WHOLE GENOME SEQUENCING OF FMDV USING NEXTERA XT KIT FROM ILLUMINA

1. PURPOSE AND SCOPE

This kit is used for the sequencing of the whole genome of Foot-and-Mouth Disease Virus.

2. DEFINITIONS

cDNA: complementary DNA

RT: reverse transcription

PCR: polymerase chain reaction

3. PRINCIPLE OF THE METHOD

After RNA extraction, the resuspension step should be performed using molecular biology grade water. It is mandatory to avoid any buffer containing EDTA which could interfer with the sequencing reaction. The first step of the analysis relies on the reverse transcription (RT) of the RNA of foot-and-mouth disease virus into complementary DNA (cDNA). The cDNA obtained is then transformed into double strand DNA and can be used for the preparation of the sequencing libraries using the Nextera XT kit from Illumina. Once generated, the libraries can be loaded on a MiSeq instrument for the sequencing run.

3. EQUIPMENT AND MATERIALS

- 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
- Magnetic strand
- Qubit quantifier
- Illumina MiSeq

4. REAGENTS AND PRODUCTS

1. DNA QUANTIFICATION

Qubit dDNA High Sensitivity kit (Thermofisher)

Qubit assay tubes

Aluminium foil

2. SEQUENCING KIT

Illumina Nextera XT kit

Illumina index and replacement caps

AMPure XP Beads or equivalent size-selection beads

96-well plate or PCR strips and associated films/caps

Mulitchannel pipette and corresponding tips

Absolute ethanol

Magnetic stand

1M NaOH

Illumina MiSeq reagent cartridge

5. PREPARATION OF SAMPLES

- Extract the viral RNA either with a manual extraction kit (QIAGEN…) or with the automated extraction robot (Ideal32, KingFisher…) and resuspend into molecular biology grade water (presence of EDTA in samples is strictly forbidden).
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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.

6. PROCEDURE

6.1 PREPARATION OF THE DOUBLE STRAND DNA FOR THE SEQUENCING STEP

The samples should be treated with DNAse to avoid any "contamination" by residual DNA (i.e. from cells…). The RNA should be placed on ice to avoid any degradation which could be critical for the sequencing step. The DNAse treatment is realized using the Dnase I Amp grade kit from Invitrogen™ (Ref: 18068015). In the assembly room, the DNase I Reaction Buffer 10X should be thawed. For one sample, prepare the reactional DNAse mix as follow (final volume of 11 µl):

- 1 µL of DNase I Reaction Buffer 10X (1X final)
- 1 µL of DNase I, Amp Grade (1U/µL) (0.1U/µL final)

For one sample: distribute 2 µl of reactional DNAse mix in one tube and put 1 µl of EDTA 25mM in an additional tube (this tube is then put on ice for a further use). Add 8 µL of RNA to the reactional DNAse mix. Mix gently by flicking the tube and centrifuge briefly. Incubate 15 minutes at room temperature. Then, add 1 µL of EDTA 25mM to inactivate the enzymatic reaction.

In the thermocycler room (or assembly room), incubate the samples at 65°C during 10 minutes, and then, put the samples at 4°C (HOLD step, equivalent to ice). Quickly proceed to the next step (reverse transcription).

In the mix room, unfroze the reagents for the RT SSIV reaction (unless enzymes that will be used at the last time): dNTP (mix (10mM), primers, SSIV buffer RT 5X (10mM) and DTT 100 mM. In the assembly room, turn on the water bath at 37°C. Then, warm the SSIV

buffer RT 5X (10mM) at least 10 minutes before use. Each reagent should be well homogenized before use. For one sample, prepare the reactional mix n°1 as follow (the final volume of the RT will be 22 µl):

- 1 µL dNTP mix (10mM)
- 0.5 µL Hexamers (100 µM)
- 1 µL NK72 (10 µM)
- 1 µL Rev6 (10 µM)

Mix well by flicking and centrifuge briefly.

In the assembly room, add 3.5 µL of the reactional mix n°1 to the 11µL of RNA denatured (10 pg à 5 µg of RNA). Homogenize well and centrifuge briefly. In the thermocycler room (or assembly room), incubate samples at 65°C during 5 minutes, and at 4°C during 2 minutes (similar to ice). Put RNA into ice and quickly proceed to the next step.

In the mix room, homogenize correctly the reagents before use. For one sample, prepare the reactional mix n°2 as follow:

- 4 µL of SSIV buffer RT 5X (10mM) previously warmed at 37°C
- 1 µL of DTT 100 mM
- 1 µL of RNaseOUT (40U/µl)
- 1 µL of Supercript IV Reverse transcriptase (200U/µl)

Mix well by flicking and then centrifuge briefly.

In the assembly room, add 7 µL of reactional mix n°2 to the mix n°1/RNA. Homogenize correctly and centrifuge briefly. In the thermocycler room, incubate samples at 23°C during 10 minutes (Hybridation of random primers), 50 minutes at 53°C (RT reaction), 10 minutes at 80°C (inactivation of polymerisation) and then add a holding step at 4°C (similar to ice). Quickly proceed to the next step (SSB)

In the mix room, unfrost Second Strand Synthesis Reaction Buffer for the SSB (unless enzymes which will be used at the last minute. Homogenize well the reagents before use. For one sample, prepare the reaction mix as follow (final volume of SSB = 80µl):

- 48 µL of water (DNase RNase Free)
- 8 µL of Second Strand Synthesis Reaction Buffer
- 4 µL o Second Strand Synthesis Enzyme Mix

Distribute 60 µL of reactional mix into strips. Briefly centrifuge. In the serology room (be careful of cDNA and assembly room), add single strand cDNA from the RT reaction (21,5 µl) to the 60 µL of reactional mix. Homogenize well and centrifuge briefly. In the thermocycler room, incubate the samples at 16°C during 2h30, and then at 4°C (HOLD step, similar to ice).

The double strand DNA could be stored at 4°C if used quickly or stored at -20°C for a longer storage. The next step will be the library preparation for the Illumina Nextera XT sequencing. You will use 1 ng of genomic DNA into 5 µl, so DNA should be quantified using the Qubit system, and correctly diluted before launching the library preparation

6.2 PREPARATION OF THE SEQUENCING LIBRARIES USING PRE-QUANTIFIED GDNA

Do not vortex any buffer of the Nextera XT kit, Invert few times to homogenate before utilization.

You need to design your entire WGS array. Fill the lab tracking sheet to assign indexes to each of your strains, so they will all have their specific code (Illumina Experiment Manager). This step is critical as a wrong assignment of indexes can lead to poor quality results.

Pre-quantify your samples after genomic extraction using the Qubit dsDNA HS kit according to the manufacturer's instructions. Samples should be diluted at 0,2 ng/µl for the library preparation.

1. Each reagent should be removed from the freezer, and let thawed for 30 minutes in ice-water bucket:
   - ATM (Amplicon Tagment Mix)
   - TD (Tagment DNA Buffer)
   - Indexes
   - NPM (Nextera PCR Master Mix)
   - RSB (Resuspension Buffer)

You can use this time to turn on thermocycler to allow it to begin warming.

2. Remove the following reagents from cooler and let them warm up at room temperature: AMPure XP beads and NT (Neutralize Tagment Buffer).

3. Following 30 minutes on ice, remove indexes from ice and allow them to begin warming to room temperature.
4. Using PCR strip tubes (or 96 well-plate), add 10 µL of TD buffer to each well to be used for this assay. Change tips each time to ensure the delivered volume is correct.
5. Add 1ng of input DNA to each well (5 µl of dilution at 0.2 ng/µl).
6. Add 5 µL of ATM buffer to each well. Be careful, as this buffer is viscous. Change tips each time to ensure the delivered volume is correct. This is a time sensitive step: too much time will result in fragments that are too small.
7. Mix well but gently and slowly by pipetting (use a 100uL pipette set to 15uL).
8. Close the tubes (or seal plate with film).
9. Centrifuge at 300xg at room temperature for one minute.
10. Incubate in thermocycler and run the following program (NEXTERA, NTA program, V=20uL):

    - 55°C for 5 min

    - hold at 10°C for 1 min.

    This step is time and temperature sensitive, do not leave the thermal cycler.
11. Add 5 µL of NT buffer in each well. Mix well by pipetting. This is also a time sensitive step, use a multichannel pipette whenever possible.
12. Close the tubes (or seal plate with film) and centrifuge at 300xg at room temperature for one minute.
13. Incubate the samples at room temperature for 5 minutes. This time can be used to mix and centrifuge the indexes.
14. Add 5 µL of the appropriate white-capped indexes in each well. Be really careful to avoid cross-contamination. Discard previous cap and replace with a new one.
15. Add 5 µL of the appropriate orange-capped indexes in each well. Be really careful to avoid cross-contamination. Discard previous cap and replace with a new one.
16. Add 15 µL of NPM in each well. Change tips each time to ensure the delivered volume is correct.
17. Mix well by pipetting.
18. Close the tubes (or seal plate) and centrifuge at 300xg at room temperature for one minute. This is a critical step. The plate needs to be tightly sealed with a microseal to avoid sample evaporation.
19. Perform the following PCR program (NEXTERA, PCR program, V=50 µL):

    - 72°C for 3 minutes

    - 95°C for 30 sec

    - 12 cycles of: 95°C for 10 sec, 55°C for 30 sec, 72°C for 30 sec

    - 72°C for 5 minutes

    - Hold at 10°C.

    Vortex the AMPure XP beads for 30 sec. This will allow time for the bubbles to settle out of the solution before use in further steps.
20. Centrifuge samples at 300xg at room temperature for one minute.

    The next steps are to be performed in the post-PCR room.
21. Transfer PCR products into a PCR strip or 96-well non-skirted plate (v=50 µL).
22. Vortex strongly the AMPure XP beads for 5 additional sec.
23. According to the number of cycles that will be used, follow the recommendations hereafter: for 2x150 runs on the Miseq, add 30 µL of AMPure XP beads in each well; or for 2x250 runs on the MiSeq, add 25 µL of AMPure XP beads in each well. Change tips each time to ensure the delivered volume is correct. Avoid bead carryover by confirming no liquid droplets are on your pipette tip. This will affect the ratio of beads to PCR reaction and affects the insert size.
24. Mix well by pipetting. Inspect the mixture; it is important that the brown beads are suspended well throughout the entire solution.
25. Incubate at room temperature for 5 to 10 minutes. Use this time to prepare 400 µL of 80% ethanol per sample (320 µL of absolute ethanol + 80 µL of pure water).
26. Place the plate or tubes on a magnetic stand for 2 minutes. It is important for the beads to collect into a pellet just below the solution's surface. This can best be achieved by not forcing the tube to the bottom of the magnetic stand or by using a spare pipette tip to wedge the tube at an appropriate angle.

27. Discard supernatant. Avoid disturbing the pellet. It may be easier to avoid the pellet with a small pipette (200 µL) for next steps.
28. Add 180 µL of 80% Ethanol solution without disrupting the beads.
29. Incubate 30 sec.
30. Discard supernatant (Avoid disturbing the pellet).
31. Add 180 µL of 80% Ethanol solution without disrupting the beads.
32. Incubate 30 sec.
33. Discard all the supernatant (Avoid disturbing the pellet).
34. Let air-dry for 3 minutes on the magnetic stand. Exceeding the maximum air-dry period can make resuspension difficult and is not recommended. Over-drying is indicated by cracks in the bead pellets. Make sure that RSB buffer is well thawed and mix well by vortexing.
35. Rehydrate the beads with 52.5 µL of RSB buffer and mix by pipetting.
36. Incubate at 37°C for 5 minutes.
37. Place the plate on the magnetic stand for 2 minutes.
38. Carefully transfer 30 µL of supernatant into a new strip of PCR tube or 96-well plate (Avoid disturbing the pellet).
39. Safe stopping point: store the samples at +4°C or -20°C.

6.3 STANDARD LIBRARY NORMALIZATION

1. Quantify the samples with Qubit dsDNA high sensitivity kit.
2. Determine the average size of the libraries using the Bioanalyzer or a TapeStation.
3. Calculate the molarity (in nM) of each library according to the table below.
4. Transfer 20 µl of each library to a new tube (or plate) and dilute in to 2 nM with RSB according to the table below.

Sample name	Concentration (ng/uL)	Average size (bp)	Molarity (nM)	Final volume (uL)	V of RSB (uL)
	Qc	*Size*	*Mc=* *(Qc / (Size * 660))*1.10⁶*	*Vf=Mc*10*	*=Vf-20*

6.4 LIBRARY POOLING AND MISEQ SAMPLE LOADING

1. Remove a MiSeq reagent cartridge from the freezer and thaw at room temperature in a water bath (Need approximately 1 hour).
2. Remove HT1 (Hybridization buffer) from freezer and thaw at room temperature.
3. In an ice bucket prepare an ice-water bath with 3 parts ice and 1 part water.
4. Prepare fresh 0.2N NaOH from a 1N frozen stock solution (40 µl H2O + 10 µl 1N NaOH).
5. Transfer 10 µl of each diluted sample in a single 1.5 ml LoBind tube.
6. Denature the 2 nM library pool by combining 5µl of the 2 nM library pool and 5 µl 0.2N NaOH.
7. Vortex briefly and spin down.
8. Incubate for 5 minutes at room temperature.
9. Add 990 µl pre-chilled HT1 to the tube containing the denatured library. The result is 1 ml of 10 pM denatured library. Keep the denatured library in ice-water until ready to load. During that time invert the MiSeq reagent cartridge several times to mix all reagents.
10. Load 600 µl of diluted libraries into the “Load Samples” reservoir of the MiSeq reagent cartridge. Prepare the MiSeq instrument and launch the sequencing run.