SANGER SEQUENCING OF FMDV

1. PURPOSE AND SCOPE

The monitoring of the worldwide epidemiological distribution of the foot-and-mouth disease virus (FMDV) and the characterization of the various circulating viral strains are of high importance for reference laboratories. To identify and classify the strains, the genomic region most frequently studied is the one encoding the viral capsid protein VP1 (gene 1D). This procedure describes the sequencing of the VP1 gene of FMDV using a Sanger sequencing approach and the SeqStudio Genetic Analyzer from Life technologies.

2. DEFINITIONS

cDNA: Complementary Deoxyribonucleic acid

dNTP: Desoxyribonucleoside triphosphate

ddNTP: Didesoxyribonucleoside triphosphate

FMD: Foot-and-Mouth Disease

RT-PCR: Reverse Transcriptase PCR

VP1: Viral Protein 1

3. PRINCIPLE OF THE METHOD

The region of the viral genome to be sequenced is amplified by an initial RT-PCR reaction to obtain cDNA copies including the sequence coding for the VP1 protein of the FMDV (Knowles et al, 2016). The initial cDNA is purified using the ExoSAP-IT PCR Product Cleanup kit (Applied Biosystems), an enzymatic mixture that captures and degrades the nucleotides and enzymes that remained free during this first RT-PCR reaction. It can also be purified on a silica filter column, for example using the GFX PCR Cleanup kit (GE Healthcare). The purified cDNA is quantified using the Qubit® fluorometer with the DNA High Sensitivity kit (Molecular Probes, Invitrogen). The quantified cDNA is amplified by a sequencing PCR using the BigDye 3.1 kit (Applied Biosystem) which contains classic nucleotides (dNTP) and dideoxynucleotides (ddNTP) that carry only one -OH group and a fluorophore specific to its associated base (A, T, C or G). The ddNTP/dNTP ratio is 1/100, crucial to allow random incorporation of ddNTP and to favor the appearance of sequencing products of all sizes. From a single primer (sense or antisense), dNTPs and ddNTPs are incorporated by Taq polymerase by complementarity into a copy strand of the initial cDNA. In this context, to obtain a good quality sequencing reaction, a primer/matrix ratio close to 10 to 1 is necessary, so it is essential to quantify and then dilute the cDNA and primers to the correct concentration beforehand. Incorporation of a ddNTP into the copy strand stops the activity of Taq polymerase. Each fragment obtained is thus marked at its 3' end by a fluorophore specific to the type of base of the last incorporated ddNTP.

A final purification of the sequencing products is carried out using the BigDye XTerminator kit (Applied Biosystem) to capture unincorporated ddNTPs, salts and other charged molecules that could interfere with the electrophoretic migration and reading of the sequencing samples. The cDNA fragments marked at their 3' ends are then separated according to their molecular mass by capillary electrophoretic migration on a high-resolution polymer incorporated into the reading cassette of the SeqStudio Genetic Analyzer. The smallest fragments migrate faster, allowing the successive classification of fragments differing by a single base. The complete sequence is reconstructed by successive reading of the fluorescence as all the fragments pass in front of the sensor in increasing size order, thereby reconstructing the nucleotide sequence of the template strand on an electropherogram

3. EQUIPMENT AND MATERIALS

- PCR microtubes 0.2 ml in 8-tube strips or 96-well PCR plates (free of detectable DNase, RNase and PCR-Inhibitor)
- Aluminium
- Single-channel and multi-channel micropipettes
- Optical cap strips or optical films
- Shaker
- Conventional thermocycler
- Centrifuge with buckets for microplates
- 1.5 ml microtubes (RNase, DNase-free)
- Dedicated racks
- Gloves
- Filter tips (RNase, DNase- and pyrogen-safe) suitable for pipetting equipment
- Ice bucket, ice
- Benchtop microcentrifuge
- IKA® MS 3 digital universal shaker
- Qubit fluorometer (Invitrogen, Thermofisher)
- 96-well plate with barcode Applied Biosystems® (Do not use MicroAmp Fast Optical 96-Well Reaction Plate to avoid damaging the cartridge)
- Septa for 96-well plate Applied Biosystems®
- SeqStudio sequencing device

4. REAGENTS AND PRODUCTS

- Specific primer aliquots (at 3.2 µM) at -20°C (+/- 5°C)
- M13 control primer (21nt) at -20°C (+/- 5°C)
- pGEM control DNA at -20°C (+/- 5°C)
- ExoSAP-IT Express PCR Product Cleanup kit (Ozyme)
- Qubit dsDNA HS assay (molecular probes) at +4°C (+/- 3°C)
- BigDye terminator® Ready reaction Mix v3.1 (Applied Biosystems)
- BigDye terminator® Sequencing buffer v1.1 v3.1 5X (Applied Biosystems)
- BigDye Xterminator® Purification kit XTerminator Solution (Applied Biosystems)
- BigDye terminator® Purification kit SAM Solution (Applied Biosystems)
- Ultra-pure water RNase/DNase free

5. PREPARATION OF SAMPLES

Sample acceptance conditions

Samples must first be analyzed by gel after RT-PCR of the VP1 gene (from RNA into cDNA). It is recommended to plan analysis series with a total number of reactions corresponding to a multiple of 4 and arrange them in columns on the plate. If there are empty wells, fill them with deionized water or formamide. A maximum of 48 wells can be analyzed in 24 hours by the sequencer without loss of reading quality between the first and last well analyzed. The number of simultaneous samples for analysis is therefore a maximum of 24, including controls. If this number is exceeded, refer to the storage conditions before analysis.

Storage of samples before analysis

Sequencing reactions can be stored for 24 hours at +5°C (+/- 3°C), one month at -21°C (+/- 5°C), or several months at < -70°C before being read on the sequencer.

Storage of samples or residues after analysis

Residuals after sequencing run can be stored for 24 hours at +5°C (+/- 3°C) or one month at -21°C (+/- 5°C), if they have spent less than 24 hours in the sequencer, for subsequent re-reading.

6. PROCEDURE

Reminder: to obtain a good quality sequencing reaction, it is necessary to have a primer/matrix ratio close to 10:1 and a ddNTP/dNTP ratio of 1:100.

Before performing the sequencing reaction, it is recommended to dilute the primers at 3,2 µM into nuclease free water.

Preparation of enzymatic purification of RT-PCR Products (cDNA) and Sanger PCR Mix:

In the mix room, depending on the number of PCR samples to be purified, introduce 2 µL of ExoSAP-IT Express PCR Product Cleanup into a strip of PCR microtubes labelled “Exo”.

Note: The purification of RT-PCR products and the Sanger PCR mix can be performed independently.

Thaw the BigDye terminator ready reaction mix v3.1, water, and primers in the dark on ice. Take the diluent (5X Sequencing Buffer) out of the refrigerator. Assemble the premix according to the following proportions for one sample:

	Premix Forward (µL)	Premix Reverse (µL)	pGEM
Ultrapure water (DNase RNase free)	3,5	3,5	4
Big Dye Terminator ready reaction mix	2,0	2,0	2
5 X Sequencing Buffer	1,0	1,0	1
Forward primer at 3,2 µM	0,5	-	(primer M13) 1
Reverse primer at 3,2 µM	-	0,5	(pGEM control) 1

Add 7 µL of premix to each tube or each well of the standard PCR plate to be prepared for the sequencing reaction. Label the plate or strips "Sanger." Place the reagents for the pGEM control reaction directly into the corresponding column.

Enzymatic Purification of RT-PCR Products (cDNA):

In the assembly room, add 5 µL of the RT-PCR product to be purified into the “EXO” strips and close the tube tightly. Mix or vortex for 2-3 seconds. Briefly centrifuge for 2-3 seconds. Incubate in a conventional thermocycler in room E/1/50T and start the EXOSAP-IT cycle (15 minutes at 37°C and 15 minutes at 80°C). The purified PCR products can be stored overnight at +4°C, for one week at -20°C, or stored at -80°C for later use.

Quantification of cDNA using Qubit system:

Use the Qubit dsDNA HS assay kit (molecular probes). Place 190 µL of Working solution in 2 Qubit tubes labelled S1 and S2. Protect from light. Add 10 µL of standard 1 or standard 2 to tubes S1 and S2 respectively. Briefly centrifuge. Prepare as many tubes as the number of samples to be quantified, containing 198 µL of Working solution. Do not prepare more than 10 tubes at a time. Add 2 µL of purified DNA sample from the EXO strip to each tube. Protecting the tubes from light, vortex, then incubate at room temperature for 2 minutes. On the Qubit, select "dsDNA" then "1X dsDNA high sensitivity". Select "Read standards". Insert tube S1 into the Qubit. Select "Read Standard" and note the value in the tracking sheet. Insert tube S2, select "Read standard" and note the value in the tracking sheet (S2 can be measured as a sample, with the expected value being 10 ng/µl). The Qubit is ready to quantify samples.

Once the 2 minutes of incubation for the tubes to be quantified have passed, insert the first tube into the Qubit and select "Run Sample". When the result appears, check "Sample volume" and set it to 2 µL. In "output sample unit", verify that the selected unit is "ng/µL" and press "read tube". Record the concentrations on the tracking sheet. Perform any suggested dilutions in Nuclease-free water.

Dilution of purified cDNA and assembly for Sanger reaction PCR:

For a VP1 PCR product amplified to the length of our fragment of interest (500-1000 bp), introduce 12.5 ng per sequencing reaction, i.e., 25 ng for both sense and antisense reactions:

Taille du produit PCR (pb)	100-200	200-500	500-1000	1000-2000	>2000	ss DNA
DNA quantity (ng)	1-3	3-10	5-20	10-40	40-100	25-50

In a standard PCR strip labeled "EXO", add 7 µL of purified DNA or, if a dilution was necessary, mix the volume of purified DNA qsp 7 µL Nuclease-free water. Add 3 µL of the purified, quantified, and diluted PCR products to the wells of the "SANGER" strip or plate.

Seal and briefly centrifuge. Start the SANGER sequencing reaction program:

Temperature (°C)	Time	Number of cycles
96°C	1 min	1
96°C	10 sec	25
50°C	5 sec	
60°C	4 min	
10°C	hold	

Purification of sequencing products:

As a reminder, the total number of reactions must be a multiple of 4. If there are empty wells, fill them only with molecular grade deionized water or formamide.

Vortex the XTerminator solution for at least 10 seconds at maximum speed. Revortex every 2 minutes during use (to prevent bead sedimentation). If particles are present, heat the SAM solution to +37°C and mix to dissolve, then cool to room temperature before use. Prepare the XTerminator bead premix (10 µL/tube) with SAM solution (40 µL/tube) in E/1/50T. Mix by aspiration/expulsion. Dispense 50 µL of the premix into the optical PCR plate or optical reading strips suitable for SeqStudio. Add 10 µL of the Sanger PCR reaction. Secure the samples with tape in the IKA® MS 3 digital shaker universal SM plate vortexer next to the sequencer (room E/1/50T). Cover the plate with aluminum foil to protect from light. Vortex at 2000 rpm for 30 minutes at room temperature. Centrifuge for 2 minutes at 1000xg. The plate can be stored at +4°C for up to 24 hours for reading. For longer storage, it is recommended to keep at -20°C.

Reading of sequencing products:

Cover the columns containing samples with septa using the tray retainer and 8-well septa for optical strips and 96-well septa for whole plates. Select the BigDye XTerminator (BDX) long run module. Samples are stable between 16 and 24 hours on the plate. If the run is completed in less than 24 hours, it is possible to rerun, obtaining new high-quality reads. The results are acceptable for obtaining additional reads and increasing confidence in subsequent analysis.