Quantification of Sudan Ebola Virus

Nucleoprotein (np) gene

For general laboratory and research use only

Advanced kit

150 tests

Introduction to Sudan Ebola Virus

Ebola viruses are the causative agents for a severe, lethal viral disease called Ebola haemorrhagic fever in humans and primates. They are long, filamentous, enveloped and non-segmented viruses with a negative stranded RNA genome, belonging to the family Filoviridae. The genus Ebolavirus is comprised of five distinct species: Bundibugyo, Ivory Coast, Reston, Sudan and Zaire. They are tubular virions, generally 80 nm in diameter and 800 nm in length. The genome is approximately 19 kb long and consists of 7 linearly arranged genes in order 3'-NP-VP35-VP40-GP-VP30-VP24-L. Sudan Ebola virus was identified in 1976 and was initially believed to be identical to the Zaire species. It was detected among cotton factory workers in Nzara, Sudan, with the first case reported as a worker exposed to a potential natural reservoir. The virus was not found in any of the local animals and insects that were tested in response.

The Ebola virus is primarily transmitted to humans through close contact with the blood, secretions, organs or other bodily fluids of infected animals. It then spreads into the community through human to human transmission, resulting from direct contact with the blood or secretions of an infected person. Initially it is commonly spread among family and friends of infected individuals. Health-care workers have frequently been infected while treating Ebola patients and outbreaks in hospital environments are common. Transmission via infected semen can also occur up to seven weeks after clinical recovery. Decomposed bodies of infected individuals can remain infectious for 3 to 4 days after death.

The incubation period of Ebola haemorrhagic fever is usually 5 to 20 days. Clinical signs of the infection include fever, headache, sore throat, joint and muscle aches, diarrhoea and weakness. It is a febrile haemorrhagic illness where the case-fatality rate varies between 25% and 90%. In severe cases of hemorrhagic fever, there can be damage to blood vessels and extensive internal and external bleeding or haemorrhage. There is no cure for Ebola hemorrhagic fever, no established drug therapy to treat Ebola infection, and no vaccine that can protect humans against Ebola.

Specificity

The PrimerDesign™ genesig Kit for Sudan Ebola Virus (SUDV) Genomes is designed for the in vitro quantification of SUDV genomes. The kit is designed to have the broadest detection profile possible whilst remaining specific to the SUDV genome.

The primers and probe sequences in this kit have 100% homology with a broad range of clinically relevant reference sequences based on a comprehensive bioinformatics analysis.

If you require further information, or have a specific question about the detection profile of this kit then please send an e.mail to enquiry@primerdesign.co.uk and our bioinformatics team will answer your question.

Kit Contents

- SUDV specific primer/probe mix (150 reactions BROWN)
  FAM labeled, BHQ quenched
- SUDV positive control template (for Standard curve RED)
- Internal extraction control RNA (150 reactions BLUE)
- Internal extraction control primer/probe mix (150 reactions BROWN)
  VIC labelled
- Endogenous ACTB primer/probe mix (150 reactions BROWN)
  FAM labeled, BHQ quenched
- Internal extraction control/SUDV/ACTB RT primer mix
  (150 reactions GREEN) Required for two step protocol only
- RNAse/DNAse free water

Reagents and equipment to be supplied by the user

Real-Time PCR Instrument

RNA extraction kit

This kit is designed to work well with all processes that yield high quality RNA with minimal PCR inhibitors.

oasig™ Lyophlised OneStep qRT-PCR MasterMix kit

Contains complete one step qRT-PCR MasterMix

Optional - Reverse Transcription kit and Mastermix

Although a one step RT-PCR protocol is recommended, this kit is designed to work well with a two step protocol. We recommend the use of PrimerDesign Precision™ Reverse Transcription kits and the use of PrimerDesign 2x Precision™ MasterMix for the two step protocol.

Pipettors and Tips

Vortex and centrifuge

Thin walled 1.5 ml PCR reaction tubes

Kit storage and stability

This kit is stable at room temperature but should be stored at -20°C on arrival. PrimerDesign does not recommend using the kit after the expiry date stated on the pack. Once the lyophilized components have been re-suspended, unnecessary repeated freeze/thawing should be avoided. The kit is stable for six months from the date of resuspension under these circumstances.

Suitable sample material

All kinds of sample material suited for PCR amplification can be used. Please ensure the samples are suitable in terms of purity, concentration, and RNA/DNA integrity (An internal PCR control is supplied to test for non specific PCR inhibitors). Always run at least one negative control with the samples. To prepare a negative-control, replace the template RNA sample with RNAse/DNAse free water.

Dynamic range of test

Under optimal PCR conditions PrimerDesign detection kits have very high priming efficiencies of >95% and can detect less than 100 copies of target template.

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Principles of the test

Real-time PCR

A SUDV specific primer and probe mix is provided and this can be detected through the FAM channel.

The primer and probe mix provided exploits the so-called TaqMan® principle. During PCR amplification, forward and reverse primers hybridize to the SUDV DNA/cDNA. A fluorogenic probe is included in the same reaction mixture which consists of a DNA probe labeled with a 5`-dye and a 3`-quencher. During PCR amplification, the probe is cleaved and the reporter dye and quencher are separated. The resulting increase in fluorescence can be detected on a range of real-time PCR platforms.

One Step vs. Two step real-time PCR

When detecting/quantifying the presence of a target with an RNA genome PrimerDesign recommend the use of a one step qRT-PCR protocol. One step qRT-PCR combines the reverse transcription and real-time PCR reaction in a simple closed tube protocol. This saves significant bench time but also reduces errors. The sensitivity of a one step protocol is also greater than a two step because the entire biological sample is available to the PCR without dilution. This kit will also work well with a two step approach (Precision™ nanoScript reverse transcription kit plus Precision™ MasterMix) if required but the use of oasig™ OneStep MasterMix is the preferred method.

Positive control

For copy number determination and as a positive control for the PCR set up, the kit contains a positive control template. This can be used to generate a standard curve of SUDV copy number / CT value. Alternatively the positive control can be used at a single dilution where full quantitative analysis of the samples is not required. Each time the kit is used, at least one positive control reaction must be included in the run. A positive result indicates that the primers and probes for detecting the target SUDV gene worked properly in that particular experimental scenario. If a negative result is obtained the test results are invalid and must be repeated. Care should be taken to ensure that the positive control does not contaminate any other kit component which would lead to false-positive results. This can be achieved by handling this component in a Post PCR environment. Care should also be taken to avoid cross-contamination of other samples when adding the positive control to the run. This can be avoided by sealing all other samples and negative controls before pipetting the positive control into the positive control well.

Negative control

To confirm the absence of contamination, a negative control reaction should be included every time the kit is used. For this reaction, the RNAse/DNAse free water should be used instead of template. A negative result indicates that the reagents have not become contaminated while setting up the run. If a positive result is obtained the results should be ignored and the test samples repeated. Possible sources of contamination should first be explored and removed.

Internal RNA extraction control

When performing RNA extraction, it is often advantageous to have an exogenous source of RNA template that is spiked into the lysis buffer. This control RNA is then co-purified with the sample RNA and can be detected as a positive control for the extraction process. Successful co-purification and real-time PCR for the control RNA also indicates that PCR inhibitors are not present at a high concentration.

A separate RT primer mix and a real-time PCR primer/probe mix are supplied with this kit to detect the exogenous RNA using real-time PCR. The PCR primers are present at PCR limiting concentrations which allows multiplexing with the target sequence primers. Amplification of the control cDNA does not interfere with detection of the pathogen target cDNA even when present at low copy number. The Internal control is detected through the VIC channel and gives a CT value of 31+/-3 depending on the level of sample dilution.

Endogenous ACTB control

To confirm extraction of a valid biological template, a primer and probe mix is included to detect the Actin Beta (ACTB) gene. Detection of ACTB is through the FAM channel and it is NOT therefore possible to perform a multiplex for ACTB and the pathogen primers. A poor ACTB signal may indicate that the sample did not contain sufficient biological material.

Carry-over prevention using UNG (optional)

Carry over contamination between PCR reactions can be prevented by including uracil-N-glycosylase (UNG) in the reaction mix. Some commercial mastermix preparations contain UNG or alternatively it can be added as a separate component. UNG can only prevent carry over from PCR reactions that include deoxyuridine triphosphate (dUTP) in the original PCR reaction. Alliance Bio Inc. recommend the application of 0.2U UNG per assay with a 15 minute incubation step at 37°C prior to amplification. The heat-labile UNG is then inactivated during the Taq polymerase activation step (95°C for 10 minutes).

Reconstitution Protocol

To minimize the risk of contamination with foreign DNA, we recommend that all pipetting be performed in a PCR clean environment. Ideally this would be a designated PCR lab or PCR cabinet. Filter tips are recommended for all pipetting steps.

1. **Pulse-spin each tube in a centrifuge before opening.**
   This will ensure lyophilised primer and probe mix is in the base of the tube and is not spilt upon opening the tube.

2. **Reconstitute the kit components in the RNase/DNase free water supplied, according to the table below:**
   To ensure complete resuspension, vortex each tube thoroughly.

Component	Volume
Pre-PCR transparent envelope	
SUDV Primer/Probe mix (BROWN)	165 µl
Internal extraction control primer/probe mix (BROWN)	165 µl
Internal extraction control/Pathogen/ACTB RT primer mix (GREEN)	165 µl
Endogenous ACTB primer/probe mix (BROWN)	165 µl
Pre-PCR heat-sealed foil	
Internal extraction control RNA (BLUE)	600 µl
Post-PCR heat-sealed foil	
Positive Control Template (RED) *	500 µl

***** This component contains high copy number template and is a VERY significant contamination risk. It must be opened and handled in a separate laboratory environment, away from the other components.

RNA extraction

The internal extraction control RNA can be added either to the RNA lysis/extraction buffer or to the RNA sample once it has been resuspended in lysis buffer.

DO NOT add the internal extraction control RNA directly to the unprocessed biological sample as this will lead to degradation and a loss in signal.

1. Add 4µl of the Internal extraction control RNA (BLUE) to each sample in RNA lysis/extraction buffer per sample.

2. Complete RNA extraction according to the manufacturers protocols.

One Step RT-PCR detection protocol

A one step approach combining the reverse transcription and amplification in a single closed tube is the preferred method.

For optimum performance and sensitivity.

All pipetting steps and experimental plate set up should be performed on ice. After the plate is poured proceed immediately to the One Step amplification protocol. Prolonged incubation of reaction mixes at room temperature can lead to PCR artifacts that reduce the sensitivity of detection.

1. **For each RNA sample prepare a reaction mix according to the table below:**

Component	Volume
2 x oasig™ OneStep qRT-PCR MasterMix	10 µl
SUDV Primer/Probe mix (BROWN)	1 µl
Internal extraction control primer/probe mix (BROWN)	1 µl
RNA sample	X µl
RNAse/DNAse free water (WHITE)	X µl
Final Volume	**20 µl**

2. **For each RNA sample prepare an endogenous ACTB control reaction according to the table below:**

This control reaction will provide crucial information regarding the quality of the biological sample.

Component	Volume
2 x oasig™ OneStep qRT-PCR MasterMix	10 µl
Endogenous ACTB primer/probe mix (BROWN)	1 µl
RNA sample	X µl
RNAse/DNAse free water (WHITE)	X µl
Final Volume	**20 µl**

Standard curve protocol

1. **For each standard curve sample prepare a reaction mix according to the table below**

Component	Volume
2 x oasig™ OneStep qRT-PCR MasterMix	10 µl
SUDV Primer/Probe mix (BROWN)	1 µl
RNAse/DNAse free water (WHITE)	4 µl
Final Volume	**15 µl**

2. **Preparation of standard curve dilution series.**

1) Pipette 900µl of RNAse/DNAse free water into 5 tubes and label 2-6
2) Pipette 100µl of Positive Control Template (RED) into tube 2
3) Vortex thoroughly
4) Change pipette tip and pipette 100 µl from tube 2 into tube 3
5) Vortex thoroughly

Repeat steps 4 and 5 to complete the dilution series

Standard Curve	Copy Number
Tube 1 Positive control (RED)	2×10^5 per µl
Tube 2	2×10^4 per µl
Tube 3	2×10^3 per µl
Tube 4	2×10^2 per µl
Tube 5	20 per µl
Tube 6	2 per µl

3. **Pipette 5µl of standard template into each well for the standard curve according to your plate set-up**

The final volume in each well is 20µl.

One Step Amplification Protocol

Amplification conditions using oasig™ lyophilised OneStep MasterMix.

	Step	Time	Temp
Standard Curve	Reverse Transcription	10 mins	42 °C
	Enzyme activation	2 mins	95 °C
50 Cycles	Denaturation	10 secs	95 °C
	DATA COLLECTION *	60 secs	60 °C

* Fluorogenic data should be collected during this step through the FAM and VIC channels

Alternative two step reverse transcription/real-time PCR protocol

Reverse Transcription

If you need to perform separate reverse transcription and amplification (two step Real Time PCR) then we recommend the PrimerDesign Precision nanoScript™ Reverse Transcription kit. A reverse transcription primer (GREEN) is included and is designed for use with the Precision nanoScript™ reverse transcription kit. A protocol for this product is available at www.primerdesign.co.uk

1. **After reverse transcription, prepare a reaction mix according to the table below for each cDNA sample**

Component	Volume
2 x Precision™ MasterMix	10 µl
SUDV Primer/Probe mix (BROWN)	1 µl
RNAse/DNAse free water (WHITE)	3 µl
Final Volume	**15 µl**

2. **Pipette 15µl of this mix into each well according to your real-time PCR experimental plate set up.**

3. **Prepare sample cDNA templates for each of your samples (suggested concentration (5ng/µl) in RNAse/DNAse free water.**
   If the concentration of RNA that was used to make the cDNA is not known, then dilute your RT reaction mix 1:5 (10µl of sample cDNA and 40µl of water).

4. **Pipette 5µl of cDNA template into each well, according to your experimental plate setup.**
   The final volume in each well is 20µl. For negative control wells use 5µl of RNAse/DNAse free water.

Amplification Protocol

Amplification conditions using PrimerDesign 2 X Precision™ MasterMix.

	Step	Time	Temp
50 Cycles	Enzyme activation	10 mins	95 °C
	Denaturation	10s	95 °C
	DATA COLLECTION *	60s	60 °C

* Fluorogenic data should be collected during this step through the FAM and VIC channels

Interpretation of Results

Target	Internal control	Negative control	Positive control	Interpretation
+ive	+ive	-ive	+ive	**+ive**
+ive	-ive	-ive	+ive	**+ive**
-ive	+ive	-ive	+ive	**-ive**
-ive	-ive	-ive	-ive	**Experiment fail**
+ive	+ive	+ive	+ive	**Experiment fail**

Internal PCR control

When used according to the above protocols, assuming a 100% extraction efficiency, that 1:10 of extracted RNA is used in the RT reaction and that 1:5 of the RT reaction is used in an individual well, then a CT value of 31 is expected. However this can vary significantly depending on the extraction efficiency, the quantity of RNA added to the RT and PCR reaction and the individual machine settings. CT values of 31±3 are within the normal range. When amplifying a SUDV sample with a high genome copy number, the internal extraction control may not produce an amplification plot. This does not invalidate the test and should be interpreted as a positive experimental result.

Endogenous ACTB control

The signal obtained from the ACTB primer and probe set will vary according to the amount of biological material present in a given sample. An early signal indicates the presence of a good yield of biological material. A late signal suggests that little biological material is present in the sample.