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RealStar®

Ebolavirus RT-PCR Kit 1.0
Instructions for Use

For Use Under the Emergency

Use Authorization (EUA) Only

Version 05/2020

RealStar®

RealStar®

Ebolavirus RT-PCR Kit 1.0

For use with

ABI Prism® 7500 SDS and 7500 Fast SDS (Applied Biosystems)

LightCycler® 480 Instrument II (Roche)

CFX96™ system/Dx real-time system (BIO-RAD)

For Use Under the Emergency Use Authorization (EUA) Only

IVD	For *in vitro* diagnostic use
EUA	For use only under Emergency Use Authorization
REF	Product No.: 581023
Σ	96 rxns
	Store at -25°C ... -15°C
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	altona Diagnostics GmbH • Mörkenstr. 12 • D-22767 Hamburg

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Content

1. Intended Use

The RealStar® Ebolavirus RT-PCR Kit 1.0 is a real-time reverse transcription polymerase chain reaction (RT-PCR) test intended for the qualitative detection of RNA from Ebolaviruses (such as *Zaire ebolavirus*, (including the *Zaire ebolavirus* strain detected in the West Africa outbreak 2014), *Sudan ebolavirus*, *Tai Forest ebolavirus*, *Bundibugyo ebolavirus*, and *Reston ebolavirus*) on specified instruments in EDTA plasma from individuals with signs and symptoms of Ebolavirus infection in conjunction with clinical and epidemiological risk factors.

Testing with the RealStar® Ebolavirus RT-PCR Kit 1.0 should not be performed unless the patient meets clinical and epidemiologic criteria for testing suspect specimens.

Assay results are for the presumptive detection of Ebolavirus. The definitive detection of Ebolavirus requires additional testing and confirmation procedures in consultation with public health or other authorities for whom reporting is required. The diagnosis of Ebolavirus infection must be made based on history, signs, symptoms, exposure likelihood, and other laboratory evidence in addition to the detection of Ebolavirus.

The level of Ebolavirus that would be present in EDTA plasma from individuals with early systemic infection is unknown. Negative results do not preclude Ebolavirus infection and should not be used as the sole basis for patient management decisions.

The RealStar® Ebolavirus RT-PCR Kit 1.0 is for use only under the Emergency Use Authorization (EUA) by laboratories certified under the Clinical Laboratory Improvement Amendments of 1988 (CLIA), 42 U.S.C. §263a, to perform high complexity tests, or by similarly qualified non-U.S. laboratories, and is limited to clinical laboratory personnel specifically instructed and trained in the techniques of real-time PCR and *in vitro* diagnostic procedures.

Notification of Public Health: Local, state and national public health agencies (for example, county and state health departments or the U.S. Centers for Disease Control and Prevention (CDC) should be notified of any patient suspected to have Ebola Virus Disease (EVD). Confirmatory testing at the state/local public health laboratory or at CDC is necessary for positive detection results and may be necessary for negative detection results. Laboratories should consult with local, state or national public health officials on any positive detection OR no detection EVD test result on the need for additional testing and appropriate transportation of specimens.

2. Kit Components

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

3. Storage

- The RealStar® Ebolavirus 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.
- Always check the expiration date and do not use reagents beyond the expiration date.
- 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.

4. Material and Devices required but not provided

- Appropriate real-time PCR instrument (chapter 6. Product Description):
  - ABI Prism® 7500 SDS (Applied Biosystems, Cat No. 4351104)
  - ABI Prism® 7500 Fast SDS (Applied Biosystems, Cat No. 4351106)
  - LightCycler® 480 Instrument II (Roche, Cat No. 05015278001)
  - CFX96™ system/Dx real-time system (BIO-RAD, Cat. No. 185-5195)
- Appropriate nucleic acid extraction system or kit:

  QIAamp® Viral RNA Mini Kit (QIAGEN, Cat. No. 52906 or 52904)
- Desktop centrifuge with a rotor for 2 ml reaction tubes (Eppendorf 5415C or equivalent)
- Centrifuge with a rotor for microtiter plates, if using 96 well reaction plates
- Vortex mixer (VWR 58810-163 or equivalent)
- Appropriate 96 well reaction plates or reaction tubes with corresponding (optical) closing material
- Pipettes (adjustable)
- Pipette tips with filters (disposable)
- Powder-free gloves (disposable)
- Nuclease-Free Water (not DEPC-Treated), Life Technologies (Cat. No 4387936) or equivalent

NOTE

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

5. Background Information

Ebolavirus is a genus within the family *Filoviridae*. Genus *Ebolavirus* contains five species: *Bundibugyo ebolavirus* (BEBOV), *Reston ebolavirus* (RESTV), *Sudan ebolavirus* (SEBOV), *Tai Forest ebolavirus* (TAFV) and *Zaire ebolavirus* (ZEBOV) [1].

All are endemic in Africa except RESTV which is endemic in South-East Asia. Natural hosts of Ebolaviruses are fruit-bats [2][3]. After transmission to humans, Ebolaviruses 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 Ebolavirus 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 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 fever [7].

Virus RNA detection using RT-PCR has successfully been done from different sample materials in the past (e.g. plasma, serum, whole blood, swabs, urine 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 Ebolavirus detection have been published, but none of them includes an internal amplification control. 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 Ebolavirus diagnostics. Nevertheless, the latest sequence information available and the occurrence of new Ebolavirus species (BEBOV) showed the need to constantly check and update the existing methods.

The Ebolavirus 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. The L gene was chosen as target sequence for the RealStar® Ebolavirus RT-PCR Kit 1.0. The concept of choosing the L gene of RNA viruses as a target for diagnostic RT-PCRs has been successfully applied in the past for Lassa virus, filoviruses and other RNA viruses [10–12].

[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, Ölschläger 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 RNA viruses, there is an inherent risk for any PCR based test system that accumulation of mutations over time may lead to false negative results.***

6. Product Description

1. The RealStar® Ebolavirus RT-PCR Kit 1.0 is an *in vitro* diagnostic test system, based on real-time PCR technology, for the qualitative detection of Ebolavirus specific RNA in human EDTA Plasma. The assay is designed to detect all Ebolavirus species which are relevant human pathogens and *Reston ebolavirus*. The reagent system includes a heterologous amplification system (Internal Control) to identify possible RT-PCR inhibition and to confirm the integrity of the reagents of 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 labelled with fluorescent reporter and quencher dyes.

   Probes specific for Ebolavirus RNA are labeled with the fluorophore FAM. The probe specific for the target of the Internal Control (IC) is labeled with the fluorophore JOE. Using probes linked to distinguishable dyes enables the parallel detection of Ebolavirus specific RNA and the Internal Control in the corresponding detector channels of the real-time PCR instrument.

   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.

2. The workflow starts with taking EDTA whole blood from the patient. Further processing of the specimen must take place following appropriate CDC guidelines for handling potentially infectious material has to take place under BSL3 or BSL4 conditions until final inactivation of the sample. Separation of cellular blood components from plasma has to be performed by centrifugation in aero-

sol-tight centrifuges. The plasma will be mixed with lysis buffer for RNA extraction. Using the QIAamp® Viral RNA Mini Kit (QIAGEN, Hilden, Germany) 140 µl of plasma have to be mixed with 560 µl of AVL. The extraction of the RNA is performed following the manufacturers instruction of the QIAamp® Viral RNA Mini Kit (QIAGEN). The extracted RNA will afterwards serve as template for analysis with the RealStar® Ebolavirus RT-PCR Kit 1.0. The temperature cycling and signal detection can be done with the real-time PCR cyclers listed as followed:

- ABI Prism® 7500 SDS and 7500 Fast SDS (Applied Biosystems)
- LightCycler® 480 Instrument II (Roche)
- CFX96™ system/Dx real-time system (BIO-RAD)

The evaluation of the results and positive or negative calling of the samples is the last step of the workflow.

3. The RealStar® Ebolavirus RT-PCR Kit 1.0 consists of:
    - Two Master reagents (Master A and Master B)
    - Template Internal Control (IC)
    - Postive Control Target EBOLA
    - PCR grade water

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

The following control materials are provided and to be used with the RealStar® Ebolavirus RT-PCR Kit 1.0:

a) Internal Control
The Internal Control contains a defined copy number of an “artificial” RNA molecule with no homologies to any other known sequences. It has to be added to the nucleic acid extraction procedure and is reverse transcribed, amplified and detected in parallel to the Ebolavirus specific RNA. The function of the Internal Control is to ensure the integrity of Ebolavirus specific real-time RT-PCR results by indicating potential RT-PCR inhibition.

b) PCR grade water
The PCR grade water is to be used as negative control for the RT-PCR-reaction. Its function is to indicate contamination of RT-PCR reagents.

c) Positive Control Target EBOLA
The “Positive Control Target EBOLA” consists of an *in vitro* transcript which contains the target sequence used by the RealStar® Ebolavirus RT-PCR Kit 1.0 for the detection of Ebolavirus specific RNA. The “Positive Control Target EBOLA” is used as positive control for the RT-PCR and verifies the functionality of the Ebolavirus RNA specific RT-PCR detection system, which is included in the RealStar® Ebolavirus RT-PCR Kit 1.0.

Apart from the controls provided with the RealStar® Ebolavirus RT-PCR Kit 1.0 a water sample (Nuclease-Free Water (not DEPC-Treated), Life Technologies (Cat. No 4387936) or equivalent) should be included in each run as Negative Process Control (NPC) to control the nucleic acid extraction procedure.

7. Test Principle

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

- Reverse transcription of target RNA 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® Ebolavirus RT-PCR Kit 1.0 is a real-time reverse transcription polymerase chain reaction (rRT-PCR) test. The Ebolavirus specific primer and probe set is designed to detect RNA from Ebolavirus in EDTA plasma specimens from patients.

One-step RT-PCR assays are one-tube assays that first reverse-transcribe specific RNA templates into cDNA copies. This cDNA then undergoes a polymerase chain reaction (PCR) that utilizes a thermocyclic heating and cooling of the reaction to logarithmically amplify a specific region of DNA. The probe anneals to a specific target sequence located between the forward and reverse primers. During the extension phase of the PCR cycle, the 5' nuclease activity of Taq polymerase degrades the probe, causing the reporter dye to separate from the quencher dye, generating a fluorescent signal. With each cycle, additional reporter dye molecules are cleaved from their respective probes, increasing the fluorescence intensity. Fluorescence intensity is monitored at each PCR cycle.

8. Limitations and Precautions

- Negative results do not preclude infection with Ebolavirus and should not be used as the sole basis of a patient treatment/management decision. All results should be interpreted by a trained professional in conjunction with review of the patient's history and clinical signs and symptoms.
- This test should not be used to test specimens from asymptomatic individuals.
- 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. False positive results may occur from cross-contamination by target organisms, their nucleic acids or amplified product.
- Appropriate specimen collection, transport, storage and processing procedures are required for the optimal performance of this test. Improper collection, storage, or transport of specimens may lead to false negative results.
- The impact of the administration of Ebolavirus vaccines and/or therapeutics on the ability to detect Ebolavirus RNA in patient specimens has not been evaluated.
- This assay must not be used on the specimen directly. Appropriate nucleic acid extraction using the QIAamp® Viral RNA Mini Kit must be conducted prior to using this assay.
- The presence of RT-PCR inhibitors may cause false negative or invalid results.
- Potential mutations within the target regions of the virus genome covered by the primer and/or probes of the test may result in failure to detect the presence of the pathogen.

9. Warnings and Precautions

- This assay is for *in vitro* diagnostic use under the FDA Emergency Use Authorization only.
- Local, state, and national public health agencies (for example, county and state health departments or the U.S. Centers for Disease Control and Prevention (CDC)) should be notified of any patient suspected to have Ebola Virus Disease (EVD). Confirmatory testing at the state/local public health laboratory or at CDC is necessary for positive detection results and may be necessary for negative detection results. Laboratories should consult with local, state or national public health officials on any positive detection OR no detection (negative) EVD test result on the need for additional testing and appropriate transportation of specimens.
- Use of this product is limited to personnel specially instructed and trained in the techniques of real-time PCR and *in vitro* diagnostic procedures.
- Use of this product is limited to specified laboratories and clinical laboratory personnel who have been trained on authorized instruments.
- Results need to be interpreted in conjunction with clinical signs, symptoms and travel history of the patient.
- Do not use reagents from other manufacturers with this assay.
- Specimens should always be treated as if infectious and/or biohazardous in accordance with safe laboratory procedures.
- Follow necessary precautions when handling specimens. Use personal protective equipment (PPE) consistent with current guidelines.
- Avoid microbial and nuclease (DNase/RNase) contamination of the specimen 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) specimen preparation, (ii) reaction set-up and (iii) amplification/detection activities. Workflow in the

laboratory should proceed in unidirectional manner. Always wear disposable gloves in each area and change them before entering different areas.

- 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 use components of the kit that have passed their expiration date.
- Discard sample and assay waste according to your local safety regulations.
- Performance of the RealStar® Ebolavirus RT-PCR Kit 1.0 has only been evaluated for EDTA plasma specimens in conjunction with the QIAamp® Viral RNA Mini Kit.
- 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.

10. Instructions for Use

10.1 RNA Extraction using the QIAamp® Viral RNA Mini Kit

Follow the manufacturer's instructions for use (QIAamp® Viral RNA Mini Handbook 04/2010). Specific details are described below.

10.1.1 Addition of carrier RNA and Internal Control template to Buffer AVL

Check Buffer AVL for precipitate, and if necessary incubate at 80°C until precipitate is dissolved. Prepare the required amount of Buffer AVL freshly. Calculate the volume of "Buffer AVL/carrier RNA/Internal Control template"-mix needed per batch of samples by selecting the number of samples to be simultaneously processed

from Table 1. Mix the reagents by inverting the tube 10 times. The number of samples is defined by the number of patient samples to be tested plus one additional Negative Process Control (NPC; Nuclease-Free Water (not DEPC-Treated), Life Technologies (Cat. No 4387936) or equivalent).

Table 1: Volumes of Buffer AVL, carrier RNA and Internal Control template required for the QIAMP® Viral RNA Mini Kit procedure

No. of Samples	Vol. of Buffer AVL[a] (ml)	Vol. of carrier RNA[a] (µl)	Vol. of IC template (green lid)[b] (µl)
1	0.56	5.60	6.00
2	1.12	11.20	12.00
3	1.68	16.80	18.00
4	2.24	22.40	24.00
5	2.80	28.00	30.00
6	3.36	33.60	36.00
7	3.92	39.20	42.00
8	4.48	44.80	48.00
9	5.04	50.40	54.00
10	5.60	56.00	60.00
11	6.16	61.60	66.00
12	6.72	67.20	72.00
13	7.28	72.80	78.00
14	7.84	78.40	84.00
15	8.40	84.00	90.00
16	8.96	89.60	96.00
17	9.52	95.20	102.00
18	10.08	100.80	108.00
19	10.64	106.40	114.00
20	11.20	112.00	120.00
21	11.76	117.60	126.00
22	12.32	123.20	132.00
23	12.88	128.80	138.00
24	13.44	134.40	144.00

[a] supplied with the QIAamp® Viral RNA Mini Kit

[b] supplied with the RealStar® Ebolavirus RT-PCR Kit 1.0

Do not forget to reconstitute buffers AW1 and AW2 with 96-100% ethanol (see manufacturer guidelines for more information).

10.1.2 Extraction process

1. Pipette 560 µl of prepared Buffer AVL containing carrier RNA and Internal Control template into a 2 ml labelled microcentrifuge tube.
2. Add 140 µl of specimen or 140 µl of water for Negative Process Control (NPC) to be extracted to the 2 ml labelled RNase-free microcentrifuge tube and mix by pulse-vortexing for 15 seconds.
3. Incubate specimen(s) and control at room temperature (15–25°C) for 10 minutes.
4. Briefly centrifuge the tubes to remove drops from the inside of the lid.
5. Add 560 µl of 96-100% ethanol to each specimen and control tube, and mix by pulse-vortexing for 15 seconds. After mixing, briefly centrifuge the tubes to remove drops from inside the lid.
6. For each specimen and control, place a QIAamp® spin column into a 2 ml collection tube (from the QIAamp® Viral RNA Mini Kit). Be sure to label the top of the columns clearly.
7. Carefully transfer 630 µl of the mixture from step 5 to the QIAamp® spin column WITHOUT moistening the rim of the column.
8. Centrifuge 1 minute at 6,000 x g. If the specimen has not cleared the filter after the first run, repeat centrifugation until the specimen has cleared the filter. Discard the collection tube containing the flow through.
9. For each specimen and control, place the QIAamp® spin column into a second, clean 2 ml collection tube (from the QIAamp® Viral RNA Mini Kit). Add the remaining mixture from step 5 to the respective spin column WITHOUT moistening the rim of the column. Pay special attention to add the remaining mixture to the correct column!

10. Centrifuge 1 minute at 6,000 x g. If the specimen has not cleared the filter after the first run, repeat centrifugation until the specimen has cleared the filter. Discard the collection tube containing the flow through.
11. For each specimen and control, place the QIAamp® spin column into another, clean 2 ml collection tube (from the QIAamp® Viral RNA Mini Kit) and add 500 µl of Buffer AW1. Discard the tube containing the filtrate from the previous step.
12. Centrifuge 1 minute at 6,000 x g. If the buffer has not cleared the filter after 1 minute, repeat centrifugation until buffer has cleared the filter.
13. Place each QIAamp® spin column into a fourth clean 2 ml collection tube (from the QIAamp® Viral RNA Mini Kit). Carefully open the QIAamp® spin column and add 500 µl of Buffer AW2.
14. Centrifuge at full speed (approx. 14,000 x g) for 3 minutes. Discard the tube containing the filtrate from the previous step.
15. To eliminate any possible Buffer AW2 carryover, place the QIAamp® spin column into a new collection tube, discard the old collection tube, and centrifuge at full speed (approx. 14,000 x g) for 3 minutes.
16. Place the QIAamp® spin column in a clean, clearly labelled 1.5 ml RNase-free microcentrifuge tube (not provided). Discard the old collection tube containing the filtrate.
17. Carefully open the QIAamp® spin column and add 60 µl of Buffer AVE that has been equilibrated to room temperature. Close the cap, and incubate at room temperature for 1 minute. Centrifuge at 6,000 x g for 1 minute, RNA is now present in the eluate and ready to test. Store extracted specimens and controls at 2-8°C until PCR master mixes are prepared.

Extracted specimens should be tested with the RealStar® Ebolavirus RT-PCR Kit 1.0 within 6 hours of completing the extraction process. Residual unextracted specimens should be stored at 2-8°C while testing is in progress. Long-term storage of extracted specimens (>6 hours) should be at -20°C. Minimize (not to exceed 3) repeated freeze-thaw cycles.

NOTES

⚠ ***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. Make sure to eliminate any traces of ethanol prior to elution of the nucleic acid.***

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

e-mail:	**support@altona-diagnostics.com**
phone USA:	**+1 614 706 178 4**
phone headquarter Hamburg:	**+49 40 548 0676 0**

10.2 Master Mix Setup

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

The RealStar® Ebolavirus RT-PCR Kit 1.0 contains a heterologous Internal Control (IC), which serves as a control of the sample preparation procedure (nucleic acid extraction) and as a RT-PCR inhibition control.

NOTE

⚠ ***Never add the Internal Control directly to the specimen!***

- 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**

10.3 Reaction Setup

- Pipette 20 µl of the Master Mix into each required well of an appropriate optical 96-well reaction plate.
- Add 1 µl of the IC into the wells that will be used for the Negative Control (PCR grade water) and the Positive Control Target EBOLA. Do not add additional IC template into the wells that will be used with any sample or control which has been extracted previously and already contains IC.
- Add 10 µl of the sample or the Negative Process Control (eluate from the nucleic acid extraction) or 10 µl of the Positive Control Target EBOLA or Negative Control (PCR grade water).
- Make sure that the Positive Control Target EBOLA and at least one Negative Control (PCR grade water) and one Negative Process Control (NPC) 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.
- 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**

11. 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® Ebolavirus RT-PCR Kit 1.0 on the LightCycler® 480 Instrument II (Roche), CFX96™ system/Dx real-time system (BIO-RAD), ABI Prism® 7500 SDS and ABI Prism® 7500 Fast SDS (Applied Biosystems) please refer to chapter 11.4 “Special remarks on the setup of authorized cyclers. For further questions please contact our Technical Support (see section 16).

11.1 Settings

- Define the following settings:

Settings	
Reaction Volume	30 µl
Ramp Rate	Default
Passive Reference	None

11.2 Fluorescent Detectors (Dyes)

- Define the fluorescent detectors (dyes):

Detection	Detector Name	Reporter	Quencher
Ebolavirus specific RNA	Ebolavirus	FAM	(None)
Internal Control	IC	JOE (LightCycler® 480 Instrument II, ABI Prism® 7500 SDS and ABI Prism® 7500 Fast SDS); VIC (CFX96™ system)	(None)

11.3 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

11.4 Special remarks on the setup of authorized cyclers

Please find below special remarks on the setup of LightCycler® 480 Instrument II (Roche), CFX96™ system/Dx real-time system (BIO-RAD), ABI Prism® 7500 SDS and ABI Prism® 7500 Fast SDS (Applied Biosystems).

11.4.1 Set-up of the LightCycler® 480 Instrument II

1. In the “Experiment settings”, select “Detection Format: Dual Color Hydrolysis Probe / UPL Probe”.
2. Make sure by checking the “Customize” field that the setting shown for the “Filter Combinations” are “FAM (465-510)” and “VIC/HEX/Yellow555 (533-580)”.

11.4.2 Set-up of the ABI Prism® 7500 SDS

Go to “Plate Setup”, “Define Targets and Samples”, “Assign Targets and Samples”:

1. Select the whole plate.
2. Click the assign-boxes for both targets. The targets should appear in the wells in the plate layout.
3. Make sure to choose “none” in the “Select the dye to use as the passive reference.” Section section (default setting is “ROX”).

11.4.3 Set-up of the ABI Prism® 7500 SDS Fast

The same settings for “Plate Setup” as for the ABI Prism® 7500 SDS apply (see above). For the Fast version, go to “Experiment properties”. The ramp speed has to be set to “Standard (~2 hours to complete a run)”. The RealStar® Ebolavirus RT-PCR Kit 1.0 is not compatible with the fast cycling conditions and the increased ramp rates.

11.4.4 Set-up of the CFX96™ system

Open the “Plate Editor” window and select all wells of the 96 well-plate. Click “Select Fluorophores”. For “Channel 1” check the box behind FAM and for “Channel 2” check the box behind VIC. Assign samples to the wells by selecting the appropriate “Sample Type “and afterwards “Load” FAM and VIC to the wells. The target name of FAM should be set to “Ebolavirus” and the target name of VIC to “Internal Control”.

12. Data Analysis

For basic information regarding data analysis on specific real-time PCR instruments, please refer to the manual of the respective instrument.
For questions regarding data analysis of the RealStar® Ebolavirus RT-PCR Kit 1.0 on authorized real-time PCR instruments please contact our Technical Support (See Section 16).

12.1 Validity of Diagnostic Test Runs

12.1.1 Valid Diagnostic Test Run

For a **valid** diagnostic test run, the following control conditions must be met:

Control ID	FAM (Ebolavirus) Detection Channel	JOE/VIC (Internal Control) Detection Channel
Positive Control Target EBOLA	POSITIVE	POSITIVE
Negative Control (PCR grade water)	NEGATIVE	POSITIVE
Negative Process Control (NPC)	NEGATIVE	POSITIVE

12.1.2 Invalid Diagnostic Test Run

A 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 the case of an **invalid** diagnostic test run, repeat testing by using the remaining purified nucleic acids or start from the original samples again.
If a test run is **repeatedly invalid** please contact our Technical Support (see section 16).

12.2 Interpretation of Results

Sample ID	FAM (Ebolavirus) Detection Channel	JOE/VIC (Internal Control) Detection Channel	Result Interpretation
A	POSITIVE	POSITIVE	Ebolavirus specific RNA detected.
B	POSITIVE	NEGATIVE	Ebolavirus specific RNA detected. High ebolavirus load in the sample leads to absent Internal Control signal.
C	NEGATIVE	POSITIVE	Ebolavirus specific RNA not detected. Sample does not contain detectable amounts of Ebolavirus specific RNA.
D	NEGATIVE	NEGATIVE	PCR inhibition or reagent failure. Repeat testing from original sample or collect and test a new sample.

13. Analytical Performance Evaluation

13.1 Analytical Sensitivity

Analytical sensitivity of the RealStar® Ebolavirus RT-PCR Kit 1.0 is defined as the concentration (plaque forming units (PFU) per ml) of Ebolavirus specific RNA molecules that can be detected with a positivity rate of ≥ 95%.

Estimation of the Limit of Detection (LoD): Serial dilutions of RNA purified from *Zaire ebolavirus* 2014/Gueckedou-C05 obtained from the Bernhard Nocht Institute for Tropical Medicine, Hamburg (Germany) were prepared in AE buffer. Each dilution was then spiked into human EDTA plasma mixed with AVL buffer, extracted with the QIAamp® Viral RNA Mini Kit (QIAGEN) in triplicate and tested with the RealStar® Ebolavirus RT-PCR Kit 1.0 on the LightCycler® 480 Instrument II Instrument (Roche). The lowest concentration at which all replicates were tested positive was treated as the estimated LoD. The results can be found in the table below:

Table 2: LoD estimate of the RealStar® Ebolavirus RT-PCR Kit 1.0

Strain tested	PFU/ml	Call rate	Replicate 1 (Cp)	Replicate 2 (Cp)	Replicate 3 (Cp)
Zaire ebolavirus 2014/ Gueckedou-C05	1000.00	3/3	24.18	24.13	24.17
	100.00	3/3	27.17	27.29	27.19
	31.62	3/3	28.32	28.60	29.06
	10.00	3/3	29.72	29.78	29.89
	3.16	3/3	30.19	30.31	30.28
	1.00	3/3	30.85	30.73	31.07

The RealStar® Ebolavirus RT-PCR Kit 1.0 in conjunction with the QIAamp® Viral RNA Mini Kit manual extraction system and the LightCycler® 480 Instrument II detected 3/3 replicates at 1 PFU/ml.

Confirmation of the Limit of Detection (LoD): Based on the results obtained from the estimation of the LoD, a dilution of RNA equivalent to 1 PFU/ml was prepared in AE buffer and then spiked into 20 individual human EDTA plasma samples mixed with AVL buffer, extracted with the QIAamp® Viral RNA Mini Kit (QIAGEN) and tested with the RealStar® Ebolavirus RT-PCR Kit 1.0 on the LightCycler® 480 Instrument II. The results can be found in the table below:

Table 3: LoD confirmation on the LightCycler® 480 Instrument II

Virus concentration: 1 PFU/ml			
Specimen	**Pos/Neg**	**Cp (FAM)**	**Cp (JOE)**
1	+	35.00	29.43
2	+	34.77	29.18
3	+	34.76	29.55
4	+	37.92	30.96
5	-	N/D	30.82
6	+	34.95	29.80
7	+	36.06	29.86
8	+	37.69	29.91
9	+	34.67	29.71
10	+	34.89	29.43
11	+	33.58	29.36
12	+	33.84	29.24
13	+	34.79	29.76
14	+	33.65	29.25
15	+	34.04	29.28
16	+	34.56	29.55
17	+	34.75	29.13
18	+	33.88	29.46
19	+	32.75	29.39
20	+	33.81	29.22
Mean Cp (n=20)		34.76	29.61
SD		1.29	0.50
CV%		3.72	1.67
Results		19/20	20/20

At the concentration of 1 PFU/ml 19/20 replicates were detected positive and thereby confirm the LoD to be 1 PFU/ml in human EDTA plasma for the RealStar® Ebolavirus RT-PCR Kit 1.0 in conjunction with the QIAamp® Viral RNA Mini Kit manual extraction system and the LightCycler® 480 Instrument II.

Confirmation of the Limit of Detection (LoD) for additional real-time PCR instruments: Based on the results obtained from the LoD confirmation using the LightCycler® 480 Instrument II, a dilution of RNA equivalent to 1 PFU/ml was prepared in AE buffer and then spiked into 20 individual human EDTA plasma samples mixed with AVL buffer, extracted with the QIAamp® Viral RNA Mini Kit (QIAGEN) and tested with the RealStar® Ebolavirus RT-PCR Kit 1.0 on the following additional real-time PCR instruments:

- CFX96™ system/Dx real-time system (BIO-RAD)
- ABI Prism® 7500 SDS (Applied Biosystems)

The results can be found in the tables below:

Table 4: LoD confirmation on the CFX96™ system

Virus concentration: 1 PFU/ml			
Specimen	**Pos/Neg**	**Cp (FAM)**	**Cp (VIC)**
1	+	31.52	29.85
2	+	30.84	29.15
3	+	33.16	29.56
4	+	34.60	30.55
5	+	34.33	30.72
6	+	33.24	29.95
7	+	33.58	30.00
8	+	32.67	30.50
9	+	32.78	29.80
10	+	32.27	29.34
11	+	31.11	29.37
12	+	30.61	29.27
13	+	33.18	29.99
14	+	32.02	29.57
15	+	31.03	29.36
16	+	31.21	29.64
17	+	31.08	29.30
18	+	31.27	29.48
19	+	31.04	29.33
20	+	31.33	29.04
Mean Cp (n=20)		32.14	29.69
SD		1.22	0.48
CV%		3.80	1.61
Results		20/20	20/20

At the concentration of 1 PFU/ml 20/20 replicates were detected positive and thereby confirm the LoD to be 1 PFU/ml in human EDTA plasma for the RealStar® Ebolavirus RT-PCR Kit 1.0 in conjunction with the QIAamp® Viral RNA Mini Kit manual extraction system and the CFX96™ system.

Table 5: LoD confirmation on the ABI Prism® 7500 SDS

Virus concentration: 1 PFU/ml			
Specimen	**Pos/Neg**	**Cp (FAM)**	**Cp (JOE)**
1	+	32.24	28.33
2	+	31.72	27.76
3	+	35.00	28.57
4	+	35.55	29.72
5	-	N/D	29.92
6	+	32.39	28.70
7	+	32.21	28.87
8	+	32.44	29.23
9	+	33.29	28.24
10	+	31.37	28.40
11	+	31.83	28.16
12	+	31.79	28.19
13	+	33.15	28.86
14	+	31.41	28.19
15	+	31.92	28.24
16	+	32.22	28.44
17	+	31.91	28.03
18	+	31.40	28.12
19	+	30.50	28.27
20	+	31.32	28.14
Mean Cp (n=20)		32.30	28.52
SD		1.23	0.56
CV%		3.82	1.96
Results		19/20	20/20

At the concentration of 1 PFU/ml 19/20 replicates were detected positive and thereby confirm the LoD to be 1 PFU/ml in human EDTA plasma for the RealStar® Ebolavirus RT-PCR Kit 1.0 in conjunction with the QIAamp® Viral RNA Mini Kit manual extraction system and the ABI Prism® 7500 SDS.

13.2 Analytical Specificity

13.2.1 Reactivity

The analytical reactivity of the RealStar® Ebolavirus RT-PCR Kit is ensured by the thorough selection of the oligonucleotides (primers and probes). The oligonucleotides were checked by sequence comparison analysis against public available sequences to ensure that all known Ebolavirus variants will be detected.

To evaluate the analytical performance of the RealStar® Ebolavirus RT-PCR Kit 1.0 with regards to reactivity genomic RNA or *in vitro* transcript (IVT) from different Ebolavirus species and strains was used. The genomic RNA was extracted from cell culture supernatant obtained from Bernhard Nocht Institute for Tropical Medicine, Hamburg (Germany). All samples were analyzed with the RealStar® Ebolavirus RT-PCR Kit 1.0 on the LightCycler® 480 Instrument II (Roche). All samples tested positive in the presence of the different *Zaire ebolavirus* strains and the other Ebolavirus species *Sudan ebolavirus, Tai Forest ebolavirus, Reston ebolavirus* and *Bundibugyo ebolavirus*, thereby showing reactivity with all known Ebolavirus species.

Table 6: Reactivity of the RealStar® Ebolavirus RT-PCR Kit 1.0

	RealStar® Ebolavirus RT-PCR Kit 1.0	
Species	Strain	Result for Ebolavirus
Zaire ebolavirus	Gabon 2003*	positive
Zaire ebolavirus	Mayinga*	positive
Zaire ebolavirus	2014/ Gueckedou-C05*	positive
Sudan ebolavirus	Gulu*	positive
*Tai Forest ebolavirus**	N/A	positive
*Reston ebolavirus**	N/A	positive
*Bundibugyo ebolavirus***	N/A	positive

*Eluates with genomic RNA from respective pathogen provided by the Bernhard Nocht Institute for Tropical Medicine, Hamburg;

***in vitro* transcript (IVT)

13.2.2 Cross-Reactivity

To evaluate the analytical specificity of the RealStar® Ebolavirus RT-PCR Kit 1.0 with regards to cross-reactivity genomic RNA or DNA from different relevant pathogens was used. The genomic RNA or DNA was extracted from cell culture supernatant or preparations from different providers (Quality Control for Molecular Diagnostics (QCMD), Glasgow, Scotland, or Bernhard Nocht Institute for Tropical Medicine (BNITM), Hamburg, Germany). All samples were analyzed with the RealStar® Ebolavirus RT-PCR Kit 1.0 on a LightCycler® 480 Instrument II (Roche).

Table 7: Analytical specificity of the RealStar® Ebolavirus RT-PCR Kit 1.0 with regards to cross-reactivity

	RealStar® Ebolavirus RT-PCR Kit 1.0	
Pathogen	Strain	Result for Ebolavirus
Japanese encephalitis virus*	N/A	negative
Saint Louis encephalitis virus*	N/A	negative
West Nile virus	NY99*	negative
West Nile virus	Uganda*	negative
Yellow Fever virus	17D*	negative
Yellow Fever Virus	French Neurotropic Virus*	negative
Murray Valley encephalitis virus*	N/A	negative
Zika virus*	N/A	negative
Tick borne encephalitis virus*	N/A	negative
Usutu virus*	N/A	negative
Dengue virus	1*	negative
Dengue virus	2*	negative
Dengue virus	3*	negative
Dengue virus	4*	negative
Hepatitis C virus	3a**	negative
Hepatitis C virus	1b**	negative
West Nile virus	NY99**	negative
Hepatitis A virus	1b, HM175**	negative
Hepatitis E virus	gg3c**	negative
Crimean-Congo hemorrhagic fever virus	Afg09-2990*	negative
Lassa virus	Nig08-A37*	negative
Lassa virus	CSF*	negative
Lassa virus	Lib05-1580/121*	negative
Lassa virus	AV*	negative

Table 7: (continuation)

	RealStar® Ebolavirus RT-PCR Kit 1.0	
Pathogen	Strain	Result for Ebolavirus
Junin virus	XJ*	negative
Machupo virus	Carvallo*	negative
Sabia virus	SPH114202*	negative
Guanarito-Virus	INH-95551*	negative
Vesicular stomatitis virus	Indiana*	negative
Rift-Valley fever virus	MP 12*	negative
Hantaan virus	76-118*	negative
Marburgvirus	Musoke*	negative
Marburgvirus	Popp*	negative
Marburgvirus	Leiden*	negative
Rotavirus	Wa***	negative
Respiratory syncytial virus	A***	negative
Respiratory syncytial virus	B***	negative
Plasmodium malariae	N/A	negative
E. coli	N/A	negative

*Eluates with genomic RNA from respective pathogen provided by the Bernhard Nocht Institute for Tropical Medicine, Hamburg

**Eluates with genomic RNA from respective pathogen sourced from QCMD EQA Panels

*** ATCC VR-2018 (Rotavirus) ATCC-VR-26D (RSV-A) and ATCC-VR-955D (RSV-B)

No cross-reactivity of the RealStar® Ebolavirus RT-PCR Kit 1.0 with genomic RNA or DNA of the selected pathogens was observed. All samples tested generated a positive Internal Control signal in the JOE channel, whereas no signal was observable in the target specific (FAM) channel.

For additional pathogens with limited or no availability, an *in silico* analysis was done showing that cross-reactivity is unlikely to occur. The primer sequences were blasted against selected species (viruses, bacteria and parasites) specified in the table below. The BLAST® algorithms were set to: blastn, word size 15, maximal target sequences 1000, match/mismatch scores 1,-2. All primers contained in the RealStar® Ebolavirus RT-PCR Kit 1.0 were blasted individually. Hits were reviewed for potential formation of PCR product through binding of the primers in close proximity and with the right orientation to each other on target nucleic acid molecules. No constellation was found that could lead to undesired amplification of potentially cross-reacting target sequences.

Table 8: Pathogens checked for cross-reactivity via *in silico* analysis

Pathogen
Influenza virus
Chikungunya virus
Adenovirus
Enterovirus
Salmonella typhimurium
Pseudomonas aeruginosa
Shigella
Coxiella burnetii
Vibrio cholerae
Borrelia recurrentis
Plasmodium falciparum
Plasmodium vivax

13.2.3 Performance testing with negative samples

To test the analytical specificity of the RealStar® Ebolavirus RT-PCR Kit 1.0 with regards to its performance with Ebolavirus negative specimens, 36 negative plasma samples from individual donors were tested. Following nucleic acid extraction with the QIAamp® Viral RNA Mini Kit (QIAGEN) samples were analyzed using the RealStar® Ebolavirus RT-PCR Kit 1.0 on a LightCycler® 480 Instrument II (Roche). Within the RT-PCR run 9 Negative Controls (PCR grade water) as well as 4 Positive Controls (provided with the RealStar® Ebolavirus RT-PCR Kit 1.0) for Ebolavirus were analyzed.
The results obtained for the 36 individual plasma samples, which have been tested with the RealStar® Ebolavirus RT-PCR Kit 1.0, are summarized in the table below.

Table 9: Specificity negative samples: RealStar® Ebolavirus RT-PCR Kit 1.0

Result for		
Negative Control	**Positive Control Target EBOLA**	**Samples**
9/9: Signal in JOE channel (IC); no signal in FAM channel	**4/4:** Signal in JOE(IC) and FAM channel	**36/36:** Signal in JOE(IC); no signal in FAM channel

All 36 samples have been tested negative for Ebolavirus specific RNA (i.e. no signal in the FAM channel) and show an Internal Control signal in the JOE channel.

13.2.4 Performance testing with negative samples across different PCR platforms

Equivalent performance of the RealStar® Ebolavirus RT-PCR Kit 1.0 on different real-time PCR instruments with respect to analytical specificity was evaluated by testing 20 plasma samples from individual donors, which were negative for Ebolavirus specific RNA. RNA extraction was carried out using the QIAamp® Viral RNA Mini Kit (QIAGEN). All samples were subjected to single extraction.

The sample input volume was 140 µl and the elution volume 60 µl. Amplification and detection were performed with one lot of the RealStar® Ebolavirus RT-PCR Kit 1.0 on the following real-time PCR instruments:

- CFX96™ system/Dx real-time system (BIO-RAD)
- ABI Prism® 7500 SDS (Applied Biosystems)

Within each run 4 Negative Controls (PCR grade water provided with the RealStar® Ebolavirus RT-PCR Kit 1.0) as well as 2 Positive Controls (provided with the RealStar® Ebolavirus RT-PCR Kit 1.0) for Ebolavirus were analyzed.

The results observed for the 20 individual plasma samples, which have been tested with the RealStar® Ebolavirus RT-PCR Kit 1.0 on the different real-time PCR instruments, are summarized in the table below.

Table 10: Real-time PCR instrument equivalence: Specificity negative samples

Real-time PCR Instrument	Negative Control	Positive Control Target EBOLA	Samples
CFX96™ system	4/4: Signal in VIC channel (IC); no signal in FAM channel	2/2: Signal in VIC (IC) and FAM channel	20/20 samples: No signal in FAM channel
ABI Prism® 7500 SDS	4/4: Signal in JOE channel (IC); no signal in FAM channel	2/2: Signal in JOE (IC) and FAM channel	20/20 samples: No signal in FAM channel

20 of the 20 samples have been tested negative for Ebolavirus specific RNA on both real-time PCR instruments used.

13.2.5 Interfering Substances

The influence of endogenous potentially interfering substances on the performance of the RealStar® Ebolavirus RT-PCR Kit 1.0 was examined by spiking selected interfering substances into simulated EDTA plasma samples containing no Ebolavirus or Ebolavirus in a concentration of the Positive Control Target EBOLA (approx. 100x LoD) and of the Positive Control Target EBOLA 1:10 diluted (approx. 10x LoD), respectively. Those samples containing interfering substances were compared to samples without interferent, which functioned as reference.
The potentially interfering substances as well as the concentrations of these used for the experiments are shown in the table below.

Table 11: Potentially interfering substances tested

Substance	Final Concentration in the Sample	Provider
Bilirubin	30 mg/dl	Calbiochem
Hemoglobin	2 g/dl	SIGMA
Triglyceride	1 g/dl	Fluka
Human Serum Albumin	6 g/dl	Fresenius Kabi
gDNA	10 µg/dl	Roche

Each sample was processed in three replicates. Following nucleic acid extraction with the QIAamp® Viral RNA Mini Kit (QIAGEN) samples were analyzed using the RealStar® Ebolavirus RT-PCR Kit 1.0 on a CFX96™ system.

The data used for the evaluation of the influence of different potentially interfering substances on the performance of the RealStar® Ebolavirus RT-PCR Kit 1.0 are summarized in the table below.

Table 12: Results of testing of samples containing different potentially interfering substances

			Reference	gDNA	Bilirubin	Hemo-globin	Trigly-ceride	Protein
Ebola-virus: nega-tive	Ebola-virus specific signal	Mean C_p value	n/a	n/a	n/a	n/a	n/a	n/a
		Δ C_p to Reference	n/a	n/a	n/a	n/a	n/a	n/a
	Internal Control signal	Mean C_p value	28.92	28.86	29.09	28.81	29.23	28.91
		Δ C_p to Reference	n/a	0.06	0.17	0.11	0.31	0.01
Ebola-virus: 10x LoD	Ebola-virus specific signal	Detection rate	3/3 pos.	3/3 pos.	3/3 pos.	3/3 pos.	3/3 pos.	3/3 pos.
		Δ C_p to Reference	n/a	n/a	n/a	n/a	n/a	n/a
	Internal Control signal	Mean C_p value	28.92	28.55	28.96	28.94	28.94	28.87
		Δ C_p to Reference	n/a	0.37	0.04	0.02	0.02	0.05
Ebola-virus: 100x LoD	Ebola-virus specific signal	Mean C_p value	25.56	25.23	25.50	25.37	25.15	25.36
		Δ C_p to Reference	n/a	0.33	0.06	0.19	0.41	0.20
	Internal Control signal	Mean C_p value	28.70	28.33	28.70	28.46	29.23	28.72
		Δ C_p to Reference	n/a	0.37	0.00	0.24	0.53	0.02

No interference was observed for samples containing elevated levels of endogenous substances (bilirubin, hemoglobin, triglycerides, human serum albumin and human gDNA). For positive samples containing a high concentration (approx. 100x LoD) of Ebolavirus specific RNA the Δ Cp values compared to the reference samples were observed to be between 0.06 and 0.41. Results obtained for Ebolavirus negative samples and the low positive samples (approx. 10x LoD) showed that all negative samples were tested negative and all low positive samples were tested positive. Since also the fluorescence intensities (FAM and VIC) were not affected, it can be concluded, that the different potentially interfering substances tested have no significant influence on the performance of the RealStar® Ebolavirus RT-PCR Kit 1.0.

14. Clinical Performance Evaluation

14.1 Mock clinical study

Data from the LoD study confirmed that the LoD of the RealStar® Ebolavirus RT-PCR Kit 1.0 for *Zaire ebolavirus* 2014/Gueckedou-C05 is 1 PFU/ml. To predict clinical performance at the 95% confidence interval (CI), viral RNA at different concentrations was prepared in AE buffer and then spiked into overall 45 samples composed of AVL buffer and independent human EDTA plasma specimens. Fifteen specimens each were spiked at 2.25x LoD (2.25 PFU/ml), at 3.0x LoD (3 PFU/ml), and at a concentration of 200x LoD (200 PFU/ml). Another 100 individual human EDTA plasma samples were left unspiked. All samples were blinded, handed to an unbiased operator and extracted using the QIAamp® Viral RNA Mini Kit (QIAGEN). The samples were analyzed with the RealStar® Ebolavirus RT-PCR Kit 1.0 on the LightCycler® 480 Instrument II (Roche), CFX96™ system (BIO-RAD) and ABI Prism® 7500 SDS (Applied Biosystems), respectively. The blinded spiking key was unmasked after the results were complete.

14.1.1 Analysis on the LightCycler® 480 Instrument II

The results of the analysis with the RealStar® Ebolavirus RT-PCR Kit 1.0 on the LightCycler® 480 Instrument II (Roche) can be found in the tables below.

Table 13: Mock Clinical Study on the LightCycler® 480 Instrument II

Spiking key		Virus concentration (PFU/ml)	Cp value	Call	IC Cp	Agreement with expected result
1	2.25 x LOD	2.25E+00	31.14	+	29.51	YES
2	200 x LOD	2.00E+02	26.56	+	27.76	YES
3		N/A	N/A	-	28.93	YES
4	200 x LOD	2.00E+02	27.21	+	28.00	YES
5		N/A	N/A	-	28.81	YES
6		N/A	N/A	-	29.11	YES
7		N/A	N/A	-	29.28	YES
8		N/A	N/A	-	29.25	YES
9	2.25 x LOD	2.25E+00	30.88	+	29.09	YES
10		N/A	N/A	-	28.95	YES
11		N/A	N/A	-	29.95	YES
12	3 x LOD	3.00E+00	30.88	+	29.19	YES
13		N/A	N/A	-	29.12	YES
14		N/A	N/A	-	29.19	YES
15	2.25 x LOD	2.25E+00	31.12	+	29.34	YES
16	3 x LOD	3.00E+00	30.50	+	29.22	YES
17		N/A	N/A	-	29.04	YES
18		N/A	N/A	-	29.31	YES
19	200 x LOD	2.00E+02	26.79	+	27.94	YES
20	2.25 x LOD	2.25E+00	31.10	+	29.27	YES
21		N/A	N/A	-	29.13	YES
22		N/A	N/A	-	28.87	YES
23		N/A	N/A	-	29.16	YES
24		N/A	N/A	-	29.18	YES
25	2.25 x LOD	2.25E+00	31.25	+	29.52	YES

Table 13: (continuation)

Spiking key		Virus concentration (PFU/ml)	Cp value	Call	IC Cp	Agreement with expected result
26		N/A	N/A	-	29.05	YES
27	200 x LOD	2.00E+02	27.98	+	28.87	YES
28		N/A	N/A	-	29.63	YES
29		N/A	N/A	-	28.94	YES
30		N/A	N/A	-	29.21	YES
31		N/A	N/A	-	29.14	YES
32	2.25 x LOD	2.25E+00	30.91	+	28.94	YES
33		N/A	N/A	-	29.22	YES
34		N/A	N/A	-	29.05	YES
35		N/A	N/A	-	29.49	YES
36	3 x LOD	3.00E+00	31.81	+	30.06	YES
37	2.25 x LOD	2.25E+00	31.70	+	29.84	YES
38		N/A	N/A	-	29.08	YES
39	200 x LOD	2.00E+02	27.56	+	28.56	YES
40	2.25 x LOD	2.25E+00	31.36	+	29.88	YES
41	3 x LOD	3.00E+00	31.17	+	29.08	YES
42	2.25 x LOD	2.25E+00	31.36	+	29.33	YES
43		N/A	N/A	-	29.29	YES
44		N/A	N/A	-	30.09	YES
45		N/A	N/A	-	29.65	YES
46		N/A	N/A	-	29.51	YES
47	2.25 x LOD	2.25E+00	30.96	+	29.24	YES
48		N/A	N/A	-	29.20	YES
49		N/A	N/A	-	29.42	YES
50		N/A	N/A	-	29.64	YES

Table 13: (continuation)

Spiking key		Virus concentration (PFU/ml)	Cp value	Call	IC Cp	Agreement with expected result
51		N/A	N/A	-	29.26	YES
52		N/A	N/A	-	29.57	YES
53		N/A	N/A	-	29.27	YES
54	200 x LOD	2.00E+02	27.66	+	28.75	YES
55		N/A	N/A	-	29.31	YES
56	3 x LOD	3.00E+00	30.97	+	29.21	YES
57		N/A	N/A	-	29.43	YES
58		N/A	N/A	-	29.33	YES
59		N/A	N/A	-	28.95	YES
60	200 x LOD	2.00E+02	27.72	+	28.53	YES
61		N/A	N/A	-	29.65	YES
62		N/A	N/A	-	29.47	YES
63		N/A	N/A	-	29.64	YES
64		N/A	N/A	-	29.48	YES
65		N/A	N/A	-	29.46	YES
66		N/A	N/A	-	30.01	YES
67		N/A	N/A	-	29.53	YES
68		N/A	N/A	-	29.98	YES
69	2.25 x LOD	2.25E+00	31.40	+	29.76	YES
70		N/A	N/A	-	29.63	YES
71		N/A	N/A	-	29.04	YES
72		N/A	N/A	-	29.25	YES
73	3 x LOD	3.00E+00	31.19	+	29.55	YES
74	200 x LOD	2.00E+02	27.05	+	27.97	YES
75		N/A	N/A	-	29.19	YES

Table 13: (continuation)

Spiking key		Virus concentration (PFU/ml)	Cp value	Call	IC Cp	Agreement with expected result
76		N/A	N/A	-	29.79	YES
77		N/A	N/A	-	29.50	YES
78		N/A	N/A	-	29.23	YES
79		N/A	N/A	-	29.80	YES
80		N/A	N/A	-	29.48	YES
81	3 x LOD	3.00E+00	31.89	+	30.20	YES
82		N/A	N/A	-	29.43	YES
83		N/A	N/A	-	29.47	YES
84		N/A	N/A	-	29.27	YES
85		N/A	N/A	-	29.06	YES
86		N/A	N/A	-	29.06	YES
87	200 x LOD	2.00E+02	27.19	+	28.23	YES
88	3 x LOD	3.00E+00	31.16	+	29.51	YES
89		N/A	N/A	-	29.27	YES
90		N/A	N/A	-	29.14	YES
91		N/A	N/A	-	29.33	YES
92		N/A	N/A	-	29.60	YES
93	2.25 x LOD	2.25E+00	30.88	+	29.04	YES
94		N/A	N/A	-	29.09	YES
95		N/A	N/A	-	29.50	YES
96		N/A	N/A	-	29.03	YES
97		N/A	N/A	-	29.44	YES
98		N/A	N/A	-	29.07	YES
99		N/A	N/A	-	29.15	YES
100	200 x LOD	2.00E+02	27.78	+	29.18	YES

Table 13: (continuation)

Spiking key		Virus concentration (PFU/ml)	Cp value	Call	IC Cp	Agreement with expected result
101		N/A	N/A	-	29.59	YES
102		N/A	N/A	-	29.11	YES
103	3 x LOD	3.00E+00	30.75	+	29.01	YES
104	3 x LOD	3.00E+00	31.09	+	29.15	YES
105	200 x LOD	2.00E+02	27.55	+	28.69	YES
106		N/A	N/A	-	29.30	YES
107		N/A	N/A	-	29.17	YES
108		N/A	N/A	-	29.25	YES
109		N/A	N/A	-	29.34	YES
110	200 x LOD	2.00E+02	27.22	+	28.16	YES
111		N/A	N/A	-	29.47	YES
112		N/A	N/A	-	29.06	YES
113		N/A	N/A	-	29.15	YES
114		N/A	N/A	-	29.14	YES
115		N/A	N/A	-	29.12	YES
116	200 x LOD	2.00E+02	27.45	+	28.49	YES
117		N/A	N/A	-	29.14	YES
118	3 x LOD	3.00E+00	31.11	+	29.31	YES
119		N/A	N/A	-	29.62	YES
120		N/A	N/A	-	29.21	YES
121		N/A	N/A	-	29.51	YES
122	200 x LOD	2.00E+02	27.25	+	28.75	YES
123		N/A	N/A	-	29.78	YES
124	2.25 x LOD	2.25E+00	31.54	+	29.85	YES
125		N/A	N/A	-	29.80	YES

Table 13: (continuation)

Spiking key		Virus concentration (PFU/ml)	Cp value	Call	IC Cp	Agreement with expected result
126	3 x LOD	3.00E+00	31.47	+	30.07	YES
127	3 x LOD	3.00E+00	31.21	+	29.66	YES
128		N/A	N/A	-	29.56	YES
129		N/A	N/A	-	29.88	YES
130	2.25 x LOD	2.25E+00	31.15	+	29.32	YES
131		N/A	N/A	-	29.78	YES
132		N/A	N/A	-	29.55	YES
133	200 x LOD	2.00E+02	26.82	+	28.30	YES
134		N/A	N/A	-	29.67	YES
135	3 x LOD	3.00E+00	31.35	+	29.71	YES
136		N/A	N/A	-	29.66	YES
137		N/A	N/A	-	29.70	YES
138		N/A	N/A	-	30.16	YES
139		N/A	N/A	-	29.67	YES
140		N/A	N/A	-	29.52	YES
141		N/A	N/A	-	29.72	YES
142	2.25 x LOD	2.25E+00	31.60	+	29.61	YES
143		N/A	N/A	-	29.96	YES
144		N/A	N/A	-	30.01	YES
145	3 x LOD	3.00E+00	31.11	+	29.25	YES

Table 14: Mock Clinical Study on the LightCycler® 480 Instrument II - Summary Statistics

RealStar® Ebolavirus RT-PCR Kit 1.0 in combination with the LightCycler® 480 Instrument II	Positive results		Negative results
Positive specimens (2.25x LOD, 15 samples)	15		0
Positive specimens (3x LOD, 15 samples)	15		0
Positive specimens (200x LOD, 15 samples)	15		0
Negative specimens (100 samples)	0		100
Total (145 samples)	45		100
			95% CI
Positive Percent Agreement	45/45	100%	92.1% - 100%
Negative Percent Agreement	100/100	100%	96.3% - 100%

The RealStar® Ebolavirus RT-PCR Kit 1.0 in conjunction with the QIAamp® Viral RNA Mini Kit manual extraction system and the LightCycler® 480 Instrument II correctly identified 45 of 45 specimens spiked with *Zaire ebolavirus* 2014/Gueckedou-C05 RNA at the concentrations shown, including concentrations near the limit of detection of the assay. No unspiked specimen rendered a positive signal.

14.1.2 Analysis on the CFX96™ system

The results of the analysis with the RealStar® Ebolavirus RT-PCR Kit 1.0 on the CFX96™ system can be found in the tables below.

Table 15: Mock Clinical Study on the CFX96™ system

Spiking key		Virus concentration (PFU/ml)	Cp value	Call	IC Cp	Agreement with expected result
1	2.25 x LOD	2.25E+00	31.71	+	29.28	YES
2	200 x LOD	2.00E+02	24.59	+	28.33	YES
3		N/A	N/A	-	28.57	YES
4	200 x LOD	2.00E+02	25.24	+	28.38	YES
5		N/A	N/A	-	28.50	YES
6		N/A	N/A	-	29.02	YES
7		N/A	N/A	-	28.91	YES
8		N/A	N/A	-	29.07	YES
9	2.25 x LOD	2.25E+00	31.14	+	28.77	YES
10		N/A	N/A	-	28.51	YES
11		N/A	N/A	-	29.31	YES
12	3 x LOD	3.00E+00	30.22	+	28.99	YES
13		N/A	N/A	-	28.63	YES
14		N/A	N/A	-	28.66	YES
15	2.25 x LOD	2.25E+00	31.15	+	29.05	YES
16	3 x LOD	3.00E+00	30.88	+	29.13	YES
17		N/A	N/A	-	28.62	YES
18		N/A	N/A	-	29.05	YES
19	200 x LOD	2.00E+02	24.66	+	28.44	YES
20	2.25 x LOD	2.25E+00	31.36	+	28.72	YES
21		N/A	N/A	-	28.93	YES
22		N/A	N/A	-	28.34	YES
23		N/A	N/A	-	28.67	YES
24		N/A	N/A	-	28.63	YES
25	2.25 x LOD	2.25E+00	31.91	+	29.40	YES

Table 15: (continuation)

Spiking key		Virus concentration (PFU/ml)	Cp value	Call	IC Cp	Agreement with expected result
26		N/A	N/A	-	28.69	YES
27	200 x LOD	2.00E+02	26.29	+	29.38	YES
28		N/A	N/A	-	29.19	YES
29		N/A	N/A	-	28.26	YES
30		N/A	N/A	-	29.03	YES
31		N/A	N/A	-	28.71	YES
32	2.25 x LOD	2.25E+00	31.24	+	28.45	YES
33		N/A	N/A	-	28.95	YES
34		N/A	N/A	-	28.39	YES
35		N/A	N/A	-	28.91	YES
36	3 x LOD	3.00E+00	31.69	+	29.71	YES
37	2.25 x LOD	2.25E+00	34.41	+	29.39	YES
38		N/A	N/A	-	28.36	YES
39	200 x LOD	2.00E+02	25.3	+	28.69	YES
40	2.25 x LOD	2.25E+00	31.46	+	29.55	YES
41	3 x LOD	3.00E+00	30.69	+	28.60	YES
42	2.25 x LOD	2.25E+00	31.17	+	28.65	YES
43		N/A	N/A	-	28.56	YES
44		N/A	N/A	-	29.65	YES
45		N/A	N/A	-	29.24	YES
46		N/A	N/A	-	29.03	YES
47	2.25 x LOD	2.25E+00	31.13	+	28.88	YES
48		N/A	N/A	-	28.58	YES
49		N/A	N/A	-	28.89	YES
50		N/A	N/A	-	28.86	YES

Table 15: (continuation)

Spiking key		Virus concentration (PFU/ml)	Cp value	Call	IC Cp	Agreement with expected result
51		N/A	N/A	-	28.59	YES
52		N/A	N/A	-	28.82	YES
53		N/A	N/A	-	28.41	YES
54	200 x LOD	2.00E+02	25.47	+	29.06	YES
55		N/A	N/A	-	28.51	YES
56	3 x LOD	3.00E+00	30.49	+	28.90	YES
57		N/A	N/A	-	28.71	YES
58		N/A	N/A	-	28.48	YES
59		N/A	N/A	-	28.38	YES
60	200 x LOD	2.00E+02	25.15	+	28.42	YES
61		N/A	N/A	-	28.62	YES
62		N/A	N/A	-	28.90	YES
63		N/A	N/A	-	28.68	YES
64		N/A	N/A	-	28.82	YES
65		N/A	N/A	-	28.66	YES
66		N/A	N/A	-	29.25	YES
67		N/A	N/A	-	28.51	YES
68		N/A	N/A	-	29.47	YES
69	2.25 x LOD	2.25E+00	31.33	+	29.19	YES
70		N/A	N/A	-	28.85	YES
71		N/A	N/A	-	28.73	YES
72		N/A	N/A	-	28.99	YES
73	3 x LOD	3.00E+00	30.84	+	28.98	YES
74	200 x LOD	2.00E+02	25.02	+	29.05	YES
75		N/A	N/A	-	29.14	YES

Table 15: (continuation)

Spiking key		Virus concentration (PFU/ml)	Cp value	Call	IC Cp	Agreement with expected result
76		N/A	N/A	-	29.72	YES
77		N/A	N/A	-	29.42	YES
78		N/A	N/A	-	29.12	YES
79		N/A	N/A	-	29.58	YES
80		N/A	N/A	-	29.06	YES
81	3 x LOD	3.00E+00	33.45	+	29.97	YES
82		N/A	N/A	-	29.38	YES
83		N/A	N/A	-	29.23	YES
84		N/A	N/A	-	29.19	YES
85		N/A	N/A	-	29.08	YES
86		N/A	N/A	-	29.02	YES
87	200 x LOD	2.00E+02	24.75	+	29.18	YES
88	3 x LOD	3.00E+00	31.47	+	29.42	YES
89		N/A	N/A	-	29.20	YES
90		N/A	N/A	-	29.13	YES
91		N/A	N/A	-	29.19	YES
92		N/A	N/A	-	29.46	YES
93	2.25 x LOD	2.25E+00	30.86	+	28.99	YES
94		N/A	N/A	-	29.11	YES
95		N/A	N/A	-	29.19	YES
96		N/A	N/A	-	29.03	YES
97		N/A	N/A	-	29.19	YES
98		N/A	N/A	-	29.06	YES
99		N/A	N/A	-	28.89	YES
100	200 x LOD	2.00E+02	24.76	+	30.16	YES

Table 15: (continuation)

Spiking key		Virus concentration (PFU/ml)	Cp value	Call	IC Cp	Agreement with expected result
101		N/A	N/A	-	29.50	YES
102		N/A	N/A	-	29.33	YES
103	3 x LOD	3.00E+00	31.05	+	28.62	YES
104	3 x LOD	3.00E+00	29.50	+	28.87	YES
105	200 x LOD	2.00E+02	24.83	+	29.48	YES
106		N/A	N/A	-	29.09	YES
107		N/A	N/A	-	28.83	YES
108		N/A	N/A	-	29.03	YES
109		N/A	N/A	-	29.11	YES
110	200 x LOD	2.00E+02	24.14	+	29.15	YES
111		N/A	N/A	-	29.54	YES
112		N/A	N/A	-	28.86	YES
113		N/A	N/A	-	28.93	YES
114		N/A	N/A	-	29.24	YES
115		N/A	N/A	-	29.27	YES
116	200 x LOD	2.00E+02	25.07	+	29.10	YES
117		N/A	N/A	-	28.87	YES
118	3 x LOD	3.00E+00	30.00	+	29.29	YES
119		N/A	N/A	-	29.64	YES
120		N/A	N/A	-	28.89	YES
121		N/A	N/A	-	29.25	YES
122	200 x LOD	2.00E+02	24.05	+	29.49	YES
123		N/A	N/A	-	29.30	YES
124	2.25 x LOD	2.25E+00	30.25	+	29.48	YES
125		N/A	N/A	-	29.46	YES

Table 15: (continuation)

Spiking key		Virus concentration (PFU/ml)	Cp value	Call	IC Cp	Agreement with expected result
126	3 x LOD	3.00E+00	29.67	+	29.84	YES
127	3 x LOD	3.00E+00	30.87	+	29.36	YES
128		N/A	N/A	-	29.31	YES
129		N/A	N/A	-	29.26	YES
130	2.25 x LOD	2.25E+00	29.64	+	29.01	YES
131		N/A	N/A	-	29.35	YES
132		N/A	N/A	-	29.17	YES
133	200 x LOD	2.00E+02	24.09	+	29.13	YES
134		N/A	N/A	-	29.15	YES
135	3 x LOD	3.00E+00	30.17	+	29.45	YES
136		N/A	N/A	-	29.22	YES
137		N/A	N/A	-	29.20	YES
138		N/A	N/A	-	29.65	YES
139		N/A	N/A	-	29.16	YES
140		N/A	N/A	-	29.22	YES
141		N/A	N/A	-	29.37	YES
142	2.25 x LOD	2.25E+00	29.53	+	29.29	YES
143		N/A	N/A	-	29.48	YES
144		N/A	N/A	-	29.49	YES
145	3 x LOD	3.00E+00	30.30	+	29.38	YES

Table 16: Mock Clinical Study on the CFX96™ system - Summary Statistics

RealStar® Ebolavirus RT-PCR Kit 1.0 in combination with the CFX96™ system	Positive results		Negative results
Positive specimens (2.25x LOD, 15 samples)	15		0
Positive specimens (3x LOD, 15 samples)	15		0
Positive specimens (200x LOD, 15 samples)	15		0
Negative specimens (100 samples)	0		100
Total (145 samples)	45		100
			95% CI
Positive Percent Agreement	45/45	100%	92.1% - 100%
Negative Percent Agreement	100/100	100%	96.3% - 100%

The RealStar® Ebolavirus RT-PCR Kit 1.0 in conjunction with the QIAamp® Viral RNA Mini Kit manual extraction system and the CFX96™ system correctly identified 45 of 45 specimens spiked with *Zaire ebolavirus* 2014/Gueckedou-C05 RNA at the concentrations shown, including concentrations near the limit of detection of the assay. No unspiked specimen rendered a positive signal.

14.1.3 Analysis on the ABI Prism® 7500 SDS

The results of the analysis with the RealStar® Ebolavirus RT-PCR Kit 1.0 on the ABI Prism® 7500 SDS (Applied Biosystems) can be found in the tables below.

Table 17: Mock Clinical Study on the ABI Prism® 7500 SDS

Spiking key		Virus concentration (PFU/ml)	Cp value	Call	IC Cp	Agreement with expected result
1	2.25 x LOD	2.25E+00	N/D	-	28.62	NO
2	200 x LOD	2.00E+02	25.82	+	27.96	YES
3		N/A	N/D	-	28.35	YES
4	200 x LOD	2.00E+02	26.63	+	28.32	YES
5		N/A	N/D	-	28.32	YES
6		N/A	N/D	-	28.53	YES
7		N/A	N/D	-	28.09	YES
8		N/A	N/D	-	28.67	YES
9	2.25 x LOD	2.25E+00	32.52	+	28.29	YES
10		N/A	N/D	-	27.98	YES
11		N/A	N/D	-	29.28	YES
12	3 x LOD	3.00E+00	32.00	+	28.86	YES
13		N/A	N/D	-	28.50	YES
14		N/A	N/D	-	28.30	YES
15	2.25 x LOD	2.25E+00	32.01	+	28.70	YES
16	3 x LOD	3.00E+00	33.64	+	28.57	YES
17		N/A	N/D	-	28.32	YES
18		N/A	N/D	-	28.55	YES
19	200 x LOD	2.00E+02	26.30	+	28.21	YES
20	2.25 x LOD	2.25E+00	33.39	+	28.62	YES
21		N/A	N/D	-	28.70	YES
22		N/A	N/D	-	28.20	YES
23		N/A	N/D	-	28.31	YES
24		N/A	N/D	-	28.40	YES
25	2.25 x LOD	2.25E+00	33.13	+	28.97	YES

Table 17: (continuation)

Spiking key		Virus concentration (PFU/ml)	Cp value	Call	IC Cp	Agreement with expected result
26		N/A	N/A	-	28.37	YES
27	200 x LOD	2.00E+02	27.49	+	29.20	YES
28		N/A	N/D	-	29.22	YES
29		N/A	N/D	-	27.90	YES
30		N/A	N/D	-	28.81	YES
31		N/A	N/D	-	28.43	YES
32	2.25 x LOD	2.25E+00	32.38	+	28.29	YES
33		N/A	N/D	-	28.52	YES
34		N/A	N/D	-	28.38	YES
35		N/A	N/D	-	28.73	YES
36	3 x LOD	3.00E+00	34.07	+	29.82	YES
37	2.25 x LOD	2.25E+00	35.15	+	29.33	YES
38		N/A	N/D	-	28.38	YES
39	200 x LOD	2.00E+02	27.01	+	28.69	YES
40	2.25 x LOD	2.25E+00	33.83	+	29.36	YES
41	3 x LOD	3.00E+00	33.01	+	28.39	YES
42	2.25 x LOD	2.25E+00	32.92	+	28.77	YES
43		N/A	N/D	-	28.50	YES
44		N/A	N/D	-	29.77	YES
45		N/A	N/D	-	29.06	YES
46		N/A	N/D	-	28.87	YES
47	2.25 x LOD	2.25E+00	32.40	+	28.81	YES
48		N/A	N/D	-	28.56	YES
49		N/A	N/D	-	28.56	YES
50		N/A	N/D	-	28.78	YES

Table 17: (continuation)

Spiking key		Virus concentration (PFU/ml)	Cp value	Call	IC Cp	Agreement with expected result
51		N/A	N/D	-	28.72	YES
52		N/A	N/D	-	28.88	YES
53		N/A	N/D	-	28.52	YES
54	200 x LOD	2.00E+02	27.03	+	29.00	YES
55		N/A	N/D	-	28.42	YES
56	3 x LOD	3.00E+00	32.95	+	28.59	YES
57		N/A	N/D	-	28.44	YES
58		N/A	N/D	-	28.43	YES
59		N/A	N/D	-	28.35	YES
60	200 x LOD	2.00E+02	27.03	+	28.70	YES
61		N/A	N/D	-	28.69	YES
62		N/A	N/D	-	28.64	YES
63		N/A	N/D	-	28.43	YES
64		N/A	N/D	-	28.66	YES
65		N/A	N/D	-	28.31	YES
66		N/A	N/D	-	29.06	YES
67		N/A	N/D	-	28.51	YES
68		N/A	N/D	-	29.26	YES
69	2.25 x LOD	2.25E+00	33.03	+	29.04	YES
70		N/A	N/D	-	28.60	YES
71		N/A	N/D	-	26.42	YES
72		N/A	N/D	-	26.75	YES
73	3 x LOD	3.00E+00	30.67	+	27.10	YES
74	200 x LOD	2.00E+02	25.04	+	26.94	YES
75		N/A	N/D	-	26.87	YES

Table 17: (continuation)

Spiking key		Virus concentration (PFU/ml)	Cp value	Call	IC Cp	Agreement with expected result
76		N/A	N/D	-	27.31	YES
77		N/A	N/D	-	27.12	YES
78		N/A	N/D	-	26.98	YES
79		N/A	N/D	-	27.48	YES
80		N/A	N/D	-	26.65	YES
81	3 x LOD	3.00E+00	34.02	+	27.99	YES
82		N/A	N/D	-	27.59	YES
83		N/A	N/D	-	27.08	YES
84		N/A	N/D	-	27.08	YES
85		N/A	N/D	-	26.72	YES
86		N/A	N/D	-	26.79	YES
87	200 x LOD	2.00E+02	24.99	+	26.95	YES
88	3 x LOD	3.00E+00	31.94	+	27.15	YES
89		N/A	N/D	-	27.10	YES
90		N/A	N/D	-	27.65	YES
91		N/A	N/D	-	27.25	YES
92		N/A	N/D	-	27.49	YES
93	2.25 x LOD	2.25E+00	30.33	+	26.69	YES
94		N/A	N/D	-	26.83	YES
95		N/A	N/D	-	26.98	YES
96		N/A	N/D	-	26.89	YES
97		N/A	N/D	-	27.14	YES
98		N/A	N/D	-	27.61	YES
99		N/A	N/D	-	27.05	YES
100	200 x LOD	2.00E+02	25.71	+	28.05	YES

Table 17: (continuation)

Spiking key		Virus concentration (PFU/ml)	Cp value	Call	IC Cp	Agreement with expected result
101		N/A	N/D	-	27.34	YES
102		N/A	N/D	-	27.09	YES
103	3 x LOD	3.00E+00	30.50	+	26.65	YES
104	3 x LOD	3.00E+00	30.68	+	26.73	YES
105	200 x LOD	2.00E+02	25.37	+	27.43	YES
106		N/A	N/D	-	27.32	YES
107		N/A	N/D	-	27.00	YES
108		N/A	N/D	-	27.12	YES
109		N/A	N/D	-	27.05	YES
110	200 x LOD	2.00E+02	24.92	+	26.95	YES
111		N/A	N/D	-	27.20	YES
112		N/A	N/D	-	26.80	YES
113		N/A	N/D	-	27.02	YES
114		N/A	N/D	-	27.29	YES
115		N/A	N/D	-	27.14	YES
116	200 x LOD	2.00E+02	25.13	+	27.12	YES
117		N/A	N/D	-	26.66	YES
118	3 x LOD	3.00E+00	30.46	+	27.21	YES
119		N/A	N/D	-	27.23	YES
120		N/A	N/D	-	26.67	YES
121		N/A	N/D	-	27.25	YES
122	200 x LOD	2.00E+02	24.74	+	27.46	YES
123		N/A	N/D	-	27.63	YES
124	2.25 x LOD	2.25E+00	32.61	+	27.81	YES
125		N/A	N/D	-	27.26	YES

Table 17: (continuation)

Spiking key		Virus concentration (PFU/ml)	Cp value	Call	IC Cp	Agreement with expected result
126	3 x LOD	3.00E+00	30.50	+	27.57	YES
127	3 x LOD	3.00E+00	29.81	+	27.15	YES
128		N/A	N/D	-	27.36	YES
129		N/A	N/D	-	27.37	YES
130	2.25 x LOD	2.25E+00	31.82	+	27.39	YES
131		N/A	N/D	-	27.56	YES
132		N/A	N/D	-	27.22	YES
133	200 x LOD	2.00E+02	24.33	+	27.03	YES
134		N/A	N/D	-	27.03	YES
135	3 x LOD	3.00E+00	30.03	+	27.14	YES
136		N/A	N/D	-	27.19	YES
137		N/A	N/D	-	27.10	YES
138		N/A	N/D	-	27.94	YES
139		N/A	N/D	-	27.35	YES
140		N/A	N/D	-	27.00	YES
141		N/A	N/D	-	27.11	YES
142	2.25 x LOD	2.25E+00	31.67	+	27.25	YES
143		N/A	N/D	-	27.11	YES
144		N/A	N/D	-	27.29	YES
145	3 x LOD	3.00E+00	29.71	+	27.01	YES

Table 18: Mock Clinical Study on the ABI Prism® 7500 SDS - Summary Statistics

RealStar® Ebolavirus RT-PCR Kit 1.0 in combination with the ABI Prism® 7500 SDS	**Positive results**		**Negative results**
Positive specimens (2.25x LOD, 15 samples)	14		1
Positive specimens (3x LOD, 15 samples)	15		0
Positive specimens (200x LOD, 15 samples)	15		0
Negative specimens (100 samples)	0		100
Total (145 samples)	44		101
			95% CI
Positive Percent Agreement	44/45	97.8%	88.4% - 99.6%
Negative Percent Agreement	100/100	100%	96.3% - 100%

The RealStar® Ebolavirus RT-PCR Kit 1.0 in conjunction with the QIAamp® Viral RNA Mini Kit manual extraction system and the ABI Prism® 7500 SDS correctly identified 44 of 45 specimens spiked with *Zaire ebolavirus* 2014/Guecke-dou-C05 RNA at the concentrations shown, one discrepant result was observed near the limit of detection of the assay. No unspiked specimen rendered a positive signal.

14.2 Comparison study using published primer sets

As no real clinical samples for a comparison study with the RealStar® Ebolavirus RT-PCR Kit 1.0 were available, samples were simulated and tested against three different reference methods at the Bernhard Nocht Institute for Tropical Medicine (BNITM), Hamburg, Germany.

Virus particles were spiked into negative patient plasma and serially diluted in pooled plasma samples. Viral strains used were *Zaire ebolavirus* Mayinga, *Zaire ebolavirus* 2014/Gueckedou-C05 and *Sudan ebolavirus* Gulu. Dilutions were prepared in the BSL-4 laboratory at BNITM up to a dilution of $1:10^8$. RNA from the samples was extracted with the QIAamp® Viral RNA Mini Kit (QIAGEN) and tested in replicates (duplicates or triplicates) with the RealStar® Ebolavirus RT-PCR Kit 1.0 and selected published tests [1, 2]. Both methods used for reference testing are real-time PCR based assays, which have been established in the PCR diagnostic laboratory of the BNITM.

The performance between the assays was compared by determining hit rates observed for the different sample dilutions.

Details are shown in the following tables.

Table 19: Results for *Zaire ebolavirus* Mayinga

***Zaire ebolavirus* Mayinga (stock: 9.72 x 10e+05 PFU/ml)**									
Dilution 10^x	**-1**	**-2**	**-3**	**-4**	**-5**	**-6**	**-7**	**-8**	**-9**
RealStar® Ebolavirus RT-PCR Kit 1.0	3/3	3/3	3/3	3/3	3/3	1/3	0/3	0/3	0/3
Panning L-Gen Screen [1]	3/3	3/3	3/3	3/3	3/3	0/3	0/3	0/3	0/3
Gibb EBOV GP [2]	2/2	2/2	2/2	2/2	0/2	0/2	0/2	0/2	0/2

Table 20: Results for *Zaire ebolavirus* 2014/Gueckedou-C05

***Zaire ebolavirus* 2014/Gueckedou-C05 (stock: 5.80 x 10e+05 PFU/ml)**									
Dilution 10^x	**-1**	**-2**	**-3**	**-4**	**-5**	**-6**	**-7**	**-8**	**-9**
RealStar® Ebolavirus RT-PCR Kit 1.0	3/3	3/3	3/3	3/3	3/3	1/3	0/3	0/3	0/3
Panning L-Gen Screen [1]	3/3	3/3	3/3	3/3	3/3	0/3	0/3	0/3	0/3
Gibb EBOV GP [2]	2/2	2/2	2/2	2/2	0/2	0/2	0/2	0/2	0/2

Table 21: Results for *Sudan ebolavirus* Gulu

***Sudan ebolavirus* Gulu (stock concentration not available)**									
Dilution 10^x	**-1**	**-2**	**-3**	**-4**	**-5**	**-6**	**-7**	**-8**	**-9**
RealStar® Ebolavirus RT-PCR Kit 1.0	3/3	2/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
Panning L-Gen Screen [1]	3/3	3/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
Gibb EBOV GP [2]	2/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2

[1] Panning, M., Laue, T., Ölschläger, S., Eickmann, M., Becker, S., Raith, S., Courbot, M.-C.G., Nilsson, M., Gopal, R., Lundkvist, A., et al. (2007). Diagnostic reverse-transcription polymerase chain reaction kit for filoviruses based on the strain collections of all European biosafety level 4 laboratories. J. Infect. Dis. 196 Suppl 2, S199–204.

[2] Gibb, T.R., Norwood, D.A., Woollen, N., and Henchal, E.A. (2001a). Development and evaluation of a fluorogenic 5' nuclease assay to detect and differentiate between Ebola virus subtypes Zaire and Sudan. J. Clin. Microbiol. 39, 4125–4130

15. Quality Control

In accordance with the altona Diagnostics GmbH DIN EN ISO13485-certified Quality Management System, each lot of RealStar® Ebolavirus RT-PCR Kit 1.0 is tested against predetermined specifications to ensure consistent product quality.

16. Technical Assistance

For customer support, please contact our Technical Support:

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17. Trademarks and Disclaimers

RealStar® (altona Diagnostics GmbH); ABI Prism® (Applied Biosystems); LightCycler® (Roche); QIAamp® (QIAGEN); CFX96™ (BIO-RAD).

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The RealStar® Ebolavirus RT-PCR Kit 1.0 is for use only under Emergency Use Authorization (EUA) by specified laboratories and clinical laboratory personnel who have been trained on authorized instruments.

Not available in all countries.

18. Explanation of Symbols

IVD	*In vitro* diagnostic medical device
EUA	For use only under Emergency Use Authorization
REF	Product number
LOT	Batch code
	Contains sufficient for “n” tests/reactions (rxns)
	Temperature limitation
	Version
	Use until
	Caution
	Consult instructions for use
	Manufacturer

Notes

Notes

always a drop ahead.

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