ADIAGENE
Bio-X Diagnostics

INSTRUCTION MANUAL

ADIAVET™ PIF FAST TIME

TEST FOR THE DETECTION OF THE FELINE CORONAVIRUS RESPONSIBLE FOR FELINE INFECTIOUS PERITONITIS (FIP) BY REAL-TIME ENZYMATIC AMPLIFICATION (RT-PCR TEST)

References:
ADI521-50 (50 reactions)
ADI521-100 (100 reactions)

VET

English version
NE521-01
2020/06

ADIAVET™ PIF FAST TIME

I. Revision history

N/A	Not Applicable (first publication)
Correction	Correction of document anomalies
Technical change product	Addition, revision and/or removal of information related to the
Administrative	Implementation of non-technical changes noticeable to the user

Note: minor typographical, grammar and formatting changes are not included in the revision history.

Release Date	Part Number	Change type	Change summary
2020/06	NE521-01		creation

II. General information

1. Purpose of the test

ADIAVET™ PIF FAST TIME kit is intended to detect the feline coronavirus using real-time Polymerase Chain Reaction (PCR) technology, from whole blood, ascites, tissue and faecal swab samples.

2. Pathogen

Feline Coronavirus (FCoV) is an RNA virus, found worldwide. It particularly infects birds and mammals, mainly young cats. The virus infects the feline in an enteric form called Feline Enteric CoronaVirus (FeCV) which causes digestive problems. This form can then mutate and become infectious (FIPV: Feline Enteric CoronaVirus). The mutated virus is responsible for Feline Infectious Peritonitis (FIP). FIP is one of the biggest killers of cats. PCR diagnosis is one of the most effective methods to detect the presence of the pathogen because it is fast, sensitive and specific.

3. Description and purpose of the test

This test is based first on the reverse transcription (RT) of RNA into complementary DNA. Then, cDNA is amplified (PCR) by a DNA polymerase using specific primers. Both enzymatic reactions occur in the same tube (One-step RT-PCR).
Amplified products are detected in real-time thanks to a specific labelled hydrolysis probe (5'-exonulease technology).

The ADIAVET™ PIF FAST TIME kit enables the simultaneous detection of:

- The feline coronavirus (probe labelled in FAM),
- The β-actine, an internal control of extraction and amplification steps specific from an endogenous RNA (probe labelled with a fluorochrome with the same spectrum as VIC and HEX). As part of cat faecal swab analysis, an additional exogenous internal control is provided with the kit.

ADIAGENE validated the test kit using RNA purification kits (Bio-X Diagnostics, Qiagen, Macherey-Nagel). Other purification kits can be used if they have been validated by the user.

Analysis options according to the specimen:

Specimen	Individual analysis
Whole blood (EDTA)	☑
Ascites	☑
Tissue swab	☑
Faecal swab	☑

III. Material and reagents

1. Reagents provided with the kit

REF ADI521-50		
A5	……..amplification solution	1 x 500 µl tube with green cap (a ready-to-use reagent)
PIF CTL+	FIP positive control	1 tube with purple cap (to reconstitute)
EPC-ext	……Exogeneous extraction control	1 x 300 µl tube with yellow cap (a ready-to-use reagent)
NF-Water	Nuclease free Water	1 x 1000 µl tube with white cap (a ready-to-use reagent)
REF ADI521-100		
A5	……..amplification solution	2 x 500 µl tubes with green cap (a ready-to-use reagent)
PIF CTL+	FIP positive control	1 tube with purple cap (to reconstitute)
EPC-ext	……Exogeneous extraction control	2 x 300 µl tubes with yellow cap (a ready-to-use reagent)
NF-Water	Nuclease free Water	1 x 1000 µl tube with white cap (a ready-to-use reagent)

2. Validity and storage

On receipt, the kit should be stored at <**-15°C**.
It is recommended to make aliquots of A5 solution if if the number of tests to be performed requires more than three thaws. **Do not defrost reagents more than 3 times.**
Realtime reagents are susceptible to light: store them in the darkness.
The A5 reagent is ready to use for PCR reaction.
Do not mix reagents of two different batches.

3. Use of EPC Ext (used for faecal swab)

Aliquot this solution and store it at <-15°C. For each extraction, use 5 µl of EPC-Ext per feacal samples. **Do not defrost each aliquot more than 3 times.**

4. Use of PIF CTL+

Add **200 µl** of **NF-Water** to the **PIF CTL+** tube included in the kit. Homogenize the tube content using a mixer such as vortex, for at least 20 seconds. Aliquot this solution by 6 or 12 µl and store them at <15°C.
For each analysis, we recommend to use **5 µl** of **PIF CTL+** in a one of the wells.

5. Equipment required but not supplied in the kit

Material should be Nuclease-free (e.g. autoclaved for 25 minutes twice at +120°C or once for 60 minutes at +121°C)

- Thermal cycler with consumables for real-time PCR: 0.2 ml PCR tubes or closed 96-wells PCR plates with optical quality
- Class II Microbiological Safety Cabinet
- A centrifuge for microtubes or 96-wells plates
- Universal laboratory mixer mill
- A heating block or water bath
- Instrument for homogenous mixing of tubes
- 1 - 10 µl pipette, 20 - 200 µl pipette and 200 - 1000 µl pipette
- Nuclease-free filter tips
- Nuclease-free microtubes: 1.5 ml and 2 ml
- Powder-free latex gloves
- 96-100% ethanol solution
- Nuclease-free water
- PBS 1X buffer pH=7.4 (recommended composition, NaCl 150 mM, Na2HPO4 5 mM, KH2PO4 1.7mM, without Ca2+, without Mg2+ - another composition could be used after a validation made by the user)

- Material needed for individual column extraction

  - QIAamp® Viral RNA kit (Qiagen, 50 extractions: ref. 52904; 250 extractions: ref. 52906)
  - Nucleospin® RNA Virus (Macherey-Nagel, 50 extractions: ref. 740956.50; 250 extractions: ref. 740956.250)

- Automated DNA/RNA extraction kit using magnetic beads

  - ADIAMAG (Bio-X Diagnostics, 200 tests, ref. NADI003)

IV. Recommendation before the analysis of samples

Before starting the test, read the entire protocol and scrupulously respect it.

1. Precautions

Adiagène has elaborated this PCR test with the use of Bio-X Diagnostics, Qiagen and Macherey-Nagel extraction kits. Other extraction kits can be used with prior validation.

Follow the supplier's instructions for the storage, the preparation and the use of the extraction reagents.

Some kits include and/or need the use of toxic reagents. These reagents should be use with gloves and within a chemical cabinet.

We strongly recommend that only appropriately trained personnel perform this test. Ensure the accuracy and precision of the micropipettes used. The quality of the obtained results depends upon rigorous respect of good laboratory practices.

The PCR generates large amount of amplified DNA. A few molecules of amplified products are sufficient to generate a positive result. It is important to reserve 2 rooms, one for manipulation of samples to be tested, and another one for amplified products analysis. Do not open the PCR tubes after amplification.
Samples for analysis should be handled and disposed of as biological waste. **Take all measures of security and confinement required for the manipulation of the concerned biological agents.**

We recommend using fractions of demineralised and saline water and to autoclave them twice for 25 minutes at +120°C or once for 60 minutes at +121°C. Take a fresh aliquot for each new manipulation in order to avoid contamination.

2. Storage of samples and DNA extracts

Samples can be stored for 1 or 2 days at +2/8°C. After 2 days, we recommend to store them at <-15°C.
Extracted RNAs are quite sensitive molecules. Extraction is made at room temperature and should be performed as fast as possible to avoid degradation. We then recommend to read the entire protocol before starting the test and to respect it rigorously. Crude extracts should be stored at the end of extraction on melting ice or at +2/8°C for a few hours, then at <-15°C.

3. Samples preparation

Bloods should be collected in an anticoagulant tube (EDTA) under a microbiological safety cabinet.
See § IV for the extraction and purification of RNA.

4. Controls to be included

The use of controls allows to verify the reliability of the results.
The controls should be included in in each trial of analysis. A trial is defined as all the samples treated in the same conditions.
The combination of these different controls allows the validation of all the steps of the analytical process (extraction+amplification), regardless of the matrices.

- The internal endogenous control (β-actine), naturally found in the samples, allows to verify the extraction and amplification steps of each sample.
- The PIF CTL+ allows to validate the amplification of the target.

Other controls that must or could be added:

- **Negative control of extraction (required)**

To verify the absence of cross-contamination, at least one negative control must be included per trial (e.g. the normative requirement and recommendation for the development and the validation of veterinary PCR NF U47-600 suggests the use of 1 negative control for 24 samples or 4 negative samples for a 96 wells-plate). This control could be a negative matrix, or a buffer used for dilutions.

- **Positive control of extraction (recommended)**

A positive control could be added in each trial. The control is a sample including the feline coronavirus. It could come from a positive sample available in the laboratory or from a negative sample spiked with a solution of feline coronavirus. This positive control should close to the limit of detection of the method. It informs about the fidelity of the obtained results between different trials.

V. Extraction and purification

1. Using QIAamp® Viral RNA kit

All the centrifugations are performed at room temperature.

<table>
<tr><th></th><th>Blood from anticoagulant tube (EDTA), Ascites</th><th>Faecal swab</th><th>Tissue</th></tr>
<tr><td>Sample</td><td>Place 140 µl in a microtube.</td><td>Add 2 ml of PBS 1X in the tube of the swab, homogenize.
Place 140 µl in microtube containing 5µl EPC-Ext.</td><td>Place 20 mg in a microtube.</td></tr>
<tr><td rowspan="2">Lysis</td><td colspan="3">Add 560 µl of buffer AVL + Carrier RNA.</td></tr>
<tr><td colspan="2">Homogenize for ~15 seconds.
Check if the mix is homogeneous.
Incubate at room temperature during 10 minutes.</td><td>Grind*
Centrifuge for 2 minutes at 6000 g.
Place the supernatant in a microtube.</td></tr>
<tr><td>Binding preparation</td><td colspan="3">Add 560 µl of ethanol 100%.
Homogenize by using a mixer such as vortex (~15 seconds).</td></tr>
<tr><td>Transfer to columns and binding to the membrane</td><td colspan="3">Identify columns, apply 630 µl of the obtained solution to the corresponding column.
Centrifuge for 1 minute at 10 000 g.
Change the collection tube, put the rest of the mix on the column and centrifuge for 1 minute at 10 000 g.</td></tr>
<tr><td>1st wash</td><td colspan="3">Change the collection tube and add 500 µl of buffer AW1.
Centrifuge for 1 minute at 10 000 g.</td></tr>
<tr><td>2nd wash</td><td colspan="3">Change the collection tube and add 500 µl of buffer AW2.
Centrifuge for 1 minute at 100000 g.</td></tr>
<tr><td>Column dry step</td><td colspan="3">Change the collection tube.
Centrifuge for 3 minutes at 10 000 g.</td></tr>
<tr><td>Elution</td><td colspan="3">Transfer the column to a microtube. Add 60 µl of buffer AVE.
Incubate for ~1 minute at room temperature and centrifuge for 1 minute at 10 000 g.</td></tr>
<tr><td>Storage</td><td colspan="3">Close the tubes, identify and store on ice if using immediately or at <-15°C.</td></tr>
</table>

* For example, using a Mixer Mill: add 1 metal bead (3 mm), grind for 2 minutes at 30 Hz.

2. Using Nucleospin® RNA Virus kit

All the centrifugations are performed at room temperature.

	Blood from anticoagulant tube (EDTA), Ascites	**Faecal swab**	**Tissue**
Sample	Place **140 µl** in a microtube.	Add **2 ml** of **PBS 1X** in the tube of the swab, homogenize. Place **140 µl** in microtube containing **5µl EPC-Ext.**	Place **20 mg** in a microtube.
Lysis	Add **560 µl** of **buffer RAV1 + Carrier RNA**.		
	Homogenize for ~15 seconds. Incubate at room temperature during 10 minutes.		Grind* Centrifuge for 2 minutes at 6000 g. Place the **supernatant** in a microtube.
Binding preparation	Add **560 µl** of **ethanol 100%**. Homogenize by pipetting (~10 times) or by using a mixer such as vortex (~15 seconds). Briefly centrifuge.		
Transfer to columns and binding to the membrane	Identify columns, apply **630 µl** of the **obtained solution** to the corresponding column Centrifuge for 1 minute at 10 000 g. Change the collection tube and put the rest of the mix on the column and centrifuge for 1 minute at 10 000 g.		
1st wash	Change the collection tube and add **500 µl** of **buffer RAW**. Centrifuge for 1 minute at 10 000 g.		
2nd wash	Change the collection tube and add **500 µl** of **buffer RAV3**. Centrifuge for 1 minute at 10000 g.		
Column dry step	Change the collection tube. Centrifuge for 3 minutes at 10 000 g.		
Elution	Transfer the column to a microtube. Add **60 µl** of **Nuclease-free water**. Incubate for ~1 minute at room temperature and centrifuge for 1 minute at 10 000 g.		
Storage	Close the tubes, identify and store on ice if using immediately or at <-15°C.		

* For example, using a Mixer Mill: add 1 metal bead (3 mm), grind 2 for minutes at 30 Hz.

3. Using ADIAMAG kit

See the NEKF user manual available on the web site mentioned on the certificate of analysis which is included in the ADIAVET™ kit applied.

VI. Amplification

a - Determine the number of samples analysed, including the controls (e.g. positive and negative extraction control, positive control of amplification (PIF CTL+) and PCR reagent control (NTC)).

b - Defrost the A5 solution reagent at room temperature. Homogenize. Dispense **10 µl** of A5 solution in each PCR tubes or PCR plate wells with a micropipette with a Nuclease-free tip.

d- **Immediately afterwards, place the tube with A5 solution back at <-15°C and protected from light**

e- For each sample, the PIF CTL+, the extraction negative control (required) and the extraction positive control (recommended): Add **5 µl** of purified extract to the **10 µl** of A5 solution.
For the PCR reagent control (NTC): do not add anything to the A5 solution.
Take care to have no bubbles in the bottom of the wells. Immediately after filling the wells, store purified RNA extracts at +2/8°C or <-15°C.

f- Store the plate or the tubes on melting ice or at +2/8°C until the cycler is programmed and start quickly the run after you have placed the plate or the tubes in the cycler.

The feline coronavirus target is read in FAM. The Internal Control is read in VIC or HEX. The Quencher is non fluorescent. The solution contains a passive reference ROX for the ABI machines. Fluorescence is read during the elongation step.

The following programs are defined for **ABI Prism** thermocyclers (like 7500, StepOne…) from **Applied Biosystems** activate the "emulation 9600" option if available), for the **MX3005P** and **ARIAMX** of **Agilent** and for **CFX96** of **BioRad**.

Standard program		Fast program	
10 min. 45°C		10 min. 45°C	
10 min. 95°C		10 min. 95°C	
15 sec. 95°C**	45 cycles	5 sec. 95°C	45 cycles
1 min. 60°C		30 sec. 60°C *	

* Note 32 secondes for the ABI7500 thermofisher
** Note 30 secondes for the MX3005P

Contact us if you wish to use other thermalcyclers.

VII. Interpretation of results

1. Definitions

The **« base line »** corresponds to the background of fluorescence and describes the non-characteristic part of the curve observed during the first cycles.

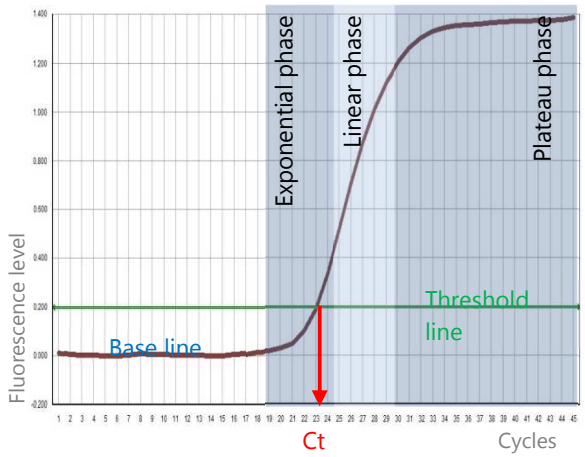
The **« Characteristic amplification curve »** describes a fluorescence curve with an exponential phase, a linear phase and a plateau phase.

The **« threshold line »** has to be placed over the background in the exponential phase of a characteristic amplification curve or a group of characteristic amplification curves.

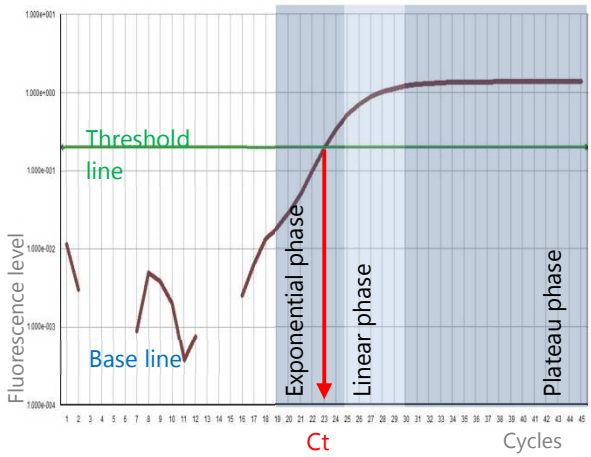
The **« threshold cycle » (Ct)** of a well corresponds, for each detected fluorophore, at the crossing point of the threshold line with the fluorescent curve. The Ct value expressed by the machine for each well depends on the threshold position and on the quantity of target sequences initially present in the PCR well.

Example of characteristic amplification curve

Arithmetic scale of Y axis



Logarithmic scale of Y axis



2. Validation and interpretation of results

Display the FAM curves from the plate and set the threshold value as indicated above. Proceed in the same mean for the VIC or HEX curves.

A. The test is valid if:

Amplification is considered to be **valid** if the following results are obtained for the controls:

Controls	Reagent control (NTC)	PIF CTL+	Extraction negative control	Extraction positive control *
FAM amplification	no	yes	no	yes
VIC/HEX amplification	no	yes	no	no/yes
Validation of	Absence of contamination for amplification	Amplification of the target	Absence of contamination for extraction	Extraction and amplification steps

* Optional

The indicative Ct values (FAM and VIC/HEX dyes) of the PIF CTL+ are indicated in the certificate of analysis of the kit.

B. Result interpretation

RNA extraction and amplification for each sample are considered to be **valid** if at least a characteristic amplification curve is observed for feline coronavirus (FAM) or for the internal control (VIC/ HEX).

Example	A	B	C	D
FAM amplification	no	yes	yes	no
VIC/HEX amplification	yes	no	yes	no
Result	negative	positive	positive	undetermined

The sample is considered as **negative** if a characteristic amplification curve is observed in VIC/HEX without any amplification in FAM (example A).

The sample is considered as **positive** if a characteristic amplification curve is observed in FAM (example B). Internal control can be co-amplified (example C).

A total absence of characteristic amplification curve for a sample (example D) shows a defective RNA extraction (lost or destruction of RNA) or a deficient real-time PCR (inhibitors in the sample, program error or no template added). In this case, we recommend first to repeat the test with pure as well as with tenfold diluted RNA in Nuclease-free water. Then, if the test is still not valid, a new extraction is recommended.

VIII. Index of symbols

Symbol	Meaning
	Catalogue number
	Manufacturer
	Upper temperature limit
	Use by date
	Batch code
	Consult Instructions for Use
	Contains sufficient for <n> tests
	Keep away from sunlight
	For veterinary in vitro use only – For animal use only

S.A.R.L. ADIAGENE
9, rue Gabriel Calloët-Kerbrat
22440 Ploufragan - France

RCS 417 876 299
Tel. +33 (0)2 96 68 40 20
www.biox.com