Primerdesign™ Ltd

Feline calicivirus

Open Reading Frame 1

(orf1)

genesig® Advanced Kit

150 tests

GENESIG

Kits by Primerdesign

For general laboratory and research use only

Introduction to Feline calicivirus

Feline calicivirus (FCV) is a single stranded RNA virus which typically causes a self-limiting oral and upper respiratory tract disease in cats. FCV belongs to the Caliciviridae family of viruses which also includes significant human pathogens such as norovirus and sapovirus. The genome is approximately 7.7 kb long, it is polyadenylated at the 3' end and bound by a virally-encoded protein at the 5'-end. It encodes three open reading frames (ORFs). ORF 1 codes for the non-structural proteins including a viral protease and an RNA-dependent RNA polymerase. ORF 2 codes for the major capsid protein and shows sequence variability depending on strain, and ORF 3 encodes a minor structural protein.

Cats can be infected with FCV via the nasal, oral or conjunctival routes. Indirect transmission is also possible, especially in an environment such as a cattery where secretions may contaminate cages, feed and personnel. The virus can persist for several weeks when on dried surfaces at room temperature and longer in colder wetter conditions. Most infected cats shed the virus in secretions within 30 days of being infected. Some remain carriers and shedders for life, although most will eliminate the disease. The virus predominantly replicates in the oral and respiratory tissues, although some strains vary in their tissue tropisms and pathogenicity and it may also affect the joints or skin.

The most common clinical sign of the disease is oral ulcerations which are usually observed on the tip of the tongue and can rupture with necrosis of the overlying epithelium. Lesions can also be observed in the joints of infected cats which can lead to lameness as a result of acute synovitis. Other symptoms that are observed include conjunctivitis, sneezing, fever, anorexia, ocular and nasal discharge, excessive salivation, gingivitis and oedema. Diagnosis of FCV is difficult without specific tests because the symptoms are similar to other feline respiratory diseases, especially feline herpes virus.

Specificity

The Primerdesign genesig Kit for Feline calicivirus (FCV) genomes is designed for the in vitro quantification of FCV genomes. The kit is designed to have a broad detection profile. Specifically, the primers represent 100% homology with over 95% of the NCBI database reference sequences available at the time of design.

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.

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

- **FCV specific primer/probe mix (150 reactions BROWN)**
  FAM labelled
- **FCV positive control template (for Standard curve RED)**
- **Internal control control primer/probe mix (150 reactions BROWN)**
  VIC labelled as standard
- **Internal extraction control RNA (150 reactions BLUE)**
- **Endogenous control primer/probe mix (150 reactions BROWN)**
  FAM labelled
- **RNase/DNase free water (WHITE)**
  for resuspension of primer/probe mix
- **Template preparation buffer (YELLOW)**
  for resuspension of positive control template and internal control template

Reagents and equipment to be supplied by the user

Real-Time PCR Instrument

Ability to detect FAM and VIC channels

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 RNA with minimal PCR inhibitors.

oasig® Lyophilised OneStep RT-qPCR Master Mix or Precision®PLUS OneStep 2X RT-qPCR Master Mix

This kit is recommended for use with Primerdesign OneStep 2X RT-qPCR master mixes.

Pipettors and Tips

Vortex and Centrifuge

Thin walled 1.5ml PCR reaction 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 RNA/DNA integrity. Always run at least one negative control with the samples. To prepare a negative control, replace the template RNA 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 1×10^8 and 1×10^2 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.

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Principles of the test

Real-Time PCR

A FCV specific primer and probe mix is provided and this can be detected through the FAM channel.

The primer/probe mix provided exploits the so-called TaqMan® principle. During PCR amplification, forward and reverse primers hybridise 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 qPCR 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 FCV 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 FCV 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 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 RNA extraction control

When performing RNA extraction, it is often advantageous to have an exogenous source of RNA template that is spiked into the lysis buffer. This control RNA is then co-purified with the sample RNA and can be detected as a positive control for the extraction process. Successful co-purification and qPCR for the control RNA also indicates that PCR inhibitors are not present at a high concentration.

A separate qPCR primer/probe mix are supplied with this kit to detect the exogenous RNA using qPCR. The PCR primers are present at PCR limiting concentrations which allows multiplexing with the target sequence primers. Amplification of the control cDNA does not interfere with detection of the FCV target cDNA 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.

Internal RNA extraction 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 FCV 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/probe mix is in the base of the tube and is not spilt upon opening the tube.

2. Resuspend the FCV 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	
FCV 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 internal control template and positive control template and in the template resuspension 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 RNA (**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.**

RNA extraction

The internal extraction control RNA can be added either to the RNA lysis/extraction buffer or to the RNA sample once it has been resuspended in lysis buffer.

DO NOT add the internal extraction control RNA 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 RNA (**BLUE**) to each sample in RNA lysis/extraction buffer per sample.

2. Complete RNA extraction according to the manufacturer's protocols.

OneStep RT-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 poured proceed immediately to the OneStep amplification protocol. Prolonged incubation of reaction mixes at room temperature can lead to PCR artifacts that reduce the sensitivity of detection.

1. **For each RNA sample prepare a reaction mix according to the table below:**
   Include sufficient reactions for positive and negative controls.

Component	Volume
oasig Onestep or PrecisionPLUS Onestep 2X RT-qPCR Master Mix	10 µl
FCV 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. **For each RNA sample prepare an endogenous control reaction according to the table below (optional):**
   This control reaction will provide crucial information regarding the quality of the biological sample.

Component	Volume
oasig OneStep or PrecisionPLUS OneStep 2X RT-qPCR Master Mix	10 µl
Endogenous control primer/probe mix (**BROWN**)	1 µl
RNase/DNase free water (WHITE)	4 µl
Final volume	**15 µl**

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

4. **Prepare sample RNA templates for each of your samples.**

5. **Pipette 5µl of RNA template into each well according to your experimental plate set up.**
   For negative control (NTC) wells use 5µl of RNase/DNase free water. The final volume in each well is 20µl.

6. **If a standard curve is included for quantitative analysis prepare a reaction mix according to the table below:**

Component	Volume
oasig OneStep or PrecisionPLUS OneStep 2X RT-qPCR Master Mix	10 µl
FCV primer/probe mix (**BROWN**)	1 µl
RNase/DNase free water (WHITE)	4 µl
Final volume	**15 µl**

7. **Preparation of standard curve dilution series.**

   1) Pipette 90µl of template preparation buffer into 5 tubes and label 2-6
   2) Pipette 10µl of positive control template (RED) into tube 2
   3) Vortex thoroughly
   4) Change pipette tip and pipette 10 µl from tube 2 into tube 3
   5) Vortex thoroughly

   Repeat steps 4 and 5 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

8. **Pipette 5µl of of standard template into each well for the standard curve according to your plate set-up**
   The final volume in each well is 20µl.

One Step RT-qPCR amplification protocol

Amplification conditions using oasig OneStep or PrecisionPLUS OneStep 2X RT-qPCR Master Mix

	Step	Time	Temp
	Reverse Transcription	10 mins	55°C
	Enzyme activation	2 mins	95°C
X 40 cycles	Denaturation	10 secs	95°C
	DATA COLLECTION*	60 secs	60°C

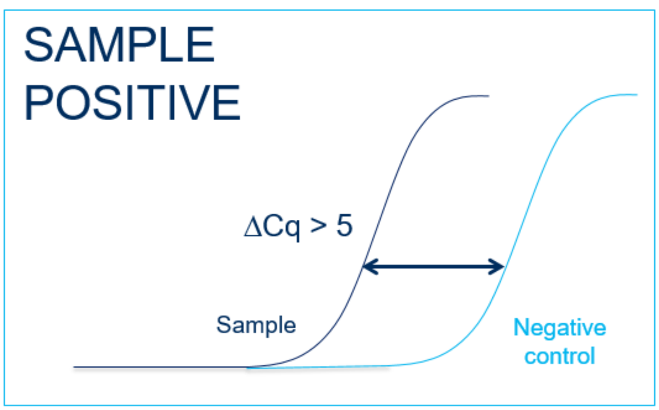
* Fluorogenic data should be collected during this step through the FAM and VIC channels.

Interpretation of results

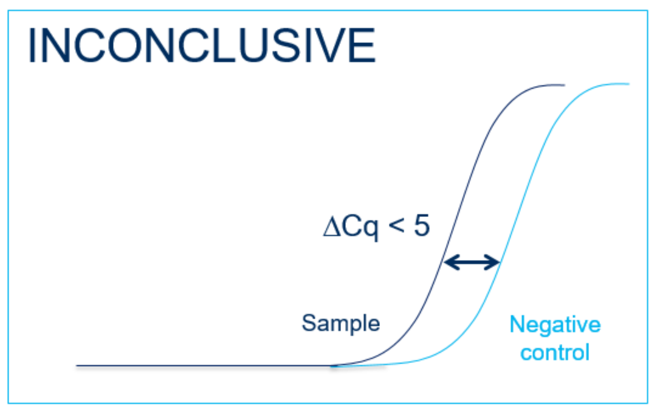
Target (FAM)	Internal control (VIC)	Positive Control	Negative Control	Interpretation
≤30	+ / -	+	-	**POSITIVE QUANITATIVE RESULT** calculate copy number
>30	+	+	-	**POSITIVE QUANITATIVE RESULT** calculate copy number
>30	-	+	-	**POSITIVE QUALITATIVE RESULT** Do not report copy number as this could be due to poor sample extraction
-	+	+	-	**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 these quality control criteria 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.

Internal PCR control

The Cq value obtained with the internal control will vary significantly depending on the extraction efficiency, the quantity of RNA added to the RT and PCR reaction and the individual machine settings. Cq values of 28±3 are within the normal range. When amplifying a FCV 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.