1.0 OBJECTIVE

The objective of this document is to lay down a method for the isolation of viral RNA from samples such as cell-free body fluids. Isolated RNA can be used in RT-qPCR.

2.0 REFERENCES

- QIAamp Viral RNA Mini Kit (Qiagen, catalog nr. 52906)
- TRIzol reagent (ThermoFischer, catalog no. 15596026)
- RNAzol RT (Sigma, catalog no.R4533)

3.0 ABBREVIATIONS

- RNA: Ribonucleic acid
- RT-qPCR: Reverse transcription quantitative polymerase chain reaction

4.0 EQUIPMENT AND MATERIALS

- QIAamp Viral RNA Mini Kit (Qiagen, catalog no. 52906)
- TRIzol reagent (ThermoFischer, catalog no. 15596026)
- RNAzol RT (Sigma, catalog no. R4533)
- Microcentrifuge
- Micropipettes p10, p20, p200 and p1000 (Gilson or similar)
- Disposable tips for micropipettes p10, p20, p200 and p1000

- Microcentrifuge tube of 1.5 and 0.5 ml (Eppendorf, catalog no. 0030120086 and 0030121023 or similar)
- Chloroform (Riedel-de Haen, catalog no. 34854)
- Isopropanol (Riedel-de Haen, catalog no. 33539 or similar)
- Ethanol (Riedel-de Haen, catalog no. 02860 or similar)
- Nuclease-free water (Promega, catalog no. P119C or similar)
- DNase I recombinant, RNase-free (Roche, catalog no. 04716728001)

5.0 SAMPLE DETAILS

Oropharyngeal (OP) and nasopharyngeal (NP) swabs in saline medium (discard swab and collect medium), or other sources of clinical material including bronchoalveolar lavage (BAL), are processed for RNA isolation as follows.

6.0 PREPARATION OF REAGENTS

75% ethanol (v/v): Mix 75 ml of absolute ethanol with 25 ml of nuclease-free water.
70% isopropanol (v/v): Mix 70 ml of absolute isopropanol with 30 ml of nuclease-free water.

7.0 PROCEDURE

7.1 General Considerations

A method for purification of viral RNA is intended for isolation of total RNA from samples such as cell-free body fluids. Three methods of RNA extraction are explained below, using QIAamp kit, RNAzol RT or TRIzol reagent followed by alcohol precipitation.

7.2 Procedure

7.2.1 Isolation of viral RNA from sample using QIAamp kit

- Pipet 560 µl of Buffer AVL containing carrier RNA into a 1.5 ml microcentrifuge tube.
  The carrier RNA is provided with the kit in lyophilised form (310 µg). Add 310 µL of Buffer AVE to the tube to obtain a solution of 1 µg/µl.
- Add 140 µl of liquid sample to the microcentrifuge tube containing Buffer AVL-carrier RNA.
  If the sample volume is larger than 140 µl, increase the amount of Buffer AVL-carrier RNA proportionally.
- Mix by vortexing for 15 seconds.
  In order to ensure efficient lysis, it is essential that the sample is mixed thoroughly with the buffer.
- Incubate at room temperature for 10 minutes.
- Briefly spin down the tube to remove drops from the inside of the lid.
- Add 560 µl of absolute ethanol to the sample, and mix by vortexing for 15 seconds. After mixing, briefly spin down the tube to remove drops from inside the lid.
  If the sample volume is larger than 140 µl, increase the amount of ethanol proportionally.
- Carefully apply 630 µl of the solution from the previous step to the QIAamp Mini Column.
- Centrifuge at 6 000 g for 1 minute and discard the flow-through.
  If the sample volume is larger than 140 µl, repeat this step until all the lysate has been loaded onto the spin column.
- Add 500 µl of Buffer AW1, and centrifuge at 6 000 g for 1 minute. Discard the flow-through.
- Add 500 µl of Buffer AW2, and centrifuge at 15 000 g for 3 minutes. Discard the flow-through.
- Place the QIAamp Mini column in a new collection tube. Centrifuge at 15 000 g for 1 minute.
- Place the QIAamp Mini column in a clean 1.5 ml microcentrifuge tube and add 60 µl of Buffer AVE. Incubate at room temperature for 1 minute and centrifuge at 15 000 g for 2 min.
- Discard the QIAamp Mini column and keep the eluted RNA.
- Proceed with DNase treatment or store the RNA at -80 °C.

Note: As an alternative to the QIAamp kit other classical methods can be used to isolate RNA from samples such treatment with RNAzol RT (Sigma, R4533) or TRIzol reagent (ThermoFischer, catalog no. 15596026) followed by alcohol precipitation (see next sections below).

7.2.2 Isolation of viral RNA from sample using RNAzol RT reagent

- Add 1000 µl RNAzol RT in the 400 µl sample.
- Shake the sample and incubate it at room temperature for 5 minutes.
- Centrifuge 15 minutes at 12 000 g at +4°C. Centrifugation separates the mixture in a semisolid pellet containing DNA, proteins and polysaccharides and supernatant containing RNA.
- Transfer supernatant containing RNA into clean microcentrifuge tube and add an equal volume of 100% isopropanol to precipitate RNA.
- Allow to stand for 10 minutes and then centrifuge at 12 000 g for 10 minutes at room temperature. Remove alcohol solution with a micropipette.
- Wash the pellet twice with 500 µl of 75% ethanol and centrifuge at 8 000 g for 3 minutes at room temperature.
- Remove alcohol solution with a micropipette.
- Solubilize the pellet of RNA, without drying, in 30 µl of nuclease-free water.
- Proceed with DNase treatment or store the RNA at -80 °C.

7.2.3 Isolation of viral RNA from sample using TRIzol reagent

- Add 750 µl TRIzol solution in the sample.
- Gentle mix the reaction by pipetting up and down and incubate at room temperature for 5 minutes.
- Add 150 µl of chloroform, vortex and incubate at room temperature for 10 minutes.

- Centrifuge 15 minutes at 11 000 g at +4°C. Three phases are visibly separated: an upper aqueous phase containing RNA, an interphase containing DNA, and a lower organic phase containing proteins, sugars and fatty acids
- Transfer an upper aqueous phase containing RNA into clean microcentrifuge tube and add 375 µl of nuclease-free water and 750 µl isopropanol. This is the step of RNA precipitation. Leave it for10 minutes at room temperature.
- Centrifuge 15 minutes at 11 000 g at +4°C.
- Remove the supernatant and wash the pellet with750 µl of 75% ethanol and centrifuge again for 15 minutes at 11 000 g at +4°C.
- Remove the supernatant and air-dry the pellet.
- Resuspended the pellet of RNA in 30 µl of nuclease-free water.
- Proceed with DNase treatment or store the RNA at -80 °C.

7.2.4. DNase treatment of viral RNA

- Treat RNA with DNase that should be RNase-free.
- Prepare reaction mix as follows

RNA	**For 30 µl sample**	**For 60 µl sample**
Buffer (10X)	4 µl	7.5 µl
DNase I (RNase-free) (1X)	4 µl	7 µl
Nuclease-free water	2 µl	0.5 µl
Total reaction volume	**40 µl**	**75 µl**

- Add the above reaction mixture to the eluted RNA, incubate for 30 minutes at room temperature.
- After 30 minutes inactivate the DNase by incubation for 5 minutes at 75 °C.
- Put RNA on ice and store in -80 °C until further use.

8.0. ADDITIONAL NOTES

- Basic expertise in working with RNA is highly recommended.
- Wear gloves.
- Use nuclease free water.
- Whenever possible, sterile disposable labware should be used in working with RNA in order to avoid degradation.
- All reagents and equipment dedicated to the work with RNA should be RNase-free.