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INTERNAL VALIDATION REPORT

ID GENE LYO™ FMDV TRIPLEX

REAL-TIME RT-PCR KIT FOR THE QUALITATIVE DETECTION OF THE FOOT AND MOUTH DISEASE VIRUS RNA

METHOD	**RT-qPCR – Triplex – Qualitative**
TARGET SEQUENCES	• **FMDV RNA:** region conserved in all FMDV serotypes: 3D gene (PCR design as prescribed by the WOAH manual) • **Endogenous Non-Target Positive Control (NTPCen)**: sequence specific to the validated animal species mentioned below (ubiquitous cellular gene) • **Exogenous Non-Target Positive Control (NTPC-FMDV)**: sequence specific to a non-pathogenic RNA virus
SAMPLE TYPES	• **Whole blood** (in EDTA tubes) and **Serum** • **Milk** • **Organs and Tissues** (e.g tongue and epithelium from unruptured or recently ruptured vesicles) • **Swab** (oral and oropharyngeal) • **Vesicular fluids** • **Viral culture Nucleic acid storage card** (Gene Saver™ 2.0 CARDS) • **Environmental samples** (boot swabs)
VALIDATED SPECIES	Sheep, cattle, goats, cervids, suids, camelids and other FMDV-susceptible species
PRODUCT CODE	IDFMDVL

With you at every step

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INTRODUCTION

Foot and mouth disease (FMD) is one of the most contagious viral diseases in ruminants, and one of the most feared transboundary diseases, causing epizootic diseases in a few weeks and with devastating economic consequences. The causative agent belongs to the genus *Aphthovirus*, family *Picornaviridae*. There are seven distinct serotypes of FMD virus (FMDV), namely, O, A, C, Asia1, SAT1, SAT2 and SAT3, and within each serotype there are numerous strains. The wide range of hosts, infectivity at low dose, rapid replication, high levels of viral excretion and multiple forms of transmission make FMD difficult to control and eradicate.

The World Organization for Animale Health (WOAH) and the Food and Agriculture Organization (FAO) have defined an international strategy for the eradication and the control of the disease (Progressive Control Pathway for Foot-and-Mouth Disease, PCP-FMD).

Since FMD can spread quickly and cannot be differentiated clinically from other vesicular diseases such as vesicular stomatitis (VS) and swine vesicular disease (SVD), rapid and specific identification of the agent is of utmost importance.

Innovative Diagnostics has developed a molecular diagnostic tool capable of detecting all known serotypes of FMDV including FMD strains recently isolated in Europe particularly in blood, serum, tissues and organs (e.g. tongue and epithelium from unruptured or recently ruptured vesicles), swabs (oral and oropharyngeal), vesicular fluids, milk, nucleic acid storage cards and environmental samples (boot swabs).

This Triplex RT-qPCR test targets a conserved region in the 3D gene of the FMDV, and **uses a design described in the WOAH manual** [1-3]. Additionnaly, the kit allows simultaneous amplification of an **ubiquitous gene** of the main affected species (**NTPCen**, see Table page 4) and of an **exogenous positive control (NTPC-FMDV)** added to all samples to **detect potential sample inhibition.** The NTPC-FMDV is of particular interest in the case of **acellular matrices** (environmental samples, surface swabs, etc…).

Samples are analysed after RNA purification, for example by automated nucleic acid extraction using the ID Gene™ Mag Fast Extraction Kit (product code: MAGFAST).

This document summarizes validation data obtained for the ID Gene Lyo™ FMDV Triplex kit (product code: IDFMDVL).

TEST PRINCIPLE

The ID Gene Lyo™ FMDV Triplex kit (product code: IDFMDVL) is based on the real time Reverse Transcription quantitative PCR method (RT-qPCR). This kit is a qualitative triplex test that simultaneously amplifies a target RNA sequence as well as an endogenous non-target positive control and an exogenous non-target positive control.

Viral RNA is extracted from a variety of matrices such as whole blood or organs before the amplification step.

For the nucleic acid extraction step, Innovative Diagnostics recommends the following method:

DESCRIPTION	PRODUCT NAME	PRODUCT CODE
Fast nucleic acid extraction kit with magnetic beads	ID Gene™ Mag Fast Extraction Kit	MAGFAST

The ID Gene Lyo™ FMDV Triplex kit includes:

- a freeze-dried Amplification Reaction Mixture (ARM-FMDVL) which allows for the amplification and the qualitative detection of the RNA of FMDV, as well as of the endogenous and exogenous controls. It contains different sets of primers, probes and a master mix (including reverse transcriptase and polymerase) required to perform the RT-qPCR amplification.
- a resuspension buffer RB2 to reconstitute the freeze-dried ARM-FMDVL before first use. It contains stabilizing agents.
- one or 2 empty tube(s) (ARM-TUBE) to transfer and store the ARM-FMDVL after reconstitution. It ensures better preservation.
- a FMDV Positive Amplification Control (product code: PAC-FMDV) which consists of synthetic nucleic acids containing the target sequence specific to FMDV, as well as sequences for endogenous and exogenous controls. It is used to validate the amplification of target RNA sequences.
- a freeze-dried Exogenous Non-Target Positive Control (product-code: NTPC-FMDV), consisting of a non-pathogenic virus. Once reconstituted, this control is to be added to each sample and negative extraction controls (NEC) at the pre-treatment stage for nucleic acids extraction. It provides the exogenous signal to assess the efficiency of the pre-treatment and nucleic acid extraction, and to identify the possible presence of inhibitors, in each sample and extraction control.

The Table below shows all the species detected by the endogenous internal control (NTPCen):

SPECIES	TESTED & VALIDATED *IN VITRO*	*IN SILICO* PREDICTION
Cattle	✓	✓
Ovine	✓	✓
Caprine	✓	✓
Cervids	✓	✓
Camelids	✓	✓
Swine	✓	✓
Wild species – Mouflon, deer, Pyrenean chamois, chamois, ibex, wild boar, Buffaloes (water, Asian and African)	✓	✓

The ID Gene Lyo™ FMDV Triplex RT-qPCR kit uses fluorescent probes relying on FAM™, VIC®/HEX™ and Cy5 fluorochromes for detection of the targets, as detailed below:

TARGET	CHANNEL CAPABLE OF READING
FMDV	FAM™
Endogenous Non-Target Positive Control (NTPCen)	VIC®/HEX™
Exogenous Non-Target Positive Control (NTPC-FMDV)	Cy5

Three amplification programs are available for the ID Gene Lyo™ FMDV Triplex RT-qPCR kit:

- the ultra-rapid program (which allows RT-qPCR to be carried out in approximately 40 minutes).
- the rapid program (which allows RT-qPCR to be carried out in approximately 50 minutes).
- the standard program (which is compatible with amplification programs offered by other reagent manufacturers and therefore allows simultaneous analysis).

AMPLIFICATION PROGRAMS	RUN LENGTH
Standard	80 minutes
Rapid	50 minutes
Ultra-rapid	40 minutes

STEP	ULTRA-RAPID PROGRAM	RAPID PROGRAM	STANDARD PROGRAM	NUMBER OF CYCLES
(1) Reverse transcription	10 min at 45°C	10 min at 45°c	10 min at 45°c	1
(2) Activation of polymerase	2 min at 95°C	2 min at 95°C	10 min at 95°C	1
(3) DNA Denaturation / Elongation	5 sec at 95°C 2 sec at 60°C	10 sec at 95°C 30 sec at 60°C	15 sec at 95°C 60 sec at 60°C	40

ANALYTICAL SPECIFICITY

IN SILICO

The analytical specificity of the ID Gene Lyo™ FMDV Triplex RT-qPCR assay was evaluated *in silico* by aligning the 3D gene target PCR systems (primers and probes) with the databases available on the NCBI (National Center for Biotechnology Information).

After alignment, a 100% *in silico* specificity was found for the target gene regions specifically selected for FMDV, which are highly conserved regions.

EXPERIMENTAL

Inclusivity study

Inclusivity was evaluated on a panel composed of 7 inactivated strains from the Pirbright Institute (United Kingdom, Woking GU24 ONF).

The results are presented in Table 1 below:

STRAIN	ORIGIN	IDFMDVL RESULTS
FMDV-Ag serotype O	Pirbright, UK	Detected
FMDV-Ag serotype A		Detected
FMDV-Ag serotype Asia 1		Detected
FMDV-Ag serotype C		Detected
FMDV-Ag SAT1		Detected
FMDV-Ag SAT2		Detected
FMDV-Ag SAT3		Detected

Table 1: Analytical specificity results obtained with the ID Gene Lyo™ FMDV Triplex kit on a panel of FMDV strains

RESULTS (Table 1) :

- The **ID Gene Lyo™ FMDV Triplex** (IDFMDVL) successfully detected **all samples from the reference panel tested,** including all serotypes of FMDV, leading to a **measured inclusivity of 100%.**

Exclusivity study

Exclusivity was evaluated on a panel of strains with phylogenetic relationship, similarities in target sequences and/or found within the same ecological niche.

Exclusivity of the ID Gene Lyo™ FMDV Triplex RT-qPCR kit was evaluated on a panel of 65 pathogens from different species.

STRAIN	IDFMDVL RESULTS
Bacteria	
Anaplasma phagoctophilum	Not detected
Campylobacter coli	Not detected
Campylobacter fetus fetus	Not detected
Chlamydophila abortus	Not detected
Escherichia coli K99	Not detected
Escherichia coli LA9A	Not detected
Histophilus somni	Not detected
*Mycobacterium avium (*subsp. *avium, paratuberculosis* and *hominissuis)*	Not detected
Mycobacterium bovis	Not detected
Mycobacterium caprae	Not detected
Mycobacterium microti	Not detected
Mycobacterium phlei	Not detected
Mycobacterium tuberculosis	Not detected
Parasites	
Cryptosporidium parvum	Not detected
Toxoplasma gondii	Not detected
Neospora caninum	Not detected
Leishmania infantum	Not detected
Virus	
SVDV (Swine Vesicular Disease Virus)	Not detected
VSV (Stomatite Vesicular Disease Virus)	Not detected
BRV (Bovine Rhinitis Virus)	Not detected
ASFV (African Swine Fever Virus)	Not detected
SuHV-1 (Aujeszky)	Not detected
Bluetongue virus (1,3,4,8,12)	Not detected
Bovine Adenovirus	Not detected
Bovine pneumovirus (A, B, 3)	Not detected
Bovine Coronavirus	Not detected
Bovine Herpes virus 1 (IBR)	Not detected
Bovine Herpes virus 2	Not detected
Bovine Herpes virus 3	Not detected
Bovine Herpes virus 4	Not detected
Capripox virus	Not detected
Bovine Para influenza 3	Not detected
Bovine Leucosis Virus	Not detected
BVDV (1a, 1c, 1d, 1e, 1g, 1h, 1l, 2a, 2b, 3,4)	Not detected
IBV	Not detected
Bovine Respiratory Syncitial Virus	Not detected
EHDV-8	Not detected
H1N1	Not detected
H5N2	Not detected
H7N1	Not detected
H9N2	Not detected
NDV (Newcastle disease)	Not detected
PPRV	Not detected
Schmallenberg Virus	Not detected
VISNA-MAEDI virus	Not detected
West Nile Virus	Not detected

Table 2: Exclusivity results obtained with the ID Gene Lyo™ FMDV Triplex kit on a panel of different pathogens

RESULTS (Table 2):

- The **ID Gene Lyo™ FMDV Triplex** kit does not detect any of the pathogens of the tested panel, leading to a **measured analytical specificity of 100%**.

ANALYTICAL SENSITIVITY – LD_{PCR}

The limit of detection of the PCR (LD_{PCR}) is the smallest number of copies of the target nucleic acid that can be detected, in 95% of the cases, per unit of volume:

- Number of copies/PCR (total volume per PCR = 20 µL)

The limit of detection in PCR-based analyses (LD_{PCR}) was determined by performing serial dilutions (8 replicates), on 3 different sessions, using a calibrated synthetic RNA containing the FMDV target sequence (product-code: PAC-FMDV).

The amplification step was performed with the ID Gene Lyo™ FMDV Triplex RT-qPCR kit using the rapid and ultra-rapid amplification programs.

The results obtained are presented in Tables 3 and 4.

NUMBER OF COPIES/PCR	**RAPID PROGRAM**	
	Nb of positives/ Nb of replicates	Detection frequency (%)
125	24/24	100%
62.5	24/24	100%
31.25	**23/24**	**95.8%**
15.6	22/24	92%
7.8	20/24	83.3%

Table 3: Determination of the LD_{PCR} of the FMDV target with the rapid amplification program

NUMBER OF COPIES/PCR	**ULTRA-RAPID PROGRAM**	
	Nb of positives/ Nb of replicates	Detection frequency (%)
50	23/24	95.8%
40	**23/24**	**95.8%**
31.25	21/24	87.5%

Table 4: Determination of the LD_{PCR} of the FMDV target with the ultra-rapid amplification program

RESULTS (Tables 3 and 4):

- The **LD_{PCR} (95%)** for the ID Gene Lyo™ FMDV Triplex kit with the **rapid amplification program** was established at **32 copies/PCR.**
- The **LD_{PCR} (95%)** for the ID Gene Lyo™ FMDV Triplex kit with the **ultra-rapid amplification program** was established at **40 copies/PCR.**

LINEARITY RANGE AND EFFICIENCY

The linearity of a quantitative test is its ability to generate results proportional to the concentration of the target within a given range of values modeled by a linear function. A linear regression exists between the instrumental response (example: Cq) and the decimal logarithm of the amount of the target (copy number of the target per PCR). The determination of the parameters of the regression line (y = a x '+ b) and the validation of the linear equation must allow to:

- determine the equation of the regression line,
- determine the efficiency (E),
- determine and validate the linear regression performances.

In theory, the number of molecules of the target sequence should double during each replication cycle, which corresponds to an amplification efficiency of 100% (E= 100%).

Amplification efficiency is calculated by performing five 10-fold serial dilutions of the PAC-FMDV (containing the specific target sequences) in 3 different runs using the rapid and ultra-rapid amplification programs.

First, the Cq values obtained are ploted in fonction of the corresponding concentrations expressed on a logarithmic scale. Then, a linear regression curve through the data points is generated to calculate the slope of the regression line to determine the efficiency of the RT-qPCR.

Efficiency is calculated using the equation: $E= -1 + 10^{(-1/slope)}$

In theory, for an efficiency between 85% and 115%, the slope should be found between -4.115 and 2.839.

EFFICIENCY RESULTS USING THE RAPID AMPLIFICATION PROGRAM

Efficiency results

RNA ASSAYED (COPIES/PCR) FMDV target	OBTAINED CQ VALUES		
	SESSION 1	SESSION 2	SESSION 3
12.10^6	15.94	15.83	16.01
12.10^5	19.01	18.92	19.40
12.10^4	22.78	22.51	23.05
12.10^3	26.14	26.23	26.82
12.10^2	29.68	29.34	29.90
120	33.10	33.47	33.94

Table 5: Efficiency results with the rapid amplification program for the FMDV target

Characteristics of the linear regression curves

RAPID PROGRAM	SLOPE (a)	Y-INTERCEPT (b)	COEFFICIENT R^2	EFFICIENCY %
Session 1	-3.5	42.1	0.99	**93.1**
Session 2	-3.6	42.2	0.99	**91.1**
Session 3	-3.6	40.3	1	**94.6**
Average	**-3.57**	**41.53**	**0.99**	**92.93**

Table 6: Linear regression curves obtained with the rapid amplification program for the FMDV target
(R^2: correlation coefficient)

RESULTS (Tables 5 and 6) :

- The average amplification efficiency of the **ID Gene Lyo™ FMDV Triplex kit** for the FMDV target is **92.9 %** with the **rapid amplification program.**
- The average **R^2** coefficient is **0.99.**

EFFICIENCY RESULTS USING THE ULTRA-RAPID AMPLIFICATION PROGRAM

Efficiency results

RNA ASSAYED (COPIES/PCR) FMDV target	OBTAINED CQ VALUES		
	SESSION 1	SESSION 2	SESSION 3
12.10^6	16.90	17	17.06
12.10^5	20.35	20.35	20.37
12.10^4	24	23.82	24.01
12.10^3	27.53	27.27	27.51
12.10^2	30.96	30.88	30.99
120	34.97	34.21	34.35

Table 7: Efficiency results with the ultra-rapid amplification program for the FMDV target

Characteristics of the linear regression curves

ULTRA-RAPID PROGRAM	SLOPE (a)	Y-INTERCEPT (b)	COEFFICIENT R^2	EFFICIENCY %
Session 1	-3.5	39.1	0.99	**93.9**
Session 2	-3.4	38.6	1	**94.9**
Session 3	-3.6	38.9	0.99	**89.3**
Average	**-3.51**	**38.9**	**0.99**	**92.69**

Table 8: Linear regression curves obtained with the ultra-rapid amplification program for the FMDV target
(R^2: correlation coefficient)

RESULTS (Tables 7 and 8) :

- The average amplification efficiency of the **ID Gene Lyo™ FMDV Triplex kit** for the FMDV target is **92.7 %** with the **ultra-rapid amplification program .**
- The average **R^2** coefficient is **0.99.**

CHARACTERISATION OF THE COMPLETE METHOD

The parameters describing properties relative to the determination of the amount of target that can be detected and quantified are known as the Limit of Detection. The characterisation of the method was performed and validated on different matrices.

METHOD DETECTION LIMIT (MDL)

The Method Detection Limit (MDL) is the lowest amount of the analyte, for a defined amount and type of sample, that can be repeatedly detected (with 100% probability) under the experimental conditions described by the method, and considering all the steps of the method of analysis (sample size, sample preparation, extraction, reverse transcription and qPCR).

The characterisation of the MDL was done by using bovine and swine whole blood samples, epithelium from bovine tongue, oral cavity swab from swine and caprine milk samples from FMDV-negative animals.

These negative matrices were spiked with a non-infectious material : FMDV-O/EGY/18/2016, provided by the Pirbright Institute and quantified at 9.10^6 copies/PCR by relative RT-PCR using quantified synthetic RNA. Nucleic acids were then extracted with the automated magnetic beads extraction system (ID Gene™ Mag Fast Extraction Kit, product code: MAGFAST) and amplified with both rapid and ultra-rapid amplification programs using the IDFMDVL RT-qPCR kit.

For the nucleic acid storage card (GENE SAVER™ 2.0 CARDS), 150 µL of spiked bovine whole blood were spread on the card and the sample was tested according to the recommendations mentioned in the instructions for use of the ID Gene™ Mag Fast Extraction Kit (FMDV-MAGFAST).

To determine the MDL, 4 replicates of 5 concentration levels were tested in 2 different sessions.

RESULTS USING THE RAPID AMPLIFICATION PROGRAM

SAMPLE	**BOVINE WHOLE BLOOD**				
Spiked with	FMDV-O quantified suspension at 9.10^6 copies/PCR				
Final concentration (copies/PCR)	9000	4500	2250	1125	885
Number of replicates	4				
Number of sessions	2				
Extraction system	Automated with magnetic beads, MAGFAST				
Amplification program	Rapid and Ultra-rapid				
Amplification kit	ID Gene Lyo™ FMDV Triplex				
MDL (copies/PCR)	**11.10^2**				
MDL (copies/mL of blood)	**36.10^4**				

Table 9: Method detection limit obtained for spiked bovine whole blood extracted with the ID Gene™ Mag Fast Extraction Kit and amplified with the IDFMDVL kit using both rapid and ultra-rapid amplification programs

SAMPLE	SWINE WHOLE BLOOD				
Spiked with	FMDV-O quantified suspension at 9.10^6 copies/PCR				
Final concentration (copies/PCR)	9000	4500	2250	1125	885
Number of replicates	4				
Number of sessions	2				
Extraction System	Automated with magnetic beads, MAGFAST				
Amplification program	Rapid and Ultra-rapid				
Amplification kit	ID Gene Lyo™ FMDV Triplex				
MDL (copies/PCR)	**11.10^2**				
MDL (copies/mL of blood)	**36.10^4**				

Table 10: Method detection limit obtained for spiked swine whole blood extracted with the ID Gene™ Mag Fast Extraction Kit and amplified with the IDFMDVL kit using both rapid and ultra-rapid amplification programs

SAMPLE	BOVINE TONGUE				
Spiked with	FMDV-O quantified suspension at 9.10^6 copies/PCR				
Final concentration (copies/PCR)	9000	4500	2250	1125	885
Number of replicates	4				
Number of sessions	2				
Extraction system	Automated with magnetic beads, MAGFAST				
Amplification program	Rapid and Ultra-rapid				
Amplification kit	ID Gene Lyo™ FMDV Triplex				
MDL (copies/PCR)	**11.10^2**				
MDL (copies/mg of tongue)	**18.10^3**				

Table 11: Method detection limit obtained for spiked bovine tongue epithelium extracted with the ID Gene™ Mag Fast Extraction Kit and amplified with the IDFMDVL kit using both rapid and ultra-rapid amplification programs

SAMPLE	ORAL SWAB FROM SWINE				
Spiked with	FMDV-O quantified suspension at 9.10^6 copies/PCR				
Final concentration (copies/PCR)	9000	4500	2250	1125	885
Number of replicates	4				
Number of sessions	2				
Extraction System	Automated with magnetic beads, MAGFAST				
Amplification program	Rapid and Ultra-rapid				
Amplification kit	ID Gene Lyo™ FMDV Triplex				
MDL (copies/PCR)	**11.10^2**				
MDL (copies/mL of swab supernatant)	**36.10^4**				

Table 12: Method detection limit obtained for spiked swine oral swab supernatant extracted with the ID Gene™ Mag Fast Extraction Kit and amplified with the IDFMDVL kit using both rapid and ultra-rapid amplification programs

SAMPLE	CAPRINE MILK				
Spiked with	FMDV-O quantified suspension at 9.10^6 copies/PCR				
Final concentration (copies/PCR)	9000	4500	2250	1125	885
Number of replicates	4				
Number of sessions	2				
Extraction system	Automated with magnetic beads, MAGFAST				
Amplification program	Rapid and Ultra-rapid				
Amplification kit	ID Gene Lyo™ FMDV Triplex				
MDL (copies/PCR)	**11.10^2**				
MDL (copies/mL of milk)	**36.10^4**				

Table 13: Method detection limit obtained for spiked caprine milk extracted with the ID Gene™ Mag Fast Extraction Kit and amplified with the IDFMDVL kit using both rapid and ultra-rapid amplification programs

SAMPLE	NUCLEIC ACID STORAGE CARD (GENE SAVER™ 2.0 CARDS)				
Spiked with	Bovine blood spiked with FMDV-O quantified suspension at 9.10^6 copies/PCR				
Final concentration (copies/PCR)	9000	4500	2250	1125	885
Number of replicates	4				
Number of sessions	2				
Extraction System	Automated with magnetic beads, MAGFAST				
Amplification program	Rapid and Ultra-rapid				
Amplification kit	ID Gene Lyo™ FMDV Triplex				
MDL (copies/PCR)	**45.10^2**				
MDL (copies/mL of blood)	**11.10^3**				

Table 14: Method detection limit obtained for spiked whole blood spread on the Nucleic acid Storage Cards, extracted with the ID Gene™ Mag Fast Extraction Kit and amplified with the IDFMDVL kit using both rapid and ultra-rapid amplification programs

RESULTS (Tables 9, 10, 11, 12, 13 and 14):

- When using the automated extraction method with magnetic beads of the **ID Gene™ Mag Fast Extraction Kit (MAGFAST)** and **the ID Gene Lyo™ FMDV Triplex kit** with both rapid and ultra-rapid amplification programs, the MDL obtained for **spiked samples** was:
  - **36.10^4 copies/mL** for **bovine and swine whole blood,**
  - **18.10^3 copies/mg** for **bovine tongue,**
  - **36.10^4 copies/mL** for **oral cavity swab from swine,**
  - **36.10^4 copies/mL** for **caprine milk,**
  - **11.10^3 copies/mL** for **Nucleic Acid Storage Cards.**

DIAGNOSTIC SENSITIVITY AND SPECIFICITY

To evaluate diagnostic sensitivity and specificity, a total of 313 samples were tested with the ID Gene Lyo™ FMDV Triplex kit.

When required, nucleic acid extraction was performed with the ID Gene™ Mag Fast Extraction Kit (MAGFAST) as per the kit instructions. Samples were then tested with the rapid amplification program.

PANEL DESCRIPTION

Panel 1

- This panel included 3 non infectious serum samples originating from cattle provided by the Pirbright Institute as part of an inter-laboratory trial organised in 2018.

Panel 2

- This panel included 160 + 50 whole blood samples from France (Occitanie region):
    - 160 bovine whole blood FMDV-negative samples,
    - 50 swine whole blood FMDV-negative samples.

Panel 3

- This panel included 100 goat milk FMDV-negative samples provided by a french departmental laboratory.

RESULTS

For all samples tested, a specific curve was observed for the endogenous Control (NTPCen), thereby allowing interpretation of results for all samples. The data obtained for the 313 samples tested are summarised in the Table 15 below.

SAMPLE TYPE	NUMBER OF SAMPLES TESTED	DIAGNOSTIC SENSITIVITY (Se)	DIAGNOSTIC SPECIFICITY (Sp)
Serum	3	3/3 **100%**	-
Whole blood	210	-	210/210 **100%**
Milk	100	-	100/100 **100%**

Table 15: Diagnostic sensitivity (Se) and specificity (Sp) results obtained with the ID Gene Lyo™ FMDV Triplex RT-qPCR kit for the different sample types tested

RESULTS (Table 15):

- For the FMDV target, diagnostic sensitivity and specificity results obtained for a panel of 313 samples extracted with magnetic beads and amplified with the ID Gene Lyo™ FMDV Triplex RT-qPCR kit, are:
    - **Measured sensitivity Se = 100%** (95% CI [51, 100], n=3),
    - **Measured specificity Sp = 100%** (95% CI [99, 100], n=310).

Note : When testing bulk milk samples, the number of individual animals that can be included was evaluated in study [4], which uses a RT-qPCR test based on the same target as the ID Gene Lyo™ FMDV Triplex (3D sequence as described in the WOAH manual). The conclusion of this study is as follow : *"based on the peak C_T values detected in this study, these findings indicate that it could be possible to identify one acutely-infected milking cow in a typical-sized dairy herd (100–1000 individuals) using milk from bulk tanks or milk tankers."*

Given that our ID Gene Lyo™ FMDV Triplex kit shows earlier Cq value than 2 other commercial RT-qPCR kits and 2 in-house RT-qPCR tests from a European NRL (see below), we can extend these findings to the ID Gene Lyo™ FMDV Triplex kit.

COMPARISON WITH OTHER RT-qPCR KITS

COMMERCIALLY AVAILABLE RT-QPCR KITS

The performances of the ID Gene Lyo™ FMDV Triplex kit were compared to two other commercially available RT-qPCR kits (Kit A and Kit B, as per manufacturer's instructions) with the rapid amplification program using:

- **3 non infectious serum samples from cattle** provided by The Pirbright Institute as part of an inter-laboratory trial organised in 2018
- **9 negative samples from different matrices, spiked at two different loading rates** (named "++" and "+" below).

The results are presented in Figure 1.

	CQ VALUE FOR THE FMDV TARGET				
	ID Gene Lyo™ FMDV Triplex	KIT A	ΔCq ID Gene Lyo™ – Kit A	KIT B	ΔCq ID Gene Lyo™ – Kit B
SAMPLE 1 (O/EGY/18/2016)	17.23	20.56	-3.33	28.12	-10.89
SAMPLE 2 (A/IRN/8/2016)	16.38	18.86	-2.48	27.62	-11.24
SAMPLE 3 (SAT2-OMN/4/2015)	18.74	21.92	-3.18	29.48	-10.74
SAMPLE 4 (NA CARDS ++)	32.98	37.08	-4.10	Non detected	-
SAMPLE 5 (caprine milk ++)	30.31	35.32	-5.01	Non detected	-
SAMPLE 6 (oral swab from swine ++)	30.57	34	-3.43	Non detected	-
SAMPLE 7 (bovine whole blood ++)	30.91	35.75	-4.84	Non detected	-
SAMPLE 8 (epithelium from bovine tongue ++)	30.74	36.15	-5.41	Non detected	-
SAMPLE 9 (swine whole blood ++)	30.9	36.39	-5.49	Non detected	-
SAMPLE 10 (epithelium from bovine tongue +)	33.83	37.85	-4.02	Non detected	-
SAMPLE 11 (oral swab from swine +)	33.49	36.55	-3.06	Non detected	-
SAMPLE 12 (caprine milk +)	33.24	37.86	-4.62	Non detected	-
Mean Cq	**28.28**	**32.36**	**-4.08**		**-10.96**

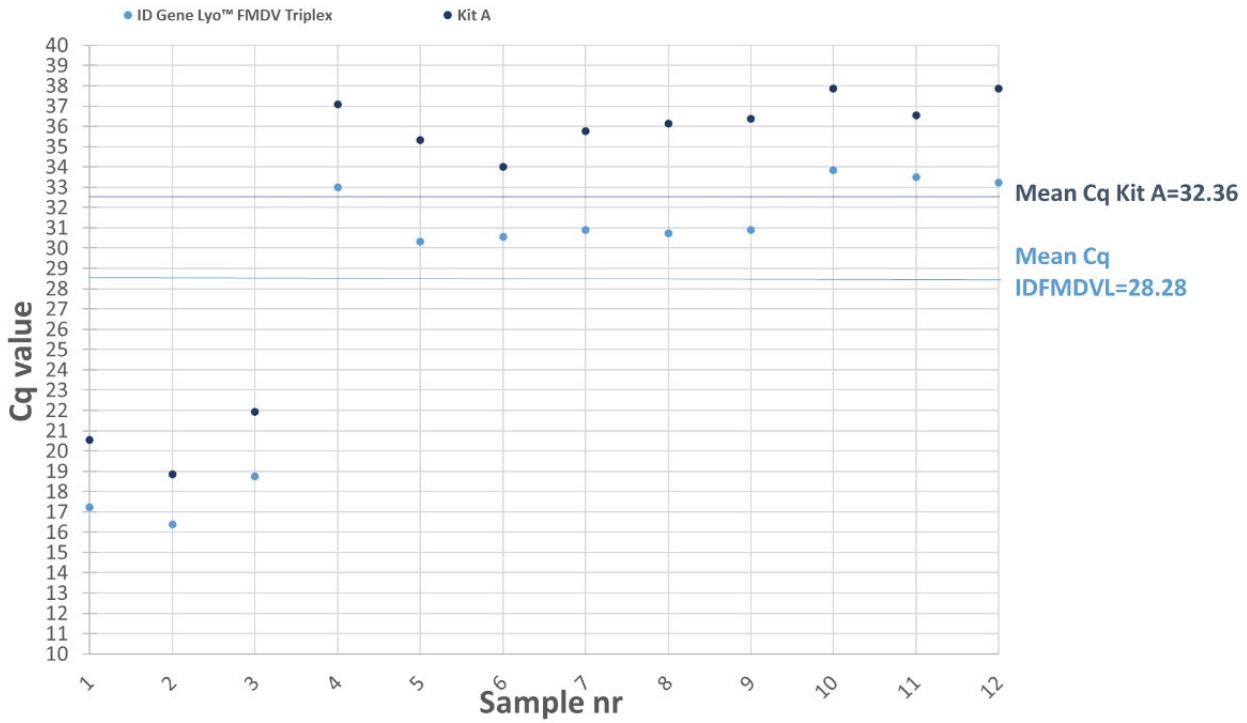

Figure 1: Comparison of results obtained for 12 positive samples using the IDFMDVL RT-qPCR kit, Kit A and Kit B

RESULTS (Figure 1):

- On the panel of 12 samples tested, the **ID Gene Lyo™ FMDV Triplex kit** gave earlier **Cq values than the competing Kit A** (ΔCq = -4.08) and **Kit B** (ΔCq = -10.96).

COMPARISON WITH 2 IN-HOUSE RT-QPCR TESTS

Five (5) different FMDV strains were tested with the ID Gene Lyo™ FMDV Triplex kit and two other RT-qPCR techniques, targetting the 3D Gene (PCR B and PCR C) performed at a European National Reference Laboratory for FMD. This comparison study was conducted by this laboratory on serially-diluted samples down to 10^{-8}.

The results are presented in Table 16 below.

	CQ VALUE FOR THE FMDV TARGET		
STRAIN	**PCR B**	**PCR C**	**ID Gene Lyo™ FMDV Triplex**
O/…/…/…. 10^{-1}	18.52	18.34	17.59
O/…/…/…. 10^{-2}	21.88	21.49	20.85
O/…/…/…. 10^{-3}	25.42	25.48	24.23
O/…/…/…. 10^{-4}	29.07	28.39	27.73
O/…/…/…. 10^{-5}	32.49	31.52	31.24
O/…/…/…. 10^{-6}	36.15	35.99	34.79
O/…/…/…. 10^{-7}	39.69	39.99	39.00
O/…/…/…. 10^{-8}	-	40.18	-
A/…/…/…. 10^{-1}	21.87	23.74	21.20
A/…/…/…. 10^{-2}	25.18	27.15	23.47
A/…/…/…. 10^{-3}	28.81	30.97	26.44
A/…/…/…. 10^{-4}	32.62	34.58	31.90
A/…/…/…. 10^{-5}	36.11	37.96	35.53
A/…/…/…. 10^{-6}	40.03	41.48	38.28
A/…/…/…. 10^{-7}	-	-	39.56
A/…/…/…. 10^{-8}	-	-	-
Asia1/…/…/…. 10^{-1}	19.27	21.32	18.18
Asia1/…/…/…. 10^{-2}	22.63	24.57	21.52
Asia1/…/…/…. 10^{-3}	26.20	29.09	24.83
Asia1/…/…/…. 10^{-4}	29.82	31.44	28.29
Asia1/…/…/…. 10^{-5}	34.12	34.83	31.94
Asia1/…/…/…. 10^{-6}	36.94	38.33	35.15
Asia1/…/…/…. 10^{-7}	40.29	-	-
Asia1/…/…/…. 10^{-8}	-	-	-
SAT1/…/…/…. 10^{-1}	18.00	18.35	16.18
SAT1/…/…/…. 10^{-2}	21.17	21.87	19.53
SAT1/…/…/…. 10^{-3}	24.76	25.27	23.00
SAT1/…/…/…. 10^{-4}	28.18	29.10	26.34
SAT1/…/…/…. 10^{-5}	31.76	33.14	29.83
SAT1/…/…/…. 10^{-6}	35.21	38.26	32.88
SAT1/…/…/…. 10^{-7}	39.66	-	36.25
SAT1/…/…/…. 10^{-8}	-	-	-
SAT2/…/… 10^{-1}	19.66	20.07	18.54
SAT2/…/… 10^{-2}	22.58	23.17	21.76
SAT2/…/… 10^{-3}	26.25	26.90	25.37
SAT2/…/… 10^{-4}	29.83	30.41	28.95
SAT2/…/… 10^{-5}	33.35	33.83	32.38
SAT2/…/… 10^{-6}	36.88	37.58	35.47
SAT2/…/… 10^{-7}	41.48	38.85	39.52
SAT2/…/… 10^{-8}	-	-	-
MEAN Cq (from 10^{-1} to 10^{-6})	**28.16**	**29.15**	**26.78**

Table 16: Comparison of results obtained for identification of 5 different FMDV strains using the ID Gene Lyo™ FMDV Triplex RT-qPCR kit, PCR B and PCR C

RESULTS (Table 16):

- The **ID Gene Lyo™ FMDV Triplex kit** gave **earlier Cq values** than two other in-house reference RT-qPCR tests from this NRL.
- The **ID Gene Lyo™ FMDV Triplex kit** has a Limit of Detection comparable to the other PCR tests performed by this NRL.

ROBUSTNESS

The robustness of the ID Gene Lyo™ FMDV Triplex kit was evaluated by testing the maximum and minimum RNA volume, amplification reaction mixture volume and temperature used in the different steps of the ultra-rapid amplification program, as defined in the instructions for use, and evaluated in the limiting conditions described below:

- 3 X LD_{PCR} for the FMDV target using the PAC-FMDV,
- FMDV-spiked oral swab from swine at the limit of detection, and extracted using the ID Gene™ Mag Fast Extraction Kit (product code: MAGFAST),
- Volume of RNA ± 10%,
- Volume of ARM-FMDVL ± 10%,
- Ultra-rapid amplification program:
  - Reverse transcription step: 10 minutes at 45°C ± 1°C,
  - Activation of the polymerase: 2 minutes at 95°C ± 1°C,
  - DNA denaturation: 5 seconds at 95°C ± 1°C,
  - Annealing step: 2 seconds at 60°C ± 1°C.

VARIATION IN RNA VOLUME

	AVERAGE OF 3 CQ VALUES FOR THE FMDV SIGNAL			
	-10%	**SET VOLUME**	**+10%**	**CV%**
SPIKED ORAL SWAB (MDL)	30.81	30.94	30.74	**1.36**
3 X LD_{PCR}	33.69	33.34	33.41	**0.78**

Table 17: Robustness of the ID Gene Lyo™ FMDV Triplex kit in response to changes in RNA volume using the ultra-rapid amplification program

VARIATION IN TEMPERATURES

	AVERAGE OF 3 CQ VALUES FOR THE FMDV SIGNAL			
	-1°C	**SET TEMPERATURE**	**+1°C**	**CV%**
SPIKED ORAL SWAB (MDL)	31.22	30.85	30.78	**1.06**
3 X LD_{PCR}	33.46	33.42	33.33	**0.91**

Table 18: Robustness of the ID Gene Lyo™ FMDV Triplex kit in response to changes in temperature using the ultra-rapid amplification program

VARIATION IN AMPLIFICATION REACTION MIXTURE VOLUME

	AVERAGE OF 3 CQ VALUES FOR THE FMDV SIGNAL			
	-10%	**SET VOLUME**	**+10%**	**CV%**
SPIKED ORAL SWAB (MDL)	31.07	31.12	31.30	**0.81**
3 X LD_{PCR}	33.21	33.40	33.59	**1.16**

Table 19: Robustness of the ID Gene Lyo™ FMDV Triplex kit in response to changes in amplification reaction mixture volume using the ultra-rapid amplification program

RESULTS (Tables 17, 18 and 19):

- Using the ultra-rapid amplification program, the ID Gene Lyo™ FMDV Triplex RT-qPCR kit gave:
  - a **CV% of less than 2%** when the **RNA volume** varied by **± 10%**,
  - a **CV% of less than 2%** when the **temperature** varied by **± 1°C,**
  - a **CV% of less than 2%** when the **amplification reaction mixture volume** varied by **± 10%.**
- The ID Gene Lyo™ FMDV Triplex kit shows **excellent robustness.**

STABILITY

DESCRIPTION

The stability of the ARM of the ID Gene Lyo™ FMDV Triplex was measured, under different conditions, before and after opening:

- Freeze-dried Amplification Reaction Mixture ARM-FMDVL:
  - stored at ≤ -16°C (recommended storage temperature)
  - stored at ≥ 37 °C (to mimic shipment at ambient/uncontrolled temperature)
- ARM-FMDVL after reconstitution with the RB2 resuspension buffer:
  - stored at ≤ -16°C (recommended storage temperature).

Assays were performed with the rapid amplification program, with:

- The **PAC-FMDV**,
- the PAC-FMDV diluted down to **3 X LD_{PCR}** for the FMDV target (96 copies/PCR),
- a **negative swine whole blood sample spiked at 10 X MDL** with non-infectious material (FMDV-O/EGY/18/2016) and extracted with the MAGFAST extraction system,
- a **negative bovine tongue epithelium sample spiked at 10 X MDL** using with non-infectious material (FMDV-O/EGY/18/2016) and extracted with the MAGFAST extraction system,
- a **negative caprine milk sample spiked at 10 X MDL** using with non-infectious material (FMDV-O/EGY/18/2016) and extracted with the MAGFAST extraction system.

RESULTS

The Cq values obtained for the FMDV target were recorded as a function of time for the ARM-FMDVL in freeze-dried form and at a storage temperature ≤ -16°C (Figure 2) or of 37°C (Figure 3), or in reconstituted form and at a storage temperature ≤ -16°C (Figure 3).

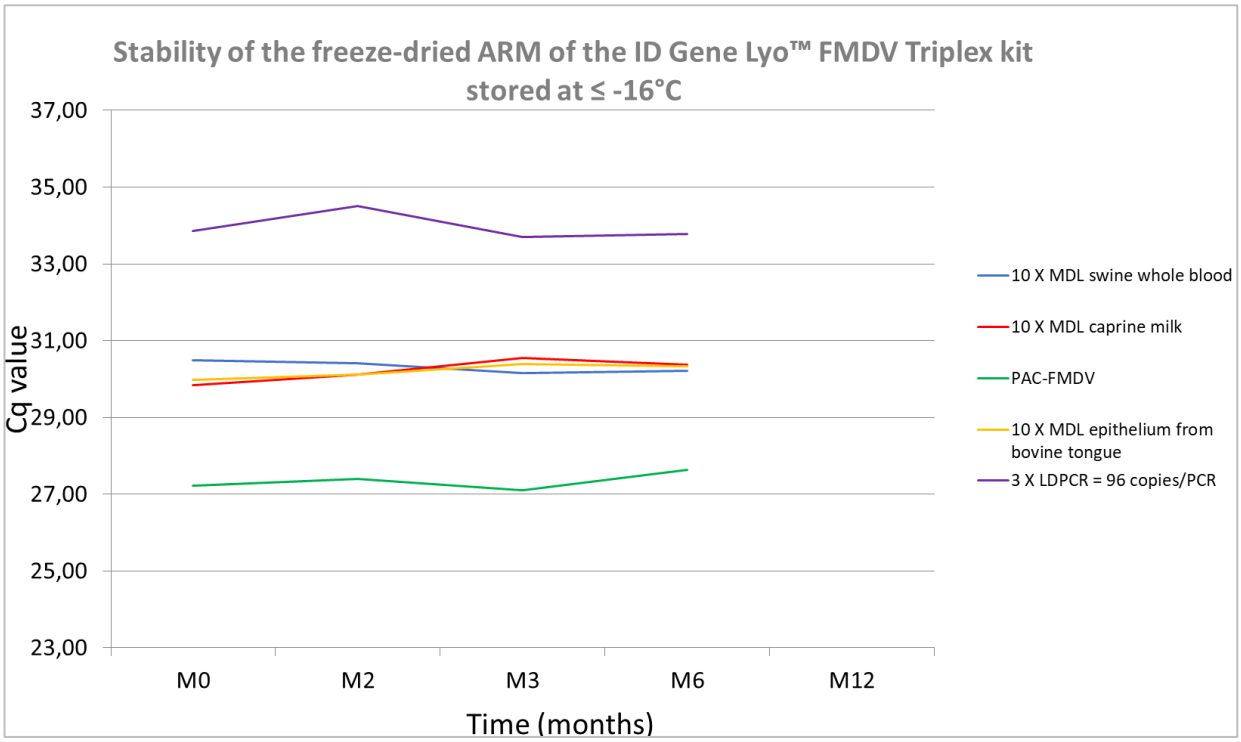

Figure 2: Stability results obtained with the ID Gene Lyo™ FMDV Triplex RT-qPCR (freeze-dried form). Real-time stability study at ≤ -16°C, FMDV target signal

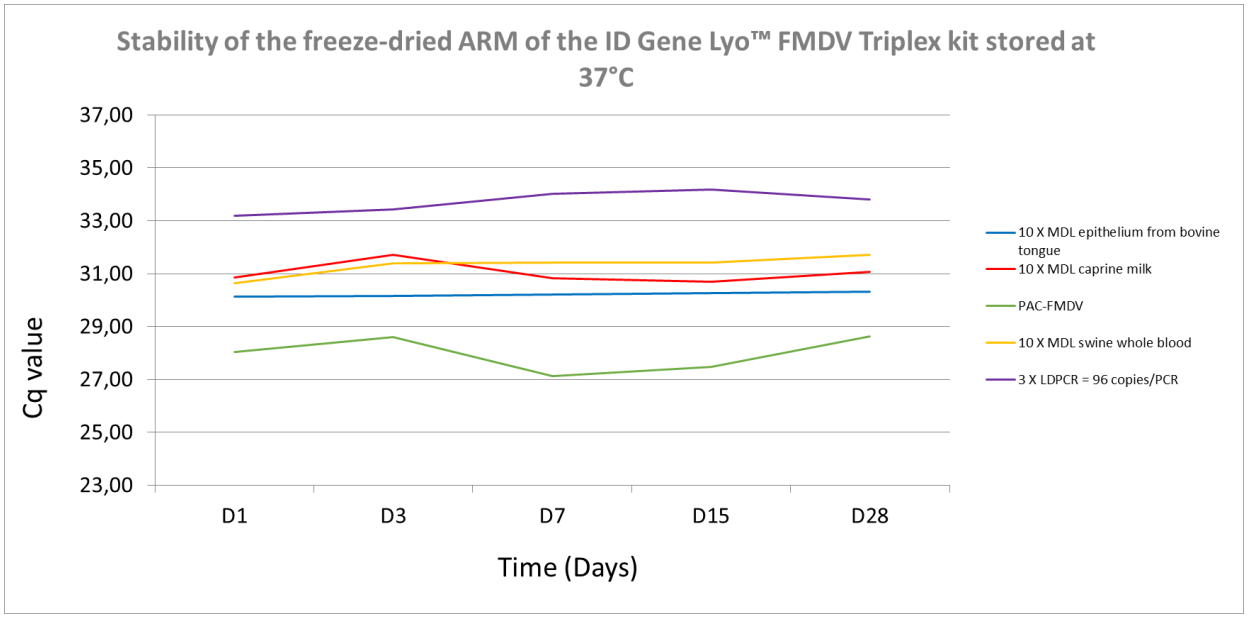

Figure 3: Stability results obtained with the ID Gene Lyo™ FMDV Triplex RT-qPCR (freeze-dried form). Real-time stability study at 37°C, FMDV target signal

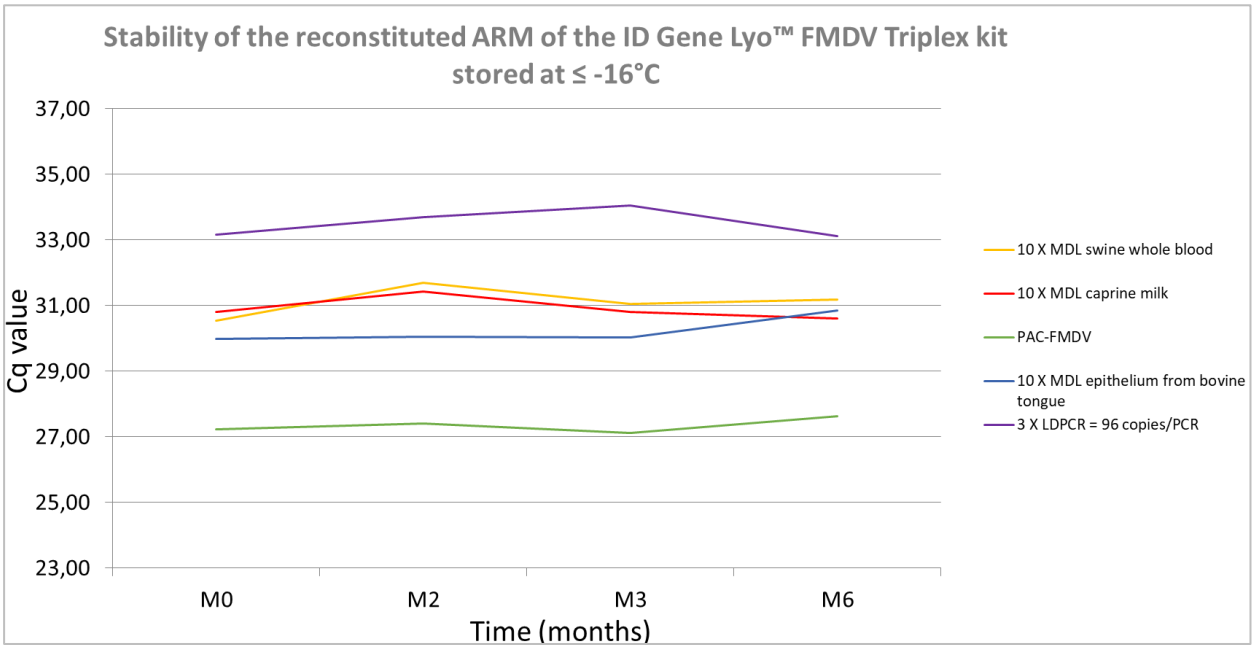

Figure 4: Stability results obtained with the ID Gene Lyo™ FMDV Triplex RT-qPCR (reconstituted form). Real-time stability study at ≤ -16°C, FMDV target signal

RESULTS (Figure 2, 3 and 4):

- The ARM-FMDVL Amplification Reaction Mixture:
  - when **stored at ≤ -16°C (recommended storage temperature)** for **6 months under freeze-dried form,** shows consistent results, with **detection of the target without loss of sensitivity.**
  - when **stored at 37°C** for **1 month under freeze-dried form,** shows consistent results, with **detection of the target without loss of sensitivity** allowing **shipping at uncontrolled temperatures.**
  - when **stored at ≤ -16°C (recommended storage temperature)** for **6 months after resuspension,** shows consistent results, with **detection of the target without loss of sensitivity.**
- The study suggests the good stability of the ID Gene Lyo™ FMDV Triplex kit.
- The study continues and data will be generated over the entire shelflife of the reagent (i.e. 3 years for the freeze-dried form and 1 year after reconstitution).

CONCLUSION

The **ID Gene Lyo™ FMDV Triplex** RT-qPCR kit:

- **does not cross react with any of the pathogens tested,** among which 65 isolates found in the same ecological sphere as the virus of interest**.**
- **efficiently detects all serotypes of the FMDV.**
- demonstrates **high performances:**
    - LD_{PCR} = 32 copies/PCR with the rapid amplification program.
    - LD_{PCR} = 40 copies/PCR with the ultra-rapid amplification program.
- offers **diagnostic and analytical specificity and sensitivity** of **100%** on the tested panels.
- demonstrates **equivalent performances compared to two other in-house RT-qPCR tests used by a national european reference laboratory** and gave earlier **Cq values than two competing commercial RT-qPCR kits** on the tested panel (ΔCq = -4,08 or -10,96).
- has an **excellent robustness (CV% < 2)** on critical parameters of the RT-qPCR reaction.
- is composed of a **freeze-dried Amplification Reaction Mixture (ARM-FMDVL)** for **greater stability over time** and **improved resistance to transport conditions.**

References

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[2] Callahan *et al.* (2002). Use of a portable real-time reverse transcriptase-polymerase chain reaction assay for rapid detection of foot-and-mouth disease virus. J. Am. Vet. Med. Assoc., 220, 1636–1642.

[3] Shaw *et al.* (2007). Protocol: Implementation of a one-step real-time RT-PCR protocol for diagnosis of foot-and-mouth disease. J. Virol. Methods, 143, 81–85.

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History of revisions

VERSION	EDIT DATE	REFERENCE	TYPE OF REVISION	REVISION MADE
0125	03/2025	doc0192	Not applicable (first version)	N/A

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