# IDEXX FIP Virus RealPCR™ Test Validation Studies

## Introduction

There are two recognized coronavirus biotypes that infect cats, each causing different biological outcomes: feline enteric coronavirus (FECV) and feline infectious peritonitis virus (FIPV). The FIP Virus RealPCR™ Test differentiates between the less virulent or nonpathogenic FECV biotype and the virulent or pathogenic FIPV biotype, allowing for more definitive diagnosis or exclusion of feline infectious peritonitis (FIP). Diagnosing FIP remains difficult for practitioners because there are many different clinical presentations and laboratory abnormalities that are not specific for FIP. The availability of this new test can help veterinarians reach a confirmed diagnosis so that cat owners can make informed decisions regarding treatment and prepare themselves for the ultimate outcome.

## Research leading to the introduction of the FIP Virus RealPCR Test

Recently, Dr. Peter Rottier and his group at Utrecht University identified two mutations in the fusion peptide of the spike (S) gene of feline coronavirus (FCoV) in cats with FIPV that were absent in cats with FECV.[1] In coronaviruses, the S protein functions in cell entry and is responsible for receptor attachment and membrane fusion. It was postulated that these virulence mutations enable FIPV to efficiently infect and replicate in macrophages and to spread into tissues more efficiently, whereas replication of FECV is restricted primarily to the epithelial cells of the gut.[1] Based on these findings and in collaboration with the Utrecht University researchers, IDEXX's molecular diagnostic laboratory developed and validated the FIP Virus RealPCR Test, which can identify each mutation separately.

## When to perform the FIP Virus RealPCR Test

The FIP Virus RealPCR Test is an additional tool that can be used to help confirm the diagnosis of FIP in cats where there are clinical signs and other compatible laboratory findings of this disease. The test should be performed on effusions (abdominal or pleural fluid) in cats with suspect wet FIP and on tissue biopsy or aspirates (internal immune organs, such as spleen and mediastinal lymph nodes) in cats with suspect dry FIP. Testing of whole blood specimens is not recommended because FCoV viral load is inconsistent and, if present, of low magnitude, which affects the success rate of biotyping. Feces will not be accepted for biotyping because of the possibility of a local gastrointestinal (GI) tract infection with a FECV strain while a FIPV strain is causing clinical signs systemically in tissues.

## Technical basis of the FIP Virus RealPCR Test

The FIP Virus RealPCR Test is based on the allelic discrimination principle: Specific short, fluorescent hydrolysis probes either detect the two single FIPV nucleotide mutations at a particular fluorescent wavelength or the FECV wild-type sequence at a different fluorescent wavelength. Fluorescent raw data is used to calculate a ratio between the two fluorescent values. The clinically characterized samples of validation study 1 (see below) were used to determine that to correctly biotype the FCoV in a specimen as either FECV or FIPV, the degree of fluorescence for the reported biotype needed to exceed twice that of the other biotype. This reduces the risk of picking up rare random mutations at these sites that are not causing FIP.

Worldwide, there are many different FCoV strains or variants that have variable spike gene nucleotide sequences. In order to cover this variability, a set of many primers is combined into the real-time polymerase chain reaction (PCR) for each mutation. In our experience, our assay detects greater than 97% of strains in Europe and North America. Studies are underway to determine how many FCoV variants we can detect in samples of Asian origin.

## Test results and interpretations of the FIP Virus RealPCR Test

All diagnostic specimens are tested for FCoV viral load using the IDEXX Feline Coronavirus (FCoV) RealPCR™ Test, which detects the 7b gene. Based on the outcome of the typing assay run in parallel with the 7b assay, there are five different FCoV biotype results possible:

| FCoV biotype result | Interpretation |
|---|---|
| **FIPV** | FCoV has mutated into the FIPV biotype. In a cat with clinical signs, this supports the diagnosis of FIP. If clinical signs are absent, the FIPV biotype indicates the cat is at high risk for developing FIP and should be monitored closely. |
| **FECV** | FCoV has not mutated and the cat is unlikely to have FIP. |
| **Mixed biotype** | Indicates that both the nonmutated FECV biotype and the mutated FIPV biotype are detectable in the specimen. This indicates that the cat is at risk for developing FIP and should be closely monitored. If clinical signs of FIP are present, repeat testing in 1–2 weeks is recommended. |
| **Indeterminate** | Indicates that a high FCoV viral load is present in the specimen submitted, which in a nonfecal specimen is suspicious for FIP. However, the FCoV could not be biotyped, and therefore, FIP cannot be confirmed. An inability to biotype in a high-viral-load specimen may reflect new strain variations or a recombinant serotype II FCoV, which carries the canine spike gene. |
| **Below limit of detection** | FCoV cannot be typed because there were insufficient viral particles to permit biotyping. FIP cannot be ruled out. This result is common with whole blood specimens but can occur with any specimen type. Consider submitting an alternate specimen type. (e.g., peritoneal, pleural or CSF fluid, or tissue biopsy or aspirate) |

# Validation studies performed for the FIP Virus RealPCR™ Test

## Validation study 1

**Scope: To validate that the FIP Virus RealPCR™ Test accurately differentiates the FECV wild-type spike gene sequence from the FIPV mutant spike gene sequence, discovered by Dr. Rottier**

**Study design:** 186 samples were collected in the Netherlands between 2006 and 2011. The diagnostic performance, predictive values, and accuracy of the FIP Virus RealPCR Test was determined from cats, who were either healthy or had confirmed FIP based on clinical presentation, histological assessment, and immunohistochemistry (IHC).

**Results:** For the 164 cats where a biotype result was obtained, the diagnostic sensitivity was 98.7% (1 cat with FIP receiving FECV biotype result), diagnostic specificity was 100% (no healthy cat receiving FIP virus biotype result), and overall accuracy of the test was 99.4%.

Healthy cats were defined as clinically nonsuspicious for FIP but FCoV PCR positive in the feces. A total of 92 cases were included, of which 85 were biotyped as FECV (absence of mutations), no samples (0%) were biotyped as FIPV, 2 samples (2.2%) contained insufficient viral load for the biotyping assay, and 5 samples (5.4%) had high enough viral load but no biotyping result could be obtained.

In the FIP group, there were 94 clinically characterized cats confirmed to have FIP by histology and IHC. Of these, 78 cases were biotyped as FIPV (presence of one of two mutations) and one as FECV. Due to low viral load,14 cases (14.9%) could not be biotyped. One sample (1.3%) had sufficient viral load but could not be biotyped.

The following table gives an overview of the FIPV typing results in both groups:

| **FIP group:** | **94** | **Samples** |
|---|---|---|
| | 14 | Below limit of detection |
| | 1 | Indeterminate |
| | 1 | FECV |
| | 78 | FIPV |
| **Healthy group:** | **92** | **Samples** |
| | 2 | Below limit of detection |
| | 5 | Indeterminate |
| | 85 | FECV |
| | 0 | FIPV |
| **Total** | **186** | **Samples** |

Based on the results of this first sample set, the diagnostic performance was calculated:

| | | **Clinical score** | | **Performance** | |
|---|---|---|---|---|---|
| | | FIP | Non-FIP | | % |
| **FIPV typing RealPCR** | FIPV | 78 | 0 | Sensitivity | 98.7 |
| | FECV | 1 | 85 | Specificity | 100.0 |
| | | | | Positive predictive value | 100.0 |
| | | | | Negative predictive value | 98.8 |
| | | | | Accuracy | 99.4 |

**Conclusion:** This study validated that the FIP Virus RealPCR Test accurately differentiates the FECV wild-type spike gene sequence from the FIPV mutant spike gene sequence, with an **overall accuracy of 99.4%**.

## Validation study 2

**Scope: To confirm that the FIPV spike gene mutations detected by the FIP Virus RealPCR Test occur in cats outside the Netherlands**

In order to develop a broadly applicable diagnostic application, we needed to determine if the spike gene mutations identified by Peter Rottier, upon which the FIP Virus RealPCR Test was based, were not geographically specific. To help determine this, we reviewed the spike gene sequences deposited in a public database from defined sick FIP or healthy FECV cases.

**Study design:** The public GenBank® database was searched for registered FCoV spike gene sequences that came from countries other than the Netherlands and that contained the nucleotide region where the spike gene mutations are located.

**Results:** At the time of the database search, there were 542 FCoV spike gene sequences registered in GenBank. However, of these only 18 FCoV spike gene sequences registered were from outside the Netherlands and contained the area of the spike gene where the identified gene mutations occur. Of these 18 registered sequences,11 were from confirmed FIP cases from the United States; 3 were from confirmed FIP cases from Japan; 2 were from confirmed FIP cases from Germany; and only 2 were from confirmed non-FIP cases, one each from the U.S. and Taiwan. The FIPV mutant spike gene sequence was identified in all the confirmed FIP cases, and the FECV wild-type spike gene sequence was present in the non-FIP cases.

**Conclusion:** This study confirms that the FIPV spike gene mutations occur in cats with FIP from the U.S., Japan, and Germany.

## Validation study 3

**Scope: To prove that the FIP virus spike gene mutations occur in Japan and can be identified in FIP-confirmed cats**

**Study design:** A total of 33 samples collected from FIP-suspect cases based on clinical and laboratory data or histologic confirmation were assessed with the FIP Virus RealPCR Test.

**Results:** 17 samples (51%) typed as FIPV; 0 samples (0%) as FECV; 12 samples (36%) as below limit of detection; 0 samples (0%) as indeterminate; and 4 samples (12%) as FCoV negative. Sample types included ascites supernatant only (29) and kidney tissue (1), plasma (2), and serum (1). FCoV viral load was low in all but one case. The low FCoV viral load is in contrast to our typical diagnostic submissions and is explained by the absence of nucleated cells in the ascites supernatant and the lack of viral particles.

**Conclusion:** Despite the low FCoV viral load due to less-than-ideal sample types, this sample set confirmed that a) the spike gene mutations occur in Japan, and b) the spike gene mutations can be identified in FIP-compatible cases.

## Validation study 4

**Scope: A retrospective study to validate the FIP Virus RealPCR™ Test can differentiate FIPV from FECV in clinically sick cats**

**Study design:** We selected 87 samples (49 effusions and 38 whole blood) submitted to the IDEXX Reference Laboratories in 2013 for the diagnostic Feline Coronavirus (FCoV) RealPCR™ Test.

**Results:** Of the 49 effusions,18 (37%) tested FCoV negative and 31 (63%) tested FCoV positive. Of the 31 FCoV PCR-positive samples, 65% biotyped as FIPV, and 13% as FECV; 19% of FCoV positive samples contained a viral load that could not be biotyped (below limit of detection), and one sample could not be biotyped despite high viral load (indeterminate result). Importantly, most effusions (81%) contained sufficient viral load to be biotyped.

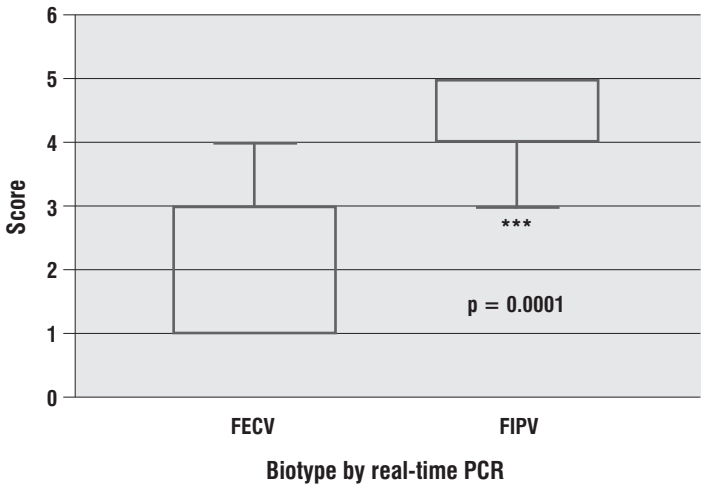
Of the 38 whole blood samples, only 8 (21%) were FCoV positive, 4 (50%) didn't contain sufficient viral load to be biotyped (below limit of detection), 2 (25%) biotyped as FIPV, one (13%) as FECV, and one (13%) could not be biotyped despite sufficiently high viral load. These results highlight why whole blood is not a recommended sample type for the FIP Virus RealPCR Test.

One important goal of this study was to obtain a negative control population of cats that were sick and had clinical signs that could possibly be attributed to FIP but that were ultimately diagnosed with another disease. History and physical examination findings were obtained from the primary veterinarian, available laboratory results were reviewed and the clinician's primary diagnosis was considered. An internal medicine specialist, Nancy Sanders, DVM, DACVIM, DACVECC, reviewed all available information and assigned a clinical score of 1–6 to each cat.

| Score | Definition | Criteria |
|---|---|---|
| 1 | Definitely not FIP | Definitive diagnosis of another disease by either histopathology, rapid response to appropriate treatment such as antibiotics, and/or cat alive and doing well after extended period of time |
| 2 | Probably not FIP | No definitive diagnosis available but strong support for another disease and/or cat alive and doing well after an extended period of time |
| 3 | Possibly not FIP | Clinical presentation and available laboratory results suggestive of another disease but not enough information classify more confidently |
| 4 | Possibly FIP | Clinical presentation and available laboratory results suggestive of FIP but not enough information to classify more confidently |
| 5 | Probably FIP | Clinical presentation, laboratory results, and fluid analysis highly supportive of diagnosis of FIP and/or histopathology supportive of FIP but immunohistochemistry not performed |
| 6 | Definitely FIP | Confirmation of FIP by histopathology and immunohistochemistry |

Statistical analysis using a Mann-Whitney t-test showed a highly significant difference between the two groups (figure 1, $p = 0.0001$), confirming a high correlation of FECV results in sick cats that did not have a clinical diagnosis of FIP, and of FIPV results in sick cats who were highly suspect or confirmed to have FIP.

**Figure 1.** Mann-Whitney t-test results.




**Conclusion:** This study confirmed that the FIP Virus RealPCR Test was able to differentiate FECV from FIPV in clinically sick cats. Importantly, no cat with a clinical score indicating absence of FIP-compatible disease was typed as FIPV.

## Validation study 5

**A prospective study to validate that the FIP Virus RealPCR Test can differentiate FIPV from FECV in clinically sick cats**

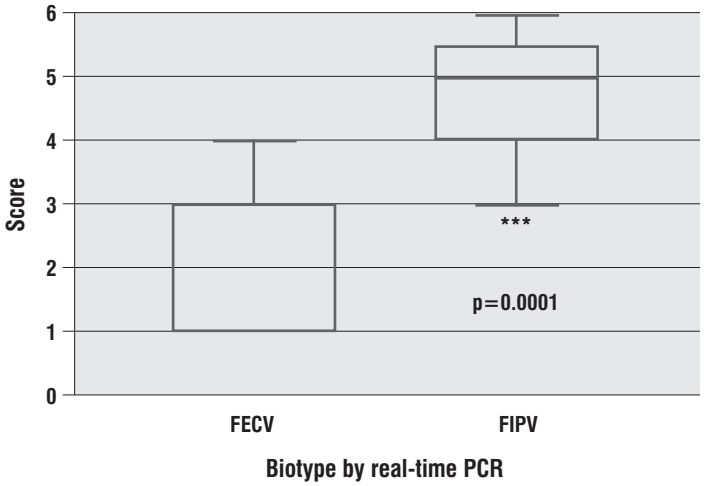
**Study design:** Nancy Sanders, DVM, DACVIM, DACVECC, an internal medicine specialist, identified during consultation with veterinarians, cats that were suspected of having FIP. Additional samples were requested from these cats for testing with the FIP Virus RealPCR Test. The data set includes 65 samples collected between October 2013 and April 2014.

**Results:** The data set included 65 samples collected through Dr. Nancy Sanders' consulting activity. From the 65 cases, 32 were FCoV negative on the sample tested, 33 were FCoV positive. Of the 33 FCoV positive samples,13 had low viral load, and 1 failed for quality control reasons. No sample typed as indeterminate; 18 samples typed as FIPV; they were included in the statistical analysis.

From the 18 FIPV-typed samples,13 had either a clinical score of 5 or 6. In 5 cats with a clinical score of 4 (possibly FIP but final diagnosis unknown), some laboratory results were considered atypical for FIP while their clinical presentations and histories were suspicious for FIP.

When results from this study were combined with the FIP-confirmed cases of study 3, the separation between the two groups became even more significant—again indicating that the typing assay is able to correctly identify the FIPV biotype associated with the correct clinical presentation of FIP.

**Figure 2.** Statistical analysis of the combined cases from the retrospective and the prospective studies.




**Conclusion:** This study confirmed that the FIP Virus RealPCR™ Test was able to differentiate FECV from FIPV in clinically sick cats.

## Overall conclusions

Diagnosing FIP in a sick cat has remained a challenge because of the lack of direct confirmatory test options. The mutation theory led to investigations at multiple research centers and at various regions of the viral genome, which have furthered the understanding that the transition from a benign to a lethal FCoV strain occurs within the spike gene. From a molecular diagnostics point of view, the spike gene mutations in the fusion peptide are an ideal target for the development of a confirmatory FIPV test, given their genetic stability and confirmed absence in FECV strains.

In our clinical studies, we aimed to confirm that the FIP Virus RealPCR Test:

- Accurately differentiates the FECV wild-type spike gene sequence from the FIPV mutant spike gene sequence, discovered by Dr. Rottier in cats from the Netherlands.
- Detects these mutations in cats with FIP in North America, Europe, and Japan.
- Differentiates FIPV from FECV in clinically sick cats.

Based on these clinical validation projects, we can confidently recommend the FIP Virus RealPCR™ Test as a confirmatory diagnostic tool in cats with suspect FIP based on clinical presentation and initial workup.

## Reference

1. Chang HW, Egberink HF, Halpin R, Spiro DJ, Rottier PJM. Spike protein fusion peptide and feline coronavirus virulence. *Emerg Infect Dis.* 2012;18(7):1089–1095.