

BLACKBOX® CCHFV IgG ELISA Kit (Crimean-Congo Hemorrhagic Fever Virus, IgG)

For Research Use Only (RUO)

Lot No. (See product label)

Size: 96-wells

Principle: IgG ICB (Immune Complex Binding) ELISA

Type: Qualitative

Storage: Store at 2-8°C for 6 months. **Note: conjugate has to be stored at -20°C**

Intended Use:

The BLACKBOX® CCHFV IgG ELISA Kit is intended for qualitative detection of IgG antibodies to CCHFV in human serum.

In conjunction with the BLACKBOX® CCHFV IgM ELISA Kit, the assay provides serological evidence of an acute or past infection with CCHFV. Test results have to be critically assessed with reference to clinical symptoms, available anamnestic information and the results of other diagnostic tests performed.

The kit is not intended for self-testing. Assay performance characteristics have not been established for automated instruments.

General Description: Crimean-Congo Hemorrhagic Fever

Crimean-Congo Hemorrhagic Fever (CCHF) is an infectious disease endemic in a variety of countries in Southeastern Europe, the Middle East and Asia as well as in Africa [1]. Causing agent for the zoonotic disease is a virus of the *Bunyaviridae* family, the Crimean-Congo Hemorrhagic Fever Virus (CCHFV). Virus transmission occurs via the bite of an infected tick but also by close contact to body fluids of infected persons or livestock. After an incubation time of up to 13 days (depending on the route of transmission), unspecific flu-like symptoms like fever, myalgia, headache and gastrointestinal symptoms are observed. After this initial phase, a severe hemorrhagic and often lethal course of the disease occurs in some patients, leading to a mortality rate of CCHF-patients of about 30 % [1]. Due to the severity of the disease, the absence of specific therapeutic options and the high risk of human to human transmission, the CCHFV is classified as a virus of the highest biological risk class (Biosafety Level 4).

Virus-specific IgM and IgG antibodies are usually detectable by the end of the first week of illness (day 5 – 7) and persist for several months (IgM) or years (IgG) [1].

Test Principle

The BLACKBOX® CCHFV IgG ELISA Kit is based on the patented IgG Immune Complex Binding (ICB) ELISA technology [2, 3]. Diluted human control sera and patient serum samples are co-incubated together with an HRP (horseradish peroxidase) - labeled recombinant CCHFV antigen in a microwell plate coated with a recombinant IgG immune complex specific capture molecule. During the incubation time, immune complexes are formed which bind specifically and with high affinity to the capture molecule. All antibodies not binding to the antigen and excess labeled antigen are removed in the subsequent washing step. The bound IgG/antigen immune complexes are visualized by application of the colorimetric HRP-substrate TMB. After stopping the enzymatic reaction, the assay result is generated by measuring the optical density of the solution in the well at 450/620 nm.

Reagents and materials provided in the kit

Component	Supplied amount/ packaging	Color coding	Storage
Microwell plate (IgG)	12 strips in sealed aluminium pouch with desiccant bag	n.a.	2°C - 8°C
Positive control	25 µl in 0.5 ml vial	red cap	2°C - 8°C
Negative control	50 µl in 0.5 ml vial	white cap	2°C - 8°C
Sample dilution buffer (SDB)	100 ml in 125 ml bottle	clear cap	2°C - 8°C
Conjugate dilution buffer (CDB)	14 ml in 15 ml bottle	blue cap	2°C - 8°C
Wash buffer 10 x	100 ml in 125 ml bottle	clear cap	2°C - 8°C
Conjugate (HRP-labelled recombinant CCHFV antigen)	70 µl in 0.5 ml vial	blue cap	-20°C
Substrate - TMB	14 ml in 15 ml amber bottle	amber cap	2°C - 8°C
Stop solution	14 ml in 15 ml bottle	clear cap	2°C - 8°C
Adhesive foil	2 pieces	n.a.	n.a.
Instruction for use	n.a.	n.a.	n.a.

Table 1. Reagents and materials provided in the kit.

The kit allows the performance of 96 reactions, including negative and positive controls. For analysis of small numbers of patient samples, provided reagents are sufficient for 12 independent tests (1 strip per test, 5 patient samples per strip).

For information on reagents' shelf life and handling/security instructions see page 7 of this manual.

Materials/instruments required but not supplied in the kit

For preparation of 1 x wash buffer:

Deionized water
Graduated cylinder
Pipetboy
Glass or plastic pipettes for volumes up to 25 ml

For preparation of other reagents/serum samples and assay performance:

Pipettes for volumes up to 10 µl, 100/200 µl and 1000 µl
Pipette tips for volumes up to 10 µl, 100/200 µl and 1000 µl
Reagent reservoirs
Microfuge tubes
Paper towels/absorbent paper
Timer
Wet chamber (sealable plastic box + paper towels)
ELISA plate reader (450 nm, 620 nm)

Optional: eight-channel pipette, automated ELISA plate washer

Specimen collection, preparation, storage and handling

The BLACKBOX® CCHFV IgG ELISA Kit has been validated using human sera. Assay performance was not tested using whole blood, plasma or other specimens. Use of hyperlipemic, hemolyzed, icteric or contaminated sera may cause erroneous results.

Due to the assay principle based on co-incubation of diluted serum sample and diluted HRP-labelled antigen, sera stabilized with NaN_3 cannot be analyzed using this test.

For serum preparation, blood samples have to be collected by approved venipuncture procedures by qualified personnel using appropriate collection tubes allowing blood clotting. For clotting, incubate blood sample for 30 min at RT (alternatively: over night at 4°C). After centrifugation (1400xg, 10 min, 4°C), aseptically transfer the supernatant (= serum) to a fresh sterile tube.

Serum samples can be kept at RT for short periods of time (< 8 hours). For storage, serum samples should be refrigerated (4°C, < 6 months) or frozen (-20°C or -80°C, long term storage). Repeated freeze/thaw cycles should be avoided. It is recommended to ship serum samples on dry ice. After thawing, serum samples have to be mixed gently but thoroughly.

The CCHFV being a virus of the highest biological safety level (BSL4), inactivation of sera prior to testing may be desirable. The BLACKBOX® CCHFV IgG ELISA Kit has been validated using CCHFV-positive sera inactivated with Triton X-100.

Test Procedure

General remarks

- Perform all pipetting steps at room temperature (20°C – 25°C) using calibrated, well maintained pipettes and strictly follow the ELISA procedure protocol described below. Deviations in assay parameters like volumes, incubation times and incubation temperatures may cause invalid results.
- Mix all reagents gently but thoroughly before use.
- Do not substitute or mix reagents from different kit lots or from other manufacturers.
- Upon arrival, **store the conjugate (E30.04) at -20°C**. For conjugate dilution (see below) remove the necessary amount of conjugate from the vial and immediately place the residual conjugate back in the freezer. Due to the addition of glycerol to the conjugate storage buffer, it is not necessary to thaw the conjugate solution before pipetting.
- **CDB and SDB** should be chilled, because the formation of immune complexes takes place at 4°C.
- To prevent condensation, the **microwell plate** (sealed in aluminium pouch with desiccant bag) has to be **equilibrated to room temperature (20°C – 25°C) at least 30 minutes** before opening the package to remove the required number of microwell strips. Unused microwell strips can be stored in presence of the desiccant bag at 4°C in the re-sealed aluminium pouch.
- **Wash Buffer, TMB Substrate and Stop Solution** should be equilibrated to room temperature (20°C – 25°C) before use.
- Plate washing can be performed manually using a multi-channel pipette, however it is preferable that an automated plate washer is used. In both cases, complete removal of wash solution after the washing steps is mandatory. Remaining buffer has to be removed by tapping the microplate face-down on an absorbent paper towel.
Important: When using an automated plate washer, account for the additional volume needed for system priming when calculating the required volume of 1 x Wash Buffer.
- Avoid cross-contamination of wells during all pipetting and washing steps.
- Avoid the formation of air bubbles during all pipetting steps. Especially air bubbles present during the OD measurement may cause false readings.

Preparation of reagents and specimen

- **1 x Wash Buffer.** Wash Buffer is provided in the kit as a 10 x stock solution. In case of salt precipitate having formed in the stock solution, the solution has to be warmed up to approximately 30°C – 40°C to completely dissolve the precipitate. To obtain 1 x Wash Buffer, dilute the required amount of 10 x Wash Buffer Stock Solution in deionized water (volumes depending on number of microwell strips used, see Table 2 below). **The 1 x Wash Buffer is stable at RT for one week.**
- **Conjugate dilution.** Prepare a **conjugate pre-dilution by adding 5 µl of conjugate stock to 500 µl CDB**. This pre-dilution has to be **further diluted** to obtain the conjugate working solution (volumes depending on number of microwell strips used, see table below).
The **conjugate is provided in a viscous storage buffer containing 50% glycerol**. Thus, **pipette the stock solution carefully** under visual control and make sure that no additional solution is attached to the outside of the pipette tip. Make sure that the 5 µl conjugate stock is transferred quantitatively to the CDB by pipetting up and down several

times and mix the 1:100 pre-dilution carefully but thoroughly before preparing the conjugate working dilution. Always prepare a fresh conjugate pre-dilution and conjugate working dilution before performing the test and discard residual pre-dilution and working dilution afterwards.

- **Control samples dilution.** Dilute the positive and the negative control sera provided with the kit in SDB

Positive control: 50 µl SDB + 1 µl control sample

Negative control: 75 µl SDB + 1.5 µl control sample.

- **Serum samples dilution.** Dilute the serum samples in SDB (500 µl SDB + 10 µl serum).
- **TMB substrate, stop solution.** Both solutions are provided in the kit ready to use (required volume depending on the number of microwell strips used, see Table 2 below).

# strips	Wash Buffer		Conjugate working dilution		TMB (ml)	Stop solution (ml)
	Wash Buffer 10x (ml)	Deionized water (ml)	Conjugate pre-dilution (µl)	CDB (µl)		
1	2	18	4	496	1	1
2	4	36	4	496	2	2
3	6	54	6	744	3	3
4	8	72	8	992	4	4
5	10	90	10	1240	5	5
6	12	108	13	1488	6	6
7	14	126	15	1735	7	7
8	16	144	17	1983	8	8
9	18	162	19	2231	9	9
10	20	180	21	2479	10	10
11	22	198	23	2727	11	11
12	24	216	25	2975	12	12

Table 2: Preparation of reagents for different numbers of microwell strips used for testing.

ELISA procedure

1. **Plate and reagents preparation.** Prepare required number of microwell strips, 1 x wash buffer, antigen dilution, control samples dilutions and serum samples dilutions as stated above in section "Preparation of reagents and specimen".
2. **Pipetting conjugate, control samples and serum samples.** Pipette 25 µl of conjugate working solution in each well, then add 25 µl of diluted control samples (1 well positive control, 2 wells negative control) and diluted serum samples into the respective wells and mix carefully by pipetting. Make sure that the bottom of the wells are completely covered by gently tapping the plate on the desktop.
3. **Incubation.** Seal plate using the adhesive foil provided with the kit and incubate over night (24h) at 4°C in a moist environment (wet chamber).
4. **Plate washing.** Wash strips 3 times with 300 µl 1 x Wash Buffer per well. Soak strips for 30 sec in between washes. Remove residual Wash Buffer by tapping the plate upside-down on paper towels and then, without letting the wells dry out, immediately proceed with step 5.

5. **Substrate incubation.** Add 100 µl TMB substrate to each well; incubate 20 min at room temperature (20°C – 25°C) in the dark. Wells generating a positive result will turn blue.
6. **Stopping.** Add 100 µl of stop solution to each well. Blue color of wells generating a positive result will turn yellow.
7. **Measurement.** Measure optical density at 450 nm and 620 nm using a microplate reader within 30 min after stopping the assay. Calculate the difference OD450 – OD620.

Evaluation of results

Test results can be accounted as valid if the following criteria are met:

OD450-OD620 of positive control > 1.5

OD450-OD620 of negative control < 0.1

Use the average absolute OD450-OD620 absorbance value for the negative control sample ($OD_{neg, av}$) to calculate the assay cut-off OD_{CO} according to the following formula:

$$OD_{neg, av} + 0.150 = OD_{CO}$$

The Index Values (IV) for the tested serum samples can then be calculated according to:

$$IV_{Sample} = OD450-OD620(sample)/OD_{CO}$$

Evaluate the obtained results according to Table 3.

Index Value	Result CCHFV IgG	Interpretation
$IV_{Sample} > 1.1$	positive	Indicative of past or current infection with CCHFV, assess positive result by additional testing and/or clinical findings.
$0.9 < IV_{Sample} < 1.1$	equivocal	Ambiguous result, repeat test for this serum and (if available) a follow-up sample of the patient taken 3-5 days later and/or analyze sample by additional testing.
$IV_{Sample} < 0.9$	negative	No anti-CCHFV IgG antibodies were detected. This finding does not exclude an acute infection with CCHFV, because IgG antibodies usually are not detectable before day 5-7 after onset of illness. If available, a follow-up sample of the patient taken several days to weeks later should be tested. Furthermore, in severe cases of CCHF, antibody response may completely fail to develop. Assess diagnosis by additional testing and/or clinical findings.

Table 3: Result classification for index values and interpretation of results.

Kit shelf life and kit component storage

- Under correct storage conditions (see below), stability of the kit is guaranteed until the expiration date label on the box. Do not use the kit after the expiration date.
- Upon receipt of the kit, **immediately freeze the conjugate (recombinant HRP-labelled CCHFV antigen) at -20°C**. For conjugate dilution remove the necessary amount of conjugate from the vial and immediately place the residual conjugate back in the freezer. Due to the addition of glycerol to the conjugate storage buffer, it is not necessary to thaw the conjugate solution before pipetting.
- All other kit components have to be stored refrigerated (+2°C to +8°C).

General safety information

- The kit is intended for research use only (RUO).
- Tests have to be performed by qualified laboratory personnel.
- Wear appropriate protective clothing (lab coat, gloves, safety glasses) when handling kit components and patient sera.
- As blood products, positive and negative control samples as well as patient samples should be treated as potentially infectious. It is recommended to handle and dispose samples and all material having been in contact with the samples (i.e. pipette tips, tubes, microwell strips) under appropriate safety conditions. The CCHFV being a virus of the highest biological safety level (BSL4), inactivation of sera prior to testing may be desirable. The BLACKBOX® CCHFV IgG ELISA Kit has been validated using CCHFV-positive sera inactivated with Triton X-100. **Do not use heat inactivation.**
- The Stop solution contains sulfuric acid which is corrosive. Contact with skin or eyes must be avoided. In case of exposure, flush with large amounts of water.

Reference List

- [1] Bente DA, Forrester NL, Watts DM, McAuley AJ, Whitehouse CA, *et al.* (2013) Crimean-Congo hemorrhagic fever: history, epidemiology, pathogenesis, clinical syndrome and genetic diversity. *Antiviral Res* 100: 159-189.
- [2] Schmitz H, Gabriel M, Emmerich P (2011) Specific detection of antibodies to different flaviviruses using a new immune complex ELISA. *Med Microbiol Immunol.* 200(4):233-9
- [3] Emmerich P, Mika A, von Possel R, Rackow A, Liu Y, *et al.* (2018) Sensitive and specific detection of Crimean-Congo Hemorrhagic Fever Virus (CCHFV)-Specific IgM and IgG antibodies in human sera using recombinant CCHFV nucleoprotein as antigen in μ -capture and IgG immune complex (IC) ELISA tests. *PLoS Negl Trop Dis* 12: e0006366.

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