INTENDED USE

The **Mouse Anti-Crimean-Congo Hemorrhagic Fever Virus (CCHFV) IgM** ELISA Kit detects and quantifies CCHFV -specific IgM in mouse 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 a 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). 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 Mouse Anti-CCHFV NP IgM ELISA kit is based on the binding of mouse anti-CCHFV IgM in samples to CCHFV NP antigen coated on the plate, and bound antibody is detected by anti-mouse IgM-specific HRP conjugate. After a washing step, chromogenic substrate (TMB) is added and color is developed by the enzymatic reaction of HRP on the substrate which is directly proportional to the amount of anti- CCHFV NP IgM present in the sample. Stopping Solution is added to terminate the reaction and A450nm is then measured using an ELISA reader. The presence of mouse IgM antibody in samples is determined relative to anti-CCHFV NP Controls.

PRODUCT SPECIFICATIONS

Specificity

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

Assay Sensitivity

The CCHFV antigen coating level and HRP conjugate concentration are optimized to differentiate anti-CCHFV IgM from background (non-antibody) signal with mouse serum samples diluted 1:100.

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 4°C for long term and ambient temperature for short term.
Sample Diluent Concentrate (20x) Cat. No. SD-20T, 10ml	Dilute the entire volume, 10ml + 190ml with distilled or deionized water into a clean stock bottle. Label as **Working Sample/Conjugate Diluent** and store at 2-8°C until the kit lot expires or is used up.
Anti-Mouse IgM - HRP Conjugate Concentrate (100x) Part: H-MsM.2a11, 0.15ml	Peroxidase conjugated anti-Mouse IgM in buffer with 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 Coated 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; diluted in buffer with antimicrobial as stabilizers.
Anti-CCHFV Positive Control	320442-PC	0.65 ml	Antiserum with anti-CCHFV reactivity; [value range on label]
Low NSB Sample Diluent (LNSD)	TBTm Reduces non-specific binding	30 ml	Buffer with protein, detergents and antimicrobial. Use as is for sample dilution. See **Assay Design**, page 3.
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-Mouse IgM HRP Concentrate.
- Stock bottle to store diluted Wash Solution; 0.2 to 1L.
- Distilled or deionized water to dilute reagent concentrates.
- Microwell plate reader at 450 nm wavelength and ELISA plate washer

ASSAY DESIGN AND SET-UP

Sample Collection and Handling
Serum and other biological fluids may be used as samples with proper dilution to avoid solution matrix interference. For **serum**, collect blood by venipuncture, allow clotting, and separate the serum by centrifugation at room temperature. If samples will not be assayed immediately, store refrigerated for up to a few weeks, or frozen for long-term storage.

Antibody Stability & Dilution
Initial dilution of serum into **Working Sample Diluent** (WSD) is recommended to stabilize antibody activity. This enhances reproducible sampling, and stabilizes the antibody activity for years, stored refrigerated or frozen. Further dilution into **Low NSB Sample Diluent** (LNSD), which provides the lowest assay background, should be at least 10 times the initial dilution and performed the same day as the assay.

Example: Initial (1:5): **10**ul serum + **40**ul WSD [or 0.1ml + 0.4ml]
Further (1:50): **10**ul initial (1:5) + **90**ul LNSD (1:50)

Assay Design

Review Interpretation of Results (p. 5-7) 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 lower than the **1 U/ml Calibrator**. This is usually 1:100 or greater dilution for Mouse serum with normal levels of IgG and IgM.
- Run the **Anti-CCHFV Positive Control**; value range is on the vial label.
- 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. Blank OD should be <0.3.
- Run a set of **Calibrators**, which validate that the assay was performed to specifications: **10 U/ml** should give a high signal (>1.5 OD); **1 U/ml** should give a low signal which can be used to discriminate at the Positive/Negative threshold (see Interpretation of Results, p. 5).
- 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 C.**
- 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 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 1-5 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, blanks, 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-Mouse IgM HRP to each well.
- Incubate for 30 minutes.
- Wash wells 5 times as in step 1.

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.

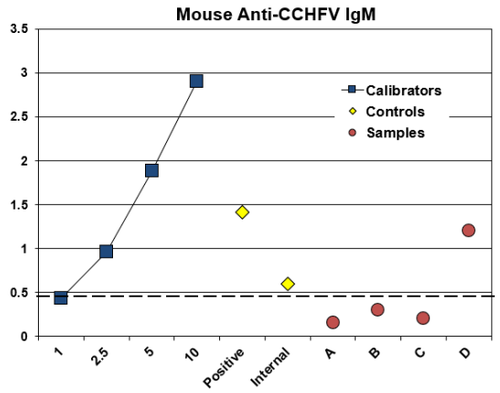
INTERPRETATION OF RESULTS

Method A. Antibody Activity Threshold Index

Compare Samples to **1 U/ml Calibrator** or **Internal Control**

=Positive/Negative Cut-off.

Example:



Results

The **sensitivity** of the assay to detect anti-CCHFV IgM, from either natural exposure or vaccination, is controlled so that the **1 U/ml Calibrator** represents a threshold OD for most true positives in mouse serum diluted to 1:100 or greater. Visual inspection of the data in the above graph shows the following:

Calibrators – dilution curve of an anti-CCHFV antibody, derived from immunization, shows the OD range of the assay; high value indicates optimal sensitivity of the assay.

1 U/ml: a 'Cut-off' line has been drawn to indicate a threshold distinguishing between **Positive/Negative**. This is not a clear-cut threshold, rather a low OD area that could represent either low positives or high background negatives.

Positive Control – antiserum reactive to CCHFV; value range is on the vial label. This Control can be used to assess reproducibility and to normalize between-assay variation.

Internal Control – a true positive from an immune mouse that represents the investigator's experience in distinguishing low positive from negative samples (not in kit). This should be run in each assay to supplement the 1 U/ml Calibrator for Positive/Negative discrimination purposes.

Samples A,B,C,D – 3 samples (A, B, C) are negative: below the threshold; 1 sample (D) is positive: clearly above the threshold.

The **1 U/ml** Calibrator can be used to calculate a **Threshold Index** that numerically discriminates Positive/Negative (see p.6):

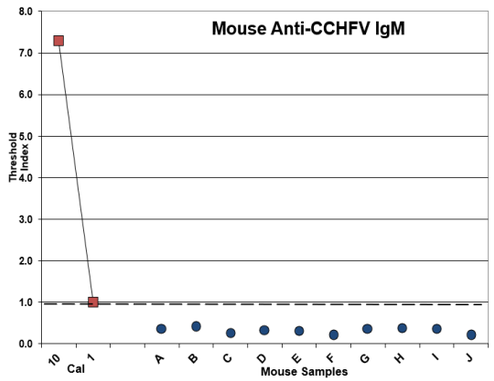
- Divide each Sample net OD by the 1 U/ml Calibrator net OD. Values above 1.0 are a measure of **Positive** Antibody Activity; below 1.0 is **Negative** for antibody.

ASSAY PERFORMANCE

Example:

Mouse Serum/Plasma IgM

A panel of non-immunized mouse sera was tested for anti-CCHFV IgM (1:100 dilution in Low NSB Sample diluent). **Threshold Index** was calculated using the **1** U/ml **Cal**.



Results

Anti-CCHFV IgM: All ten samples were negative (below the 1.0 threshold) at 1:100 dilution.

Notes:

1. **Positives** may be due to prior encounter with the virus or non-CCHFV proteins with common epitopes, or may be an aspect of the innate immune repertoire.
2. When the **Positive Index** is **above 5.0**, using a dilution curve to calculate titer is a more accurate quantitation method (see Method C).
3. The **sensitivity** of the assay may be adjusted by changing the sample dilutions: a) increase dilution (e.g., 1/200) to lower the signals of borderline positives to negative; b) decrease dilution (e.g., 1/50) to convert borderline samples to positive. With the latter, the values of negatives may increase, so an alternative threshold should be considered using known negatives to develop a **Positive Index** (see below) or use an **Internal Control** (Page 5).

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.

INTERPRETATION OF RESULTS (cont)

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 sera dilution.

This calculation also **quantifies** the positive Antibody Activity level, assigning a higher value to samples with higher Antibody Activity, and vice versa.

Method C. Titers from Sample Dilution Curves

The titer of elevated 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. The Positive and Sensitivity Control values 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

= IgM Antibody Activity Units

PRECAUTIONS AND SAFETY INSTRUCTIONS

Calibrators, Sample Diluent, and Antibody HRP contain bromonitrodioxane (BND: 0.05%, w/v). Stop Solution contains dilute sulfuric acid. Follow good laboratory practices and avoid ingestion or contact of any reagent with skin, eyes, or mucous membranes. All reagents may be disposed of down a drain with copious amounts of water. MSDS for TMB, sulfuric acid, and BND can be requested or obtained from the ADI website.

Instruction Manual #M-AE-320410-1

Mouse Anti-Crimean-Congo Hemorrhagic Fever Virus (CCHFV) IgM

Cat. #AE-320410-1

For Quantitation of Anti-CCHFV IgM In Mouse Serum or Plasma

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

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ELISA Kit Components	Amount	Part
CCHFV Coated Microwell Strip Plate	8-well strips (12)	320421
Anti- CCHFV Positive Control	0.65 ml	320442PC
Anti- CCHFV Calibrator 1 U/ml	0.65 ml	320442B
Anti- CCHFV Calibrator 2.5 U/ml	0.65 ml	320442C
Anti- CCHFV Calibrator 5 U/ml	0.65 ml	320442D
Anti- CCHFV Calibrator 10 U/ml	0.65 ml	320442E
Anti-Mouse IgM HRP Conjugate (100X)	0.15 ml	H-MsM.2a11
Sample Diluent (20X)	10 ml	SD20T
Low NSB Sample Diluent	30 ml	TBTm
Wash Solution Concentrate (100X)	10 ml	WB-100
TMB Substrate	12 ml	80091
Stop Solution	12 ml	80101
Product Manual	1 ea	M-AE-320410-1