Primer Design Ltd

R01059

Equine Strangles

Kit version: 1

Target regions:

S. equi–equi (eqbE_3 gene)

S. equi zooepidemicus (fabG_2 gene)

genesig®PLEX Kit

100 tests

GENESIG

Kits by Primerdesign

For general laboratory and research use only

Introduction to Streptococcus equi

Streptococcus equi are gram-positive, coccoid bacteria with a genome of approximately 2.3 Mb. Belonging to the Streptococcus genus and consisting of two subspecies, Streptococcus equi subspecies equi (S. equi-equi) and Streptococcus equi subspecies zooepidemicus (S. equi zooepidemicus) they typically appear in pairs or long chains under a microscope. They are catalase-negative, an important test to differentiate streptococci from staphylococci and represent as beta-haemolytic when cultured on blood agar. Determination can be difficult as colony morphology between the subspecies can be identical. They are characterised as Lancefield group C and considered to rarely cause disease in humans.

S. equi-equi is an infectious, contagious disease that is highly adapted to and produces clinical disease in the Equidae family (horses, donkeys and mules). S. equi-equi is highly contagious and produces high morbidity and low mortality in susceptible populations. It is characterised by the formation of abscesses in the lymph tissue of the upper respiratory tract. The formation of abscesses and inflammation that occurs restricts the airways, causing difficulty breathing and swallowing. The resulting strained respiratory noises are responsible for the common name ‘strangles’. Symptoms of ‘Strangles’ include high fever, nasal discharge, abscesses on other areas of the body (Bastard Strangles). Transmission occurs via expelled fomites and direct contact with infectious material such as mucus. Clinically ill horses should be isolated to prevent the spread of the pathogen.

S. equi zooepidemicus is closely related to S. equi-equi with genome similarity of ~90%. Studies have indicated that S. equi-equi is derived from S. equi zooepidemicus. It is an opportunistic pathogen that is a normal part of equine bacterial flora. It is responsible for a wide variety of diseases in susceptible horses including, pneumonia, abortion, chronic skin wounds and epididymitis (the structure that collects and stores sperm). Studies have shown that zoocins released by S. equi zooepidemicus can kill S. equi-equi, therefore strangles abscesses that rupture can quickly become colonised by S. equi zooepidemicus.

Specificity

The genesig®PLEX kit for Equine Strangles is designed for the in vitro detection of Streptococcus equi. subsp equi and Streptococcus equi. subsp zooepidemicus. At the time of most recent review the assays within this kit are predicted to detect over 95% of sequences available from the NCBI database.

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

- **2x Multiplex primer/probe mix (50 reactions, BROWN)**
  FAM, ROX and VIC labelled (see table below).

Target	Fluorophore
S. equi-equi	FAM
S. equi zooepidemicus	ROX
Internal control (IEC)	VIC

- **1x Multiplex positive control template (RED)**
- **1x Internal extraction control DNA (150 reactions, BLUE)**
- **2x Lyophilised oasig® Master Mix (50 reactions, SILVER)**
- **2x oasig® resuspension buffer (BLUE)**
- **1x RNase/DNase free water (WHITE)**
  for resuspension of primer/probe mixes
- **1x Template preparation buffer (YELLOW)**
  for resuspension of positive control template

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.

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

Individual primers and probes designed for each target have been combined into a single reaction and these can be detected through 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. 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 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 S. equi-equi signal through the FAM channel and an S. equi zooepidemicus 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 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. 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 VIC channel and gives a Cq value of 28+/-3.

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 mix 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	
Multiplex primer/probe mix (**BROWN**)	55 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	
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.**

4. **Resuspend the lyophilised OneStep Master Mix in oasig® resuspension buffer, according to the table below:**

Component - resuspend in template preparation buffer	Volume
Lyophilised oasig® Master Mix (SILVER)	525 µl

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.**

2. **Complete DNA extraction according to the manufacturer's 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
Lyophilised oasig® Master Mix (SILVER)	10 µl
Multiplex primer/probe mix (**BROWN**)	1 µl
RNase/DNase free water (WHITE)	4 µl
Final Volume	**15 µl**

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

3. **Pipette 5µl 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). The final volume in each well is 20 µl.

4. **Pipette 5µl of positive control template into each well, according to your experimental plate set up.**
   For positive control contains templates for S. equi-equi and S. equi zooepidemicus. The final volume in each well is 20 µl.

qPCR amplification protocol

Amplification conditions when using oasig® Master Mix.

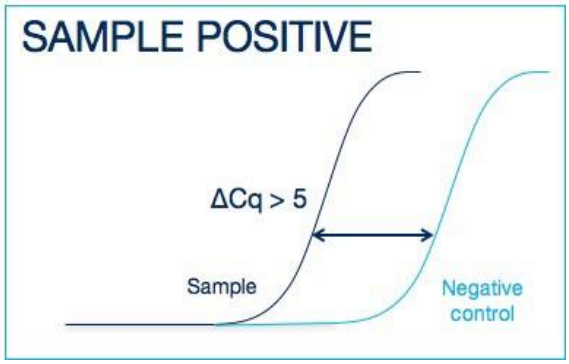
	Step	Time	Temp
	Enzyme activation	2 min	95 °C
Cycling x50	Denaturation	10 s	95 °C
	DATA COLLECTION *	60 s	60 °C

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

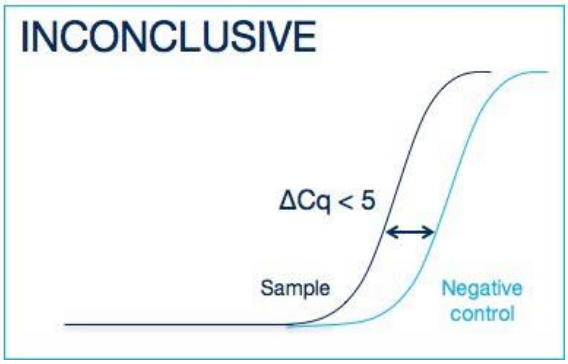
Interpretation of results

Target	Internal control (VIC)	Positive control	Negative control	Interpretation
FAM+	**+ / -**	**+**	**-**	**S. equi-equi POSITIVE RESULT**
ROX+	**+ / -**	**+**	**-**	**S. equi zooepidemicus POSITIVE RESULT**
-	**+**	**+**	**-**	**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 well should give an amplification plot through the FAM channel (S. equi-equi) and the ROX channel (S. equi zooepidemicus). There is no internal control template within the positive control so the VIC 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 plot) 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 and ROX channels may be present.

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