each well, according to your experimental plate set up.**
   For negative control wells use 5 µl of RNase/DNase free water (WHITE). For positive control wells use 5 µl of the positive control template (**RED**). The final volume in each well is 20 µl.

5. (Optional) Standard curve preparation for quantitative analysis.

For quantitative analysis of the samples a standard curve dilution series can be prepared using the positive control template (**RED**). This is not required for qualitative analysis.

5.1 Reaction mix preparation for the standard curve.

Include sufficient reactions for each dilution of the standard curve.

Component	Volume
oasig® lyophilised 2X qPCR Master Mix (SILVER)	10 µl
P.jirovecii primer/probe mix (**BROWN**)	1 µl
RNase/DNase free water (WHITE)	4 µl
Final Volume	**15 µl**

5.2 Preparation of a 10-fold standard curve dilution series.

a. Pipette 90 µl of template preparation buffer (YELLOW) into 5 tubes and label them tube 2-6. The neat positive control tube (**RED**) is considered tube 1.

b. Pipette 10 µl of positive control template (**RED**) into tube 2.

c. Vortex thoroughly.

d. Change pipette tip and pipette 10 µl from tube 2 into tube 3.

e. Vortex thoroughly.

Repeat steps d and e to complete the dilution series.

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

5.3. Pipette 15 µl of reaction mix and 5 µl of the respective standard into each well for the standard curve according to your plate set up.

The final volume in each well is 20 µl.

qPCR amplification protocol

Recommended amplification conditions when using oasig® lyophilised 2X qPCR Master Mix.

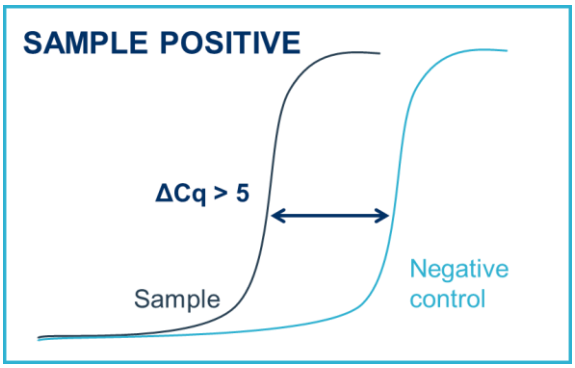
	Step	Time	Temp
	Enzyme activation	2 min	95 °C
Cycling x50	Denaturation	10 s	95 °C
	DATA COLLECTION *	60 s	60 °C

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

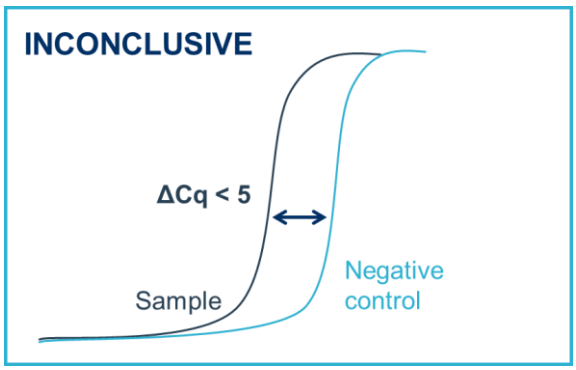
Interpretation of results

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

*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.

Positive Control

The positive control template is expected to amplify between Cq 16 – 23 in the FAM channel. Failure to satisfy this quality control criterion is a strong indication that the experiment has been compromised and 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 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.

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.

ROX Reference Dye Handling Protocol

ROX is recommended for platforms that use ROX as a passive reference dye. Use the table below to see if ROX addition is required for your hardware platform. If ROX is required, then follow the instructions below.

- Resuspend the Lyophilised ROX (**BROWN**) in the correct volume of the oasig® resuspension buffer (**BLUE**) according to the table below.
- Add resuspended ROX to each Master Mix vial at the correct level.

Real time PCR platform	ROX resuspension volume	ROX addition per Master Mix vial
Applied Biosystems 7700, 7000, and 7900, 7300 StepOne, StepOnePLUS and ViA7 platforms, Roche capillary Lightcyclers	100 µl	20 µl
All Stratagene platforms	200 µl	15 µl
Applied Biosystems 7500 platform Quantstudio™	700 µl	10 µl
All other machines	NOT REQUIRED	NOT REQUIRED

Once ROX passive reference dye has been added to the oasig® lyophilised 2X qPCR Master Mix (**SILVER**), proceed with the rest of the protocol.

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 app ropriate regulatory authorities in the country of use. During the warranty period Primer Design Ltd 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.

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