For Professional Use Only

Ebola Zaire Real-TM Handbook

Real-Time PCR test for qualitative detection of Ebola Virus

50

REF V69-50FRT

NAME
Ebola Zaire Real-TM

INTRODUCTION
Ebola virus (EBOV, formerly designated Zaire ebolavirus) is the sole member of the Zaire ebolavirus species, and the most dangerous of the five known viruses within the genus Ebolavirus. The virus and its species were both originally named for Zaire (now the Democratic Republic of Congo), the country where it was first described. The species is a virological taxon species included in the genus Ebolavirus, family Filoviridae (whose members are called Filovirus), order Mononegavirales. Its natural reservoir is believed to be bats, particularly fruit bats, and it is primarily transmitted between humans and from animals to humans, through body fluids.
The EBOV genome is approximately 19,000 base pairs long. It encodes seven structural proteins: nucleoprotein (NP), polymerase cofactor (VP35), (VP40), GP, transcription activator (VP30), VP24, and RNA polymerase (L). It's is difficult to study due to the virulent nature of the virus.
Because of its high mortality rate, EBOV is also listed a select agent, World Health Organization Risk Group 4 Pathogen (requiring Biosafety Level 4-equivalent containment), a U.S. National Institutes of Health/National Institute of Allergy and Infectious Diseases Category A Priority Pathogen, U.S. CDC Centers for Disease Control and Prevention Category A Bioterrorism Agent, and listed as a Biological Agent for Export Control by the Australia Group.

INTENDED USE
Ebola Zaire Real-TM is a Real-Time test for the qualitative detection of Ebola Virus RNA in the blood, plasma, urine, saliva, tissue. EBOLA RNA is extracted from specimens, amplified using RT-amplification and detected using fluorescent reporter dye probes specific for Ebola or Ebola IC.
Ebola Zaire Real-TM assay is for the presumptive detection of Ebola Zaire virus on specified instruments in individuals in affected areas with signs and symptoms of Ebola virus infection or who are at risk for exposure or may have been exposed to the Ebola Zaire virus (detected in the West Africa outbreak in 2014) in conjunction with epidemiological risk factors. The level of Ebola Zaire virus (detected in the West Africa outbreak in 2014) present in blood from individuals with early systemic infection is unknown. Negative results do not preclude Ebola Zaire virus infection and should not be used as the sole basis for patient management decisions. The diagnosis of Ebola Zaire virus (detected in the West Africa outbreak in 2014) infection must be made based on history, signs, symptoms, exposure likelihood, and other laboratory evidence in addition to the identification of the Ebola Zaire virus by this test. The **Ebola Zaire Real-TM** is for use only by specified laboratories and clinical laboratory personnel who have been trained on authorized instruments.

PRINCIPLE OF ASSAY
Ebola virus detection by the polymerase chain reaction (PCR) is based on the amplification of the pathogen genome specific region by using specific primers. In real-time PCR, the amplified product is detected using fluorescent dyes. These dyes are linked to oligonucleotide probes that bind specifically to the amplified product. The real-time monitoring of the fluorescence intensities during the real-time PCR allows the detection of accumulating product without re-opening of the reaction tubes after the PCR run. **Ebola Zaire Real-TM** PCR kit is a qualitative test that contains the Internal Control (IC), which must be used in the extraction procedure in order to control the extraction process of each individual sample and to identify possible reaction inhibition. **Ebola Zaire Real-TM** PCR kit uses "hot-start", which greatly reduces the frequency of nonspecifically primed reactions. "Hot-start" is guaranteed by separation of nucleotides and Taq-polymerase by using a chemically modified polymerase (TaqF), which is activated by heating at 95 °C for 15 min.

MATERIALS PROVIDED

Reagent	*Description*	*Volume, ml*	*Quantity*
RT-G-mix-2	colorless clear liquid	0.015	1 tube
RT-PCR-mix-1-FRT *EBOV*	colorless clear liquid	0.6	1 tube
RT-PCR-mix-2	colorless clear liquid	0.3	1 tube
Polymerase (TaqF)	colorless clear liquid	0.03	1 tube
TM-Revertase (MMlv)	colorless clear liquid	0.015	1 tube
Positive Control cDNA EBOV / IC STI	colorless clear liquid	0.2	1 tube
RNA-buffer	colorless clear liquid	0.2	1 tube
Positive Control EBOV	colorless clear liquid	0.1	1 tube
Negative Control (C–)*	colorless clear liquid	1.2	1 tube
Internal Control STI-87-rec**	colorless clear liquid	0.5	1 tube

* *must be used in the isolation procedure as Negative Control of Extraction.*

** *add 10 µl of Internal Control (IC) to each sample during the RNA purification procedure directly to the sample/lysis mixture*

MATERIALS REQUIRED BUT NOT PROVIDED

- RNA extraction kit.
- TRIzol® LS reagent (Life Technologies Cat. No. 10296-010, 100 mL) or TRI Reagent® LS (Sigma Cat. No. T3934-100mL, 100mL)
- Disposable powder-free gloves and laboratory coat.
- Pipettes (adjustable).
- Sterile pipette tips with aerosol barriers.
- Tube racks.
- Vortex mixer.
- Desktop centrifuge with a rotor for 2-ml reaction tubes.
- PCR box.
- Personal Real Time PCR thermocycler.
- Disposable polypropylene microtubes for PCR or PCR-plate**.**
- Refrigerator for 2–8 °C.
- Deep-freezer for ≤ –16 °C.
- Waste bin for used tips.

WARNINGS AND PRECAUTIONS

RUO For Research Use Only

⚠ Samples from Ebola patients are an extreme biohazard risk. Protocol should be performed in a Class II or higher Biosafety Cabinet (BSC).

1. Wear disposable gloves, laboratory coats and eye protection when handling specimens and reagents. Thoroughly wash hands afterward.
2. Do not pipette by mouth.
3. Do not eat, drink, smoke, apply cosmetics, or handle contact lenses in laboratory work areas.
4. Do not use a kit after its expiration date.
5. Dispose of all specimens and unused reagents in accordance with local regulations.
6. Biosafety Level 2 should be used for materials that contain or are suspected of containing infectious agents.
7. Clean and disinfect all spills of specimens or reagents using a disinfectant such as 0,5% sodium hypochlorite, or other suitable disinfectant.
8. Avoid contact of specimens and reagents with the skin, eyes and mucous membranes. If these solutions come into contact, rinse immediately with water and seek medical advice immediately.
9. Material Safety Data Sheets (MSDS) are available on request.
10. Use of this product should be limited to personnel trained in the techniques of DNA amplification.
11. PCR reactions are sensitive to contamination. Measures to reduce the risk of contamination in the laboratory include physically separating the activities involved in performing PCR in compliance with good laboratory practice.
12. Workflow in the laboratory must proceed in a uni-directional manner, beginning in the Extraction Area and moving to the Amplification and Detection Area. Do not return samples, equipment and reagents in the area where you performed previous step.

⚠ Sampling of biological materials for PCR-analysis, transportation, and storage are described in detail in the handbook of the manufacturer. It is recommended that this handbook is read before beginning of the work.

PRODUCT USE LIMITATIONS

All reagents may exclusively be used in in vitro diagnostics. Use of this product should be limited to personnel trained in the in Vitro diagnostic procedures (UNI EN ISO 18113-2:2012). Strict compliance with the user manual is required for optimal PCR results. Attention should be paid to expiration dates printed on the box and labels of all components. Do not use a kit after its expiration date.

QUALITY CONTROL

In accordance with Sacace's ISO 13485-Certified Quality Management System, each lot is tested against predetermined specifications to ensure consistent product quality.

STORAGE INSTRUCTIONS

All components of the **Ebola Zaire Real-TM** PCR kit (except for RT-G-mix-2, RT-PCR-mix-1-FRT EBOV, RT-PCR-mix-2, polymerase (TaqF), and TM-Revertase (MMlv)) are to be stored at 2–8 ºC. All components of the **Ebola Zaire Real-TM** PCR kit are stable until the expiration date on the label. The shelf life of reagents before and after the first use is the same, unless otherwise stated.

RT-G-mix-2, RT-PCR-mix-1-FRT *Ebola virus,* RT-PCR-mix-2-FEP/FRT, polymerase (TaqF), and TM-Revertase (MMlv) are to be stored at ≤ –16 °C

RT-PCR-mix-1-FRT *Ebola virus* is to be kept away from light.

Ebola Zaire Real-TM PCR kit should be transported at 2–8 °C for no longer than 5 days but should be stored at 2-8 and -16°C immediately on receipt.

STABILITY

Ebola Zaire Real-TM Test is stable up to the expiration date indicated on the kit label. The product will maintain performance through the control date printed on the label. The shelf life of reagents before and after the first use is the same, unless otherwise stated. Exposure to light, heat or humidity may affect the shelf life of some of the kit components and should be avoided. Repeated thawing and freezing of these reagents should be avoided, as this may reduce the sensitivity. Components stored under conditions other than those stated on the labels may not perform properly and may adversely affect the assay results.

SAMPLE COLLECTION, STORAGE AND TRANSPORT

Because of its high mortality rate, it's recommended to treat all suspected for EBOV whole blood samples (EDTA anticoagulant) and plasma (EDTA anticoagulant) with Trizol by adding 3 parts Trizol with 1 part whole blood or plasma (or other fluid) following the method below:

a. Add 0,75 ml of Trizol to microcentrifuge tube.
b. Add 0,25 ml of biological fluid (blood, plasma) and vortex for 5-7 seconds
c. Incubate at room temperature for 5 minutes.
d. Handle obtained samples following appropriate safety precautions.

Ebola Zaire Real-TM can analyze RNA extracted from:

- *Whole blood samples* (EDTA anticoagulant)
- *Plasma* (EDTA anticoagulant)
- *Saliva*
- *Tissue*

Specimens can be stored at +2-8°C for no longer than 12 hours, or frozen at -20°C to -80°C.
Transportation of clinical specimens must comply with country, federal, state and local regulations for the transport of etiologic agents.

RNA ISOLATION

Any commercial RNA/DNA isolation kit, if IVD-CE validated for the specimen types indicated herein at the "SAMPLE COLLECTION, STORAGE AND TRANSPORT" paragraph, could be used.
Sacace Biotechnologies recommends to use the following kits:

⇒ **Ribo-Virus** (Sacace, REF K-2/C) (150 *µl* of sample)
⇒ **SaMag Viral Nucleic Acids Extraction Kit** (Sacace, REF SM003) for plasma and cell free body fluids (400 *µl* of sample);
⇒ **Magno-Virus** (Sacace, REF K-2-16/1000) (1000 *µl* of sample).

Please carry out the RNA extraction according to the manufacturer's instructions.
During extraction, use the following controls:

- Internal Control STI-87-rec: *add 10 µl of IC RNA during the RNA isolation procedure directly to the sample/lysis mixture;*
- Negative Control (C–): *add 150* (400*) µl of Negative Control (C–) to the tube labeled Negative Control of Extraction;*
- Positive Control EBOV: *add 140 (390) µl of Negative Control (C-) and 10 µl of Positive Control EBOV to the tube labeled Positive Control of Extraction.*

REAGENTS PREPARATION (REACTION VOLUME 25 µL):

1. Prepare required quantity of reaction tubes.
2. Prepare for each sample in the new sterile tube **Reaction Mix**: add **10 µl** of **RT-PCR-mix-1**, **5 µl** of **RT-PCR-mix-2, 0,25 µl** of **RT-G-mix-2**, **0,50 µl** of **TaqF Polymerase** and **0,25 µl** of **M-MLV Revertase**. Vortex thoroughly and centrifuge for 5 sec. This mix must be used immediately. Don't store the prepared mix!

Reagents volume x 1 reaction (µl)		**10,0**	**5,00**	**0,25**	**0,50**	**0,25**
N RNA samples[1]	N reactions[2]	RT-PCR-mix-1	RT-PCR-mix-2	RT-G-mix-2	TaqF Polymerase	M-MLV Revertase
4	6	60	30	1,5	3,0	1,5
6	8	80	40	2,0	4,0	2,0
8	10	100	50	2,5	5,0	2,5
10	12	120	60	3,0	6,0	3,0
12	14	140	70	3,5	7,0	3,5
...58	60	600	300	15,0	30,0	15,0

[1] specimens plus 1 extraction control (N+1)
[2] specimens plus extraction and amplification controls (N+1+2)

3. Add **15 µl** of **Reaction Mix** into each tube.
4. Add **10 µl** of **extracted RNA** sample including Positive and Negative Controls of Extraction to appropriate tubes with Reaction Mix and mix well by pipetting.
5. Prepare for each panel 2 controls of amplification:
   - NCA: add **10 µl** of **RNA-buffer** to the tube labeled Negative Control of Amplification;
   - C+: add **10 µl** of **Positive Control cDNA EBOV** / **IC STI** to the tube labeled Positive Control of Amplification;

The results are interpreted through the presence of crossing of fluorescence curve with the threshold line.

Ebola cDNA is detected on the JOE(Yellow)/HEX channel, IC DNA on the FAM (Green) channel

AMPLIFICATION

Program the real-time instrument according to manufacturer's manual.

Amplification program for rotor-type instruments[1]

Step	*Temperature, °C*	*Time*	*Fluorescence detection*	*Repeats*
Hold	50	30 min	–	1
Hold 2	95	15 min	–	1
Cycling	95	10 s	–	5
	55	20 s	–	
	72	15 s	–	
Cycling2	95	10 s	–	40
	55	20 s	FAM/Green, JOE/Yellow	
	72	15 s		

Fluorescence is detected at the 2nd step of Cycling 2 stage (55°C) in FAM/Green and JOE/Yellow fluorescence channels.

[1] For example Rotor-Gene™ 3000/6000/Q (Corbett Research, Qiagen)

Amplification program for plate-and modular type instruments[2]

Step	*Temperature, °C*	*Time*	*Fluorescence detection*	*Repeats*
1	50 °C	30 min	–	1
2	95 °C	15 min	–	1
3	95 °C	10 s	–	5
	55 °C	20 s	–	
	72 °C	15 s	–	
4	95 °C	10 s	–	40
	55 °C	30 s	FAM, HEX/Cy3/Joe	
	72 °C	15 s		

Fluorescence is detected at stage 4 (55°C) in FAM and HEX fluorescence channels.

[2] For example, SaCycler-96™ (Sacace), CFX/iQ5™ (BioRad); Mx3005P™ (Agilent), ABI® 7300/7500/StepOne Real Time PCR (Applied Biosystems), SmartCycler® (Cepheid)

Rotor-type instruments

Settings

Channel	Calibrate/Gain Optimisation…	Threshold	More Settings/ Outlier Removal	Slope Correct	Dynamic tube
FAM/Green	*from 5 Fl to 10 Fl*	0.05	5 %	On	On
JOE/Yellow	*from 5 Fl to 10 Fl*	0.05	10-30 %	On	On

Plate-type instruments

Settings

Channel	Threshold
FAM	The threshold line should cross only sigmoid curves of signal accumulation of positive samples and should not cross the baseline; otherwise, the threshold level should be raised. Set the threshold at a level where fluorescence curves are linear and do not cross curves of the negative samples.
HEX/Joe/Cy3	

DATA ANALYSIS

Accumulation of ***Ebola virus*** **cDNA** amplification product is detected in the **JOE/Yellow/HEX** channel, Internal Control amplification product is detected in the **FAM/Green** channel.
The results are interpreted by the software of the PCR instrument used by the crossing (or not crossing) of the fluorescence curve with the threshold line.
The results of analysis are considered reliable only if the results obtained for Positive and Negative Controls of Amplification as well as for the Negative Control of Extraction are correct.

Results for controls

Control	Stage for control	Ct in channel		Interpretation
		FAM/Green	JOE/Yellow/HEX	
NCE	RNA extraction	< 35	Neg	OK
PCE	RNA extraction	< 35	< 36	OK
NCA	RT-PCR	Neg	Neg	OK
C+	RT-PCR	<35	< 36	OK

1. The sample is considered **positive** if its Ct value detected in the JOE/Yellow/HEX channel does not exceed the boundary Ct value (< 40 for clinical samples) and the Ct value detected in the FAM/Green channel does not exceed the value specified for the Internal Control (Ct < 35). The fluorescence curve should have a typical sigmoid shape and cross the threshold line once in the region of significant fluorescence increase.
2. The sample is considered **negative** if its Ct in the JOE/Yellow/HEX channel is not detected (the fluorescence curve does not cross the threshold line) and the Ct value detected in the FAM/Green channel does not exceed the boundary Ct value specified for the Internal Control (Ct < 35).

QUALITY CONTROL PROCEDURE

A defined quantity of Internal Control (IC) is introduced into each sample and control at the beginning of sample preparation procedure in order to control the extraction process of each individual sample and to identify possible reaction inhibition.
A negative control of extraction (NCE), negative control of amplification (NCA), positive control of extraction (PCE) and positive control of amplification (C+) are required for every run to verify that the specimen preparation, the amplification and the detection steps are performed correctly.
If the controls are out of their expected range (see table Results for Controls), all of the specimens and controls from that run must be processed beginning from the sample preparation step.

PERFORMANCE CHARACTERISTICS

Analytical specificity

The analytical specificity of **Ebola Zaire Real-TM** PCR kit is ensured by selection of specific primers and probes as well as stringent reaction conditions. The primers and probes were checked for possible homologies to all sequences published in gene banks by sequence comparison analysis.

Analytical sensitivity

The kit **Ebola Zaire Real-TM** allows to detect *EBOLA* RNA in 100% of the tests with a sensitivity of not less than $2X10^3$ GE/ml* using Magno-Virus extraction kit (ref. K-2-16-1000).

Target region: Nucleoprotein

* Genome equivalents (GE) of the pathogen agent per 1 ml of a sample

TROUBLESHOOTING

The results of analysis are not taken into account in the following cases:

1. If the Ct value of a clinical sample detected in the JOE/Yellow/HEX channel exceeds the boundary Ct value (>40), the result is considered **equivocal**. It is necessary to repeat the analysis twice. If a reproducible positive Ct value is detected, the sample is considered to be **positive**.
2. If any Ct value is detected for the Negative Control of Amplification (NCA) in both channels or the Ct value is detected for Negative Control of Extraction (C–) in the JOE/Yellow/HEX channel, this indicates the contamination of reagents or samples. In this case, the results of analysis of all samples are considered **invalid**. It is necessary to repeat the analysis of all tests and to take measures to detect and eliminate the source of contamination.
3. If the Ct value is absent for the Positive Control of Extraction (PCE), this indicates improper extraction procedure. RNA extraction should be repeated for all samples.
4. If the Ct value is absent for the Positive Control of RT-PCR (C+), this indicates errors in carrying out PCR or an incorrect amplification program. RT-PCR should be repeated for all samples.
5. If the Ct value of a clinical sample is absent or greater than the boundary Ct value (>40) for the JOE/Yellow/HEX channel and the Ct value in the FAM/Green channel is greater than the Ct values specified for the Internal Control (>35), the result is **invalid**. Analysis of such samples should be repeated starting from the RNA extraction stage.

KEY TO SYMBOLS USED

REF	List Number		Caution!
RUO	For Research Use Only		Contains sufficient for <n> tests
	Store at	VER	Version
	Manufacturer	**C–**	Negative control of Extraction
	Consult instructions for use	**NCA**	Negative Control of Amplification
	Expiration Date	**IC**	Internal Control
LOT	Lot Number		

* SaCycler™ is a registered trademark of Sacace Biotechnologies
* CFX™ and iQ5™ are registered trademarks of Bio-Rad Laboratories
* Rotor-Gene™ is a registered trademark of Qiagen
* MX3005P® is a registered trademark of Agilent Technologies
* ABI® is a registered trademark of Applied Biosystems
* SmartCycler® is a registered trademark of Cepheid

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