WHO Emergency Quality Assessment Mechanism for EVD IVDs
PUBLIC REPORT

Product: RealStar® Filovirus Screen RT-PCR Kit 1.0
Application number: EAE 0425-153-00

Abstract

In order to respond to the urgent need for quality-assured in vitro diagnostics in the event of Ebola Virus Disease (EVD) outbreak, WHO has established a WHO Emergency Quality Assessment Mechanism of In Vitro Diagnostics (IVDs) for EVD. It consists of review of any existing evidence of safety and performance; desktop review of selected manufacturing and quality management systems documentation and limited laboratory evaluation of the product.

RealStar® Filovirus Screen RT-PCR Kit 1.0 with product code 441013 manufactured by **altona Diagnostics GmbH**, Mörkenstraße 12, 22767 Hamburg, Germany (CE marked regulatory version) was listed as eligible for WHO procurement on 25 November 2014. This public report was amended on 31 January 2019 to reflect the inclusion of the latest Instructions for Use.

The RealStar® Filovirus Screen RT-PCR Kit 1.0 is a manual in vitro diagnostic test, based on real-time PCR technology, for the qualitative detection and differentiation of Ebola- and Marburgvirus specific RNA in human EDTA plasma. The assay is designed to detect all filovirus species which are relevant human pathogens and Restonvirus. It includes a heterologous amplification system (Internal Control) to identify possible RT-PCR inhibition and to confirm the integrity of the reagents of the kit.

Real-time RT-PCR technology utilizes reverse-transcriptase (RT) reaction to convert RNA into complementary DNA (cDNA), polymerase chain reaction (PCR) for the amplification of specific target sequences and target specific probes for the detection of the amplified DNA. The probes are labelled with fluorescent reporter and quencher dyes. Probes specific for Ebolavirus RNA are labelled with the fluorophore FAM™ whereas the probes specific for Marburgvirus RNA are labelled with a fluorophore showing similar characteristics to Cy®5. The probe specific for the Internal Control (IC) is labelled with the fluorophore JOE™. Using probes linked to distinguishable dyes enables the parallel detection of Ebolavirus and Marburgvirus specific RNA as well as the detection of the Internal Control in corresponding detector channels of the real-time PCR instrument.

The test consists of three processes in a single tube assay:

- Reverse transcription of target and Internal Control RNA to cDNA
- PCR amplification of target and Internal Control cDNA
- Simultaneous detection of PCR amplicons by fluorescent dye labelled probes

RealStar® Filovirus Screen RT-PCR Kit 1.0 is validated to be used with QIAamp® Viral RNA Mini Kit (QIAGEN) to extract the viral RNA.

RealStar® Filovirus Screen RT-PCR Kit 1.0 was developed and validated to be used with the following real-time PCR instruments:

- Mx 3005P™ QPCR System (Stratagene)
- VERSANT® kPCR Molecular System AD (Siemens)
- ABI Prism® 7500 SDS and 7500 Fast SDS (Applied Biosystems)
- LightCycler® 480 Instrument II (Roche)
- Rotor-Gene® 6000 (Corbett Research)
- Rotor-Gene® Q 5/6 plex Platform (QIAGEN)
- CFX96™ Real-Time PCR Detection System (Bio-Rad)

RealStar® Filovirus Screen RT-PCR Kit 1.0 Components

Lid Colour	Blue	Purple	Green	Red	Orange	White
Component	Master A	Master B	Internal Control	Positive Control Target Ebola	Positive Control Target Marburg	PCR grade Water
Number of Vials	8	8	1	1	1	1
Volume [µl/Vial]	60	180	1000	250	250	500

Master A and Master B reagents contain all components (buffer, enzymes, primers, and probes) to allow reverse transcription, PCR mediated amplification and target detection (Filovirus specific RNA and Internal Control) in one reaction setup.

Material and devices required but not provided

Material	Description
Appropriate real-time PCR instrument	As listed above
Appropriate nucleic acid extraction system or kit	See IFU for details
Desktop centrifuge with a rotor for 2 ml reaction tubes	
Centrifuge with a rotor for microtiter plates, if using 96 well reaction plates	
Vortex mixer	
Appropriate 96 well reaction plates or reaction tubes with corresponding (optical) closing material	
Pipettes (adjustable)	
Pipette tips with filters (disposable)	
Powder-free gloves (disposable)	

Storage: The RealStar® Filovirus Screen RT-PCR Kit 1.0 is shipped on dry ice. All components should be stored between -25°C and -15°C.

Limitation of use: Using the Cepheid Smartcycler II platform is “off label use” and not recommended, since the RealStar® Filovirus Screen RT-PCR Kit 1.0 has neither been

developed nor validated for use with this real-time PCR instrument.

Background information

altona Diagnostics GmbH submitted an expression of interest for WHO emergency quality assessment of RealStar® Filovirus Screen RT-PCR Kit 1.0 on 16 October 2014.

1. Product dossier assessment

altona Diagnostics GmbH submitted documentation in support of safety and performance for RealStar® Filovirus Screen RT-PCR Kit 1.0 as per the "Invitation to Manufacturers of Ebola Virus In Vitro Diagnostics to Submit an Expression of Interest (EOI) for Emergency Assessment by WHO".[1] The information submitted in the product application was reviewed by WHO staff and external experts (reviewers) appointed by WHO. The findings of the reviews were reported in accordance with "Emergency Quality Assessment Mechanism of In Vitro Diagnostics for Ebola Virus Protocol for the Review of Documentary Evidence of Safety, Quality and Performance" (document number WHO PQDx_0188 v0.2).

Safety and performance documentation assessment conclusion: acceptable.

Based on the review of the submitted documentation, a recommendation was made to consider RealStar® Filovirus Screen RT-PCR Kit 1.0 as eligible for WHO procurement.

2. Review of quality management documentation

To establish the eligibility for WHO procurement, altona Diagnostics GmbH was asked to provide up-to-date information about the status of their quality management system.

Based on the review of the submitted quality management system documentation, it was established that sufficient information was provided by altona Diagnostics GmbH to fulfil the requirements described in the "Invitation to manufacturers of Ebola Virus In Vitro Diagnostics to submit an Expression of Interest (EOI) for Emergency Assessment by WHO".

Quality management documentation assessment conclusion: acceptable.

Based on the review of the submitted documentation, a recommendation was made to consider RealStar® Filovirus Screen RT-PCR Kit 1.0 as eligible for WHO procurement.

3. Laboratory evaluation

Given the quality and extent of the data submitted as part of the product dossier to support the claims for its intended use, RealStar® Filovirus Screen RT-PCR Kit 1.0 assay will not be required to undergo additional laboratory evaluation for the purpose of this quality assessment.

[1] Invitation to manufacturers of Ebola virus in vitro diagnostics to submit an Expression of Interest (EOI) for emergency assessment by WHO. Accessed on 24 November 2014 at http://www.who.int/diagnostics_laboratory/141002_revised_invitation_to_mx_of_ebola_virus_diagnostics_rc.pdf?ua=1

The assay was evaluated independently by the Bernhard Nocht Institute for Tropical Medicine (BNITM) in Hamburg, Germany which is a WHO Collaborating Centre for Arbovirus and Haemorrhagic Fever Reference and Research. The sensitivity of the RealStar® Filovirus Screen RT-PCR Kit 1.0 was compared to published assays, cell culture supernatant of EBOV Mayinga, EBOV 2014/Gueckedou-C05, MARV Leiden 2008, and SUDV Gulu serially diluted in negative plasma in the biosafety level 4 laboratory. The supernatants were inactivated and the RNA was extracted. The eluates were tested in replicates with the RealStar Filovirus Screen RT-PCR Kit 1.0, the pan-filovirus assay published by Panning et al. 2007[2], and an EBOV/SUDV-specific assay published by Gibb et al. 2001[3] using in-house protocols used for routine testing. The RealStar Filovirus Screen RT-PCR Kit 1.0 was able to detect virus RNA in comparable or lower concentrations compared to the published reference assays. Additionally, no cross-reactivity with RNA of other human pathogenic viruses including haemorrhagic fever viruses, e.g. Lassa virus, Yellow fever virus, Rift-Valley fever virus, and Crimean Congo haemorrhagic fever virus was observed.

Laboratory evaluation conclusion: acceptable.

Based on the review of the submitted data, a recommendation was made to consider RealStar® Filovirus Screen RT-PCR Kit 1.0 as eligible for WHO procurement.

Scope and duration of procurement eligibility

RealStar® Filovirus Screen RT-PCR Kit 1.0 with product code 441013 manufactured by altona Diagnostics GmbH is considered to be eligible for WHO procurement. The assay may be used to test symptomatic individuals for EVD. This listing does not infer that the product meets WHO prequalification requirements and does not mean that the product is listed as WHO prequalified.

As part of the on-going requirements for listing as eligible for WHO procurement, altona Diagnostics GmbH must engage in post-market surveillance activities to ensure that the product continues to meet safety, quality and performance requirements. altona Diagnostics GmbH is required to notify WHO of any complaints, including adverse events related to the use of the product within 7 days. Furthermore, WHO will continue to monitor the performance of the assay in the field.

WHO reserves the right to rescind eligibility for WHO procurement, if additional information on the safety, quality and performance comes to WHO's attention during post-market surveillance activities.

[2] Panning M., et al., Diagnostic reverse-transcription polymerase chain reaction kit for filoviruses based on the strain collections of all European biosafety level 4 laboratories. *J Infect Dis*. 2007 Nov 15; 196 Suppl 2:S199-204.

[3] Gibb TR., et al., Development and evaluation of a fluorogenic 5' nuclease assay to detect and differentiate between Ebola virus subtypes Zaire and Sudan. *J Clin Microbiol*. 2001 Nov;39(11):4125-30.

Labelling

1. Instructions for use

altona DIAGNOSTICS

Instructions for Use

RealStar®
Filovirus Screen RT-PCR Kit 1.0

11/2018 EN

RealStar®

RealStar®

Filovirus Screen RT-PCR Kit 1.0

For use with

Mx 3005P™ QPCR System (Stratagene)

VERSANT® kPCR Molecular System AD (Siemens Healthcare)

ABI Prism® 7500 SDS (Applied Biosystems)

ABI Prism® 7500 Fast SDS (Applied Biosystems)

Rotor-Gene® 6000 (Corbett Research)

Rotor-Gene® Q5/6 plex Platform (QIAGEN)

CFX96™ Real-Time PCR Detection System (Bio-Rad)

LightCycler® 480 Instrument II (Roche)

CE

IVD

REF 441013

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11 2018

altona Diagnostics GmbH • Mörkenstr. 12 • D-22767 Hamburg

MAN-441010-EN-S04

Content

1. Intended Use

The RealStar® Filovirus Screen RT-PCR Kit 1.0 is a manual *in vitro* diagnostic test, based on real-time PCR technology, for the qualitative detection and differentiation of Ebola- and Marburgvirus specific RNA in human EDTA plasma. It is intended to be used as an aid for diagnosis in individuals with signs and symptoms of infection in conjunction with clinical and epidemiological risk factors **[1]**. The test is intended to be used by qualified personnel in appropriately equipped laboratories following the guidelines on laboratory biosafety **[2]**.

[1] Case definition recommendations for Ebola or Marburg Virus Diseases. World Health Organization, 09 August 2014. (http://www.who.int/csr/resources/publications/ebola/ebola-case-definition-contact-en.pdf?ua=1).

[2] Laboratory diagnosis of Ebola virus disease. World Health Organization, 19 September 2014; WHO reference number: WHO/EVD/GUIDANCE/LAB/14.1. (http://www.who.int/csr/resources/publications/ebola/laboratory-guidance/en/).

2. Kit Components

Lid Color	Component	Number of Vials	Volume [µl/Vial]
Blue	Master A	8	60
Purple	Master B	8	180
Green	Internal Control	1	1000
Red	Positive Control Target Ebola	1	250
Orange	Positive Control Target Marburg	1	250
White	Water (PCR grade)	1	500

3. Storage

- The RealStar® Filovirus Screen RT-PCR Kit 1.0 is shipped on dry ice. The components of the kit should arrive frozen. If one or more components are not frozen upon receipt, or if tubes have been compromised during shipment, contact altona Diagnostics GmbH for assistance.
- All components should be stored between -25°C and -15°C upon arrival.
- Repeated thawing and freezing of Master reagents (more than twice) should be avoided, as this might affect the performance of the assay. The reagents should be frozen in aliquots, if they are to be used intermittently.
- Storage between +2°C and +8°C should not exceed a period of two hours.
- Protect Master A and Master B from light.

4. Material and Devices required but not provided

- Appropriate real-time PCR instrument (see chapter 6.1 Real-Time PCR Instruments)
- Appropriate nucleic acid extraction system or kit (see chapter 8.1 Sample Preparation)
- Desktop centrifuge with a rotor for 2 ml reaction tubes
- Centrifuge with a rotor for microtiter plates, if using 96 well reaction plates
- Vortex mixer
- Appropriate 96 well reaction plates or reaction tubes with corresponding (optical) closing material
- Pipettes (adjustable)
- Pipette tips with filters (disposable)
- Powder-free gloves (disposable)

NOTE

Please ensure that all instruments used have been installed, calibrated, checked and maintained according to the manufacturer´s instructions and recommendations.

NOTE

It is highly recommended to use the 72-well rotor with the appropriate 0.1 ml reaction tubes, if using the Rotor-Gene® 6000 (Corbett Research) or the Rotor-Gene® Q 5/6 plex (QIAGEN).

5. Background Information

Ebola- and *Marburgvirus* are genera within the family *Filoviridae*. Genus *Marburgvirus* contains a single species termed *Marburg marburgvirus* (MARV). Genus *Ebolavirus* contains five species: *Bundibugyo ebolavirus* (BEBOV), *Reston ebolavirus* (RESTV), *Sudan ebolavirus* (SEBOV), *Tai Forest ebolavirus* (TAFV) and *Zaire ebolavirus* (ZEBOV) **[1]**.

All known *Ebola*- and *Marburgvirus* species are endemic in Africa except RESTV which is endemic in South-East Asia. Natural hosts of filoviruses are fruit-bats **[2] [3]**. After transmission to humans, filoviruses can cause a severe hemorrhagic fever with a relatively high mortality rate of 20-90% (depending on the species and strain in the single outbreaks) **[4]**. The mode of transmission is often difficult to determine. Hunting, slaughtering and consumption of infected wild animals are likely ways of introduction of the virus into the human population. Direct contact to bats has also been shown to be a possible way of infection **[5]**. Many different mammalian species are susceptible to filovirus infections. In particular chimpanzees and gorillas have been largely affected by *Ebolavirus* epidemics resulting in significant reduction of the great apes populations **[6]**.

Symptoms are rather unspecific at the beginning of the disease including general malaise, fever and pain in different body parts **[7]**. At the beginning of outbreaks, the disease is therefore often mistaken for Malaria, Typhoid fever or other febrile diseases common in Sub-Saharan Africa.

Infectious virus titer and RNA-titer during acute disease are usually high and the level of viremia is negatively correlated with the outcome of disease **[8]**. Bleeding and other hemorrhages are also indicators for fatal outcome of Ebola and Marburg fever **[7]**.

Laboratory diagnostics is preferably done using RT-PCR from plasma, serum or even whole blood samples. Serological tests are helpful as supporting diagnostic tools but are not useful for primary diagnosis of the disease. In fact, it has been shown that many patients (especially with fatal outcome) do not develop detectable antibody titers during the course of the disease at all **[9]**.

Several real-time RT-PCR protocols for filovirus detection have been published, but none of them includes an internal amplification control or is able to detect and type *Ebola*- and *Marburgvirus* in a single RT-PCR reaction. The protocol published by Panning and colleagues in 2007 targets the *L* gene and was shown to be a sensitive and specific assay **[10]**. Since then, it has been used by several reference laboratories worldwide for filovirus diagnostics. Nevertheless, the latest sequence information available and the occurrence of new Ebola species (BEBOV) showed the need to constantly check and update the existing methods. The 2007 L gene assay has certain weaknesses and therefore a new assay based on the L gene of filoviruses was developed by altona Diagnostics GmbH.

The *filovirus L* gene, coding for the viral polymerase, contains highly conserved sequence elements. Mutations in regions coding for enzymatically active sites will usually result in loss of function. These mutants will disappear from the viral quasispecies and have no negative impact in the specificity of the RT-PCR based assay. Therefore we decided to use the *L* gene as target sequence for the RealStar® Filovirus Screen RT-PCR Kit 1.0. The concept of choosing the L gene of RNAviruses as a target for diagnostic RT-PCRs has been successfully applied in the past for *Lassa virus*, *filoviruses* and other RNA-viruses **[10–12]**.

The RealStar® Filovirus Screen RT-PCR Kit 1.0 is recommended as a first line diagnostic test. It is designed to detect all relevant filovirus species. A second line assay is also available from altona Diagnostics GmbH. The RealStar®Filovirus Type RT-PCR Kit 1.0 targets other sequences, within the virus genome, and therefore offers the possibility to generate a confirmatory diagnostic result. Furthermore, the RealStar® Filovirus Type RT-PCR Kit 1.0 allows differentiation of all relevant filoviruses down to the species level.

Suspicion and confirmation of filovirus infections have a great impact on public health and case management. All cases have to be reported immediately to the respective authorities responsible for public health, biosafety and biosecurity (within Germany: Robert Koch Institut, Berlin; and the local “Landesgesundheitsämter”). The diagnostic procedure (e.g. recommended differential diagnosis, possible use of A- and B-sample) has to be discussed with expert reference institutions.

[1] Carroll SA, Towner JS, Sealy TK, McMullan LK, Khristova ML, Burt FJ, et al. Molecular Evolution of Viruses of the Family Filoviridae Based on 97 Whole-Genome Sequences. J Virol 2013;87:2608–16.

[2] Towner JS, Amman BR, Sealy TK, Carroll SAR, Comer JA, Kemp A, et al. Isolation of Genetically Diverse Marburg Viruses from Egyptian Fruit Bats. PLoS Pathog 2009;5:e1000536.

[3] Leroy EM, Epelboin A, Mondonge V, Pourrut X, Gonzalez J-P, Muyembe-Tamfum J-J, et al. Human Ebola Outbreak Resulting from Direct Exposure to Fruit Bats in Luebo, Democratic Republic of Congo, 2007. Vector-Borne Zoonotic Dis 2009;9:723–8.

[4] Kortepeter MG, Bausch DG, Bray M. Basic Clinical and Laboratory Features of Filoviral Hemorrhagic Fever. J Infect Dis 2011;204:S810–S816.

[5] Van Paassen J, Bauer MP, Arbous MS, Visser LG, Schmidt-Chanasit J, Schilling S, et al. Acute liver failure, multiorgan failure, cerebral oedema, and activation of proangiogenic and antiangiogenic factors in a case of Marburg haemorrhagic fever. Lancet Infect Dis 2012;12:635–42.

[6] Leroy EM, Rouquet P, Formenty P, Souquière S, Kilbourne A, Froment J-M, et al. Multiple Ebola virus transmission events and rapid decline of central African wildlife. Science 2004;303:387–90.

[7] Roddy P, Howard N, Van Kerkhove MD, Lutwama J, Wamala J, Yoti Z, et al. Clinical Manifestations and Case Management of Ebola Haemorrhagic Fever Caused by a Newly Identified Virus Strain, Bundibugyo, Uganda, 2007–2008. PLoS ONE 2012;7:e52986.

[8] Towner JS, Rollin PE, Bausch DG, Sanchez A, Crary SM, Vincent M, et al. Rapid Diagnosis of Ebola Hemorrhagic Fever by Reverse Transcription-PCR in an Outbreak Setting and Assessment of Patient Viral Load as a Predictor of Outcome. J Virol 2004;78:4330–41.

[9] Gupta M, MacNeil A, Reed ZD, Rollin PE, Spiropoulou CF. Serology and cytokine profiles in patients infected with the newly discovered Bundibugyo ebolavirus. Virology 2012;423:119–24.

[10] Panning M, Laue T, Ölschlager S, Eickmann M, Becker S, Raith S, et al. Diagnostic Reverse-Transcription Polymerase Chain Reaction Kit for Filoviruses Based on the Strain Collections of all European Biosafety Level 4 Laboratories. J Infect Dis 2007;196:S199–S204.

[11] Blasdell KR, Adams MM, Davis SS, Walsh SJ, Aziz-Boaron O, Klement E, et al. A reverse-transcription PCR method for detecting all known ephemeroviruses in clinical samples. J Virol Methods 2013;191:128–35.

[12] Vieth S, Drosten C, Lenz O, Vincent M, Omilabu S, Hass M, et al. RT-PCR assay for detection of Lassa virus and related Old World arenaviruses targeting the L gene. Trans R Soc Trop Med Hyg 2007;101:1253–64.

NOTE

Due to the relatively fast molecular evolution of RNA viruses, there is an inherent risk for any RT-PCR based test system that accumulation of mutations over time may lead to false negative results.

6. Product Description

The RealStar® Filovirus Screen RT-PCR Kit 1.0 is a manual *in vitro* diagnostic test, based on real-time PCR technology, for the qualitative detection and differentiation of Ebola- and Marburgvirus specific RNA in human EDTA plasma.

The assay is designed to detect all filovirus species which are relevant human pathogens and Restonvirus.

It includes a heterologous amplification system (Internal Control) to identify possible RT-PCR inhibition and to confirm the integrity of the reagents of the kit.

Real-time RT-PCR technology utilizes reverse-transcriptase (RT) reaction to convert RNA into complementary DNA (cDNA), polymerase chain reaction (PCR) for the amplification of specific target sequences and target specific probes for the detection of the amplified DNA. The probes are labelled with fluorescent reporter and quencher dyes.

Probes specific for Ebolavirus RNA are labelled with the fluorophore FAM™ whereas the probes specific for Marburgvirus RNA are labelled with a fluorophore showing similar characteristics to Cy®5. The probe specific for Internal Control (IC) is labelled with the fluorophore JOE™.

Using probes linked to distinguishable dyes enables the parallel detection of Ebolavirus and Marburgvirus specific RNA as well as the detection of the Internal Control in corresponding detector channels of the real-time PCR instrument.

The test consists of three processes in a single tube assay:

- Reverse transcription of target and Internal Control RNA to cDNA
- PCR amplification of target and Internal Control cDNA
- Simultaneous detection of PCR amplicons by fluorescent dye labelled probes

The RealStar® Filovirus Screen RT-PCR Kit 1.0 consists of:

- Two Master reagents (Master A and Master B)
- Internal Control (IC)
- Two Positive Controls:
  - Positive Control Ebolavirus
  - Positive Control Marburgvirus
- PCR grade water

Master A and Master B contain all components (PCR buffer, reverse transcriptase, DNA polymerase, magnesium salt, primers and probes) to allow reverse transcription, PCR mediated amplification and detection of Ebolavirus specific RNA,Marburgvirus specific RNA and Internal Control in one reaction setup.

6.1 Real-Time PCR Instruments

The RealStar® Filovirus Screen RT-PCR Kit 1.0 was developed and validated to be used with the following real-time PCR instruments:

- Mx 3005P™ QPCR System (Stratagene)
- VERSANT® kPCR Molecular System AD (Siemens Healthcare)
- ABI Prism® 7500 SDS (Applied Biosystems)
- ABI Prism® 7500 Fast SDS (Applied Biosystems)
- Rotor-Gene® 6000 (Corbett Research)
- Rotor-Gene® Q5/6 plex Platform (QIAGEN)
- CFX96™ Real-Time PCR Detection System (Bio-Rad)
- LightCycler® 480 Instrument II (Roche)

CAUTION

Usage of real-time PCR instruments not listed above may result in a reduced assay performance.

7. Warnings and Precautions

Read the Instructions for Use carefully before using the product.

- Before first use check the product and its components for:
  - Integrity
  - Completeness with respect to number, type and filling (see chapter 2. Kit Components)
  - Correct labelling
  - Frozenness upon arrival
- Use of this product is limited to personnel specially instructed and trained in the techniques of real-time PCR and *in vitro* diagnostic procedures.
- Clinical specimens should always be treated as infectious and/or biohazardous in accordance with safe laboratory procedures. Refer to the WHO guideline "Laboratory diagnosis of Ebola virus disease" (World Health Organization, 19 September 2014; WHO reference number: WHO/EVD/GUIDANCE/LAB/14.1; http://www.who.int/csr/resources/publications/ebola/laboratory-guidance/en/).
- Wear protective disposable powder-free gloves, a laboratory coat and eye protection when handling specimens.
- Avoid microbial and nuclease (DNase/RNase) contamination of the specimens and the components of the kit.
- Always use DNase/RNase-free disposable pipette tips with aerosol barriers.
- Always wear protective disposable powder-free gloves when handling kit components.
- Use separated and segregated working areas for (i) sample preparation, (ii) reaction setup and (iii) amplification/detection activities. The workflow in the laboratory should proceed in unidirectional manner. Always wear disposable gloves in each area and change them before entering a different area.
- Dedicate supplies and equipment to the separate working areas and do not move them from one area to another.
- Store positive and/or potentially positive material separated from all other components of the kit.

- Do not open the reaction tubes/plates post amplification, to avoid contamination with amplicons.
- Additional controls may be tested according to guidelines or requirements of local, state and/or federal regulations or accrediting organizations.
- Do not autoclave reaction tubes after the PCR, since this will not degrade the amplified nucleic acid and will bear the risk to contaminate the laboratory area.
- Do not use components of the kit that have passed their expiration date.
- Discard sample and assay waste according to your local safety regulations. Refer also to "Fact Sheet: Safe Handling, Treatment, Transport and Disposal of Ebola-Contaminated Waste." (Occupational Safety and Health Administration (OSHA), OSHA-DEM FS-3766, 03.2016; https://www.osha.gov/pls/publications/publication.athruz?pType=Industry&pID=527).

8. Procedure

8.1 Sample Preparation

The following specimen type is validated for use with the RealStar® Filovirus Screen RT-PCR Kit 1.0:

- Human EDTA plasma

For guidance with respect to sample processing refer to "Guidelines for the collection of clinical specimens during field investigation of outbreaks" (World Health Organization, 2000; WHO reference number: WHO/CDS/CSR/EDC/2000.4; http://www.who.int/ihr/publications/WHO_CDS_CSR_EDC_2000_4/en/).

Extracted RNA is the starting material for the RealStar Filovirus Screen RT-PCR Kit 1.0.

The quality of the extracted RNA has a profound impact on the performance of the entire test system. It has to be ensured that the system used for nucleic acid extraction is compatible with real-time PCR technology. The following kits and systems are suitable for nucleic acid extraction:

- QIAamp® Viral RNA Mini Kit (QIAGEN)
- QIAsymphony® (QIAGEN)
- NucliSENS® easyMag® (bioMérieux)
- MagNA Pure 96 System (Roche)
- m2000sp (Abbott)
- Maxwell® 16 IVD Instrument (Promega)
- VERSANT® kPCR Molecular System SP (Siemens Healthcare)

Alternative nucleic acid extraction systems and kits might also be appropriate. The suitability of the nucleic acid extraction procedure for use with RealStar® Filovirus Screen RT-PCR Kit 1.0 has to be validated by the user.

Nucleic acid extracts should be stored at 2°C to 8°C and tested with the RealStar® Filovirus Screen RT-PCR Kit 1.0 within 6 hours after completion of the extraction process. Long-term storage of the extracted nucleic acids (i.e. storage for >6 hours) should be performed at -25°C to -15°C.

If using a spin column based sample preparation procedure including washing buffers containing ethanol, it is highly recommended to perform an additional centrifugation step for 10 min at approximately 17000 x g (~ 13000 rpm), using a new collection tube, prior to the elution of the nucleic acid.

CAUTION

If your sample preparation system is using washing buffers containing ethanol, make sure to eliminate any traces of ethanol prior to elution of the nucleic acid. Ethanol is a strong inhibitor of real-time PCR.

CAUTION

The use of carrier RNA is crucial for extraction efficiency and stability of the extracted nucleic acid.

For additional information and technical support regarding pre-treatment and sample preparation please contact our Technical Support (see chapter 14. Technical Assistance).

8.2 Master Mix Setup

All reagents and samples should be thawed completely, mixed (by pipetting or gentle vortexing) and centrifuged briefly before use.

The RealStar® Filovirus Screen RT-PCR Kit 1.0 contains a heterologous Internal Control (IC), which can either be used as a RT-PCR inhibition control or as a control of the sample preparation procedure (nucleic acid extraction) and as a RT-PCR inhibition control.

- If the IC is used as a RT-PCR inhibition control, but not as a control for the sample preparation procedure, set up the Master Mix according to the following pipetting scheme:

Number of Reactions (rxns)	1	12
Master A	5 µl	60 µl
Master B	15 µl	180 µl
Internal Control	1 µl	12 µl
Volume Master Mix	**21 µl**	**252 µl**

- If the IC is used as a control for the sample preparation procedure and as a RT-PCR inhibition control, add the IC during the nucleic acid extraction procedure.
- No matter which method/system is used for nucleic acid extraction, the IC **must not** be added directly to the specimen. The IC should always be added to the specimen/lysis buffer mixture. The volume of the IC which has to be added, always and only depends on the elution volume. It represents 10% of the elution volume. For instance, if the nucleic acid is going to be eluted in

60 µl of elution buffer or water, 6 µl of IC per sample must be added into the specimen/lysis buffer mixture.

► If the IC was added during the sample preparation procedure, set up the Master Mix according to the following pipetting scheme:

Number of Reactions (rxns)	1	12
Master A	5 µl	60 µl
Master B	15 µl	180 µl
Volume Master Mix	**20 µl**	**240 µl**

CAUTION

If the IC (Internal Control) was added during the sample preparation procedure, at least the negative control must include the IC.

CAUTION

No matter which method/system is used for nucleic acid extraction, never add the IC directly to the specimen.

8.3 Reaction Setup

► Pipette 20 µl of the Master Mix into each required well of an appropriate optical 96-well reaction plate or an appropriate optical reaction tube.

► Add 10 µl of the sample (eluate from the nucleic acid extraction) or 10 µl of the controls (Positive or Negative Control).

Reaction Setup	
Master Mix	20 µl
Sample or Control	10 µl
Total Volume	**30 µl**

- ► Make sure that each Positive Control and at least one Negative Control is used per run.
- ► Thoroughly mix the samples and controls with the Master Mix by pipetting up and down.
- ► Close the 96-well reaction plate with appropriate lids or optical adhesive film and the reaction tubes with appropriate lids.
- ► Centrifuge the 96-well reaction plate in a centrifuge with a microtiter plate rotor for 30 seconds at approximately 1000 x g (~ 3000 rpm).

9. Programming the Real-Time PCR Instrument

For basic information regarding the setup and programming of the different real-time PCR instruments, please refer to the user manual of the respective instrument.

For detailed programming instructions regarding the use of the RealStar® Filovirus Screen RT-PCR Kit 1.0 on specific real-time PCR instruments please contact our Technical Support (see chapter 14. Technical Assistance).

9.1 Settings

- ► Define the following settings:

Settings	
Reaction Volume	30 µl
Ramp Rate	Default
Passive Reference	None

9.2 Fluorescence Detectors (Dyes)

► Define the fluorescence detectors (dyes):

Target	Detector Name	Reporter	Quencher
Ebolavirus specific RNA	Ebolavirus	FAM™	(None)
Marburgvirus specific RNA	Marburgvirus	Cy®5	(None)
Internal Control	IC	JOE™	(None)

9.3 Temperature Profile and Dye Acquisition

► Define the temperature profile and dye acquisition:

	Stage	Cycle Repeats	Acquisition	Temperature [°C]	Time [min:sec]
Reverse Transcription	Hold	1	-	55	20:00
Denaturation	Hold	1	-	95	02:00
Amplification	Cycling	45	-	95	00:15
			yes	58	00:45
			-	72	00:15

10. Data Analysis

For basic information regarding data analysis on specific real-time PCR instruments, please refer to the user manual of the respective instrument.

For detailed instructions regarding the analysis of the data generated with the RealStar® Filovirus Screen RT-PCR Kit 1.0 on different real-time PCR instruments please contact our Technical Support (see chapter 14. Technical Assistance).

10.1 Validity of Diagnostic Test Runs

10.1.1 Valid Diagnostic Test Run (qualitative)

A **qualitative** diagnostic test run is **valid**, if the following control conditions are met:

Control ID	Detection Channel		
	FAM™	Cy®5	JOE™
Positive Control Ebolavirus	+	-	+/-*
Positive Control Marburgvirus	-	+	+/-*
Negative Control	-	-	+

* The presence or absence of a signal in the JOE™ channel is not relevant for the validity of the test run.

10.1.2 Invalid Diagnostic Test Run (qualitative)

A **qualitative** diagnostic test run is **invalid**, (i) if the run has not been completed or (ii) if any of the control conditions for a **valid** diagnostic test run are not met.

In case of an **invalid** diagnostic test run, repeat testing by using the remaining purified nucleic acids or start from the original samples again.

10.2 Interpretation of Results

10.2.1 Qualitative Analysis

Detection Channel			Result Interpretation
FAM™	Cy®5	JOE™	
+	-	+*	Ebolavirus specific RNA detected.
-	+	+*	Marburgvirus specific RNA detected.
-	-	+	Neither Ebolavirus nor Marburgvirus specific RNA detected. The sample does not contain detectable amounts of Ebolavirus or Marburgvirus specific RNA.
-	-	-	RT-PCR inhibition or reagent failure. Repeat testing from original sample or collect and test a new sample.

* Detection of the Internal Control in the JOE™ detection channel is not required for positive results either in the FAM™ detection channel or in the Cy®5 detection channel. A high Ebolavirus and/or Marburgvirus RNA load/s in the sample can lead to reduced or absent Internal Control signals.

11. Performance Evaluation

11.1 Analytical Sensitivity

Analytical sensitivity of the RealStar® Filovirus Screen RT-PCR Kit 1.0 is defined as the concentration (copies/µl of the eluate) of *Ebola*- or *Marburgvirus* specific RNA molecules that can be detected with a positivity rate of 95%. The analytical sensitivity was determined by analysis of dilution series of MARV Popp, SEBOV Gulu and ZEBOV Gabon 2003 specific *in vitro* transcripts (IVT) of known concentration.

The data generated for the calculation of the 95% LoD are summarized in Table 1, 2 and 3 below for MARV Popp, ZEBOV Gabon 2003 and SEBOV Gulu respectively.

Table 1: RT-PCR results used for the calculation of the analytical sensitivity with respect to the detection of MARV specific RNA

Input Conc. [copies/µl]	Number of Replicates	Number of Positives	Hit Rate [%]
31.622	12	12	100
10.000	12	12	100
3.162	12	12	100
1.000	12	12	100
0.316	12	8	67
0.100	12	2	17
0.032	12	0	0
0.010	12	1	8

Table 2: RT-PCR results used for the calculation of the analytical sensitivity with respect to the detection of ZEBOV specific RNA

Input Conc. [copies/µl]	Number of Replicates	Number of Positives	Hit Rate [%]
31.622	12	12	100
10.000	12	12	100
3.162	12	12	100
1.000	12	11	92
0.316	12	7	58
0.100	12	4	33
0.032	12	1	8
0.010	12	0	0

Table 3: RT-PCR results used for the calculation of the analytical sensitivity with respect to the detection of SEBOV specific RNA

Input Conc. [copies/µl]	Number of Replicates	Number of Positives	Hit Rate [%]
31.622	12	12	100
10.000	12	12	100
3.162	12	7	58
1.000	12	1	8
0.316	12	0	0
0.100	12	0	0
0.032	12	0	0
0.010	12	0	0

The analytical sensitivity of the RealStar® Filovirus Screen RT-PCR Kit 1.0 was determined by Probit analysis:

- For the detection of MARV specific RNA, the analytical sensitivity is 1.16 copies/µl [95% confidence interval (CI): 0.22 - 11.67 target copies/µl]
- For the detection of ZEBOV specific RNA, the analytical sensitivity is 1.39 copies/µl [95% confidence interval (CI): 0.69 - 5.32 target copies/µl]
- For the detection of SEBOV specific RNA, the analytical sensitivity is 6.75 copies/µl [95% confidence interval (CI): 4.25 - 24.58 target copies/µl]

11.2 Analytical Specificity

The analytical specificity of the RealStar® Filovirus Screen RT-PCR Kit 1.0 is ensured by the thorough selection of the oligonucleotides (primers and probes). The oligonucleotides were checked by sequence comparison analysis against publicly available sequences to ensure that all relevant Ebola- and Marburg viruses genotypes will be detected.

The analytical specificity of the RealStar® Filovirus Screen RT-PCR Kit 1.0 was evaluated by testing a panel of genomic RNA/DNA extracted from different pathogens that are related to Marburg- and Ebolavirus and/or can cause similar symptoms.

The RealStar® Filovirus Screen RT-PCR Kit 1.0 did not cross-react with any of the following pathogens:

- CCHFV Afg09-2990
- Dengue virus serotype 1
- Dengue virus serotype 2
- Dengue virus serotype 3
- Dengue virus serotype 4
- Hantaan virus 76-118
- Hepatitis A virus
- Hepatitis C virus
- Hepatitis E virus
- Japanese encephalitis virus
- Junin virus XJ
- Lassa virus AV
- Lassa virus CSF
- Lassa virus Lib05-1580/121
- Lassa virus Nig08-A37
- Machupo virus Carvallo
- Murray Valley encephalitis virus
- Rift Valley fever virus MP 12
- Sabia virus SPH114202
- St. Louis encephalitis virus
- Tick-borne encephalitis virus
- Usutu virus
- VSV Indiana
- West Nile virus, NY99 D
- West Nile virus, NY99
- West Nile virus, Uganda
- Yellow fever virus
- Zika virus

11.3 Precision

Precision of the RealStar® Filovirus Screen RT-PCR Kit 1.0 was determined as intra-assay variability (variability within one experiment), inter-assay variability (variability between different experiments) and inter-lot variability (variability between different production lots). Total variability was calculated by combining the three analyses.

The variability data are expressed in terms of standard deviation and coefficient of variation based on threshold cycle (C_t) values. At least six replicates per sample were analysed for intra-assay variability, inter-assay and inter-lot variability.

Table 4: Precision data for the detection of ZEBOV specific RNA

ZEBOV	Sample Concentration [copies/µl]	Average Conc. (copies/µl)	Standard Deviation	Coefficient of Variation [%]
Intra-Assay Variability	30	31.56	0.17	0.54
	10	32.80	0.12	0.36
Inter-Assay Variability	30	31.85	0.32	1.02
	10	33.07	0.34	1.04
Inter-Lot Variability	30	31.76	0.40	1.26
	10	33.03	0.44	1.35
Total Variability	30	31.70	0.35	1.10
	10	32.95	0.38	1.16

Table 5: Precision data for the detection of MARV specific RNA

MARV	Sample Concentration [copies/µl]	Average Conc. (copies/µl)	Standard Deviation	Coefficient of Variation [%]
Intra-Assay Variability	30	30.71	0.27	0.88
	10	31.82	0.25	0.80
Inter-Assay Variability	30	30.86	0.24	0.79
	10	32.06	0.32	0.99
Inter-Lot Variability	30	30.83	0.21	0.69
	10	32.03	0.30	0.93
Total Variability	30	30.79	0.23	0.75
	10	31.96	0.29	0.92

The precision data generated for the IC detection system are summarized in tables 6 and 7 for samples containing 30 and 10 viral target copies, respectively.

Table 6: Precision data for the detection of the Internal Control, analyzing samples with 30 target copies/µl

ZEBOV and MARV	30 copies/µl	Average Conc. (copies/µl)	Standard Deviation	Coefficient of Variation [%]
Intra-Assay Variability	ZEBOV	28.98	0.05	0.19
	MARV	28.90	0.09	0.32
Inter-Assay Variability	ZEBOV	29.32	0.36	1.23
	MARV	29.26	0.39	1.32
Inter-Lot Variability	ZEBOV	29.26	0.43	1.48
	MARV	29.22	0.43	1.47
Total Variability	ZEBOV	29.16	0.37	1.29
	MARV	29.11	0.38	1.31

Table 7: Precision data for the detection of the Internal Control, analyzing samples with 10 target copies/µl

ZEBOV and MARV	10 copies/µl	Average Conc. (copies/µl)	Standard Deviation	Coefficient of Variation [%]
Intra-Assay Variability	ZEBOV	29.09	0.05	0.17
	MARV	29.02	0.07	0.26
Inter-Assay Variability	ZEBOV	29.41	0.34	1.16
	MARV	29.34	0.34	1.17
Inter-Lot Variability	ZEBOV	29.32	0.43	1.48
	MARV	29.28	0.41	1.40
Total Variability	ZEBOV	29.24	0.37	1.26
	MARV	29.19	0.35	1.21

11.4 Mock Clinical Study

To evaluate the clinical performance of the RealStar® Filovirus Screen RT-PCR Kit 1.0 genomic RNA from *Zaire ebolavirus* 2014/Gueckedou-C05 was diluted in AE buffer and then spiked into overall 45 independent Ebola- and Marburgvirus negative human EDTA plasma samples. Fifteen specimens each were spiked to a final concentration of 2.25 PFU/ml, 3 PFU/ml, and 200 PFU/ml, respectively. In addition 100 Ebola- and Marburgvirus negative individual EDTA plasma samples were tested. All samples were blinded, handed to an unbiased operator and extracted using the QIAamp® Viral RNA Mini Kit (QIAGEN). The extracted nucleic acids were analyzed with the RealStar® Filovirus Screen RT-PCR Kit 1.0 on the LightCycler® 480 Instrument II (Roche), the CFX96™ Real-Time PCR Detection System (Bio-Rad) and the ABI Prism® 7500 SDS (Applied Biosystems). The blinded spiking key was unmasked after the results were complete.

The results of the analysis with the RealStar® Filovirus Screen RT-PCR Kit 1.0 are summarized in Table 8 below.

Table 8: Mock Clinical Study - Summary statistics

RealStar® Filovirus Screen RT-PCR Kit 1.0 used in combination with	CFX96™ Real-Time PCR Detection System		Light Cycler® 480 Instrument II		ABI Prism® 7500 SDS	
Result	Positive	Negative	Positive	Negative	Positive	Negative
Positive Specimens (2.25 PFU/ml, 15 samples)	15	0	15	0	14	1
Positive Specimens (3 PFU/ml, 15 samples)	15	0	15	0	15	0
Positive Specimens (200 PFU/ml, 15 samples)	15	0	15	0	15	0
Negative Specimens (100 samples)	0	100	0	100	0	100
Total (145 samples)	45	100	45	100	44	101
Positive Percent Agreement	100% (45/45)	92.1% - 100%*	100% (45/45)	92.1% - 100%*	97.8% (44/45)	88.4% - 99.6%*
Negative Percent Agreement	100% (100/100)	96.3% - 100%*	100% (100/100)	96.3% - 100%*	100% (100/100)	96.3% - 100%*

* 95% CI (= Confidence Interval)

The RealStar® Filovirus Screen RT-PCR Kit 1.0 in conjunction with the QIAamp® Viral RNA Mini Kit manual extraction system and the LightCycler® 480 Instrument II, the CFX96™ Real-Time PCR Detection System and the ABI Prism® 7500 SDS instrument, respectively, correctly identified 97.8% to 100% of the *Zaire ebolavirus* 2014/Gueckedou-C05 RNA positive samples. No unspiked specimen rendered a positive signal.

12. Limitations

- Strict compliance with the Instructions for Use is required for optimal results.
- Use of this product is limited to personnel specially instructed and trained in the techniques of real-time PCR and *in vitro* diagnostic procedures.
- Good laboratory practice is essential for proper performance of this assay. Extreme care should be taken to preserve the purity of the components of the kit and reaction setups. All reagents should be closely monitored for impurity and contamination. Any suspicious reagents should be discarded.
- Appropriate specimen collection, transport, storage and processing procedures are required for the optimal performance of this test. Refer to “Guidelines for the collection of clinical specimens during field investigation of outbreaks” (World Health Organization, 2000; WHO reference number: WHO/CDS/CSR/EDC/2000.4; http://www.who.int/ihr/publications/WHO_CDS_CSR_EDC_2000_4/en/).
- This assay must not be used on the specimen directly. Appropriate nucleic acid extraction methods have to be conducted prior to using this assay.
- The presence of RT-PCR inhibitors (e.g. heparin) may cause false negative or invalid results.
- Potential mutations within the target regions of the Ebolavirusand Marburgvirus genome covered by the primers and/or probes used in the kit may result in failure to detect the presence of the pathogens.
- As with any diagnostic test, results of the RealStar® Filovirus Screen RT-PCR Kit 1.0 need to be interpreted in consideration of all clinical and laboratory findings.

13. Quality Control

In accordance with the altona Diagnostics GmbH ISO EN 13485-certified Quality Management System, each lot of RealStar® Filovirus Screen RT-PCR Kit 1.0 is tested against predetermined specifications to ensure consistent product quality.

14. Technical Assistance

For customer support, please contact our Technical Support:

e-mail: **support@altona-diagnostics.com**

phone: **+49-(0)40-5480676-0**

15. Literature

Versalovic, James, Carroll, Karen C.,Funke, Guido, Jorgensen, James H., Landry, Marie Louise and David W. Warnock (ed). Manual of Clinical Microbiology. 10th Edition. ASM Press, 2011.

Cohen, Jonathan, Powderly, William G, and Steven M Opal. Infectious Diseases, Third Edition. Mosby, 2010.

16. Trademarks and Disclaimers

RealStar® (altona Diagnostics); ABI Prism® (Applied Biosystems); ATCC® (American Type Culture Collection); CFX96™ (Bio-Rad); Cy® (GE Healthcare); FAM™, JOE™, ROX™ (Life Technologies); LightCycler® (Roche); SmartCycler® (Cepheid); Maxwell® (Promega); Mx 3005P™ (Stratagene); NucliSENS®, easyMag® (bioMérieux); Rotor-Gene®, QIAamp®, MinElute®, QIAsymphony® (QIAGEN); VERSANT® (Siemens Healthcare).

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The RealStar® Filovirus Screen RT-PCR Kit 1.0 is a CE-marked diagnostic kit according to the European *in vitro* diagnostic directive 98/79/EC.

Product not licensed with Health Canada and not FDA cleared or approved.

Not available in all countries.

17. Explanation of Symbols

Symbol	Explanation
IVD	*In vitro* diagnostic medical device
LOT	Batch code
CAP	Cap color
REF	Catalogue number
CONT	Content
NUM	Number
COMP	Component
GTIN	Global trade identification number
	Consult instructions for use
	Contains sufficient for “n” tests/reactions (rxns)
	Temperature limit
	Use-by date
	Manufacturer
	Caution
	Note
	Version

Notes:

Notes:

Notes:

always a drop ahead.

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