STANDARD OPERATING PROCEDURE
FMD VP1 AMPLICATION

1. PURPOSE AND SCOPE OF APPLICATION

This test is used for the virological diagnosis of foot-and-mouth disease (FMD) in cases of clinical suspicion (as a second-line test) or for epidemiological surveillance in the context of a diagnostic or research project. This test allows the amplification of the VP1 coding region of the FMD virus genome. The sequence analysis of this region allows to perform phylogenetic study and the characterization of the serotype/topotype/lineage of the FMD virus.

2. BIBLIOGRAPHIC REFERENCES

Analytical method recommended for molecular epidemiological study by the "Office International des Epizooties" (OIE) in the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals.

Bibliographic references:

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3) Valarcher JF, Knowles NJ, Zakharov V, Scherbakov A, Zhang Z, Shang YJ, Liu ZX, Liu XT, Sanyal A, Hemadri D, Tosh C, Rasool TJ, Pattnaik B, Schumann KR, Beckham TR, Linchongsubongkoch W, Ferris NP, Roeder PL, Paton DJ. Multiple origins of foot-and-mouth disease virus serotype Asia 1 outbreaks, 2003-2007. Emerg Infect Dis. 2009 Jul; 15 (7):1046-51. Appendix.

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5) Wadsworth J, Knowles NJ, Swabey KG, Stirling JM, Statham RJ, Li Y, Hutchings GH, Ferris NP, Paton DJ. Recent spread of new strains of foot-and-mouth disease virus type A in the Middle East and North Africa. Appendix 15 (http://www.fao.org/ag/againfo/commissions/docs/research_group/paphos/app15.pdf)

6) Knowles NJ and Samuel AR. RT-PCR and Sequencing Protocols for the Molecular Epidemiology of Exotic Virus Diseases of Animals. 1998. (http://www.wrlfmd.org/fmd_genotyping/genotyping_protocol.pdf).

3. PRINCIPLE OF THE METHOD

After RNA extraction, the test is first based on reverse transcription (RT) of the FMD virus RNA into complementary DNA (cDNA). The cDNA obtained is then amplified by PCR using a DNA polymerase that uses primers targeting the VP1 region that varies according to the virus serotype, thus allowing the specific amplification of the 7 serotypes of FMDV. The two enzymatic reactions are performed successively in the same tube (one-step RT-PCR).

Due to the high rate of mutations during the replication of RNA viruses, it is recommended to use simultaneously at least 2 of the 3 pairs of primers specific to the serotype searched

4. REAGENTS AND PRIMERS

4.1 Reagents

One-step RT-PCR kit, QIAGEN, ref 210212, stored at – 21 °C (± 5 °C).
Ultra pure agarose, Invitrogen (or equivalent)
Loading buffer 6x, Biolabs ref B7021S (or equivalent)
TAE 1X prepared from concentrated TAE 50X, Euromedex ref EU0201-B (or equivalent)
Molecular weight marker, Marker VI, Roche, ref 11062590001 (or equivalent)
Ethidium bromide, BIOPROBE, ref. ETBC01, diluted at 1/10 000 in distilled water (or equivalent).

4.2 Primers

All primer stocks are at 100 µM, aliquoted by 100 µL / tube maximum and stored at -21°C (± 5°C). As soon as the penultimate aliquot is used up, a new primer tube should be ordered (alarm stock = 2 aliquots).
The primers used for the amplification of the genome fragment to be sequenced are described in tables 1 and 2.

Serotype	Sens	Name	Sequence (5'-3')	Region	Size expected	Alignment	Ref.
O	+	O-1C-244F	GCAGCAAAACACATGTCAAACACCTT	VP3	1180 bp	AY593823.1	1, 2
O	+	O-1C-272F	TBGCRGGNCTYGCCCAGTACTAC	VP3	1152 bp	AY593823.1	1, 2
O	+	O-1C-283F	GCCCAGTACTACACACAGTACAG	VP3	1141 bp	AY593823.1	1, 2
A	+	A-1C-562F	TACCAAATTACACACGGGAA	VP3	874 bp	AY593762.1	1, 2
A	+	A-1C-612F	TAGCGCCGGCAAAGACTTTGA	VP3	824 bp	AY593762.1	1, 2
C	+	C-1C-536F	TACAGGGATGGGTCTGTGTGTACC	VP3	945 bp	AY593804.1	1, 2
C	+	C-1C-616F	AAAGACTTTGAGCTCCGGCTACC	VP3	865 bp	AY593804.1	1, 2
Asia1	+	As1-1C505F	TACACTGCTTCTGACGTGGC	VP3	888 bp	JF739177.1	3
Asia1	+	As1-1C530F	CCACRAGTGTGCARGGATGGGT	VP3	863 bp	JF739177.1	3
O, A, C, Asia	-	EUR-2B52R	GACATGTCCTCCTGCATCTGGTTGAT	2B	/	/	1, 2, 3

Table1: Recommended primers for amplification and sequencing of the VP1 region of serotypes O, A, C and Asia.

Serotype	Sens	Name	Sequence (5'-3')	Region	Size expected	Alignment	Ref.
SAT1	+	SAT1-1C-559F	GTGTATCAGATCACAGACACACA	VP3	1042bp	AY593845	1, 2
SAT1	+	SAT1-1U-OSF	GTGTACCAGATCACTGACAC	VP3	1042bp	AY593845	1, 2
SAT1	+	SAT1-P1-1228F	AACCTGCACTTCATGTACAC	VP3	1285bp	AY593845	2
SAT2	+	SAT2-1C-445F	TGGGACACMGGIYTGAACTC	VP3	1125bp	AF540910	1, 2
SAT2	+	SAT2-P1-1223F	TGAACTACCACTTCATGTACACAG	VP3	1259bp	AF540910	1, 2
SAT2	+	SAT2-VP3-AB F	CACTGCTACCACTCRGAGTG	VP3	1143bp	AF540910	1
SAT3	+	SAT3-1C-1222F	AATCTGCATTTCATGTACAC	VP3	1128bp	AY593850	2
SAT3	+	SAT3-1C-559F	CTGTACCAAATYACAGACAC	VP3	1280bp	AY593850	2
SAT1-2-3	-	SAT2B208R	ACAGCGGCCATGCACGACAG	2B	/	/	1, 2

Table 2: Recommended primers for amplification and sequencing of the VP1 region of serotypes SAT1, 2 and 3.

5. SAMPLES PREPARATION

If the viral RNAs are not already extracted, extract total RNA according to your usual procedure.
As an example, the following strains can be used as positive controls, depending on the targeted serotypes:

Serotype	Positive control strain
O	O Manisa
A	A 22
C	C Noville
Asia 1	Asia 1 Shamir
SAT 1	SAT 1 Botswana
SAT 2	SAT 2 Zimbabwe
SAT 3	SAT3/ZIM/1981

Table 3: Identification of positive controls to be used according to the targeted serotypes.

6. OPERATING PROCEDURE

8.1 Mix preparation

Prepare the mix for all reactions to be performed, including a NTC (Non Template Control), a positive control and for 1 or 2 additional reactions. Final volume is 25 µL per reaction, containing, for each selected primer pair, the following reagents:

Reagents	Final concentration	Volume (µL)
DNase RNase free water	/	12,6
One-Step RT-PCR 5 X buffer	1 X	5,0
dNTPs (10 mM each)	400 µM	1,0
Forward primer (100 µM)	0,8 µM	0,2
Reverse primer (100 µM)	0,8 µM	0,2
One-Step RT-PCR Enzyme (1 U / µL)	1 U	1,0
Total to be dispensed per well (µL)	**20**	
RNA volume to be dispensed per well (µL)	**5**	

Table 4 : Mix composition

Dispense 20 µL of mix into each tube and add 5 µL of RNA extract. Close the tubes and centrifuge briefly using a microcentrifuge.

8.2 RT-PCR thermal cycles

The detailed programs, adapted to the different primer pairs, are described in Table 5.
After assembly, put the tubes directly into the thermal cycler and start the program quickly.

	Type O		Types A, C et Asia1		Types SAT1-2-3	
Reverse Transcription (RT)	30 min at 50°C		30 min at 50°C		30 min at 50°C	
Hot Start Taq activation	10 min at 95°C		10 min at 95°C		10 min at 95°C	
Denaturation	1 min at 95°C	x 35 cycles	1 min à 95°C	x 35 cycles	1 min at 95°C	x 35 cycles
Hydridization	**1 min at 60°C**		**1 min at 55°C**		**1 min at 50°C**	
Elongation	1.5 min at 72°C		1.5 min at 72°C		1.5 min at 72°C	
Final elongation	5 min at 72°C		5 min at 72°C		5 min at 72°C	

Table 5: Thermal cycles used for the amplification of the VP1 region

Rq: It is possible to use other primer pairs referenced in paragraph 9 and/or in the literature or designed in-house. In this case, it is necessary to adapt the RT-PCR program applied to the hybridization temperatures of the primers, and the elongation time to the expected size of the amplified fragment.

7. VISUALIZATION OF RESULTS

Prepare a 2% agarose gel in 1 X TAE. For each sample, deposit 5 µL of amplified DNA mixed with 1 µL of loading buffer.
For each migration, add at least one molecular weight marker.

Allow 50 minutes of migration at 120 V (maximum amperage). Agarose gel migration conditions may vary depending on agarose concentration, gel size and amplified DNA size.

Immerse the gel in the ethidium bromide bath for 10-20 minutes. Visualize the amplified DNA under UV light using the imager. Save and export the gel pictures in jpeg format.

8. EXPRESSION OF RESULTS

8.1. TEST VALIDATION

The assay is validated if:

- No band is visible for negative RT-PCR controls (NTC)
- A band of the expected size is visible for positive RT-PCR controls

8.2. EXPRESSION OF RESULTS

The expression of the results depends on the bands obtained:

-**Presence of a band at the expected size**: specific amplification. The DNA can be used for sequencing (in-house or outsourced).

-**No visible band**: negative result. Test other primers or other amplification conditions.

-**Presence of a band not corresponding to the expected size in addition to the specific band**: non-specific amplification. The DNA can be used for sequencing but it is preferable to test other primers or other amplification conditions.

9. ADDITIONAL PRIMERS

The primers described in Table 6 are called additional primers. They can be used to amplify VP1 or to sequence the amplification product obtained with the primers previously mentioned.

Serotype	Sens	Name	Sequence (5'-3')	Region	Ref.
Pan FMD	-	NK72	GAAGGGCCCAGGGTTGGACTC	2A/2B	1, 2, 3
Pan FMD	-	NK61	GACATGTCCTCCTGCATCTG	2B	3
O	+	O-CRH2F	GAYTACGCSTACACSGCGTC	VP3	1,2
O	+	O-1D293F	TGGAYAACACCACYAAYCCAAC	VP1	1,2
O	+	O-1D296F	ACAACACCACCAACCCAAC	VP1	1,2
O	+	O-1D296aF	ATAACACCACTAATCCAAC	VP1	2
O	+	O-1D296bF	ACAACACCACCAATCCAAC	VP1	1,2
O	+	O-1D296cF	ATAACACCACCAATCCAAC	VP1	2
O	+	O-1C605aF	TGGCTAGTGCTGGTAAAGACTTTGAG	VP3	2
O	+	O-1C605bF	TGGCTAGTGCCGGCAAGGACTTTGAG	VP3	2
O	+	O-1C605cF	TGGCTAGCGCCGGCAAGGACTTTGAG	VP3	2
O	+	O-1C605dF	TGGCTAGCGCCGGAAAGGACTTTGAG	VP3	2
O	-	O-1D628bR	GTTGGGTTGGTGGTGTTTT	VP1	2
O	-	O-1D628R	GTTGGGTTGGTGGTGGTTGT	VP1	1,2
O	-	O-1D628aR	GTTGGATTAGTGGTGTTAT	VP1	1,2
O	+	O-1C564F	AATTACACATGGCAAGGCCGACGG	VP3	6
A	-	A-1D523R	CGTTTCATRCGCACRAGRA	VP1	1
A	+	A-1D202aF	TCAGCCACCTACTATTTCTCTGA	VP1	2
A	+	A-1D202bF	GCAGCAACATACTACTTCTCTGA	VP1	2
A	+	A-1D202cF	GCAGCAACCTACTATTTCTCTGA	VP1	2
A	+	A-1D202dF	GCGGCCACTTACTACTTCTCTGA	VP1	2
A	+	A-1D202eF	GCGGCCACCTACTATTTTTCTGA	VP1	2
A	+	A-1D202fF	GCGGCCACCTACTACTTTTCTGA	VP1	2
A	+	A-1D205F	GCNACNTACTAYTTYTC	VP1	5
A	-	A-1D478aR	CAGTGCTCCGTAGTTAAAGGATGA	VP1	2
A	-	A-1D478bR	AATTGCACCGTAATTGAAGGATGC	VP1	1,2

A	-	A-1D478cR	GAGTGCACCATAGTTGAAAGACGC	VP1	2
A	-	A-1D478dR	AATCGCACCAAAGTTGAAGGAAGT	VP1	2
A	-	A-1D478eR	AACTGCGCCGTAGTTGAAGGAGGC	VP1	2
A	-	A-1D478fR	AATTGCGCCGTAGTTGAAGGATGC	VP1	2
C	-	C-1D535R	ARAGYTCIGCICGYTTCAT	VP1	1,2
Asia	+	As1-1D205F	GCRACGTACTACTTYTCRGACCT	VP1	2,3
Asia	-	As1-1D370R	GTTGTAYACTGTYGCCAGCACACG	VP1	2,3
SAT 1	+	SAT1-1D200F	TGCGYGCIGCCACGTACTAYTTCTC	VP1	1,2
SAT 1	+	SAT1-1D200aF	TGCGTGCGGCCACGTATTATTTCTC	VP1	2
SAT 1	+	SAT1-1D200bF	TGCGCGCTGCTACGTACTACTTCTC	VP1	2
SAT 1	+	SAT1-1D200cF	TGCGYGCIGCCACGTACTAYTTCTC	VP1	2
SAT 1	+	SAT1-1D200dF	TGCGTGCTTCCACGTACTACTTCTC	VP1	2
SAT 1	+	SAT1-1D200eF	TGCGYGCGGCCACGTACTACTTCTC	VP1	2
SAT 1	-	SAT1-1D394R	GGYTTGTACTTRCARTCACCGTTGTA	VP1	1,2
SAT 1	-	SAT1-1D394aR	GGTTTGTAYTTGCAGTYGCCRTTGTA	VP1	2
SAT 1	-	SAT1-1D394bR	GGCTTGTACTTACAGTCACCATTGTA	VP1	2
SAT 1	-	SAT1-1D394cR	GGTTTGTAYTTGCAGTTGCCRTTGTA	VP1	2
SAT 1	-	SAT1-1D394dR	GGCYTGTACTTRCAGTCACCGTTGTA	VP1	2
SAT 1	-	SAT1-1D394eR	GGTTTGTAYTTGCARTCACCGTTGTA	VP1	2
SAT 2	+	SAT2-P1-1223F	TGAACTACCACTTCATGTACACAG	VP3	1,2
SAT 2	-	SAT2-D	GGTGCGCCGTTGGGTTGCCA	VP1	1,2
SAT 2	+	SAT2-1D209aF	CCACTTACTACTTTTGTGACCTTGA	VP1	2
SAT 2	+	SAT2-1D209bF	CCACCTACTACTTTTGTGACCTTGA	VP1	2
SAT 2	+	SAT2-1D209cF	CCACCTACTATTTCTGTGACCTGGA	VP1	1,2
SAT 2	+	SAT2-1D209dF	CCACGTACTACTTCTGTGACCTGGA	VP1	2
SAT 2	+	SAT-1D209F	CCACATACTACTTTTGTGACCTGGA	VP3	4
SAT 2	+	SAT2-1C513aF	CACACACACAGACACACCCGCGATGGC	VP3	2
SAT 2	+	SAT2-1C513bF	CACACACACTGACACACCTGCGATGGC	VP3	2
SAT 2	+	SAT2-1C513cF	CACCCACACAGACACACCGGCCATGGC	VP3	2
SAT 2	+	SAT2-1C513dF	CACGCACACAGACACCCCGGCCATGGC	VP3	2
SAT 2	+	SAT2-1C513dG	CACGCACACGGACACTCCCGCGATGGC	VP3	2
SAT 2	+	SAT2-1C513dH	CTCTCACACGGACACTCGCGCGAAGGC	VP3	2

Table 6: Additional primers used for sequencing or amplification of the VP1 region of FMD virus.