Burundi Rift Valley fever virus diagnostics training report

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International Livestock Research Institute, Kenya

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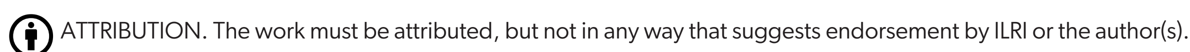

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Summary

From 13 to 17 June, two staff from the International Livestock Research Institute (ILRI) held a five-day hands-on training on Rift Valley fever (RVF) and Brucella diagnostics at laboratoire vétérinaire (LABOVET) Bujumbura, Burundi. The training was made possible by the generous support of the ILRI-led One Health Research, Education and Outreach Centre in Africa (OHRECA). Three staff from LABOVET's virology unit, one from Institut des Sciences Agronomiques du Burundi (ISABU) and a vet from ILRI Burundi attended the training. Participants mainly comprised laboratory technicians and a field veterinary officer all of whom were engaged directly in disease diagnostics starting from sample collection in the field to laboratory analyses and communication of results.

Participants received theoretical and practical training on basic enzyme-linked immunosorbent assay (ELISA) techniques and molecular biology techniques including manual ribonucleic acid (RNA) purification from serum, tissues and swabs; reverse transcription-quantitative polymerase chain reaction (RT-qPCR); qualitative steps such agarose gel, quantification; and initial steps of RVF library preparation using the Oxford Nanopore Technologies to be used for sequencing workflows. The last day was dedicated to theoretical discussions on samples, reagents and data management aimed at ensuring the generation of reliable and reproducible results. Lastly, participants were trained on the use of the Magnetic Induction Cycler (MIC qPCR) machine, which is the latest addition of the diagnostic equipment that the laboratory has procured for their routine testing.

Background

Following reports of an outbreak of an unknown disease in Burundi, ILRI-OHRECA was contacted to offer support on disease diagnostics. The clinical signs reported had led to suspicion of RVF as the primary causative agent and thus this required laboratory confirmations. To support this, ILRI dispatched a set of primers to be used in real-time PCR detection of the pathogen. As a follow-up, the team from Burundi requested technical support in setting up the assays and troubleshooting the issues they were having with their serological assays that were giving invalid ELISA results. It is against this background that OHRECA dispatched two research associates to join the team in Burundi and conduct an intensive five-day training on RVF diagnostics.

The main goal of the training was to equip the trainees with the requisite skills in RVF molecular diagnostics and genomics technologies for efficient response to disease outbreaks in Burundi. The training also aimed at ensuring the workflow was reproducible and generated data that could adequately be used to inform proper control measures in Burundi and the region at large.

Scope of the training

The training was mainly a hands-on practical session alongside which the general theoretical backgrounds were delivered on the bench before or after the assays were set. The training encompassed sample registration in the laboratory and archiving of aliquots, ELISA testing and data analysis of optical densities and results interpretation, RNA extraction, qPCR and testing of co-infections, quality control of results received and scoring samples for targeted RVF genome sequencing.

Training objectives

The objectives of the training were:

1. RVF ELISA set-up and results interpretation using the IgG and IgM kits.
2. Sample management and archiving.
3. Reagents management.
4. RNA extraction and RT-qPCR
5. Testing for co-infections such as brucellosis.
6. Library preparation for Oxford Nanopore sequencing

Training preparation and organization

The training was planned and coordinated by ILRI-OHRECA. It was held at laboratoire vétérinaire, Bujumbura, Burundi. Overall, in attendance, there were five trainees from Burundi and two trainers from ILRI Nairobi.

The list of trainees, trainers and resource personnel is annexed at the end of this report.

Training details

Day one

StepOne™ ABI real-time PCR system instrument calibration

- Calibrated for optics
- Background
- Spectral calibration dyes (FAM, VIC, JOE, SYBR and ROX)

The outcome of the calibration was successful, calibration status was automatically updated, and the next calibration date is 13 December 2023.

ELISA results troubleshooting

Given the ELISA outcome, the first observation was the storage condition of the kits. The fridge temperature was reading 16°C both on the digital display and the thermometer located inside the fridge. This could compromise the validity of the plates since the achievement of optimal optical densities ODs as specified by the manufacturer cannot be guaranteed. The team checked on the outcomes from previous results that had been reported as invalid and tried to troubleshoot the source of the invalidity. It was established that one crucial step was missed when adding the nucleoprotein: both duplicate wells for each sample had been incubated with a nucleoprotein and thus giving equivalent figures. The team was advised to rectify the error. However, since the kits had run out, we were unable to have a test run with the team. The lab team was also taken through the analysis templates (Annex 1) used at the ILRI labs, which they would use to analyse future results thereby ensuring consistency in ELISA results management.

Day two

Ribonucleic acid (RNA) extraction

The lab team was taken through the theoretical basis of RNA extraction followed by a practical demonstration (Annex 1). Available sample types included serum, swabs and tissue samples collected in the outbreak areas.

The demonstration included the preparation of the tissue samples for RNA extraction. The two methods were demonstrated by making two incisions on the tissue and swabbing these incisions followed by a normal RNA extraction procedure from the swab. The next method involved the actual extraction from the tissue itself which starts

with the maceration of a pea-sized tissue which is then incubated with proteinase K for digestion, which then follows the normal procedure of extraction as described by the manufacturer.

Following this procedure, the team extracted RNA from a total of 100 samples which included 12 swabs and 6 tissue samples. The extracted RNA was stored at -20°C in a freezer awaiting real-time PCR amplification.

Day three

Real-time PCR screening of the samples for RVF

The day started with a recap of the RNA extraction process. This was followed by the theoretical background of real-time PCR testing. The trainers explained the difference between conventional and real-time PCR including real-time reverse transcription PCR. The participants were taken through the process of preparing the Oligomix and advised on handling the primer stock. There were demonstrations on the preparation of PCR master mix reagents (Annex 1), dispensing onto the 48-well PCR plate and RNA samples were loaded. There were demonstrations on setting up a real-time PCR run on the applied biosystems step-one real-time PCR thermocycler and initiating a PCR run. The staff were also taken through the theory of RNA/DNA quality check. For sequencing purposes, the staff were taken through the process of preparing TAE buffer to be used in gel electrophoresis. The trainers also demonstrated the preparation of agarose gel for electrophoresis and loading the samples onto the gel. There was a session on checking and interpreting of real-time PCR results and a quick session on troubleshooting failures in real-time PCR.

DAY four

The day started with a recap of the PCR done on day three. The staff were then introduced to the protocol for preparing ONT libraries for sequencing (Annex 1). The staff were taken through the preparation of master mix for complementary DNA (cDNA) synthesis and the procedure of setting up the conventional thermocycler for cDNA synthesis. As the cDNA synthesis was ongoing, the staff were taken through the process of preparing the master mix for targeted RVF whole genome sequencing, primer pool preparation, setting up the cycling conditions and initiating the run. In the afternoon the staff were guided to conduct real-time qPCR analysis to detect Brucella DNA since there was already the extracted RNA, which was also shown to contain some DNA on the agarose electrophoresis gel analysis.

Day five

The gel was prepared to check on the success of targeted cDNA amplification PCR. Samples with the 400 bp amplicons were to be pooled together and cleaned up using AMPure beads before the commencement of library preparation.

The staff were taken through the sample and data management procedure to enhance their capacity in handling the mentioned items. These included handling of PCR reagents and the process of choosing a particular reagent for PCR, qPCR, or conventional PCR and choosing master mix for RT-qPCR. This was coupled with a recap of ELISA analysis and results compilation.

The staff were taken through the process of using the MIC-PCR for them to be able to utilize it once theirs was cleared from the airport. The main advantage of MIC-PCR is that it does not require calibration hence eliminating the challenges experienced on day one where we had to start by calibrating the qPCR instrument.

The training ended with a session of questions and answers.

Recommendations based on trainers observations

There is a need to routinely check the fridge temperatures to ensure the ELISA kits and other reagents are stored at the right temperature to ensure optical densities are obtained and valid test results. There is also a need to enforce calibration of the pipettes and purchase of adjustable pipettes since most of the pipettes were fixed volume pipettes. A list has been appended in Annex 4 for critical equipment the lab may consider procuring.

Notable challenges

Overall, the workshop was a great success, and the participants felt that the training addressed their needs. Some of the challenges they had were :

- Time: Many participants felt they needed more time to internalize the skills they had learned in the five days.
- Lack of critical equipment such as a thermomixer or a water bath with a shaker meant that complete tissue analysis was not achievable.

Annexes

Annex 1 – Protocols

- Elisa Standard operating procedures
- Oxford Nanopore Technologies (ONT) Protocol
- RNA extraction

1. ELISA Standard operating procedure

PART ONE: Generating the optical densities (OD)

- The ELISA assays are done following the manufacturer's instructions as outlined in the kit insert

NOTE: To get the accurate results, ensure all the instructions are followed to the letter including the incubation times, addition of the specified volumes of reagents and adding the reagents to the specified wells in the reaction place following the sequence as specified by the manufacturer.

- At the end of all the incubation steps and addition of the stop solution, be sure to read the absorbance at the specified wavelength and label the OD files appropriately for easier referencing in the future incase one might need to go back to the raw OD reads.

PART TWO: OD results interpretation

- We have provided analysis templates for RVF IgM kits and RVF IgG ELISA kits from IDVet, the templates include two sheets for easier and reproducible interpretation of the absorbance reads. The interpretation formulas as described by the kit manufacturer have been incorporated into the analysis templates and thus you should avoid editing, modifying the original template unless it is necessary.
- To begin analyzing the results, open the respective analysis template and go to the 1st worksheet labelled (raw_data), in this sheet there are two provisions where you are supposed to copy and paste the ODs for a particular ELISA test plate and the sample list for that plate.
- After pasting the ODs in the worksheet named (raw_data), navigate to the second worksheet named (analysed_data). This sheet contains five sections
    - Topmost section which has the plate description where you will be required to insert the metadata which includes the date of the analysis, the name of the technician, the Lot and REF number for each kit.
    - The second section contains the formula to check the validity of the plate
    - The third section contain the plate layout where the sample layout in the plate will appear.
    - The fourth section will contain the plate ODs as they will appear in (raw_data) sheet.
    - The fifth section will contain the analysed results and the interpretation of the results.

- The first thing to check after pasting the raw ODs is the validity of the plate. This will be indicated whether the plate is "valid" or "invalid". We will only check the sample interpretations whether we have positive or negative samples when the plate passes the validity test and the second section described in 4 (a) above is displayed as "valid".
- Once we have a valid plate, we will the proceed section 4 (e) as described above and check the sample positivity to see which samples either will be positive, negative or borderlines(doubtful). All the doubtful samples should be re-tested and if they remain to be doubtful then they should be considered negative upon the outcome being doubtful in the two tests.
- After verifying the contents of this file, click (SAVE AS) and give the file a unique name to be saved with. This avoids overwriting the contents of the original analysis template.
- Repeat the steps for all the ELISA plates tested and save all the new.

PART THREE: Compiling the results

- Once we have done PART TWO successfully, we need to compile the results for presentation and further statistical analysis.
- Open a new excel file and save it with an appropriate name for instance the name of the project/ study under which the samples were collected.
- Copy the results from PART TWO 3(e) and paste them in this new excel, this will include the headers i.e the samples tested, OD1, OD2, final_OD, sample positivity and interpretation.
- Repeat step 3 above for all the plates that were tested and then save the file.

2. Oxford Nanopore Technologies (ONT) Protocol

1. Scope and application

This procedure ensures library preparation for RVF samples for sequencing using the ONT platform.

2. Safety and risk assessment

Samples are potentially infectious, ensure all proper protective equipment are used.

3. Definition

Long-read sequencing devices from Oxford Nanopore Technologies (ONT) promise significant improvements in turnaround time, portability, and cost, compared to established short-read sequencing platforms for viral WGS (e.g., Illumina) sequencing. Further, they provide an effective, unbiased way to identify new strains and other pathogens without prior knowledge of organisms.

4. Principle

Nanopore sequencing is a unique, scalable technology that enables direct, real-time analysis of long DNA or RNA fragments. It works by monitoring changes to an electrical current as nucleic acids are passed through a protein nanopore. The resulting signal is decoded to provide the specific DNA or RNA sequence.

5. Equipment, Reagents and Apparatus.

- Package 1: Store at –20°C.

5.1.1 (lilac) LunaScript® RT SuperMix

5.1.2 (lilac) Q5® Hot Start High-Fidelity 2X Master Mix

5.1.3 (green) NEBNext Ultra II End Prep Enzyme Mix

5.1.4 (green) NEBNext Ultra II End Prep Reaction Buffer

5.1.5 (red) Blunt/TA Ligase Master Mix

5.1.6 (red) NEBNext Quick T4 Ligase

5.1.7 (red) NEBNext Quick Ligation Reaction Buffer

5.1.8 Primer Mix 1

5.1.9 Primer Mix 2

5.1.10 (white) Nuclease-free water

- Package 2: Store at room temperature. Do not freeze

5.1.11 NEBNext Sample Purification Beads

5.1.12 Required Materials Not Included

5.1.13 80% Ethanol (freshly prepared)

5.1.14 DNA LoBind Tubes (Eppendorf® #022431021)

5.1.15 Oxford Nanopore Technologies Native Barcoding Expansion kits 1-12 (EXP-NBD104) and 13-24 (EXP-NBD114)

5.1.16 Oxford Nanopore Technologies Ligation Sequencing Kit (SQK-LSK109)

5.1.17 Oxford Nanopore Technologies SFB Expansion Kit (EXP-SFB001)

5.1.18 Qubit® dsDNA HS Assay Kit (Thermo Fisher Scientific, Inc.® Q32851)

- Equipment

5.1.19 Magnetic rack/stand(NEB #S1515,Alpaqua®,cat. #A001322 or equivalent)

5.1.20 Thermal cycler

5.1.21 Vortex Mixer/ Microcentrifuge

5.1.22 Agilent® Bioanalyzer®or similar fragment analyzer and associated consumables (#4150 or #4200 TapeStation System)

5.1.23 DNase RNase free PCR strip tubes (USA Scientific 1402-1708)

5.1.24 1.5 ml tube magnet stand (NEB #S1506)

6. Procedure.

6.1 cDNA synthesis:

Gently mix 10 times by pipetting and spin down the LunaScript RT SuperMix reagents (contains primers). Prepare the cDNA synthesis reaction as described below and mix by pipetting up and down 10 times or by flicking the pipette gently.

Component	Volume
RNA Sample*	8 µl
(lilac) LunaScript RT SuperMix	2 µl
Total Volume	10 µl

Incubate reactions in a thermocycler with lid temperature at 105°C with the following steps:

Cycle step	Temp	Time	Cycle
Primer Annealing	25°C	2 minutes	1
cDNA Synthesis	55°C	20 minutes	1
Heat Inactivation	95°C	1 minute	1
Hold	4°C	∞	

Note: Safe stop: Samples can be stored at -20 °C if they are not used immediately.

6.2 Targeted cDNA amplification:

6.2.1. Gently mix Q5 Hot Start High Fidelity 2X master mix 10 times by pipetting and spin down reagents. Prepare the split pool amplification reactions as described below:

For Pool Set A:

Component	Volume
cDNA (Step 8.1)	4.5 µl
(lilac) Q5 Hot Start High-Fidelity 2X MM	6.25 µl
Primer Mix 1*	1.75 µl
Total Volume	12.5 µl

For Pool Set b:

Component	Volume
cDNA (Step 8.1)	4.5 µl
(lilac)Q5 Hot Start High-Fidelity 2X MM	6.25 µl
Primer Mix 2*	1.75 µl
Total Volume	12.5 µl

6.2.2.Mix by flicking the tube or by pipetting up and down 10 times followed by a quick spin.

Incubate reactions in a thermocycler* with the following steps:

Cycle step	Temp	Time	Cycle
Initial Denaturation	98°C	30 seconds	1
Denature	95°C	15 seconds	35
Annealing/Extension	63°C **	5 minutes	
Hold	4°C	∞	1

** It is very important to set up the annealing and extension temperature to 63°C.
Note: Samples can be stored at -20 °C if they are not used immediately

6.3 Cleanup of cDNA amplicons:

SPRIselect or AMPure ® XP Beads can be used as well. If using AMPure XP Beads, allow the beads to warm to Room temperature for at least 30 minutes before use. These bead volumes may not work properly for a cleanup at a different step in the workflow. For cleanups of samples contained in different buffer conditions, the volumes may need to be experimentally determined.

6.3.1. For each sample, combine pool A and pool B PCR Reactions.

6.3.2. Vortex SPRIselect or NEBNext Sample Purification Beads to resuspend.

6.3.3. Add 20 µl (0.8X) resuspended beads to the combined PCR reaction. Mix well by flicking the tube or pipetting up and down 10 times to mix and a very short 2-3 seconds quick centrifugation. Be sure to stop the centrifugation before the beads start to settle out.

6.3.4. Incubate samples at Room temperature for 00:10:00.

6.3.5. Place the tubes on an appropriate magnetic stand to separate the beads from the supernatant. If necessary, quickly spin the sample 00:00:01 to collect the liquid from the sides of the tube before placing on the magnetic stand

6.3.6. After 2 minutes (or when the solution is clear), carefully remove and discard the supernatant without disturbing the beads.

CAUTION: do not discard the beads-they contain DNA

6.3.7. Add 500 µl 80% freshly prepared ethanol to the tube while in the magnetic stand. Incubate at Room temperature for 00:00:30, and then carefully remove and discard the supernatant. DO NOT disturb the beads that contain DNA targets.

6.3.8. Repeat previous step once for a total of two washes. Be sure to remove all visible liquid after the second wash. If necessary, briefly spin the tube for 00:00:01, place back on the magnetic stand and re-move traces of ethanol with a p10 pipette tip

6.3.9. Air dry the beads for 00:00:30 while the tube is on the magnetic stand with the lid open.

CAUTION: Do not over-dry the beads. This may result in lower recovery of DNA. Elute the samples when the beads are still dark brown and glossy looking. When the beads turn lighter brown and start to crack, they are too dry.

6.310. Remove the tube from the magnetic stand. Elute the DNA target from the beads by adding 21 µl Nuclease-free water.

6.3.11. Mix well by flicking the tube or pipetting up and down 10 times to mix and followed by a very short centrifugation. Incubate for 00:10:00 at Room temperature. If necessary, quickly spin the sample to collect the liquid from the sides of the tube or plate wells before placing back on the magnetic stand.

6.3.12. Place the tube on the magnetic stand. After 2 minutes (or when the solution is clear), transfer 20 µl to clean PCR tubes.

6.3.13. Assess the concentration of the DNA targets. We recommend using a Qubit fluorometer for concentration assessment. Use 1 µl of sample for the Qubit fluorometer. Amplicons may also be run on a Bioanalyzer® or a TapeStation to confirm 400 bp size of amplicons.

6.4 NEBNext end prep

6.4.1. Use the Qubit readings from Step 6.3.13 to dilute 50 ng of the Targeted cDNA Amplicons sample with nuclease-free water to a final volume of 12.5 µl (4 ng/µl).

6.4.2 Add the following components to a PCR tube (End Prep Reaction and Buffer can be pre-mixed and stable on ice for 4 hours):

Component	Volume
Targeted cDNA Amplicons (Step 8.7)	12.5 µl
(green) NEBNext Ultra II End Prep Reaction Buffer	1.75 µl
(green) NEBNext Ultra II End Prep Enzyme Mix	0.75 µl
Total volume	15 l

6.4.3 Flick the tube or pipet up and down 10 times to mix the solution. Perform a quick spin to collect all liquid from the sides of the tube.

- Place in a thermocycler, and run the program below:

Temperature	Time
@ 20 °C	00:10:00
@ 65 °C	00:10:00
@ 4 °C	∞

- heated lid set to ⇢ 75°C

6.5 Barcode ligation

6.5.1 Add the following components directly to a sterile nuclease-free PCR tube.

Component	Volume
(white) Nuclease-free water	6 µl
End-prepped DNA (Previous Step)	1.5 µl
Native Barcode*	2.5 µl
(red) Blunt/TA Ligase Master Mix**	10 µl
Total volume	20 µl

** Mix the Blunt/TA Ligase Master Mix by pipetting up and down several times prior to adding to the reaction.

6.5.2. Flick the tube or pipet up and down 10 times to mix solution. Perform a quick spin to collect all liquid from the sides of the tube.

Caution: The Blunt/TA Ligase Master Mix is very viscous. Care should be taken to ensure adequate mixing of the ligation reaction, as incomplete mixing will result in reduced ligation efficiency.

6.5.3. Incubate in the following temperatures.

Temperature	Time
Room temperature	00:20:00
@ 65 °C	00:10:00
On ice	00.01.00

6.5.4. Pool all barcoded samples into one 1.5 ml DNA LoBind Tube.

6.6 Cleanup of barcoded DNA.

The volumes of SPRIselect or NEBNext Sample Purification Beads provided here are for use with the sample contained in the exact buffer at this step. AMPure XP Beads can be used as well. If using AMPure XP Beads, allow the beads to warm to Room temperature for at least 30 minutes before use.

6.6.1 Vortex NEBNext Sample Purification Beads to resuspend.

6.6.2 Add 0.4X resuspended beads to pooled, barcoded samples (Step 6.5.4), for example, if you are pooling 24 libraries (which amounts to 480 µl total), add 192 µl of resuspended Sample Purification beads to the 480 µl of pooled sample.

6.6.3. Flick the tube or pipet up and down 10 times to mix to resuspend pellet. Perform a quick spin for 00:00:01 to collect all liquid from the sides of the tube.

6.6.4. Incubate samples on bench top for 00:10:00 at Room temperature.

6.6.5 Place the tube on a 1.5 ml magnetic stand to separate the beads from the supernatant.

6.6.6. After 2 minutes (or when the solution is clear), carefully remove and discard the supernatant.

Do not disturb the beads - contain DNA targets.

6.6.7. Wash the beads by adding 250 µl Short Fragment buffer (SFB). Flick the tube or pipet up and down to mix to resuspend pellet. If necessary, quickly spin the sample for 00:00:01 to collect the liquid from the sides of the tube or plate wells before placing back on the magnetic stand.

6.6.8. Place the tube on an appropriate magnetic stand for 2 minutes (or until the solution is clear) to separate the beads from the supernatant. Remove the supernatant.

6.6.9. Repeat previous 2 steps once for a total of two washes.

Be sure to remove all visible liquid after the second wash. If necessary, briefly spin the tube, place back on the magnetic stand, and remove traces of SFB with a p10 pipette tip.

6.6.10. Add 200 µl 80% freshly prepared ethanol to the tube while on the magnetic stand. Incubate at Room temperature for 00:00:30, and then carefully remove and discard the supernatant.

6.6.11. Be careful not to disturb the beads that contain DNA targets. Perform a quick spin and place the sample tube on the magnetic stand, remove any residual ethanol.

6.6.12. Air dry the beads for 00:00:30 while the tube is on the magnetic stand with the lid open.

CAUTION: Do not over-dry the beads. This may result in lower recovery of DNA target. Elute the samples when the beads are still dark brown and glossy looking, but when all visible liquid has evaporated. When the beads turn lighter brown and start to crack, they are too dry.

6.6.13. Remove the tube from the magnetic stand. Elute the DNA target from the beads by adding 33 µl Nuclease-free water.

6.6.14. Resuspend the pellet by flicking the tube or pipetting up 10 times and down to mix. Incubate for at least 2 minutes at Room temperature. If necessary, quickly spin the sample for 00:00:01 to collect the liquid from the sides of the tube before placing back on the magnetic stand.

6.6.15. Place the tube on the magnetic stand. After 2 minutes (or when the solution is clear), transfer 32 µl to a new 1.5 ml Eppendorf DNA LoBind Tube or PCR tube.

6.6.16. Use 1 µl for the Qubit fluorometer to assess cDNA concentrations with a Qubit fluorometer

Note: Samples can be stored at -20 °C if they are not used immediately.

6.7 Adapter ligation

6.7.1. Use the Qubit readings from Step 6.6.16 to dilute 60 ng of the Native barcoded DNA pool with nuclease-free water to a final volume of 30 µl (2 ng/µl).

6.7.2. Add the following components into a 1.5 ml Eppendorf DNA LoBind Tube or nuclease-free PCR tube:

Component	Volume
Native barcoded and purified DNA (Step 8.12.12)	30 µl
Adapter Mix II (AMII)**	5 µl
(red) NEBNext Quick Ligation Reaction Buffer *	10 µl
(red) NEBNext Quick T4 Ligase	5 µl
Total volume	50 µl

* Mix the NEBNext Quick Ligation Reaction Buffer by pipetting up and down several times prior to adding to the reaction.

6.7.3. Flick the tube to mix solution. Perform a quick spin for 00:00:01 to collect all liquid from the sides of the tube.

CAUTION: The NEBNext Quick Ligation Buffer is viscous. Care should be taken to ensure adequate mixing of the ligation reaction, as incomplete mixing will result in reduced ligation efficiency.

6.7.4. Incubate at 25 °C or at Room temperature for 00:20:00

6.8 Cleanup of adapter ligated DNA

6.8.1 Vortex NEBNext Sample Purification Beads or the Ampure XP Beads to resuspend.

6.8.2 Add 50 µl (1X) resuspended beads to the ligation mix. Mix well by flicking the tube to mix followed by a quick spin for 00:00:01. Incubate samples for 00:10:00 at Room temperature.

6.8.3 Place the tube on a magnetic stand to separate the beads from the supernatant.

6.8.4 After 2 minutes (or when the solution is clear), carefully remove and discard the supernatant. DO NOT disturb the beads that contain DNA targets.

a. CAUTION: do not discard the beads.

6.8.5 Wash the beads by adding 250 µl Short Fragment Buffer (SFB). Flick the tube to mix to resuspend pellet. If necessary, quickly spin the sample to collect the liquid from the sides of the tube or plate wells then place the tube on an appropriate magnetic stand.

6.8.6 Wait for 2 minutes (or until the solution is clear) to separate the beads from the supernatant. Remove the supernatant.

6.8.7 Repeat previous 2 steps once for a total of two washes.

a. Be sure to remove all visible liquid after the second wash. If necessary, briefly spin the tube/plate, place back on the magnet and remove traces of SFB with a p10 pipette tip.

6.8.8 Remove the tube from the magnetic stand. Elute the DNA target from the beads by adding 15 µl Elution Buffer (EB) provided in SQK-LSK109 kit from Oxford Nanopore

6.8.9 Resuspend the pellet well in EB buffer by flicking the tube. Incubate for 00:10:00 at Room temperature. If necessary, quickly spin the sample to collect the liquid from the sides of the tube or plate wells before placing back on the magnetic stand.

6.8.10 Place the tube/plate on the magnetic stand. After 2 minutes (or when the solution is clear), transfer 15 µl to a new DNA LoBind tube.

6.8.11 Use Qubit to quantify 1 µl library sample. Follow Oxford Nanopore Protocol SQK-LSK109 to prepare MinION ® flow cell and DNA library sequencing mix using up to 20 ng adapter-ligated cDNA sample (previous step).

NOTE: After normalizing the DNA to 20 ng, if the volume is less than 12 µl, then top up the sample volume to 12 µl with EB.

7. Quality control

7.1 Quality of the sample and the reagents must be maintained by storing and working with then under the right temperatures recommended by the protocol.

7.2 Disinfect the working bench using 70% ethanol, DNA AWAY™ Surface Decontaminant and RNase AWAY

7.3 Run UV in the biosafety cabinet before and after the using it.

7.4 Gel electrophoresis, Tapestation, and Qubit are done after every step to ensure quality of the sample is maintained.

8. Resources

https://www.neb.com//media/nebus/files/manuals/manuale7660.pdf?rev=48c42313dcb64b0dbb16c4bfd1563a27

3. RNA extraction

1. Introduction

The kit-based method leverages the property of RNA to bind silica and glass (the membrane is composed of silica) therefore it is referred to as “solid-phase extraction”.

The kit combines the selective binding properties of a silica-based membrane with the speed of microspin or vacuum technology and is highly suited for simultaneous processing of multiple samples.

The sample is first lysed under highly denaturing conditions to inactivate RNases and to ensure isolation of intact viral RNA. Buffering conditions are then adjusted to provide optimum binding of the RNA to the membrane, and the

sample is loaded onto the spin column. The RNA binds to the membrane, and contaminants are efficiently washed away in 2 steps using 2 different wash buffers. High quality RNA is eluted in a special RNase-free buffer, ready for direct use or safe storage. The purified RNA is free of protein, nucleases and other contaminants and inhibitors.

The QIAamp Viral RNA Mini Kit is not designed to separate viral RNA from cellular DNA, and both will be purified in parallel if present in the sample. To avoid co-purification of cellular DNA, the use of cell-free body fluids for preparation of viral RNA is recommended. Samples containing cells, such as cerebrospinal fluid, bone marrow, urine and most swabs, should first be filtered, or centrifuged for 10 min at 1500 x g and the supernatant used. If RNA and DNA have been isolated in parallel, the eluate can be DNase digested using RNase-free DNase, followed by heat treatment (15 min, 70°C) to inactivate the DNase.

RNA is extremely sensitive to RNases and should always be prepared with due care. Hands and dust particles may carry bacteria and molds and are the most common sources of RNase contamination. The sample is first lysed under the highly denaturing conditions provided by Buffer AVL to inactivate RNases and to ensure isolation of intact viral RNA. Carrier RNA, added to Buffer AVL, improves the binding of viral RNA to the membrane especially in the case of low-titer samples, and limits possible degradation of the viral RNA due to any residual RNase activity. Carrier RNA serves 2 purposes. Firstly, it enhances binding of viral nucleic acids to the membrane, especially if there are very few target molecules in the sample. Secondly, the addition of large amounts of carrier RNA reduces the chance of viral RNA degradation in the rare event that RNase molecules escape denaturation by the chaotropic salts and detergent in Buffer AVL. If carrier RNA is not added to Buffer AVL this may lead to reduced viral RNA recovery.

Procedure

2. Specimen

   2.1 Nasal Wash Bulb (NWB)

   2.2 Nasal Pharyngeal Aspirate (NPA)

   2.3 Nasal Pharyngeal Swab (NPS)

   2.4 Nasal Flocked Swab (NFS)

   2.5 Oral Pharyngeal Swab (OPS)

   2.6 Induced sputum

   2.7 NP/OP

   2.8 Plasma

   2.9 Serum

   2.10 Culture supernatants

3. Materials

   3.1 Barrier tips (10µl,100µl,1000µl)

   3.2 Pipettes (10µl,100µl,1000µl)

   3.3 1.5ml Eppendorf

   3.4 Qiagen RNA Extraction Kit

3.5 Vortex

3.6 Microcentrifuge

3.7 96-100% ethanol

3.8 Plastic racks

4. Procedures

4.1 Use the table below as a guide for preparing lysis buffer (AVL and carrier RNA) based on the number of samples.

Ration of AVL to carrier RNA is summarized in the table below:

No. samples Volume AVL (ml) Volume carrier RNA-AVE (µl) No. samples Volume AVL (ml) Volume carrier RNA-AVE (µl)

No. samples	Volume AVL (ml)	Volume carrier RNA-AVE (µl)	No. samples	Volume AVL (ml)	Volume carrier RNA-AVE (µl)
1	0.56	5.6	14	7.84	78.4
2	1.12	11.2	15	8.4	84
3	1.68	16.8	16	8.96	89.6
4	2.24	22.4	17	9.52	95.2
5	2.8	28	18	10.08	100.8
6	3.36	33.6	19	10.64	106.4
7	3.92	39.2	20	11.2	112
8	4.48	44.8	21	11.76	117.6
9	5.04	50.4	22	12.32	123.2
10	5.6	56	23	12.88	128.8
11	6.16	61.1	24	13.44	134.4
12	6.72	67.2	25	14	140
13	7.28	72.8	26	14.56	145.6

4.2 Pipette 560µl of prepared buffer containing carrier RNA into a 1.5ml microcentrifuge tube.

4.3 Vortex the sample and add 140µl of it to the Buffer AVL-carrier RNA in microcentrifuge

4.4 Mix by pulse vortexing for15s. Incubate at room temperature (15-25°C) for 10 min.

4.5 Briefly centrifuge the tube at (8000rpm for 10s) to remove drops from the inside of the lid.

4.6 Add 560µl of ethanol (96-100%) to the sample and mix by pulse vortexing for15s. after mixing briefly, centrifuge the tube to remove drops from the inside of the lid.

4.7 Carefully apply 630µl of the solution from step 6.4 to the QIAmp Mini spin column (with the 2ml collection tube attached) without wetting the rim. Close the cap and centrifuge at 8000rpm for 1 min. Place the QIAmp Mini spin column into a clean 2ml collection and discard the tube containing the filtrate.

4.8 Carefully open the QIAmp Mini spin column and repeat step 6.7.

4.9 Carefully open the QIAmp Mini spin column and add 500µl of buffer AW1.Close the cap and centrifuge at 8000rpm for 1min. Place the QIAmp Mini spin column into a clean 2ml collection tube and discard the tube containing the filtrate.

4.10 Carefully open the QIAmp Mini spin column and add 500µl of buffer AW2. Close the cap and centrifuge at 14000rpm for 3min.

NOTE: In case the flow through come into contact with the spin column e.g. when removing the QIAmp mini column, discard the flow through at the collection tube and reuse the spin column. Centrifuge at 14000rpm for 1min.

4.11 Place the QIAmp Mini spin column into a clean 1.5ml microcentrifuge tube and discard the tube containing the filtrate. Carefully open the QIAmp Mini spin column and add 60µl of Buffer AVE. Close the cap and incubate at room temperature for 1 min.

4.12 Centrifuge at 8000rpm for 1 min. Discard the QIAmp Mini spin column, store the RNA at -80°C for long term storage.

5. Reference

QIAamp Viral RNA Mini Handbook (July 2020)

6. Appendix

6.1 When a new kit is opened

- Add 96-100% ethanol to Buffer AW1 and AW2 before use, see bottles/ kit manual for volume. Tick the bottle at the check box on the lid and at the sides to indicate the buffer is reconstituted and the date. Make aliquots in 50ml falcon tubes.
- Add 1550µl of Buffer AVE to Lyophilized Carrier RNA.

6.2 Addition of dissolved carrier RNA to buffer AVL.

For larger numbers of samples, calculate volumes using the following sample calculation:

n x 0.56 ml = y ml

y ml x 10 µl/ml = z µl

Where: n = number of samples to be processed simultaneously

y = calculated volume of Buffer AVL

z = volume of carrier RNA–Buffer AVE to add to Buffer AVL

Gently mix by inverting the tube 10 times. To avoid foaming, do not vortex.

Annex 2 – Participants list

No	Name	Name of organisation
	Mbazumutina Magnififue	Laboratoire National Vétérinaire
	Iradukunda Carita	Laboratoire National Vétérinaire
	Ntawuyankira Neilla	Institut des Sciences Agronomiques du Burundi (ISABU)
	Ndayikeza Evelyn	Laboratoire National Vétérinaire
	Niyokwizera Pascal	Laboratoire National Vétérinaire
	Richard Nyamota	International Livestock Research Institute
	Reuben Mwangi	International Livestock Research Institute

Annex 3 – Recommended equipment and consumables

RECOMENDED KITS, REAGENTS AND EQUIPMENT				
	Disease	Kit description	Product code	URL link
1	IDvet Brucellosis	ID Screen® Brucellosis Serum Indirect Multi-species,	BRUS-MS-10P	https://www.id-vet.com/produit/id-screen-q-fever-indirect-multi-species/
2	IDvet RVF ELISA	ID Screen® Rift Valley Fever Competition Multi-species	RIFTC-10P	https://www.id-vet.com/produit/id-screen-rift-valley-fever-competition-multi-species/
3	IDvet RVF IgM	ID Screen® Rift Valley Fever IgM Capture		https://www.id-vet.com/produit/id-screen-rift-valley-fever-igm-capture/
4	**Mosquito traps**			**Comments**
	1.	CDC Mini light traps with incandescent lights		Dr lionel may require this for field epidemiological study for RVF.
	2.	Biogents BG sentinel traps		
5	**Mosquito trap accesories**			
	1.	Hanging dry ice (carbondioxide) dispensers for CDC light trap		
	2.	12V batteries to power the traps and their chargers		
6	**Equipment for the Molecular Laboratory**			**Comments**
	1	minus (-)80 °C Upright Freezers		
	2	Thermomixers for 1.5ml Microcentrifuge tubes		It may come with other adapters such as for 0.2ml tubes
	3	Water bath with a shaker		
	4	Adjustable pipettes (Gilson or any other brand)		
	5	Centrifuge with changeable rotor for pCR plates and tubes		
	6	Spinner centrifuge		for 1.5 and 2ml tubes preferably a spinner and vortexer
	7	Microspin centrifuge for 0.2ml strip tubes		Should vortex and spin down
	8	UPS's		Power back-up for critical instruments such PCR istruments and Minion
	9	PCR plate sealer		
	10	Ice buckets		Rubber type with lid
	11	Gel documentation system		Azure c200 imaging system may be easier to use with clear gel images
	12	Computer		For the lab staff for logging in samples
	13	Barcode printer		This will assist in sample management in the lab
	14	Barcode scanner		

7	**Consumables and reagents**			
	1	2ml cryotubes		With external thread screw caps
	2	cryoboxes		With a capacity of holding 100 cryotubes for storage of samples
	3	Colour coded lab coats		To reduce risk of contamination, each lab may have unique labcoats different from others (Extraction room and PCR and post PCR)
	4	100X TE buffer		
	4	Amoce		Q Fever real-time PCR