RealStar®
Filovirus Type RT-PCR Kit 1.0

08/2014

RealStar®

Filovirus Type RT-PCR Kit 1.0

For research use only!

(RUO)

Product No.: 451003

96 rxns

Store at -25°C ... -15°C

August 2014

altona Diagnostics GmbH • Mörkenstraße 12 • D-22767 Hamburg

INS-451000-EN-02

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The RealStar® Filovirus Type RT-PCR Kit 1.0 is a reagent system, based on real-time PCR technology, for the detection of filovirus specific RNA. The assay is designed to detect *Ebola- and Marburgvirus* and differentiate the five *Ebolavirus* species: *Bundibugyo ebolavirus* (BEBOV), *Reston ebolavirus* (RESTV), *Sudan ebolavirus* (SEBOV), *Tai Forest ebolavirus* (TAFV) and *Zaire ebolavirus* (ZEBOV).

1. Kit Components

The kit contains 4 different RT-PCR assays with 24 reactions each.
It contains five different *Ebolavirus* controls representing each species and one Positive Control for *Marburgvirus*.

Marburgvirus (MARV) assay:

Lid Color	Blue	Purple	Red
Component	Master A MARV	Master B MARV	Positive Control *Marburgvirus*
Number of Vials	2	2	1
Volume [µl/Vial]	60	180	250

Zaire Ebolavirus (ZEBOV) assay:

Lid Color	Blue	Purple	Red
Component	Master A ZEBOV	Master B ZEBOV	Positive Control ZEBOV
Number of Vials	2	2	1
Volume [µl/Vial]	60	180	250

Sudan ebolavirus (SEBOV)/ *Bundibugyo ebolavirus* (BEBOV) assay:

Lid Color	Blue	Purple	Red	Red
Component	Master A SEBOV/BEBOV	Master B SEBOV/BEBOV	Positive Control SEBOV	Positive Control BEBOV
Number of Vials	2	2	1	1
Volume [µl/Vial]	60	180	250	250

Reston ebolavirus (RESTV)/ *Tai Forest ebolavirus* (TAFV) assay:

Lid Color	Blue	Purple	Red	Red
Component	Master A RESTV/TAFV	Master B RESTV/TAFV	Positive Control RESTV	Positive Control TAFV
Number of Vials	2	2	1	1
Volume [µl/Vial]	60	180	250	250

Additional components provided:

Lid Color	Green	White
Component	Internal Control	PCR grade Water
Number of Vials	1	1
Volume [µl/Vial]	1000	500

2. Storage

- The RealStar® Filovirus Type 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 at -20°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 at +4°C should not exceed a period of two hours.
- Protect Master A and Master B from light.

3. 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].

The phylogenetic situation is depicted in figure 1. All of the above mentioned species are endemic in Africa except of 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 finally 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. Especially 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 is 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 by 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].

The glycoprotein (GP) and the nucleoprotein gene have already been used in the past to differentiate filovirus species by real-time RT-PCR [10,11]. The RealStar® Filovirus Type RT-PCR Kit 1.0 targets these genes and is designed as a second line diagnostic test for confirmatory diagnostics and to identify the causative filovirus down to the species level. altona Diagnostics GmbH offers another Filovirus specific RT-PCR assay, the RealStar® Filovirus Screen RT-PCR Kit 1.0, designed to serve as a first line diagnostic assay.

Suspicion and confirmation of filovirus infections has a great impact on public health and case management. All cases have to be immediately reported 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.

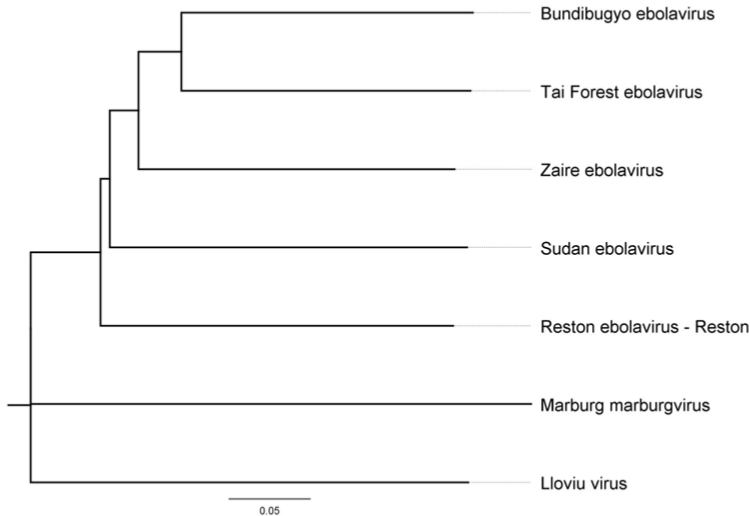


Figure 1: Neighbor-joining phylogenetic tree based on full-length sequences (HKY-model). Lloviu virus was chosen as out-group. Tree was built using Geneious version 6.1.3 created by Biomatters. Available from http://www.geneious.com/

[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 proangio-genic 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.

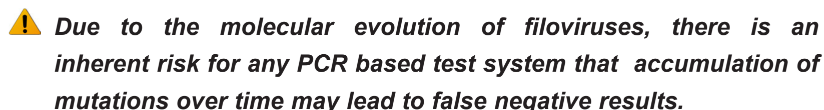
[9] Gupta M, MacNeil A, Reed ZD, Rollin PE, Spiropoulou CF. Serology and cytokine profiles in patients infected with the newly discovered Bundibugyo ebolavirus. Virology 2012;423:119–24.

[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 molecular evolution of filoviruses, there is an inherent risk for any PCR based test system that accumulation of mutations over time may lead to false negative results.

4. Product Description

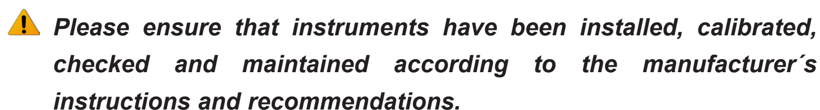
The RealStar® Filovirus Type RT-PCR Kit 1.0 is a reagent system, based on real-time PCR technology, for the detection of filovirus RNA. The assay is designed to detect *Ebola*- and *Marburgvirus* and differentiate the five *Ebolavirus* species. The reagent system includes a heterologous amplification system (Internal Control) to identify possible RT-PCR inhibition and to confirm the integrity of the reagents or the kit.
The test is based on real-time RT-PCR technology, utilizing 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 labeled with fluorescent reporter and quencher dyes. Using probes linked to distinguishable dyes enables the parallel detection and discrimination of the respective *Ebola- or Marburgvirus* specific RNA as well as the detection of Internal Control in the corresponding detector channels of the real-time PCR instrument.
The kit contains four different master PCR-reagents. Master 1 detects MARV in the FAM channel. Master 2 detects ZEBOV in the FAM channel. Master three detects SEBOV in the FAM and BEBOV in the Cy5 channel. Master 4 detects RESTV in the FAM and TAFV in the Cy5 channel. The probe specific for the target of the Internal Control (IC) is labeled with the fluorophore JOE in every Master (1-4).

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. The assay design is based on the sequence information published in Genebank as of December 2013. Occurrence of new strains and species yet unknown might make an update of primer/probe sets necessary.

The RealStar® Filovirus Type RT-PCR Kit 1.0 can be used with the following real-time PCR instruments:

- Versant™ kPCR Molecular System AD (Siemens)
- ABI Prism® 7500 SDS and 7500 Fast SDS (Applied Biosystems)
- LightCycler® 480 Instrument II (Roche)
- Rotor-Gene™ 3000/6000 (Corbett Research)
- Rotor-Gene Q 5/6 plex Platform (QIAGEN)
- CFX96 system/Dx real-time system (BIORAD)

NOTE



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

For additional information and technical support regarding pre-treatment and sample preparation please contact our Technical Support:

e-mail:	**support@altona-diagnostics.com**
phone:	**+49-(0)40-5480676-0**

5. Sample Preparation

Extracted RNA is the starting material for the RealStar® Filovirus Type 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 nucleic acid extraction kit is recommended:

- QIAamp® Viral RNA Mini Kit (QIAGEN)

If using a spin column based sample preparation procedure including washing buffers containing ethanol, 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 is highly recommended.

NOTE

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

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

6. 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 Type 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, the Master Mix is set up 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, the IC has to be added 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 depends always and only 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 to the specimen/lysis buffer mixture.

NOTE

Never add the Internal Control directly to the specimen!

- If the IC was added during the sample preparation procedure, the Master Mix is set up 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**

7. 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).
- Make sure that each of the Positive Controls and at least one Negative Control is used per run.
- Thoroughly mix the samples and controls with the Master Mix by up and down pipetting.
- Close the 96-well reaction plate with an appropriate optical adhesive film, 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).

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

8. Programming the Real-Time PCR Instruments

For basic information regarding the setup and programming of the different real-time PCR instruments, please refer to the manual of the respective instrument. For detailed programming instructions regarding the use of the RealStar® Filovirus Type RT-PCR Kit 1.0 on specific real-time PCR instruments please contact our Technical Support.

8.1 Settings

- Define the following settings:

Settings	
Reaction Volume	30 µl
Ramp Rate	Default
Passive Reference	None

8.2 Fluorescent Detectors (Dyes)

	Probe label		
Master Mix	**FAM**	**JOE**	**Cy5**
1.) MARV	MARV	IC	-
2.) ZEBOV	ZEBOV	IC	-
3.) SEBOV/BEBOV	SEBOV	IC	BEBOV
4.) RESTV/TAFV	RESTV	IC	TAFV

- Define the fluorescent detectors (dyes):

Master 1

Detection	Detector Name	Reporter	Quencher
Marburgvirus specific RNA	MARV	FAM	(None)
Internal Control	IC	JOE	(None)
n.a.	n.a.	Cy5	(None)

Master 2

Detection	Detector Name	Reporter	Quencher
Zaire ebolavirus specific RNA	ZEBOV	FAM	(None)
Internal Control	IC	JOE	(None)
n.a.	n.a.	Cy5	(None)

Master 3

Detection	Detector Name	Reporter	Quencher
Suden ebolavirus specific RNA	SEBOV	FAM	(None)
Internal Control	IC	JOE	(None)
Bundibugyo ebolavirus	BEBOV	Cy5	(None)

Master 4

Detection	Detector Name	Reporter	Quencher
Reston ebolavirus specific RNA	RESTV	FAM	(None)
Internal Control	IC	JOE	(None)
Tai Forest ebolavirus specific RNA	TAFV	Cy5	(None)

8.3 Temperature Profile and Dye Acquisition

- Define the temperature profile and dye acquisition:

	Stage	Cycle Repeats	Acquisition	Temperature	Time
Reverse Transcription	Hold	1	-	55 °C	20:00 min
Denaturation	Hold	1	-	95 °C	2:00 min
Amplification	Cycling	45	-	95 °C	0:15 min
			√	58 °C	0:45 min
			-	72 °C	0:15 min

9. Data Analysis

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

For detailed instructions regarding data analysis of the RealStar® Filovirus Type RT-PCR Kit 1.0 on different real-time PCR instruments please contact our Technical Support.

10. Interpretation of Results

MARV MASTERMIX

Sample ID	FAM Detection Channel	JOE Detection Channel	Cy5 Detection Channel	Result Interpretation
A	POSITIVE	POSITIVE*	n.a.	*Marburgvirus* specific RNA detected.
B	NEGATIVE	POSITIVE	n.a.	No *Marburgvirus* specific RNA detected.
C	NEGATIVE	NEGATIVE	n.a.	RT-PCR inhibition or reagent failure. Repeat testing from original sample or collect and test a new sample.

RESTV / TAFV MASTERMIX

Sample ID	FAM Detection Channel	JOE Detection Channel	Cy5 Detection Channel	Result Interpretation
A	POSITIVE	POSITIVE*	NEGATIVE	*Reston ebolavirus* specific RNA detected.
B	NEGATIVE	POSITIVE*	POSITIVE	*Tai Forest ebolavirus* specific RNA detected.
C	NEGATIVE	POSITIVE	NEGATIVE	Neither RESTV nor TAFV specific RNA detected. Sample does not contain detectable amounts of RESTV or TAFV specific RNA.
D	NEGATIVE	NEGATIVE	NEGATIVE	RT-PCR inhibition or reagent failure. Repeat testing from original sample or collect and test a new sample.

SEBOV / BEBOV MASTERMIX

Sample ID	FAM Detection Channel	JOE Detection Channel	Cy5 Detection Channel	Result Interpretation
A	POSITIVE	POSITIVE*	NEGATIVE	*Sudan ebolavirus* specific RNA detected.
B	NEGATIVE	POSITIVE*	POSITIVE	*Bundibugyo ebolavirus* specific RNA detected.
C	NEGATIVE	POSITIVE*	NEGATIVE	Neither SEBOV nor BEBOV specific RNA detected. Sample does not contain detectable amounts of SEBOV or BEBOV specific RNA.
D	NEGATIVE	NEGATIVE	NEGATIVE	RT-PCR inhibition or reagent failure. Repeat testing from original sample or collect and test a new sample.

ZEBOV MASTERMIX

Sample ID	FAM Detection Channel	JOE Detection Channel	Cy5 Detection Channel	Result Interpretation
A	POSITIVE	POSITIVE*	n.a.	*Zaire ebolavirus* specific RNA detected.
B	NEGATIVE	POSITIVE	n.a.	No ZEBOV specific RNA detected.
C	NEGATIVE	NEGATIVE	n.a.	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 Cy5 detection channel. High filovirus load in the sample can lead to reduced or absent Internal Control signals.

11. Technical Assistance

For customer support, please contact our Technical Support:

e-mail: **support@altona-diagnostics.com**
phone: **+49-(0)40-5480676-0**

12. Trademarks and Disclaimers

RealStar® (altona Diagnostics GmbH); Mx 3005P™ (Stratagene); ABI Prism® (Applied Biosystems); LightCycler® (Roche); Rotor-Gene™, QIAamp® (QIAGEN); VERSANT™ (Siemens); CFX96™ (BIO-RAD).

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For research use only! Not for use in diagnostic procedures.

Not available in all countries.

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13. Explanation of Symbols

Product number

Batch code

Contains sufficient for “n” tests/reactions (rxns)

Temperature limitation

Version

Use until

Caution

Consult instructions for use

Manufacturer