Primerdesign™ Ltd

Taylorella equigenitalis, Klebsiella Pneumonia and Pseudomonas aeruginosa, TKP

genesig®PLEX kit

100 tests

GENESIG

Kits by Primerdesign

For general laboratory and research use only

Introduction to T.equigenitalis, K.pneumoniae and P.aeruginosa

T.equigenitalis, K.pneumoniae and P.aeruginosa are common pathogens associated with horse breeding and STDs.

Affected stallions with seminal vesiculitis shed bacteria into their semen with *Pseudomonas aeruginosa* and *Klebsiella pneumonia* being reported associated as common pathogens causing seminal vesiculitis. Additionally, *Pseudomonas aeruginosa* and *Klebsiella pneumonia* are often environmental contaminants and can be mistaken for pathogens. Therefore, simply culturing them from a semen sample or the penile tissues may have no significance. It is significant when there are neutrophils in the semen, and mares bred with this semen develop signs of post breeding endometritis that match the organism obtained from the stallion. Cultures of a precollection urethral sample that does not have the pathogen, followed by a post collection sample of the urethra suggests the organism is being emitted with the semen. The history may also show that the mares bred to this stallion return to estrus early or have a higher incidence of endometritis. These historical features are the indications that the stallion has a STD type problem.

The disease caused by T.equigenitalis, Contagious Equine Metritis is an inflammatory disease of the reproductive tract of the mare which usually results in temporary infertility. It is a nonsystemic infection, the effects of which are restricted to the reproductive tract of the mare. When present in the mare, clinical signs include endometritis, cervicitis and vaginitis of variable severity and vaginal discharge.

K. pneumoniae bacteria can be spread by overzealous cleaning of the stallion's genitalia. Using disinfectant, instead of water and mild soap, the normal, commensal flora that should be present are removed, allowing the Klebsiella bacteria to propagate. It can also be transferred to the mare via artificial insemination and poses a significant problem if it ascends to the stallion's internal organs, causing lifelong shedding. Most often, Klebsiella pneumoniae results in endometritis, which requires treatment with antibiotics.

Carrier mares and stallions act as reservoirs of T.equigenitalis, but stallions, because they mate with numerous mares, play a much more important role in dissemination of the bacterium. The urogenital membranes of stallions become contaminated at coitus, leading to a carrier state that may persist for many months or years. Failure to observe appropriate hygienic measures when breeding mares and stallions may also spread the organism.

Specificity

The genesigPLEX kit is designed for the detection of *T.equigenitalis, K.pneumoniae and P.aeruginosa.*

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. They therefore have a very broad detection profile.

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

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

Target	Fluorophore
K.pneumoniae	FAM
P.aeruginosa	VIC
T.equigenitalis	ROX
Internal control	Cy5

- **Multiplex positive control template (RED)**
- **Internal control template (BLUE)**
- **Lyophilised oasig™PLEX Master Mix (BLUE)**
- **oasigPLEX™ resuspension buffer (BLUE)**
- **Template preparation buffer (YELLOW)**
  for resuspension of 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.

Pipettors and tips

Vortex and centrifuge

Thin walled 1.5ml tubes

qPCR plates

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 $1X10^8$ and $1X10^2$ copies of target template.

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Principles of the test

Real-time PCR

Individual primer and probes designed for each target 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 three targets in the test. 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 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 primer and probe mix are supplied multiplexed within this kit to detect the exogenous DNA template (also provided) 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 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 under optimal extraction conditions.

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	
Multiplex primer/probe mix (**BROWN**)	110µl

3. **Resuspend the positive control template and internal extraction 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
Post-PCR heat-sealed foil	
Positive control template (**RED**)*	500µl
Internal extraction control template (**BLUE**) *	600ul

*** 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 oasigPLEX Master Mix in oasigPLEX resuspension buffer, according to the table below:**

Component – resuspend in oasigPLEX resuspension buffer	Volume
Lyophilised oasigPLEX Master Mix (**BLUE**)	525µl

DNA extraction

1. Complete the DNA extraction according to the manufacturer's recommended protocols.

RT-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
oasigPLEX Master Mix (**BLUE**)	10µl
Multiplex primer/probe mix (**BROWN**)	1µl
RNase/DNase free water (**WHITE**)	4µl
Final volume	**15µl**

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

3. **Pipette 5µl of RNA 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 template for all three targets. The final volume in each well is 20µl.

RT-qPCR amplification protocol

Amplification conditions using oasigPLEX 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 positive control well should give an amplification plot through the FAM channel (*K.pneumoniae),* VIC channel (*P.aeruginosa*) and ROX channel (*T.equigenitalis*). There is no internal extraction 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 bacteria.

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 extraction control

The signal obtained from the internal extraction control reaction will vary according to the yield from the extraction. Early signal indicates the presence of good DNA yield, whilst a late signal suggests that the extraction may have been poor.

Sample data

Presence of the bacterial targets are detected in the channels indicated in the kit contents section. Positive signals indicate positive tests for those bacteria. It may be possible for samples to contain more than one bacteria therefore positive results in across multiple channels may be present.

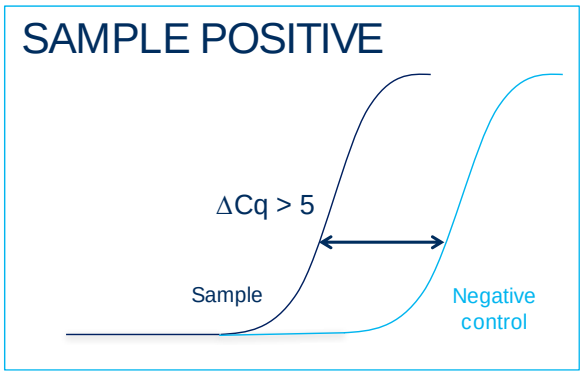
Summary of data interpretation

Target (FAM/VIC/ROX)	Internal extraction control (Cy5)	Positive Control	Negative Control	Interpretation
FAM +	**+ / -**	**+**	**-**	**K.pneumoniae POSITIVE RESULT**
VIC +	**+ / -**	**+**	**-**	**P.aeurginosa POSITIVE RESULT**
ROX +	**+ / -**	**+**	**-**	**T.equigenitalis POSITIVE RESULT**
-	**+**	**+**	**-**	**NEGATIVE RESULT**

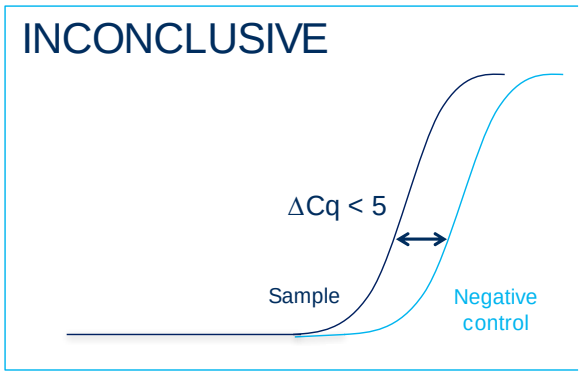
+ / -	**+ / -**	**+**	**≤35**	**EXPERIMENT FAILED** Due to test contamination
+ / -	**+ / -**	**+**	**>35**	*****
-	**-**	**+**	**-**	**SAMPLE PREPARATION FAILED**
+ / -	**+ / -**	**-**	**+ / -**	**EXPERIMENT FAILED**

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.