IVD For *in Vitro* Diagnostic Use

CE

# Congo Crimea Real-TM

# Handbook

## Real-Time PCR test for the qualitative detection of *Crimean-Congo hemorrhagic fever virus* (CCHFV)

**REF V22-50FRT**

**Σ 50**

## NAME

**Congo-Crimea Real-TM**

## INTRODUCTION

Crimean-Congo hemorrhagic fever virus (CCHF) is a widespread tick-borne viral disease, a zoonosis of domestic animals and wild animals, that may affect humans. The disease was first characterized in the Crimea in 1944 and given the name Crimean hemorrhagic fever. It was then later recognized in 1969 as the cause of illness in the Congo, thus resulting in the current name of the disease. Crimean-Congo hemorrhagic fever is found in Eastern Europe, particularly in the former Soviet Union. It is also distributed throughout the Mediterranean, in northwestern China, central Asia, southern Europe, Africa, the Middle East, and the Indian subcontinent.

Ixodid (hard) ticks, especially those of the genus, *Hyalomma*, are both a reservoir and a vector for the CCHF virus. Numerous wild and domestic animals, such as cattle, goats, sheep and hares, serve as amplifying hosts for the virus. Transmission to humans occurs through contact with infected animal blood or ticks. CCHF can be transmitted from one infected human to another by contact with infectious blood or body fluids. Documented spread of CCHF has also occurred in hospitals due to improper sterilization of medical equipment, reuse of injection needles, and contamination of medical supplies.

The onset of CCHF is sudden, with initial signs and symptoms including headache, high fever, back pain, joint pain, stomach pain, and vomiting. Red eyes, a flushed face, a red throat, and petechiae (red spots) on the palate are common. Symptoms may also include jaundice, and in severe cases, changes in mood and sensory perception. As the illness progresses, large areas of severe bruising, severe nosebleeds, and uncontrolled bleeding at injection sites can be seen, beginning on about the fourth day of illness and lasting for about two weeks.

Laboratory tests that are used to diagnose CCHF include antigen-capture enzyme-linked immunosorbent assay (ELISA), real time polymerase chain reaction (RT-PCR), virus isolation attempts, and detection of antibody by ELISA (IgG and IgM).

## INTENDED USE

**Congo-Crimea Real-TM** is a Real-Time PCR test for the qualitative detection of *Crimean-Congo hemorrhagic fever virus* (CCHFV) in clinical materials (plasma, serum) and ticks by using real-time hybridization-fluorescence detection.

## PRINCIPLE OF ASSAY

**Congo-Crimea Real-TM** Test is based on three major processes: isolation of *virus* RNA from specimens together with the internal control sample (IC), one-step reverse transcription of the RNA and Real Time amplification of the cDNA. *CCHFV* detection by the polymerase chain reaction (PCR) is based on the amplification of pathogen genome specific region using specific primers and detection via fluorescent dyes. These dyes are linked with probes of oligonucleotides which bind specifically to the amplified product. The real-time PCR monitoring of fluorescence intensities allows the accumulating product detection without reopening of reaction tubes after the PCR run. **Congo-Crimea Real-TM** PCR kit is a qualitative test which contain the Internal Control (IC). It must be used in the isolation procedure in order to control the process of each individual sample extraction and serves also to identify possible reaction inhibition.

## MATERIALS PROVIDED

### Module No.1: Real Time PCR kit (V22-50FRT)

Part N°1 – **"Controls"**

- **Negative Control (C-)***, 1,6 ml
- **Pos *CCHFV* RNA-rec****, 5 x 0,03 ml
- **CCHFV IC RNA*****, 5 x 0,12 ml
- **RNA-buffer**, 2 x 0,6 ml
- **cDNA CCHFV/IC (C+)**, 0,1 ml

Part N°2–**"Congo-Crimea Real-TM"**

- **RT-G-mix-2**, 0,15 ml
- **RT-PCR-mix-1 CCHFV**, 0,6 ml
- **RT-PCR-mix-2**, 0,3 ml
- **TaqF Polymerase**, 0,03 ml
- **M-MLV Revertase**, 0,015 ml
- **tRNA 1 µg/µl******, 5 x 0,06 ml

Contains reagents for 55 tests

* must be used in the isolation procedure as Negative Control of Extraction.
** must be used in the isolation procedure as Positive Control of Extraction.
*** add 10 µl of Internal Control RNA during the RNA purification procedure directly to the sample/lysis mixture
**** must me used during RNA extraction from ticks using Ribo-Zol isolation kit (REF K-2-3)

## MATERIALS REQUIRED BUT NOT PROVIDED

- RNA purification kit
- Real Time Thermalcycler
- Workstation
- Pipettes (adjustable)
- Sterile RNase/DNase-free pipette tips with aerosol barriers
- Tube racks
- Vortex mixer
- Desktop centrifuge
- PCR box
- Disposable 0.2-ml polypropylene microtubes for PCR or PCR plate
- Refrigerator for 2–8 °C.
- Deep-freezer for ≤ –16 °C.

## STORAGE INSTRUCTIONS

Part N° 1 – "**Controls**" must be stored at +2-8°C.

Part N° 2 – "**Congo-Crimea Real-TM**" must be stored at -20°C.

The kit can be shipped at 2-8°C for 3-4 days but should be stored at -20°C immediately on receipt.

## STABILITY

**Congo-Crimea Real-TM** 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. 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.

## 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.

## WARNINGS AND PRECAUTIONS

IVD

***In Vitro* Diagnostic Medical Device**

For *In Vitro* Diagnostic Use Only

The user should always pay attention to the following:

- Use sterile pipette tips with aerosol barriers and use new tip for every procedure.
- Store extracted positive material (samples, controls and amplicons) away from all other reagents and add it to the reaction mix in a separate area.
- Thaw all components thoroughly at room temperature before starting an assay.
- When thawed, mix the components and centrifuge briefly.
- Use disposable gloves, laboratory coats and eye protection when handling specimens and reagents. Thoroughly wash hands afterwards.
- Do not eat, drink, smoke, apply cosmetics, or handle contact lenses in laboratory work areas.
- Do not use a kit after its expiration date.
- Dispose of all specimens and unused reagents in accordance with local authorities' regulations.
- Specimens should be considered potentially infectious and handled in a biological cabinet in accordance with appropriate biosafety practices.
- Clean and disinfect all sample or reagent spills using a disinfectant such as 0.5% sodium hypochlorite, or other suitable disinfectant.
- Avoid sample or reagent contact with the skin, eyes, and mucous membranes. If skin, eyes, or mucous membranes come into contact, rinse immediately with water and seek medical advice immediately.
- Material Safety Data Sheets (MSDS) are available on request.
- Use of this product should be limited to personnel trained in the techniques of DNA amplification.
- The laboratory process must be one-directional, it should begin in the Extraction Area and then move to the Amplification and Detection Areas. Do not return samples, equipment and reagents to the area in which the previous step was performed.

Some components of this kit contain sodium azide as a preservative. Do not use metal tubing for reagent transfer.

## 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 techniques of DNA amplification (EN375). 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.

## SAMPLE COLLECTION, STORAGE AND TRANSPORT

**Congo-Crimea Real-TM** can analyze RNA extracted from:

- ***Plasma***

Whole blood collected in EDTA should be separated into plasma and cellular components by centrifugation at 800-1600 x g for 20 min within six hours. The isolated plasma has to be transferred into a sterile polypropylene tube. Plasma may be stored at 2-8°C for an additional 3 days. Alternatively, plasma may be stored at -18°C for up to one month or 1 year when stored at -70°C.

- ***Ticks***

Before ticks pretreatment, pools of ticks should be formed. Each pool can contain 5-7 non-sated ticks or 2-3 ticks of semi-sated ones. Fully sated ticks should be analyzed individually. Use sterile porcelain mortars and sterile pestles for ticks suspension preparation. If automatic homogenizer TissueLyser LT is used the following parameters are set: beads diameter – 7 mm; frequency – 50 Hz/sec; time of homogenization – 12-15 min; buffer volume – 700 µl (non-sated tick), 1000-1500 µl (sated tick and pools). In case of sated ticks, they should be punctured with sterile disposable needle prior to homogenization. Ticks can be washed in 70% ethanol if needed. Use sterile porcelain mortars and sterile pestles for ticks suspension preparation. Grind the ticks in 100 µl (if sample consist of 1 non-sated tick), in 1-1.5 ml (for pool or sated tick) of 0.15 M sodium chloride solution or PBS buffer. Mix solution with ticks by two portions. Centrifuge obtained suspension 1 min at 10000 g. It is acceptable to store material before analysis for 1 day at the temperature 2-8 C or 1 week at the temperature not more than minus 16 °C. Subsequent storage should be at the temperature not more than minus 68 °C.

Transportation of clinical specimens must comply with country, federal, state and local regulations for the transport of etiologic agents.

## RNA ISOLATION

The following isolation kits are recommended:

⇒ **DNA/RNA Prep** (Sacace, REF K-2-9) – for blood plasma or tick suspension (non-sated or semi-sated ones);

⇒ **Ribo Virus 50** – spin column extraction kit (Sacace, REF K-2-C) – for blood plasma ;

⇒ **RIBO-Zol** (REF K-2-3) – for sated ticks

- RNA is extracted from each clinical sample in the presence of **Internal Control** (**10 µl** of **IC** is added to each sample).
- Transfer **100 µl** of **Negative Control** to the tube labeled C-.
- Transfer **90 µl** of **Negative Control** and **10 µl** of **Pos *CCHFV* RNA-rec** to the tube labeled PCE.

Extract RNA according to the manual provided by the manufacturer.

## RT AND AMPLIFICATION

Total reaction volume is **25 µl**, the volume of RNA sample is **10 µl**.

1 Prepare the reaction mix for required number of samples.

2 For N reactions mix in a new tube:

**10*(N+1) µl of RT-PCR-mix-1,**

**5.0*(N+1) µl of RT-PCR-mix-2**

**0.5*(N+1) µl of TaqF Polymerase**

**0.25*(N+1) µl of RT-G-mix-2**

**0.25*(N+1) µl of MMlv**

3 Vortex the tube, then centrifuge shortly. Add **15 µl** of prepared reaction mix into each tube.

4 Using tips with aerosol filter add **10 µl** of RNA samples obtained at the stage of RNA isolation and mix carefully by pipetting**.**

5 Prepare for each panel 2 controls:

- add **10 µl** of **RNA-buffer** to the tube labeled Amplification Negative Control;
- add **10 µl** of **cDNA CCHFV/IC (C+)** to the tube labeled $C_{pos/IC}$;

### Amplification

Create a temperature profile on your Real-time instrument as follows:

| | Rotor type instruments[1] | | | | Plate type or modular instruments[2] | | | |
|---|---|---|---|---|---|---|---|---|
| **Stage** | **Temp, °C** | **Time** | **Fluorescence detection** | **Cycle repeats** | **Temp,°C** | **Time** | **Fluorescence detection** | **Cycle repeats** |
| Hold | 50 | 30 min | – | 1 | 50 | 30 min | – | 1 |
| Hold | 95 | 15 min | – | 1 | 95 | 15 min | – | 1 |
| Cycling | 95 | 10 s | – | 5 | 95 | 10 s | – | 5 |
| | 54 | 25 s | – | | 54 | 30 s | – | |
| | 72 | 15 s | – | | 72 | 15 s | – | |
| Cycling 2 | 95 | 10 s | – | 45 | 95 | 10 s | – | 45 |
| | 50 | 25 s | FAM(Green), JOE(Yellow) | | 50 | 35 s | FAM, JOE/HEX/Cy3 | |
| | 72 | 15 s | – | | 72 | 15 s | – | |

[1] *For example Rotor-Gene™ 3000/6000/Q (Corbett Research, Qiagen)*
[2] *For example, SaCycler-96™ (Sacace), iQ5™ (BioRad); Mx3005P™ (Agilent), ABI® 7300/7500/StepOne Real Time PCR (Applied), SmartCycler® (Cepheid), LineGeneK® (Bioer)*

## INSTRUMENT SETTINGS

### Rotor-type instruments

| Channel | *Threshold* | *More Settings/Outlier Removal* | *Slope Correct* | *Calibrate/Gain Optimisation…* | *Eliminate Cycles before* |
|---|---|---|---|---|---|
| **FAM/Green** | 0.03 | 10 % | on | from 5Fl to 10Fl | 5 |
| **JOE/Yellow** | 0.05 | 10 % | on | from 5Fl to 10Fl | 5 |

### Plate-type instruments

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.

## RESULTS ANALYSIS

1. The results are interpreted by the device software through the presence of crossing of fluorescence curve with the threshold line. Put the threshold line at such level where curves of fluorescence are linear.

   *cDNA of CCHFV* is detected on the JOE (Yellow)/HEX/Cy3 channel and *IC* on the FAM (Green) channel.

Results are accepted as relevant if both positive and negative controls of amplification along with negative control of extraction are passed (see table 1).

**Table 1. Results for controls**

| Control | Stage for control | Ct channel Fam (Green) | Ct channel Joe (Yellow)/ HEX/Cy3 | Interpretation |
|---|---|---|---|---|
| **NCE** | RNA isolation | Pos (< 31) | Neg | Valid result |
| **Pos *CCHFV*-RNA-rec** | RNA isolation | Pos (< 31) | Pos (< 33) | Valid result |
| **NCA** | Amplification | Neg | Neg | Valid result |
| **cDNA CCHFV/IC (C+)** | Amplification | Pos (< 31) | Pos (< 31) | Valid result |

**Interpretation of results for clinical samples**

- *CCHFV* cDNA is **detected** in a sample if its Ct value is defined in the result grid in the JOE channel (it should be less than Ct value specified in Table 2). Moreover, the fluorescence curve should cross the threshold line in the area of exponential fluorescence growth.
- *CCHFV* cDNA is **not detected** in a sample if its Ct value defined in the result grid in the FAM channel is less than the specified boundary Ct value (see Table 2), whereas the Ct value in the JOE channel is not defined.
- The result is **invalid** if the Ct value of a sample in the JOE channel is absent whereas the Ct value in the FAM channel is either absent or greater than the specified boundary Ct value. It is necessary to repeat the PCR test for such a sample.

**Table 2. Results for test samples**

| Detection channel | Matrix | Boundary Ct values |
|---|---|---|
| FAM | IC detection | **< 31** (for blood serum and blood plasma, cerebro-spinal fluid) |
| | | **< 33** (for homogenates of mosquitoes, ticks, internal organs, urine) |
| JOE | *CCHFV* detection | **< 39** |

## PERFORMANCE CHARACTERISTICS

### Analytical sensitivity

The kit **Congo-Crimea Real-TM** allows to detect *CCHFV* in 100% of the tests with a sensitivity of not less than $10^3$ copies/ml.

### Analytical specificity

The analytical specificity of the primers and probes was validated with negative samples. They did not generate any signal with the specific *CCHFV* primers and probes. The specificity of the kit **Congo-Crimea Real-TM** was 100%. The potential cross-reactivity of the kit **Congo-Crimea Real-TM** was tested against the group control

- Flaviviruses (West Nile fever virus);
- Herpesviruses (herpes simplex virus types 1 and 2, human herpes virus type 6, cytomegalovirus; Epstein-Barr virus, Varicella-Zoster virus), enteroviruses (*ECHO*, *Coxsackie*);
- *rickettsiae* of spotted fever group (*Rickettsia conorii* ssp*. caspia*, *Coxiella burnetii);*
- Orthobunyaviruses
- Hantaviruses (Puumala virus, Dobrava-Belgrade virus)
- Thogotoviruses (Batken virus)

It was not observed any cross-reactivity with other pathogens.

## TROUBLESHOOTING

1. Weak or absent signal of the IC (Fam (Green) channel): retesting of the sample is required.
    - The PCR was inhibited.
        ⇒ Make sure that you use a recommended RNA extraction method and follow the manufacturer's instructions.
    - The reagents storage conditions didn't comply with the instructions.
        ⇒ Check the storage conditions
    - The PCR conditions didn't comply with the instructions.
        ⇒ Check the PCR conditions and for the IC detection select the fluorescence channel reported in the protocol.
    - The IC was not added to the sample during the pipetting of reagents.
        ⇒ Make attention during the RNA extraction procedure.
2. Weak (Ct > 39) signal on the Joe (Yellow)/Cy3/HEX channel: retesting of the sample is required.
3. Joe (Yellow)/Cy3/HEX signal with Negative Control of extraction.
    - Contamination during RNA extraction procedure. All samples results are invalid.
        ⇒ Decontaminate all surfaces and instruments with sodium hypochlorite and ethanol.
        ⇒ Use only filter tips during the extraction procedure. Change tips among tubes.
        ⇒ Repeat the RNA extraction with the new set of reagents.
4. Any signal with Negative PCR Control.
    - Contamination during PCR preparation procedure. All samples results are invalid.
        ⇒ Decontaminate all surfaces and instruments with sodium hypochlorite and ethanol or special DNA decontamination reagents.
        ⇒ Pipette the Positive controls at the end.
        ⇒ Repeat the PCR preparation with the new set of reagents.

## KEY TO SYMBOLS USED

| | | | |
|---|---|---|---|
| REF | List Number |  | Caution! |
| LOT | Lot Number |  | Contains sufficient for <n> tests |
| IVD | For *in Vitro* Diagnostic Use | VER | Version |
|  | Store at | **NCA** | Negative Control of Amplification |
|  | Manufacturer | **NCE** | Negative control of Extraction |
|  | Consult instructions for use | **C+** | Positive Control of Amplification |
|  | Expiration Date | **IC** | Internal Control |




***Sacace Biotechnologies Srl***
*via Scalabrini, 44 – 22100 – Como – Italy Tel +390314892927 Fax +390314892926*
*mail:* ***info@sacace.com*** *web:* ***www.sacace.com***