INTENDED USE

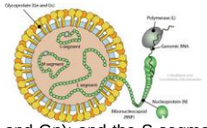
The Human Anti-Crimean-Congo Hemorrhagic Fever Virus (**CCHFV**) IgM ELISA Kit detects and quantifies CCHFV-NP specific IgM in human serum or plasma of vaccinated, immunized and/or infected hosts. This immunoassay is suitable for:

- Determining **immune status** relative to non-immune controls;
- Assessing efficacy of **vaccines**, including dosage, adjuvantcy, route of immunization and timing;
- Qualifying and standardizing vaccine batches & protocols.

This kit is for research use only (RUO), not for diagnostic use.

GENERAL INFORMATION

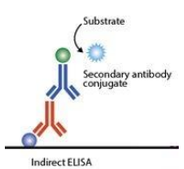
Crimean-Congo hemorrhagic fever (**CCHF**) is a widespread tick-borne viral disease, a zoonosis of domestic animals and wild animals, that may affect humans. The pathogenic virus, especially common in East and West Africa, is a member of the Bunyaviridae family of RNA viruses. Clinical disease is rare in infected mammals, but is commonly severe in infected humans, with a 30% mortality rate. Outbreaks of illness are usually attributable to handling infected animals or humans. CCHF is distributed throughout Eastern Europe, the Mediterranean, northwestern China, central Asia Africa, the Middle East, and the Indian subcontinent.



The virus genome is circular, ambisense RNA in three parts – Small (S), Middle (M) and Large (L). The L segment encodes the RNA polymerase; the M segment encodes the envelope proteins (Gc and Gn); and the S segment encodes the nucleocapsid protein. The envelope protein is initially translated as a glycoprotein precursor which is then cleaved into two smaller proteins. Based on the sequence data seven genotypes have been recognized: Africa 1 (Senegal), Africa 2 (Democratic Republic of the Congo and South Africa), Africa 3 (southern and western Africa), Europe 1 (Albania, Bulgaria, Kosovo, Russia and Turkey), Europe 2 (Greece) Asia 1 (the Middle East, Iran and Pakistan) and Asia 2 (China, Kazakhstan, Tajikistan and Uzbekistan).

Vaccines: A Turkish research team led by Refik Saydam Health Institute has developed treatment-serum derived from blood of several CCHF-patients, which have been proven to be %90 effective in CCHF patients. The vaccine is pending for FDA approval. ADI has cloned, expressed and purified CCHFV nucleoprotein (482-aa, ~55 kDa) that is being used as a candidate for newer subunit vaccine for CCHF.

PRINCIPLE OF THE TEST



The Human Anti-Crimean-Congo/CCHFV NP IgM ELISA kit is based on the binding of human anti-CCHFV IgG in samples to CCHFV NP antigen immobilized on the microwells, and anti- CCHFV NP IgM antibody is detected by anti-human IgM-HRP conjugate. After a washing step, chromogenic substrate (TMB) is added and color (blue) is developed, which is directly proportional to the amount of antibody present in the sample. Stopping Solution is added to terminate the reaction (converts blue to yellow color), and A450nm is then measured using an ELISA reader. The presence of antibody in samples is determined relative to anti-CCHFV IgM Controls.

KIT CONTENTS

The microtiter well plate and all other reagents, if unopened, are stable at 2-8°C until the expiration date printed on the box label. Stabilities of the working solutions are indicated under Reagent Preparation.

To Be Reconstituted: Store as indicated.

Component	Preparation Instructions
Wash Solution Concentrate (100x) Cat. No. WB-100, 10ml	Dilute the entire volume 10ml + 990ml with distilled or deionized water into a clean stock bottle. Label as **Working Wash Solution** and store at ambient temperature until kit is used entirely.
Sample Diluent Concentrate (20x) Cat. No. SD-20T, 10ml	Dilute 0.5ml + 9.5ml with distilled or deionized water as needed for HRP Conjugate and Sample Dilution. Label as **Working Sample/Conjugate Diluent** and store at 2-8°C until the kit lot expires or is used up.
Anti-Human IgG - HRP Conjugate Concentrate (100x) Part No. H-HuG.211, 0.15ml	Peroxidase conjugated anti-Human IgG in buffer with protein, detergents and antimicrobial as stabilizers. Dilute fresh as needed; 10ul of concentrate to 1ml of **Working Sample/Conjugate Diluent** is sufficient for 1 8-well strip. Use within the working day and discard. Return 100X to 2-8°C storage.

Ready For Use: Store as indicated on labels.

Component	Part	Amt	Contents
CCHFV Microwell Strip Plate	320421	8-well strips (12)	Coated with recombinant CCHFV antigen, and post-coated with stabilizers.
Anti-CCHFV Calibrators			
1 U/ml 2.5 U/ml 5 U/ml 10 U/ml	320442B 320442C 320442D 320442E	0.65 ml 0.65 ml 0.65 ml 0.65 ml	Four (4) vials, each containing anti-CCHFV levels in arbitrary activity Units; diluted in buffer with protein, detergents and antimicrobial as stabilizers.
TMB Substrate	80091	12 ml	Chromogenic substrate for HRP containing TMB and peroxide.
Stop Solution	80101	12 ml	Dilute sulfuric acid.

Materials Required But Not Provided:

- Pipettors and pipettes that deliver 100ul and 1-10ml. A multi-channel pipettor is recommended.
- Disposable glass or plastic 5-15ml tubes for diluting samples and Anti-Human IgG HRP Concentrate.
- Graduated cylinder to dilute Wash Concentrate; 0.2 to 1L.
- Stock bottle to store diluted Wash Solution; 200ml to 1L.
- Distilled or deionized water to dilute reagent concentrates.
- Microwell plate reader at 450 nm wavelength.

ASSAY DESIGN AND SET-UP

Sample Collection and Handling

For **serum**, collect blood by venipuncture, allow clotting, and separate the serum by centrifugation at room temperature. For other samples, including **tissue culture media**, clarify the sample by centrifugation and/or filtration prior to dilution in Sample Diluent.

Caution: Human serum and other bodily fluids may contain infectious material. Always wear gloves when handling human samples, including the standards and controls (which have been tested non-reactive for HbsAg and Anti-HIV), and dispose of these samples and containers as biohazard waste.

Assay Design

Review Calculation of Results and Limits of the Assay (p5-7) before proceeding:

- Select the proper sample dilutions accounting for expected potency of positives and minimizing non-specific binding (NSB) and other matrix effects; for example, net signal for non-immune samples should be <0.5 OD. This is usually 1/200 or greater dilution for rabbit sera with normal levels of IgG and IgM.
- Run a Sample Diluent **Blank**. This signal is an indicator of proper assay performance, especially of washing efficacy, and is used for net OD calculations, if required (**See Methods A&B)**.
- Run a set of **Calibrators**. Calibrators validate that the assay was performed to specifications; results can be used to normalize between-assay variation for enhanced precision. Reading values off a Calibrator curve has limitations. See **Method C**.
- Run a range of sample dilutions for expected higher positives that allows calculation of antibody **Titer** (when specific titer is at least 4-fold higher than non-immune)**. See Method D.**
- Run samples in duplicate if used for quantitation; non-immunes that are significantly lower than immunes may be run in singlicate. The Calibrators that are used for quantitation, e.g., for between-assay normalization, should be run in duplicate. When determining titer from a dilution curve, singlicates can be run if more than two dilution points are used for titer calculations.

Plate Set-up

Bring all reagents to room temperature (18-30° C) equilibration (at least 30 minutes).

- Determine the number of wells for the assay run. Duplicates are recommended, including 8 Calibrator wells and 2 wells for each sample and control to be assayed.
- Remove the appropriate number of microwell strips from the pouch and return unused strips to the pouch. Reseal the pouch and store refrIgerated.
- Add 200-300ul Working Wash Solution to each well and let stand for about 5 minutes. Aspirate or dump the liquid and pat dry on a paper towel before sample addition.

Assay Procedure

ALL STEPS ARE PERFORMED AT ROOM TEMPERATURE. After each reagent addition, gently tap the plate to mix the well contents prior to beginning incubation.

1. 1st Incubation [100ul – 60 min; 4 washes]

- Add 100ul of calibrators, samples and controls each to pre-determined wells.
- Tap the plate gently to mix reagents and incubate for 60 minutes.
- Wash wells 4 times and pat dry on fresh paper towels. As an alternative, an automatic plate washer may be used. Improper washes may lead to falsely elevated signals and poor reproducibility.

2. 2nd Incubation [100ul – 30 min; 5 washes]

- Add 100ul of diluted Anti-Human IgG HRP to each well.
- Incubate for 30 minutes.
- Wash wells 5 times as in step 2.

3. Substrate Incubation [100ul – 15 min]

- Add 100ul TMB Substrate to each well. The liquid in the wells will begin to turn blue.
- Incubate for 15 minutes in the dark, e.g., place in a drawer or closet.

Note: If your microplate reader does not register optical density (OD) above 2.0, incubate for less time, or read OD at 405-410 nm (results are valid).

4. Stop Step [Stop: 100ul]

- Add 100ul of Stop Solution to each well.
- Tap gently to mix. The enzyme reaction will stop; liquid in the wells will turn yellow.

5. Absorbance Reading

- Use any commercially available microplate reader capable of reading at 450nm wavelength. Use a program suitable for obtaining OD readings, and data calculations if available.
- Read absorbance of the entire plate at 450nm using a single wavelength within 30 minutes after Stop Solution addition. If available, program to subtract OD at 630nm to normalize well background.

PRECAUTIONS AND SAFETY INSTRUCTIONS

Calibrators, Sample Diluent, Positive Control, and Antibody HRP contain bromonitrodioxane (BND: 0.05%, w/v). Stop Solution contains 1% sulfuric acid. Follow good laboratory practices, and avoid ingestion or contact of any reagent with skin, eyes or mucous membranes. MSDS for TMB, sulfuric acid and BND can be requested or obtained from the ADI website:http://4adi.com/commerce/info/showpage.jsp?page_id=1060&category_id=2430&visit=10

INTERPRETATION OF RESULTS

Calculation of Results
Consider several data reduction methods to best represent the relationships among experimental and control groups, to determine **Positive Immune** and **Negative Non-immune**, and to **Quantitate** positive antibody levels.

Method A. Antibody Activity[ELISA Signal & Sample Dilution]
Represent data as net OD units (A450 signal; blank subtracted)÷ dilution = **Total Activity Units**.

A Calibrator value in the mid-OD range (e.g., 5 U/ml) can be used to normalize inter-assay values.

Method B. Positive Index
Experimental sample values may be expressed relative to the values of Control or Non-immune samples, by calculation of a **Positive Index**. One typical method is as follows:

1. Calculate the net OD mean + 2 SD of the Control/Non-immune samples = **Positive Index**.

2. Divide each sample net OD by the Positive Index. Values above 1.0 are a measure of **Positive** Antibody Activity; below 1.0 are **Negative** for antibody.

A sample value would be **Positive** if significantly above the value of the pre-immune serum sample or a suitably determined non-immune panel or pool of samples, tested at the same sample dilution. This calculation **quantifies** the positive Antibody Activity level.

Example:

	Assay Net OD		Calculated Antibody Activity	
Sample	Control	Exptl	Control	Exptl
1	0.243	2.358	0.49	**4.79**
2	0.351	0.597	0.71	**1.21**
3	0.286	1.421	0.58	**2.89**
4	0.357	1.268	0.73	**2.58**
5	0.512	0.857	**1.04**	**1.74**
6	0.342	1.296	0.70	**2.63**
7	0.298	0.608	0.61	**1.24**
8	0.285	0.369	0.58	0.75
9	0.157	0.864	0.32	**1.76**
10	0.187	0.543	0.38	**1.10**
Mean	0.302			
SD	0.095			
Mean +2 SD	**0.492**	= **Positive Index**		

CALCULATION OF RESULTS(continued)

Method C. Use of a Calibrator Curve

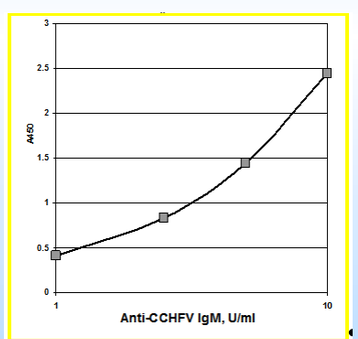
When the dilution curves of samples are parallel to the Calibrator curve (not valid if not parallel), the anti-CCHFV activity units may be determined by interpolation from the Calibrator curve, as follows:

1. The results may be calculated using any immunoassay software package. If software is not available, anti-CCHFV activity concentrations may be determined as follows:
2. Calculate the mean OD of duplicate samples.
3. On graph paper plot the mean OD of the calibrators (y-axis) against the concentration (U/ml) of anti-CCHFV (x-axis). Draw the best fit curve through these points to construct the calibrator curve. A point-to-point construction is most common and reliable.
4. The anti-CCHFV activity concentrations in unknown samples and controls can be determined by interpolation from the calibrator curve.
5. Multiply the values obtained for the samples by the dilution factor of each sample.
6. Samples producing signals higher than the 10 U/ml calibrator should be further diluted and re-assayed.

Typical Results:

Wells	Calibrators & Samples		A450 nm
A1,2	**Negative Diluent Blank**		0.08
B1,2	1 U/ml	**Calibrator**	0.41
C1,2	2.5 U/ml	**Calibrator**	0.83
D1,2	5 U/ml	**Calibrator**	1.40
E1,2	10 U/ml	**Calibrator**	2.44
F1,2	Sample	**1:200**	1.65

Sample Result: **5.8** U/ml x 200 dilution = **1160** U/ml



Calibrator Values

The Calibrators are dilutions of anti-CCHFV antibody. Values are assigned as arbitrary anti-CCHFV activity units.

CALCULATION OF RESULTS (continued)

Method D. Titers from Sample Dilution Curves
The titer of antibody activity calculated from a dilution curve of each sample is recommended as the most accurate quantitative method. Best precision can be obtained using the following guidelines:

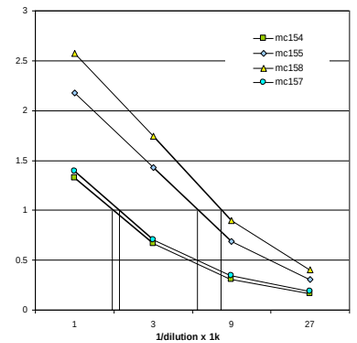
1. Use an OD value Index in the mid-range of the assay (2.0 – 0.5 OD); this provides the best sensitivity and reproducibility for comparing experimental groups and replicates. An arbitrary 1.0 OD is commonly used.
2. Prepare serial dilutions of each sample to provide a series that will produce signals higher and lower than the selected index. With accurate diluting, duplicates may not be required if at least 4 dilutions are run per sample.
3. A 5-fold dilution scheme is useful to efficiently cover a wide range which produces ODs both above and below 1.0 OD. The dilution scheme can be tightened to 3-fold or 2-fold for more precise comparative data.
4. A Calibrator value in the mid-OD range (e.g., 5 U/ml) can be used to normalize inter-assay values.

Calculations
1. On a log scale of inverse of Sample Dilution as the x-axis, plot the OD values of the two dilutions of each positive sample having ODs above and below the OD value of the Index (arbitrary or selected Calibrator).
2. From a point-to-point line drawn between the two sample ODs, read the dilution value (x-axis) corresponding to the OD of the selected Index

=IgG Antibody Activity Units

Example:

II. A **1.0 OD** Index was used to determine titer of 4 antibodies.



Titer Values

mc154 = 1.72 kU **mc155 = 5.70 kU**
mc157 = 1.85 kU **mc158 = 7.90 kU**

PRODUCT SPECIFICATIONS

Specificity

Purified recombinant (his tagi) CCHFV nucleoprotein (NP, 55 kda) is used to coat the microwells; thus, no other antibody specificity is detectable in the assay. The anti-Human IgG HRP conjugate specifically detects IgG, with no reaction with IgM, IgA or IgE class antibodies.

Instruction Manual No. M-AE-320430-1

Human Anti-Crimean-Congo Hemorrhagic Fever Virus (CCHFV) IgM ELISA Kit

AE-320430-1, 96 Tests

For Quantitation of Anti-CCHFV IgM in Human Serum or Plasma or other biological fluids

For research use only, not for diagnostic or therapeutic use.

ALPHA DIAGNOSTIC INTERNATIONAL

6203 Woodlake Center Drive•San Antonio•Texas78244•USA.
Phone (210) 561-9515 • Fax (210) 561-9544
Toll Free (800) 786-5777
Email: service@4adi.com
www.4adi.com

ELISA Kit Components	Amount	Part
CCHFV Coated Microwell Strip Plate	8-well strips (12)	320421
Anti-CCHFV Calibrator, 1 U/ml,	0.65 ml,	320422C
Anti-CCHFV Calibrator, 2.5 U/ml,	0.65 ml,	320422D
Anti-CCHFV Calibrator, 5 U/ml,	0.65 ml,	320422E
Anti-CCHFV Calibrator, 10 U/ml,	0.65 ml,	320422F
Anti-Human IgM HRP Conjugate (100X),	0.15 ml,	H-HuM.211
Wash Solution Concentrate (100X)	10 ml	WB-100
TMB Substrate	12 ml	80091
Stop Solution	12 ml	80101
Product Manual	1 ea	M-AE-320430-1