

## INTENDED USE

The **Rabbit Anti-influenza A M2 Protein (Flu A M2) IgA ELISA Kit** is an immunoassay suitable for quantifying IgA antibody activity specific for influenza A M2 protein in serum, plasma or other qualified biological samples from vaccinated, immunized and/or infected hosts.

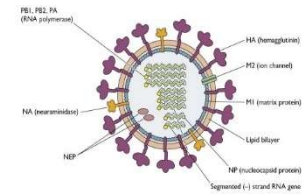
This immunoassay is suitable for:

- 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.

The assay is for research use only (RUO) and is not intended nor validated for diagnosing disease.

## GENERAL INFORMATION

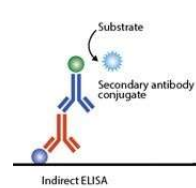
**Influenza A (Flu A)** viruses are negative-sense, ssRNA viruses of the family *Orthomyxoviridae*; subtypes cause disease in a variety of hosts, including humans, birds, swine, equine and canine. Antigenic drift of the two immunodominant surface proteins, neuraminidase (NA) and hemagglutinin (HA) of the influenza A virus makes it necessary to match the viral strains every year and then prepare the vaccine for the next season. This puts huge constraints on the vaccine production and incurs high cost. A universal vaccine will overcome these shortcomings of the current vaccines.



The extracellular domain (**M2e**, 23 a.a.) of the matrix 2 surface protein (**M2**), a proton-ion channel protein required for Flu A infectivity, is highly conserved among various strains of influenza viruses (H1N1, H5N1, H9N1 etc).

Therefore, M2 protein offers an attractive target to develop a universal Flu A vaccine. Currently used M2 vaccines include peptide carrier conjugates, baculovirus-expressed M2, M2 fusion proteins, multiple antigenic peptides, and M2 DNA-expression vaccines. Among these are: **ACAM-FLU-ATM** (Acambis), 3 M2e domains on a hepatitis B core antigen; **N8295** (Dynavax), conserved NP and M2e antigens with a TLR9 agonist; **VAX102** (Vaxinate), a recombinant fusion protein with 4 copies of M2e linked to *S. typhimurium* flagellin, with a TLR5 ligand as adjuvant; **M2-OMPC** (Merck) M2 conjugated with OMPC. Effectiveness of the various vaccines to produce protection against live Flu A challenge has been variable, with considerable host specificity in response.

## PRINCIPLE OF THE TEST



The Anti-M2 IgA/IgG/IgM ELISA kits are based on the binding of antibodies in samples to the purified M2 antigen immobilized on the microwells. Bound antibody is detected by anti-Rabbit IgA or IgG or IgM-HRP conjugate. After a washing step, chromogenic substrate (TMB) is added and color (blue), which is directly proportional to the amount of antibody present in the sample. Stop Solution is added (converts blue to yellow color), and A450nm is then measured using an ELISA reader. The activity of antibody in samples is calculated relative to supplied calibrators.

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## 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
<b>Wash Solution Concentrate (100x)</b> Cat. No. WB-100, 10ml	Dilute the entire volume 10ml + 990ml with distilled or deionized water into a clean stock bottle. Label as <b>Working Wash Solution</b> and store at 4°C for long term and ambient temp. for short term.
<b>Sample Diluent Concentrate (20x)</b> Cat. No. SD-20T, 10ml	Dilute the entire volume, 10ml + 190ml with distilled or deionized water into a clean stock bottle. Label as <b>Working Sample/Conjugate Diluent</b> and store at 2-8°C until the kit lot expires or is used up.
<b>Anti-Rabbit IgA-HRP Conjugate Concentrate (100x)</b> Part: H-HuA.211, 0.15ml	Peroxidase conjugated anti-Rabbit IgA in buffer with detergents and antimicrobial as stabilizers. Dilute fresh as needed; 10ul of concentrate to 1ml of <b>Working Sample/Conjugate Diluent</b> 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
<b>Flu A M2 Peptide Coated Strip Plate</b>	920201	8-well strips (12)	Coated with Flu A M2 peptide, and post-coated with stabilizers.
<b>Anti-Flu A M2 Calibrators</b>			
1 U/ml	920203B	0.65 ml	Four (4) vials, each containing anti-Flu A M2; in buffer with antimicrobial.
2.5 U/ml	920203C	0.65 ml	
5 U/ml	920203D	0.65 ml	
10 U/ml	920203E	0.65 ml	
<b>Rabbit x-Flu A M2 IgA Positive Control</b>	920200-PC	0.65 ml	Rabbit serum with anti-Flu A M2 IgA reactivity;  Net OD > 0.5
<b>Low NSB Sample Diluent</b>	TBTm  Not for HRP Conjugate dilution.	30 ml	Buffer with protein, detergents and antimicrobial as stabilizers. Use as is for sample dilution. See <b>Assay Design</b> , page 3.
<b>TMB Substrate</b>	80091	12 ml	Chromogenic substrate for HRP containing TMB and peroxide.
<b>Stop Solution</b>	80101	12 ml	Dilute sulfuric acid.

**Materials Required But Not Provided:**

- Pipettors and pipettes that deliver 100ul and 1-10ml.
- Disposable glass or plastic 5-15ml tubes for diluting samples and Anti-Rabbit IgA HRP Concentrate.
- 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.

**Caution:** Rabbit serum and other bodily fluids may contain infectious material. Always wear gloves when handling Rabbit 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.

### Sample Dilution & Antibody Stability

Prepare an initial sample dilution (1:10 or 20 ul sample into 180 ul) of **Working Sample Diluent** in order to stabilize antibody activity. This enhances reproducible sampling, and stabilizes the antibody activity for months, stored refrigerated or frozen. Additional dilution (1:10 of the initial stock for a final dilution of 1:200) into **Low NSB Sample Diluent** provides low assay background and good discrimination of specific signal. It is possible to change the testing dilution to 1:50-1:500 depending upon the actual sample background. All sample dilutions in Low NSB should be at least 5 times the initial dilution and performed the same day as the assay. Do not store test dilutions.

### Assay Design

Review Interpretation of Results (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 lower than the **1 U/ml Calibrator**. This is usually 1:100 or greater dilution for Rabbit serum with normal levels of IgG and IgA.
- Run the Rabbit Anti-Flu A M2 IgA Positive Control; net OD > **0.5**.
- 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).

### 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 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. 1<sup>st</sup> 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. 2<sup>nd</sup> Incubation [100ul – 30 min; 5 washes]

- Add 100ul of diluted Anti-Rabbit IgA 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, 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: [http://4adi.com/commerce/info/showpage.jsp?page\\_id=1060&catogory\\_id=2430&visit=10](http://4adi.com/commerce/info/showpage.jsp?page_id=1060&catogory_id=2430&visit=10)

# Rabbit Anti-Influenza A M2 Protein (Flu A M2) IgA ELISA Kit

## ELISA KIT # 920-240-MR, 96 tests

For Quantitation of Anti-Flu A M2 Protein IgA in Serum or Plasma or other biological fluids

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



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ELISA Kit Components	Amount	Part
Flu A M2 Peptide Coated Strip Plate	8-well strips (12)	920201
Rabbit Anti- Flu A M2 IgA	0.65 ml	920200-PC
Anti- Flu A M2 Calibrator	1 U/ml	0.65 ml 920203B
Anti- Flu A M2 Calibrator	2.5 U/ml	0.65 ml 920203C
Anti- Flu A M2 Calibrator	5 U/ml	0.65 ml 920203D
Anti- Flu A M2 Calibrator	10 U/ml	0.65 ml 920203E
Anti-Rabbit IgA HRP Conjugate (100X)	0.15 ml	H-HuA.211
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	920-240-MRA

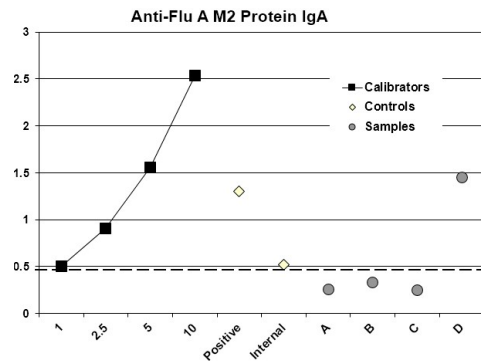
### INTERPRETATION OF RESULTS

#### 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-M2 IgA, from either natural infection or vaccination, is controlled so that the 1 U/ml Calibrator represents a threshold OD for most true positives in Rabbit 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-Flu A M2 antibody, derived from M2 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** – a Rabbit serum showing natural reactivity to Flu A M2; net OD > 0.5. This Control can be used to normalize between-assay variation.

**Internal Control** – a true positive from an immune Rabbit 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 p6):

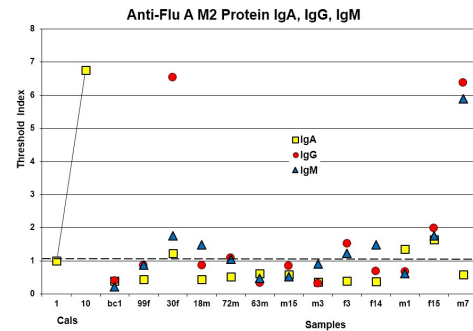
- ❖ Divide each Sample net OD by the 1 U/ml Calibrator net OD. Values above 1.0 are a measure of **Positive** Antibody Activity; below 1.0 are **Negative** for antibody.

### INTERPRETATION OF RESULTS (cont)

#### Example:

#### Rabbit Serum/Plasma IgA, IgG and IgM

A panel of Rabbit serum/plasma of unknown history were tested for anti-Flu A M2 protein IgA, IgG and IgM (1:100 dilution). **Threshold Index** was calculated using the 1 U/ml Cal.



#### Results

**Anti-Flu A M2 IgA:** most samples were negative at 1:100 (lower than 1.0 index); several were borderline or low positive.

**Anti-Flu A M2 IgG:** many samples were negative; several were low or borderline positive; 2 samples were significantly elevated.

**Anti-Flu A M2 IgM:** most samples were negative or borderline positive; 1 sample was elevated positive.

#### Notes:

- Positives** may be due to prior encounter with the virus or influenza immunization.
- The **sensitivity** of the assay may be adjusted by changing the sample dilutions: a) increase dilution (e.g., 1/500) 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).
- Other biological specimens, such as nasal swabs, may be used for antibody determination. Sample dilutions and positive/negative thresholds should be determined using specimens from non-immune or pre-immune populations.

#### 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:

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

- 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.
- 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.
- 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.
- The Positive and Sensitivity Control values can be used to normalize inter-assay values.

#### Calculations

- 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).
- 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

= IgA Antibody Activity Units

### PRODUCT SPECIFICATIONS

#### Specificity

M2 protein is 100% conserved in various strains of Influenza A: H1N1, H1N2, H2N3, H3N2, H3N9, H4N6, H5N1, H5N2, H5N7, H6N3, H6N8, H9N1, H7N6, H9N2, H10N4, H14N6, H16N3. Some strains of H5N1 (96%, A/chicken/Jilin/hn/2003(H5N1); H1N1, A/ruddy turnstone/NJ/51/1985(H11N1).

Purified peptide representing the M2 extracellular region of the Flu A M2 protein is used to coat the microwells; thus the assay is specific for antibodies directed to M2 antigens. The Anti-Rabbit IgA HRP conjugate reacts specifically with Rabbit IgA class antibodies that bind to M2 antigen on the plate. IgG, IgM and IgE antibody would not be measured above background signals.

#### Assay Sensitivity

The M2 peptide coating level, HRP conjugate concentration, and Low NSB Sample Diluent are optimized to differentiate anti-Flu A M2 from background (non-antibody) signal with Rabbit serum samples diluted 1:100.