DUPLEX ONE-STEP REAL-TIME RT-PCR ASSAYS FOR DIAGNOSIS OF FOOT-AND-MOUTH DISEASE

1. PURPOSE AND SCOPE

This test is used for the virological diagnosis of foot-and-mouth disease by the detection of the viral genome.

2. NORMATIVE REFERENCES

Bibliographic references:

1. *M. Reid, N. Ferris, G. Hutchings, Z. Zhang, G. Belsham and S. Alexandersen.* Detection of all seven serotypes of foot-and-mouth disease virus by real-time, fluorogenic reverse transcription polymerase chain reaction assay. *J. Virol. Methods, 2002, 105: 67-80*

2. *J.F. Toussaint, C. Sailleau, E. Bréard, S. Zientara and K. De Clercq*. Bluetongue virus detection by two real-time RT-qPCR targeting two different genomic segments. *J. Virol. Methods, 2007, 140: 115-128*

3. *N.P. Ferris, D.P. King, S.M. Reid, G.H. Hutchings, A.E. Shaw, D.J. Paton, N. Goris, B. Haas, B. Hoffmann, E. Brocchi, M. Bugnetti, A. Dekker and K. De Clercq.* Foot-and-mouth disease virus: a first inter-laboratory comparison trial to evaluate virus isolation and RT-PCR detection methods. *Vet. Microbiol., 2006, 117: 130-140*

4*. Johnny D Callaham; Fred Brown; Fernando A. Osorio; Jung H.Sur; Ed Kramer; Gary W. Long; Juan Lubroth; S.J. Ellis, K.S. Shoulars, K.L. Gaffney, D.L. Rock, W.M. Nelson.* Use of a portable real-time reverse transcriptase-polymerase chain reaction assay for rapid detection of foot-and-mouth disease virus. *YAVMA, 2002, 220, 11:1636-42.*

5*. N.Goris, F. Vandenbussche, C. Herr, J. Villiers, Y. Van der Stede, K. De Klercq.* Validation of two real-time RT-PCR methods for foot-and-mouth disease diagnosis: RNA-extraction, matrix effect, uncertainty of measurement and precision. *J. of Virol. Met, 2009, 160, 157-162.*

6. K. Gorna, A. Relmy, A. Romey, S. Zientara, S. Blaise-Boisseau, L. Bakkali-Kassimi. Establishment and validation of two duplex one-step real-timeRT-PCR assays for diagnosis of foot-and-mouth disease. *J. of Virol. Met. 235 (2016) 168–175*

3. DEFINITIONS

Spectrum: all susceptible species (artiodactyla)

Matrix: milk, serum, epithelial homogenate supernatant from lesions (vesicles), oropharyngeal samples, whole blood, organ homogenate, viral suspension from infected cell culture.

Analyte: genomic RNA of foot-and-mouth disease virus (all serotypes)

RT: reverse transcription

PCR: polymerase chain reaction

4. PRINCIPLE OF THE METHOD

After RNA extraction, the test first relies on the reverse transcription (RT) of the RNA of foot-and-mouth disease virus into complementary DNA (cDNA). The cDNA obtained then undergoes PCR amplification by a DNA polymerase that uses primers and a TaqMan® probe, labelled with a FAM or VIC fluorochrome at the 5' end. The two enzymatic reactions are performed successively in the same tube (one-step, real-time RT-PCR).

Due to the high level of mutations generated during the replication of RNA viruses, it is recommended to use two specific primer pairs in parallel targeting two different regions of the FMD genome: the IRES region and the encoding region for the RNA-dependent RNA polymerase 3D.

In addition, a third pair of primers is used to detect the endogenous β-actin cellular gene (present in the extracted cellular RNA fraction). Detection of this gene acts as a control for the extraction step and absence of PCR inhibitors.

This is a duplex reaction. In a single reaction, two targets are detected simultaneously: either the IRES + β-actin targets, or the 3D + β-actin targets. The probe for the FMD target is labelled with a FAM fluorochrome and the probe for the β-actin target with a VIC fluorochrome.

5. EQUIPMENT AND MATERIALS

- Class II microbiological safety cabinet (MSC)
- Cold pan
- Crushed ice
- Benchtop microcentrifuge
- Vibration shaker
- Pipetting and micro-pipetting materials
- 10, 20, 200 and 1000 µl RNase- and DNase-free filter tips
- 0.2 ml, 1.5 ml and 2 ml RNase- and DNase-free microtubes
- Plastic consumables (microplates, microtubes, rods, etc.) compatible with the real-time thermocycler
- Aluminium
- Real-time thermocycler

6. REAGENTS AND PRODUCTS

6.1. RT-PCR KIT

- AgPath-ID™ One-Step RT-PCR Reagents (Applied Biosystems)
- Ultrapure, DEPC-treated, RNase-/DNase-free H_2O for primers and probes

6.2. PRIMERS AND PROBES

- All of the master stocks are at 100 µM, aliquoted and stored at -20°C.
- The primer working stocks are at 10 µM (except for that of the IRES reverse primer which is at 100 µM), aliquoted and stored at -20°C.
- The probe working stocks are at 10 µM (3D) or 5 µM (IRES and β-actin), aliquoted and stored at -20°C. **Important: the aliquoted primer tubes at the working-stock concentration must not be frozen/thawed more than five times.**
  When a new lot of primers or probes is purchased, the solutions from the new prepared lots are checked by undertaking rtRT-PCR with the internal control.

Primers/probe	Sequence (5'- 3')	Location	Ref.
FMD-IRES-F forward primer	**5'- CAC YTY AAG RTG ACA YTG RTA CTG GTA C -3'**	nt 515-542	1
FMD-IRES-R reverse primer	**5'- CAG ATY CCR AGT GWC ICI TGT TA -3'**	complementary to nt 611-589	1
FMD-IRES-P probe	FAM 5'- CCT CGG GGT ACC TGA AGG GCA TCC -3' TAMRA	complementary to nt 588-555	1
FMD-3D-F forward primer	**5'- ACT GGG TTT TAC AAA CCT GTG A -3'**	nt 7485-7506	4
FMD-3D-R reverse primer	**5'- GCG AGT CCT GCC ACG GA -3'**	complementary to nt 7591-7575	4
FMD-3D-P probe	**FAM 5'- TCC TTT GCA CGC CGT GGG AC -3' TAMRA**	nt 7536-7555	4
ACT-F forward primer	**5'- CAG CAC AAT GAA GAT CAA GAT CAT C -3'**	nt 1005-1029	2
ACT-R reverse primer	**5'- CGG ACT CAT CGT ACT CCT GCT T -3'**	complementary to nt 1135-1114	2
ACT-P probe	**VIC 5'- TCG CTG TCC ACC TTC CAG CAG ATG T -3' TAMRA**	1081-1105	2

Where Y = C or T W = A or T R = A or G I = inosine

Note: The numbering of nucleotides (nt) for viral RNA refers to the publication by *Forss S et al, NAR, 1984, 12: 6587-6601* on the sequencing of the O1K strain, GenBank accession number: X00871.1. The numbering of nucleotides (nt) for the β-actin gene refers to the publication by *Toussaint, et al. J. Virol. Methods, 2007.*

7. PREPARATION OF SAMPLES

- Extract the viral RNA either with the QIAGEN extraction kit, from 100 to 140 µl of suspension, by eluting the RNA in 100 µl of the elution buffer included in the kit, or else with the extraction robot, from 100 to 140 µl of suspension, by performing the lysis step in the MSC and by eluting the RNA in 60 to 100 µl.
- For each run of one to 12 samples, include a sample of ultrapure water (negative extraction control) in the extraction.

Recommendations:

- Follow the usual recommendations to avoid contamination (aliquot the samples; aliquot the reagents; separate the workstations; use filter tips; wear gloves and replace them as needed).
- Work with sterile, single-use and RNase-free consumables.
- If possible, add negative extraction controls to ensure the absence of inter-sample contamination by producing a control from a negative sample corresponding to the same matrix as the test samples (= negative control). These extraction controls will then be treated as samples.
- Add the internal positive control (IPC) (positive RNA = positive PCR control).

8. PROCEDURE

8.1 PREPARATION OF THE MIX

- Preparation of the RT-PCR mix: Prepare the mix away from light for all the reactions (plan on one or two additional reactions), for a final volume of 25 µl per reaction containing for each target gene (IRES and β-actin, 3D and β-actin) the following reagents:

Reagent	FMD detection mix			
	(FMD IRES/β-actin) Per reaction		**(FMD 3D/β-actin)** Per reaction	
	Final concentration	Volume (µl)	Final concentration	Volume (µl)
Ultrapure water (DNase-/RNase-free)		1.350		1.4
2X buffer	1x	12.5	1x	12.5
FMD forward primer (IRES and 3D 10 µM)	0.4 µM	1	0.4 µM	1
FMD reverse primer (IRES **100 µM**) and (3D 10 µM)	(PS*) 1.2 µM	0.3	0.4 µM	1
Probe (FAM-TAMRA) (IRES 5 µM) and (3D 10 µM)	0.25 µM	1.250	0.2 µM	0.5
β-actin forward primer 10 µM	0.4 µM	1	0.4 µM	1
β-actin reverse primer 10 µM	0.4 µM	1	0.4 µM	1
Probe (VIC-TAMRA) (β-actin) 5 µM	0.12 µM	0.6	0.12 µM	0.6
25X RT-PCR mix (Enzyme)	1x	1	1x	1
Total to be dispensed per well	20 µl		20 µl	
RNA volume to be dispensed per well	5 µl		5 µl	

*Primary stock at 100 µM

- Dispense 20 µl of mix to each tube and add 5 µl of extracted RNA.
- For each test, it is essential to run a non-template control (NTC) containing 20 µl of mix and 5 µl of water from the same kit. This control is used to check that the reagents are not contaminated by the targets (negative control for RT-PCR).

Close the tubes with optical-cap strips or seal the microplates with optical film. Check that they are properly closed and briefly centrifuge with a microcentrifuge.

8.2 PROGRAMMING THE REAL-TIME PCR INSTRUMENT

It is best to programme the thermocycler before preparing the RT-PCR mix. Otherwise, store the plate or tubes on melting ice or at +5°C (± 3°C), away from light, until the thermocycler is programmed. Quickly start the run after installing the plate or tubes in the thermocycler (LSA-INS-0938, LSA-INS-0940, LSA-INS-1075).

All the wells are read through FAM and VIC. The quencher (Tamra) is non-fluorescent. The mixture contains an ROX passive reference for ABI instruments that normalises the signal emitted by other fluorochromes during the PCR reaction. Fluorescence is read at the end of the elongation step.

Steps	Temperature	Time	Number
Reverse transcription	45°C	10 min	1x
RT inactivation and DNA polymerase activation	95°C	10 min	1x
Denaturation	95°C	15 sec	45x
Hybridisation, elongation and reading	60°C	1 min	

9. EXPRESSION OF RESULTS

9.1. READING

Fluorescence is read, during each cycle, at the end of the elongation step. The cycle threshold (Ct) is defined as the number of cycles required for the fluorescent signal to cross the threshold. This threshold is set manually or automatically (by the instrument's software).

9.2. TEST VALIDATION

The test is validated if:

- The NTCs and the negative extraction controls all have an undetermined (UNDET) Ct value
- The Ct value for the internal positive control fulfils the validation criteria.

It is important to check the appearance of the amplification curve for each target.

The result for each sample is validated if:

- The Ct obtained for the sample with the β-actin RT-PCR is less than 40. *If the Ct is equal to or greater than 40, or still undetermined (UNDET), dilute the total RNA in RNase- and DNase-free water (1:5 dilution) and repeat the FMD and β-actin RT-PCR reactions using this dilution.*
- *If the Ct for β-actin is again equal to or greater than 40, or still undetermined, repeat the extraction of total RNA by diluting the sample in PBS, pH 7.4 (containing no calcium or magnesium, 1:2 dilution).*
- *If the Ct is again equal to or greater than 40, or undetermined, the sample will be considered as unusable (presence of RT-PCR inhibitors; lysed or putrefied sample, etc.).*

***Note**:* There may be some samples with low cell counts, e.g. plasma or viral suspension from infected cell culture. In this case, the Ct values for the β-actin gene could be greater than 40 or undetermined.

9.4. EXPRESSION OF RESULTS

- The sample is considered negative when the Ct obtained with the FMD PCR is **"Und"**. The result is reported as **"foot-and-mouth disease virus genome, undetected"**.
- The sample is considered positive when a Ct is obtained with a characteristic amplification curve (sigmoidal). The result is reported as **"detection of the foot-and-mouth disease virus genome"**.
- The sample can be considered questionable if a Ct is obtained with a non-characteristic curve. In this case, the test should be repeated to define the sample's infectivity status or else compared with other tests undertaken with the sample.
- Due to the high level of mutations generated during the replication of RNA viruses (and therefore FMD virus), a positive result can only be obtained for one of the two FMD targets (either IRES or 3D). In this case, the test should either be repeated to confirm this result or compared with other tests undertaken with the sample.