ELISA kits available from ADI (see details at the web site)

950-100-AHA Human Anti-Adenovirus IgA ELISA kit, 96 tests, Quantitative
AE-327210 Human Anti-Adenovirus IgG ELISA kit, 96 tests, Quantitative
950-120-AHM Human Anti-Adenovirus IgM ELISA kit, 96 tests, Quantitative

AE-320500-1 Mouse Anti-Zaire Ebola virus Nucleoprotein (NP) IgG ELISA Kit, 96 tests, Quantitative
AE-320510-1 Mouse Anti-Zaire Ebola virus Nucleoprotein (NP) IgM ELISA Kit, 96 tests, Quantitative
AE-320520-1 Human Anti-Zaire Ebola virus Nucleoprotein (NP) IgG ELISA Kit, 96 tests, Quantitative
AE-320530-1 Human Anti-Zaire Ebola virus Nucleoprotein (NP) IgM ELISA Kit, 96 tests, Quantitative
AE-320540-1 Rabbit Anti-Zaire Ebola virus Nucleoprotein (NP) IgG ELISA Kit, 96 tests, Quantitative
AE-320550-1 Monkey/Chimp Anti-Zaire Ebola virus Nucleoprotein (NP) IgG ELISA Kit, 96 tests, Quantitative
AE-320560-1 Monkey/Chimp Anti-Zaire Ebola virus Nucleoprotein (NP) IgM ELISA Kit, 96 tests, Quantitative

AE-320600-1 Mouse Anti-Zaire Ebola virus glycoprotein (GP) IgG ELISA Kit, 96 tests, Quantitative
AE-320610-1 Mouse Anti-Zaire Ebola virus glycoprotein (GP) IgM ELISA Kit, 96 tests, Quantitative
AE-320620-1 Human Anti-Zaire Ebola virus glycoprotein (GP) IgG ELISA Kit, 96 tests, Quantitative
AE-320630-1 Human Anti-Zaire Ebola virus glycoprotein (GP) IgM ELISA Kit, 96 tests, Quantitative
AE-320640-1 Rabbit Anti-Zaire Ebola virus glycoprotein (GP) IgG ELISA Kit, 96 tests, Quantitative
AE-320650-1 Monkey/Chimp Anti-Zaire Ebola virus glycoprotein (GP) IgG ELISA Kit, 96 tests, Quantitative
AE-320650-PC Monkey/Chimp Anti-Zaire Ebola virus glycoprotein (GP) IgG positive control
AE-320660-1 Monkey/Chimp Anti-Zaire Ebola virus glycoprotein (GP) IgM ELISA Kit, 96 tests, Quantitative
AE-320670-1 Dog Anti-Zaire Ebola virus glycoprotein IgG ELISA Kit, 96 tests, Quantitative
AE-320680-1 Pig Anti-Zaire Ebola virus glycoprotein IgG ELISA Kit, 96 tests, Quantitative

AE-320700-1 Mouse Anti-Zaire Ebola virus VP40 IgG ELISA Kit, 96 tests, Quantitative
AE-320710-1 Mouse Anti-Zaire Ebola virus VP40 IgM ELISA Kit, 96 tests, Quantitative
AE-320720-1 Human Anti-Zaire Ebola virus VP40 IgG ELISA Kit, 96 tests, Quantitative
AE-320730-1 Human Anti-Zaire Ebola virus VP40 IgM ELISA Kit, 96 tests, Quantitative
AE-320740-1 Rabbit Anti-Zaire Ebola virus VP40 IgG ELISA Kit, 96 tests, Quantitative
AE-320750-1 Monkey/Chimp Anti-Zaire Ebola virus VP40 IgG ELISA Kit, 96 tests, Quantitative
AE-320760-1 Monkey/Chimp Anti-Zaire Ebola virus VP40 IgM ELISA Kit, 96 tests, Quantitative

AE-320800-1 Zaire Ebola Virus Glycoprotein (EBOV GP antigen) ELISA Kit, 48 tests, Quantitative
AE-320800-96 Zaire Ebola Virus Glycoprotein (EBOV GP antigen) ELISA Kit, 96 tests, Quantitative
AE-320805-RT-10 Zaire Ebola Virus antigen (GP) rapid test (visual results in 2-10 mins), 10 cassettes/pk
AE-320810-1 Humanized (plant expressed) Anti-Ebola GP IgGs ELISA kit, 96 tests, Quantitative
AE-320815-1 Anti-Humanized Ebola GP IgGs (Plant expressed) (Anti-drug antibody/ADA) ELISA kit, 96 tests,
AE-320880-RT-25 Humanized Ebola IgG (humanized IgGs expressed in tobacco or other plants) rapid test (results in 2-10 min), 25 cassettes/pk

AE-321600-1 Mouse Anti-Sudan Ebola virus glycoprotein (GP) IgG ELISA Kit, 96 tests, Quantitative
AE-321610-1 Mouse Anti-Sudan Ebola virus glycoprotein (GP) IgM ELISA Kit, 96 tests, Quantitative
AE-321620-1 Human Anti-Sudan Ebola virus glycoprotein (GP) IgG ELISA Kit, 96 tests, Quantitative
AE-321630-1 Human Anti-Sudan Ebola virus glycoprotein (GP) IgM ELISA Kit, 96 tests, Quantitative
AE-321640-1 Rabbit Anti-Sudan-Ebola virus glycoprotein (GP) IgG ELISA Kit, 96 tests, Quantitative
AE-321650-1 Monkey/Chimp Anti-Sudan Ebola virus glycoprotein (GP) IgG ELISA Kit, 96 tests, Quantitative
AE-321660-1 Monkey/Chimp Anti-Sudan Ebola virus glycoprotein (GP) IgM ELISA Kit, 96 tests, Quantitative

AE-322600-1 Mouse Anti-Marburg (Angola) virus glycoprotein (GP) IgG ELISA Kit, 96 tests, Quantitative
AE-322610-1 Mouse Anti-Marburg (Angola) glycoprotein (GP) IgM ELISA Kit, 96 tests, Quantitative
AE-322620-1 Human Anti-Marburg (Angola) glycoprotein (GP) IgG ELISA Kit, 96 tests, Quantitative
AE-322630-1 Human Anti-Marburg (Angola) glycoprotein (GP) IgM ELISA Kit, 96 tests, Quantitative
AE-322640-1 Rabbit Anti-Marburg (Angola) glycoprotein (GP) IgG ELISA Kit, 96 tests, Quantitative
AE-322650-1 Monkey/Chimp Anti-Marburg (Angola) glycoprotein (GP) IgG ELISA Kit, 96 tests, Quantitative
AE-322660-1 Monkey/Chimp Anti-Marburg (Angola) glycoprotein (GP) IgM ELISA Kit, 96 tests, Quantitative

AE-323620-1 Human Anti-Reston Ebola virus glycoprotein (GP) IgG ELISA Kit, 96 tests, Quantitative

AE-324620-1 Human Anti-Bundibugyo virus glycoprotein (GP) IgG ELISA Kit, 96 tests, Quantitative

AE-325600-XH Human Anti-Zaire+Sudan+Reston+ Bundibugyo Glycoproteins combo IgG ELISA Kit, 96 tests,
AE-325600-XM Monkey Anti-Zaire+Sudan+Reston+ Bundibugyo Glycoproteins combo IgG ELISA Kit, 96

AE-327100-1 Mouse Anti-Adenovirus hexon antibody (hAdV Hxn) IgG ELISA Kit, 96 tests, Quantitative
AE-327110-1 Human Anti-Adenovirus hexon antibody (hAdV Hxn) IgG ELISA Kit, 96 tests, Quantitative
AE-327120-1 Monkey/Chimp Anti-Adenovirus hexon antibody (hAdV Hxn) IgG ELISA Kit, 96 tests, Quantitative
AE-327200-1 Mouse Anti-VSV Indiana Matrix (M) antibody (VSVIM) IgG ELISA Kit, 96 tests, Quantitative
AE-327210-1 Human Anti-VSV Indiana Matrix (M) antibody (VSVIM) IgG ELISA Kit, 96 tests, Quantitative
AE-327300-1 Mouse Anti-VSV Indiana Matrix (M) antibody (VSVIM) IgG ELISA Kit, 96 tests, Quantitative
AE-327310-1 Human Anti-VSV Indiana Glycoprotein (GP) antibody (VSVIG) IgG ELISA Kit, 96 tests, Quantitative
AE-327320-1 Monkey/Chimp Anti-VSV Indiana Glycoprotein (GP) antibody (VSVIG) IgG ELISA Kit, 96 tests, Quantitative

Instruction Manual No. AE-327210-1

Human Anti-Vesicular Stomatitis Virus Indiana Matrix (M) antibody (VSVIM) IgG Cat. # AE-327210-1

For detecting human IgG antibodies against VSVIM in Serum or Plasma
For In Vitro Research Use Only

ALPHA DIAGNOSTIC INTERNATIONAL

6203 Woodlake Center Drive • San Antonio• Texas 78244 • USA.
Phone (210) 561-9515 • Fax (210) 561-9544
Toll Free (800) 786-5777
Email: service@4adi.com
Web Site: www.4adi.com

DRAFT MANUAL: PLEASE CONSULT THE MANUAL SUPPLIED WITH THE KIT FOR ANY LOT SPECIFIC CHNAGES.

Human Anti-VSVIM IgG ELISA KIT Cat. # AE-327210-1 (96 tests)

Kit Components (96 tests)	Cat #
VSVIM antigen coated strip plate, (8x12 strip or 96 wells) # 327211	1 plate
Human Anti-VSVIM IgG (5 U/ml) 1 ml #327212A	1 vial
Human Anti-VSVIM IgG (10 U/ml) 1 ml #327212B	1 vial
Human Anti-VSVIM IgG (20 U/ml) 1 ml #327212C	1 vial
Human Anti-VSVIM IgG (40 U/ml) 1 ml #327212D	1 vial
Human Anti-VSVIM IgG (80 U/ml) 1 ml #327212E	1 vial
Human Anti-VSVIM IgG Positive Control, 1 ml #327213-PC	1 vial
Anti-Human IgG-HRP Conjugate, (10X, 1.2 ml) #10119-210	1 vial
Sample Diluent, 10 ml #SD-20T	1 bottle
LowNSB Diluent, 30 ml #TBTm (green solution)	1 bottle
Wash buffer (100X) 10 ml # WB-100	1 bottle
TMB Substrate Solution, 12 ml #80091 (brown bottle)	1 bottle
Stop Solution, 12 ml # 80101 (red cap)	1 bottle
Complete Instruction Manual # M-327210-1	1

Intended Use

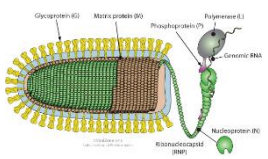
Human Anti-VSV (Indiana) Matrix protein (VSVIM) IgG ELISA Test Kit has been designed for the detection and measurement of IgG class of antibodies against VSVIM in serum, plasma or other biological samples. This immunoassay is suitable for:

- 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 not intended diagnostic or therapeutic use.

Introduction

Vesicular stomatitis is a viral disease caused by two distinct serotypes of **vesicular stomatitis virus** (**VSV) —New Jersey (VSNJV) and Indiana (VSIV)**. Vesiculation, ulceration, and erosion of the oral and nasal mucosa and epithelial surface of the tongue, coronary bands, and teats are typically seen in clinical cases, along with crusting lesions of the muzzle, ventral abdomen, and sheath. **Clinical disease has been seen in cattle, horses, and pigs and very rarely in sheep, goats, and llamas. Serologic evidence of exposure has been found in many species, including cervids, nonhuman primates, rodents, birds, dogs, antelope, and bats**. The clinical symptoms are similar to the very important foot and mouth disease virus (FMDV).



The viruses are members of the family Rhabdoviridae and genus Vesiculovirus. VSV are the prototypes of the Vesiculovirus genus. They are bullet shaped and generally 180 nm long and 75 nm wide. The genomic structure is a single strand of negative-sense RNA (11.1 kb) composed of five genes (**N, P, M, G, and L, representing the nucleocapsid protein, phosphoprotein, matrix protein, glycoprotein, and the large protein**, which is a component of the viral RNA polymerase). The G protein mediates both viral binding and

Human Sample Testing

A random population of non-vaccinated samples were tested in VSV Indiana (for GP and Matrix Protein IgG) and VSV NJ (GP IgG) at 1:100 sample dilution. For Indian strain, samples tend to positive for both Matrix and GP IgG.

Sample #	Human Anti-VSVI GP IgG	Human Anti-VSVI Matrix (M) IgG	Human Anti-VSV NJ GP IgG
109	0.524	0.869	0.556
110	0.742	0.968	0.708
111	0.620	1.442	0.870
112	1.118	1.29	0.914
113	0.289	0.678	0.390
114	1.457	2.891	0.807
115	2.485	1.31	1.549
Blanks	0.027	0.103	0.029

We recommend that users establish their own sample profile and/or compare vaccinated Vs non-vaccinated samples.

Quality Control

The test results are only valid if the test has been performed following the instructions. All standards and kit controls, if supplied, must be found within the acceptable ranges as stated on the vials. If criteria are not met, the run is not valid and should be repeated. Each laboratory should use known samples as further controls. In case of any deviation the following technical issues should be proven (reagents, protocol, equipments, etc).

PERFORMANCE CHARACTERISTICS

Intra-Assay-Precision 8.1 % **Inter-Assay-Precision** 11.3 %
Analytical Sensitivity ~2.5 U/mL

Interferences

No interferences to bilirubin up to 0.3 mg/mL; Hemoglobin up to 8.0 mg/mL and triglycerides up to 5.0 mg/mL.

Specificity and Species Reactivity

Recombinant, full length, highly purified (>95%) VSV Indiana strain, Matrix protein (M) is used in the kit as antigen. It is 60% conserved in the VSV New Jersey strain. VSV Chandipura Virus shows only 30% conservation with the Indian Matrix protein.

This kit detects only the human IgG isotype VSVIM antibodies and not the IgM or IgA. ADI has separate ELISA kits to detect IgM and IgA antibodies. Similar ELISA kits for mouse, rabbit, and monkey etc are also available.

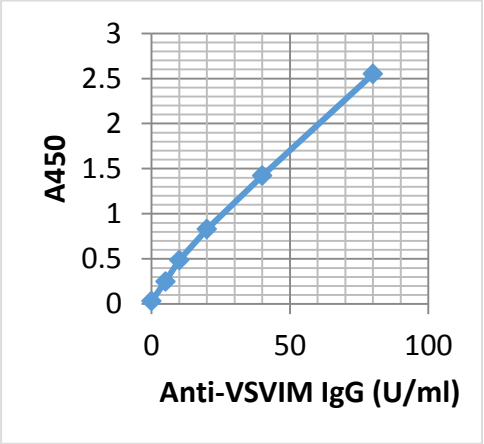
This kit is especially suited to detect and measure VSVIM antibodies as a results of VSV-vectored vaccines or VSV-Ebola vaccines.

References: .Steven M (2005) Nature Med. 11, 720-721; Heinz F (2007) PLOS Pathogens 3, e2. doi:10.1371/journal.ppat.0030002; Qiu X (2009) PLoS One 8, e5447; Geisbert T (2008) Vaccine 26, 6894-9000; Chad E (2014) PLos One 9, e94355;

WORKSHEET OF A TYPICAL ASSAY

Wells	Stds/samples	Mean A450	Net A450	Results
A1, A2	**Blanks**	0.100	-	-
B1, B2	Anti-VSVIM IgG (5 U/ml)	0.351	0.251	
C1, C2	Anti-VSVIM IgG (10 U/ml)	0.586	0.486	
D1, D2	Anti-VSVIM IgG (20 U/ml)	0.983	0.83	
D1, D2	Anti-VSVIM IgG (40 U/ml)	1.522	1.422	
E1, E2	Anti-VSVIM IgG (80 U/ml)	2.65	2.55	

NOTE: These data are for demonstration purpose only. It must not be used to determine the sample results.



Arif/4_ADI_ELISA

CALCULATION OF RESULTS:

The obtained A450 of the standards (y-axis, linear) are plotted against their concentration (x-axis) either on a graph paper or using an automated method. A good fit is provided with cubic spline, 4 parameter logistics or Logit-Log. For the calculation of the standard curve apply each signal of the standards (one obvious outlier of duplicates might be omitted and the more plausible single value might be used). The concentration of the samples can be read from the standards curve. The initial dilution has been taken into consideration when reading the results from the graph. Results of samples of higher dilution have to be multiplied with the dilution factor. Samples showing concentrations above the highest standard have to be diluted more and re-tested.

Interpretation of Results

The information is- based upon human samples.

Positive Samples **>10 U/ml**
Negative samples: For a value below the cut-off standard (arbitrary units =10 U/ml) the sample should be interpreted as a negative result.

All samples with values above the Cut-off are considered positive. Positive control values must be at least twice the values of the Cut-off control for the test to be valid.

host cell fusion with the endosomal membrane following endocytosis. The L and P proteins are subunits of the viral RNA-dependent RNA polymerase. Although there are many members of the Vesiculovirus genus, the New Jersey and Indiana serotypes are of particular interest in the Western hemisphere. These two viruses are similar in size and morphology but generate distinct neutralizing antibodies in infected animals. They have both been isolated in recent outbreaks in the USA. The virus can be transmitted through direct contact with infected animals with clinical disease (those with lesions) or by blood-feeding insects. In the southwestern USA, black flies (Simulidae) are the most likely biologic insect vector. In endemic areas, sand flies (Lutzomyia) are proven biologic vectors. The prevalence of clinical cases in a herd is generally low (10%–20%), but seroprevalence within the herd may approach 100%.

VSV diagnosis is based on the presence of typical signs and either antibody detection through serologic tests, viral detection through isolation, or detection of viral genetic material by molecular techniques. Three commonly used serologic tests are competitive ELISA, virus neutralization, and complement fixation. PCR tests may also be used to identify the virus. There is no treatment for vesicular stomatitis as animals will typically recover on their own. Control of outbreaks is dependent upon rapid recognition of initial cases, quarantine and restriction of movement of infected and in-contact animals, and insect control. The New Jersey serotype (VSNJV) is responsible for the majority of US cases in animals, and outbreaks caused by Indiana virus (VSIV) have been reported in the USA on only two occasions in the past 40 years, 1966 and 1997–1998. There are no commercially-available **VSV vaccines** in the U.S., but an autologous vaccine was made in 1995 to help control that outbreak. Several inactivated vaccines containing both the Indiana and New Jersey serotypes are used in Central and South America.

The simple structure and rapid high-titer growth of VSV in mammalian and many other cells has made it a useful tool in the fields of cellular, molecular biology, virology, and a shuttle vector for many vaccines. VSV-GP (Indiana, 511-aa) is 53% conserved VSV-GP (new Jersey strain 517-aa). The VSIV matrix protein M (Indiana, 229-aa) also is 61% conserved in New Jersey strain (229-aa). The VSV-GP and M antibodies are not cross-reactive within the Indiana and New Jersey strains. VSV-Ebola vaccine is constructed by swapping the wild type VSV-GP (Indiana strain) with the Ebola-GP. It is also referred as **VSVΔG/ZEBOVGP** (for Zaire Ebola strain GP). The modified virus is called a "**Trojan horse**" virus. VSV-based vaccines induce strong protective T cell and antibody responses after a single dose. Vesicular stomatitis viruses are easily propagated in cell culture. Recombinant VSVs expressing foreign proteins have been studied as vaccine vectors for a number of pathogens, including HIV, influenza virus, hepatitis C virus, hepatitis B virus (HBV), measles virus, respiratory syncytial virus, severe acute respiratory syndrome virus, Yersinia pestis, papillomavirus, Ebola virus, and Marburg virus. VSIV has low prevalence of preexisting antibodies so it makes VISV a suitable vector for the Ebola vaccine.

Use of ADI's VSV Antibody ELISA Kits

VSIV-Ebola GP vaccines will produce antibodies to VSIV proteins (N, P, M, and L) and also to the Ebola GP protein. Therefore, it is necessary to the establish basal level of antibodies as well as vaccine-induced levels VSIV proteins such as Matrix M protein and G Protein. The efficacy to VSV-Ebola vaccine or other vaccine can then be correlated with the VSIV vector antibodies in subjects receiving the vaccines. High level of preexisting VSIV antibodies could potentially neutralize the VISV-Ebola vaccine. ADI has made ELISA kits to measure antibodies to VSIV M and G antibodies. ELISA kits for ZEBOV GP are also available.

PRINCIPLE OF THE TEST

Alpha Diagnostic's VSVIM IgG Antibody ELISA Test Kit is based on the principle of the enzyme indirect ELISA. VSVIM antigen is bound on the surface of the microtiter strips. Diluted patient serum or ready-to-use standards are pipetted into the wells of the microtiter plate. A binding between the antibodies of the serum and the immobilized VSV antigen takes place. After a one hour incubation at room temperature, the plate is rinsed with diluted wash solution, in order to remove unbound material. Then ready-to-use anti-Mouse-IgG peroxidase conjugate is added and incubated for 30 minutes. After a further washing step, the substrate (TMB) solution is pipetted and incubated for 20 minutes, inducing the development of a blue dye in the wells. The color development is terminated by the addition of a stop solution, which changes the color from blue to yellow. The resulting dye is measured spectrophotometrically at the wavelength of 450 nm. The concentration of the IgG is directly proportional to the intensity of the color.

MATERIALS AND EQUIPMENT REQUIRED

Adjustable micropipet (5µl, 100µl, 500µl) and multichannel pipet with disposable plastic tips. Bidistilled water, reagent troughs, Orbital shaker, plate washer (recommended) and ELISA plate Reader.

PRECAUTIONS

The usual laboratory safety precautions as well as the prohibition of eating, drinking and smoking in the lab have to be followed. All sera and plasma or buffers based upon, have been tested respective to HBsAg, HIV and HCV with recognized methods and were found negative. Nevertheless precautions like the use of latex gloves have to be taken. Serum and reagent spills have to be wiped off with a disinfecting solution (e.g. sodium hypochlorite, 5%) and have to be disposed of properly. All reagents have to be brought to room temperature (18 to 25 °C) before performing the test. All reagents have to be used within the expiry period. In accordance with a Good Laboratory Practice (GLP) or following ISO9001 all laboratory devices employed should be regularly checked regarding the accuracy and precision.

Applicable **MSDS**, if not already on file, for the following reagents can be obtained from ADI web site.
TMB (substrate), H2SO4 (stop solution), and Prolcin-300 (0.1% v/v in standards, sample diluent and HRP-conjugates).
http://4adi.com/commerce/info/showpage.jsp?page_id=1060&category_id=2430&visit=10

SPECIMEN COLLECTION AND HANDLING

Principally serum or plasma (EDTA, heparin) can be used for the determination. Serum is separated from the blood, which is aseptically drawn by venipuncture, after clotting and centrifugation. The serum or plasma samples can be stored refrigerated (2-8°C) for up to 48 hours, for a longer storage they should be kept at -20 °C. The samples should not be frozen and thawed repeatedly. Lipemic, hemolytic or bacterially contaminated samples can cause false positive or false negative results. For the performance of the test the samples (not the standards) have to be diluted (1:100 or more, see below).

Sample Dilution & Antibody Stability

Prepare an initial sample dilution (1:10 or 20 ul sample into 180 ul) of **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 1:1**00**) into **Low NSB Sample Diluent** (green 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.

REAGENTS PREPARATION

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 (more >2 days) and ambient temp for short term use (1-2 days)
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 Diluent** and store at 2-8°C until the kit lot expires or is used up.
Anti-Human IgG- HRP Conjugate Concentrate (10x); 1.2ml	Peroxidase conjugated anti-human IgG in buffer with detergents and antimicrobial as stabilizers. **Dilute fresh as needed**; 100 ul of concentrate to 0.9 ml of **1x Sample Diluent** (SD-20T) is sufficient for one 8-well strip. Prepare 10 ml for a full plate. Use within the working day and discard. Return 10X conjugate to 2-8° C storage.

STORAGE AND STABILITY

The microtiter well plate and all other reagents are stable at 2-8°C until the expiration date printed on the label.

TEST PROCEDURE *(ALLOW ALL REAGENTS TO REACH ROOM TEMPERATURE BEFORE USE).*

Important: If you have not used this kit before, we recommend to use 1 or 2 strips to run the standards alone to get familiar with the test and not runt the risk of making mistakes and lose sample or the whole kit.

Remove required number of coated strips and arrange them on the plate. Store unused strips in the bag. **All samples should be diluted 1:100 (or more)**. Prepare 1X wash buffer, 1x Sample diluent, and 1X antibody-HRP conjugate. It is recommended to prepare a parallel replica plates containing all sample for quick transfer to the coated plate.

1. Label or mark the microtiter well strips to be used on the plate.
2. Dispense **100 ul sample diluent** in 1 well to be used as blank. Pipet **100 ul of** Prediluted calibrators, controls, and diluted samples into appropriate wells in *duplicate*. See worksheet of a typical set-up on page 5. Cover the plate, mix gently for 5-seconds and incubate at room temp for **60 min.**
3. Aspirate the well contents and blot the plate on absorbent paper. Immediately, **wash the wells 4 times** with 300 ul of 1X wash buffer. We recommend using an automated ELISA plate Washer for better consistency. Failure to wash the wells properly will lead to high blank or zero values. If washing manually, plate must be tapped over paper towel between washings to ensure proper washing.
4. Add **100 ul Antibody-HRP conjugate** to all wells leaving one empty for the substrate blank. Mix gently for 5-10 seconds. Cover the plate and incubate for **30 minutes** at room temp (25-28oC).
5. Wash the wells 3 times as in step 3.
6. Add **100 ul TMB substrate solution**. Mix gently for 5-10 seconds. Cover the plate and incubate for **15 minutes** at room temp. Blue color develops in positive controls and samples.
7. Stop the reaction by adding **100 ul of stop solution** to all wells. Mix gently for 5-10 seconds to have uniform color distribution (blue color turns yellow).
8. Measure the **absorbance at 450 nm** using an ELISA reader within 15 min.

NOTES:
Read instructions carefully before the assay. Do not allow reagents to dry on the wells. Careful aspiration of the washing solution is essential for good assay precision. Since timing of the incubation steps is important to the performance of the assay, pipet the samples without interruption and it should not exceed 5 minutes to avoid assay drift. If more than one plate is being used in one run, it is recommended to include a standard curve on each plate. The unused strips should be stored in a sealed bag at 4°C. Do not touch the bottom of the wells.