Primerdesign™ Ltd

Streptococcus equi subspecies *equi* & *Streptococcus equi* subspecies *zooepidemicus*

genesig®PLEX kit

100 tests

GENESIG

Kits by Primerdesign

For general laboratory and research use only

Introduction

S.equi-equi

Streptococcus equi subspecies *equi* (*S.equi-equi*) is an infectious, contagious disease of *Equidae* characterized by abscesses forming in the lymph tissue of the upper respiratory tract. The resultant respiratory difficulty gives rise to the common name of 'Strangles'. *S.equi-equi* is highly contagious and produces high morbidity and low mortality (~1%) in susceptible populations. Transmission occurs via expelled fomites and direct contact with infectious exudates such as mucus. Carrier animals are important for maintenance of the bacteria and initiation of outbreaks on premises previously free of disease. The incubation period is 3–14 days, and then presents with a fever (39.4°–41.1°C). 2 days after fever presents the further symptoms of nasal discharge, swollen lymph nodes beneath the jaw and depressed mood can become apparent. The submandibular lymphadenopathy can cause difficulty in breathing and swallowing, resulting in strained respiratory noises and giving rise to the common name of 'Strangles' for the disease.

S.equi-zooepidemicus

Streptococcus equi subspecies *zooepidemicus* is a non-motile, non-sporulating, encapsulated, Gram-positive coccoid bacterium. *S.equi zooepidemicus* is one of the two subspecies of *Streptococcus equi*; it is suggested to be the species from which subspecies *equi* has been derived and is part of the normal bacterial flora in horses. The similarity between the DNA of the two subspecies *zooepidemicus* and *equi*, is over 92%. It is isolated from wound infections of horses, and it has been isolated from other mammals such as cows, rabbits, and swine, and in some cases, also isolated from humans through throat swabs.
Streptococcus equi zooepidemicus is a pathogen of animals primarily affecting horses, in whom it can cause diseases in the upper respiratory tract, uterus, umbilicus, and wounds. In cows, *S. equi zooepidemicus* can cause mastitis, an inflammation in mammalian breast. In humans, *S. zooepidemicus* may cause glomerulonephritis and rheumatic fever. Meningitis and purulent arthritis have also been reported. As for other animals such as rabbits and swine, it can cause septicemia, which is a systematic inflammatory response to infections. This microorganism has also caused fibrinous pericarditis, fibrinous pleuritis, and pneumonia in sheep.

Specificity

The genesigPLEX kit is designed for the in vitro detection of *Streptococcus equi* subspecies *equi* (**SE**) and *Streptococcus equi* subspecies *zooepidemicus* (**SZ**).

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 and Cy5 labelled (see table below)

Target	Fluorophore
S.equi-equi (**SE**)	FAM
S.equi-zooepidemicus (**SZ**)	VIC
Internal extraction control	Cy5

- **Endogenous control primer/probe mix (150 reactions BROWN)**
- **Multiplex positive control template (RED)**
- **Internal extraction control DNA (BLUE)**
- **Lyophilised oasig™PLEX Master Mix (BLUE)**
- **oasig™ 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 RNA/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.

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

Primerdesign™ is a trademark of Primerdesign Ltd.

genesig® is a registered trademark of Primerdesign Ltd.

oasig™ is a trademark of Primerdesign Ltd.

The PCR process is covered by US Patents 4,683,195, and 4,683,202 and foreign equivalents owned by Hoffmann-La Roche AG. BI, ABI PRISM® GeneAmp® and MicroAmp® are registered trademarks of the Applera Genomics (Applied Biosystems Corporation). BIOMEK® is a registered trademark of Beckman Instruments, Inc.; iCycler™ is a registered trademark of Bio-Rad Laboratories, Rotor-Gene is a trademark of Corbett Research. LightCycler™ is a registered trademark of the Idaho Technology Inc. GeneAmp®, TaqMan® and AmpliTaqGold® are registered trademarks of Roche Molecular Systems, Inc., The purchase of the Primerdesign™ reagents cannot be construed as an authorization or implicit license to practice PCR under any patents held by Hoffmann-LaRoche Inc.

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 cDNA. 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 2 targets in the test. The kit positive control will give an SE (*S.equi-equi*) signal through the FAM channel and an SZ (*S.equi-zooepidemicus*) signal through the VIC 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 pathogen 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 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 amplification of the control DNA also indicates that PCR inhibitors are not present at a high concentration. The primers and probe necessary to detect the internal extraction control are included in the multiplex primer and probe mix. The amplification of the internal control does not affect the sensitivity of the test and is detected separately through the Cy5 channel. The Internal control will give a Cq value of 28+/-3 but this can vary greatly depending on the efficiency of sample extraction and level of sample dilution.

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

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 mixes 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
Endogenous control primer/probe mix (**BROWN**)	165µl

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**)	600µl
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 oasigPLEX Master Mix in oasig resuspension buffer, according to the table below:**

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

DNA extraction

The internal extraction control DNA can be added to either 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 4ul of the Internal extraction DNA (**BLUE**) to each sample in DNA lysis/extraction buffer.

2. Complete the DNA extraction according to the manufacturer's recommended 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
oasigPLEX Master Mix (**BLUE**)	10µl
Multiplex primer/probe mix (**BROWN**)	1µl
RNase/DNase free water (**WHITE**)	4µl
Final volume	**15µl**

2. **For each DNA sample prepare an endogenous control reaction according to the table below (optional):**
   This control reaction provides information regarding the quality of the biological sample. The test is run in a separate well and is not part of the multiplex.

Component	Volume
oasigPLEX Master Mix (**BLUE**)	10µl
Endogenous control primer/probe mix (**BROWN**)	1µl
RNase/DNase free water (**WHITE**)	4µl
Final volume	**15µl**

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

4. **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.

5. **Pipette 5µl of positive control template into each well according to your plate set up.**
   The positive control contains template for SE and SZ. The final volume in each well is 20µl.

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 and Cy5 channels.

Interpretation of results

Positive control

The positive control well should give an amplification plot through the FAM channel (SE) and the VIC channel (SZ). 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 pathogen.

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 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 BKV 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 reaction will vary according to the amount of biological material present in each 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.

Sample data

Presence of the pathogens are detected in the channels indicated in the kit contents section. Positive signals indicate positive tests for those pathogens. It may be possible for samples to contain both pathogens, therefore positive results in the FAM and VIC channels may be present.

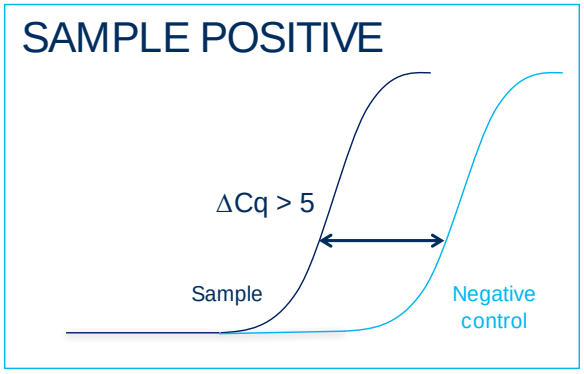
Summary of data interpretation

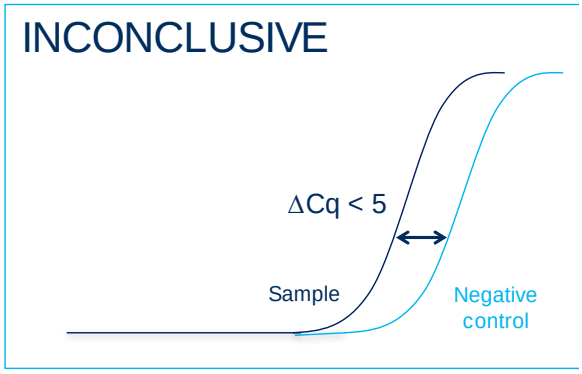
Target (FAM/VIC)	Internal Extraction Control (Cy5)	Positive Control	Negative Control	Interpretation
FAM +	**+ / -**	**+**	**-**	**SE POSITIVE RESULT**
VIC +	**+ / -**	**+**	**-**	**SZ POSITIVE RESULT**
-	**+**	**+**	**-**	**NEGATIVE RESULT**

+ / -	**+ / -**	**+**	**≤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.