Version 22-04/24

User's Manual

Mouse Anti-Histone ELISA Kit

REF **DEIA120J**

Σ **96T**

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This product is for research use only and is not intended for diagnostic use.

For illustrative purposes only. To perform the assay the instructions for use provided with the kit have to be used.

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PRODUCT INFORMATION

Intended Use

The Mouse Anti-Histone Total Ig ELISA Kit is an immunoassay suitable for quantifying or titering total antibody activity (IgG, IgA and IgM) specific for histone in serum or plasma. Other biological fluids, including tissue culture medium, may be validated for use.

Principles of Testing

Antibodies reactive with autologous nuclear components, such as DNA and histones, can represent an autoim mune basis for pathological conditions such as systemic lupus erythematosis (SLE) in humans. In mice homozygous for the lymphoproliferation spontaneous mutation (Fas^{lpr}), a systemic autoimmunity develops with age which includes elevated levels of anti-dsDNA and other anti-nuclear antibodies (ANA), including anti-histone. The expanded use in the drug industry of biological modifiers has been associated with production of autoantibodies, of which mice, and possibly also other hosts such as humans and monkeys, are susceptible. A prototype disease in mice is lupus caused by the drug minocycline, with elevated anti-dsDNA among other autoantibodies and pathological conditions. Recent investigations have focused on the role of innate immune mechanisms, including Toll-like receptors (TLRs) and TH2 immunity, responding to the damage-associated molecular patterns of dying cells, as underlying cause of autoimmunity; these may be induced by drugs, including vaccines and adjuvants, with aging, or with other health conditions.

Reagents And Materials Provided

1. Histone Coated Microwell Strip Plate, 8-well strips. Coated with histone antigens, and post-coated with stabilizers. 2. Mouse Anti-Histone Calibrator, 100 U/ml, 0.65 ml 3. Mouse Anti-Histone Calibrator, 250 U/ml, 0.65 ml 4. Mouse Anti-Histone Calibrator, 500 U/ml, 0.65 ml 5. Mouse Anti-Histone Calibrator, 1000 U/ml, 0.65 ml

Four (4) vials of calibrators, each containing mouse antihistone IgG levels in arbitrary activity Units; diluted in buffer with protein, detergents and antimicrobial as stabilizers.

6. Anti-Mouse Ig HRP Conjugate (100×), 0.15 ml 7. Sample Diluent (20×), 10 ml 8. Low NSB Sample Diluent, 30 ml, Buffer with protein, detergents and antimicrobial as stabilizers. Use as is for sample dilution. Not for HRP Conjugate dilution. 9. Wash Solution Concentrate (100×), 10 ml 10. TMB Substrate, 12 ml. Chromogenic substrate for HRP containing TMB and peroxide. 11. Stop Solution, 12 ml. Dilute sulfuric acid.

Materials Required But Not Supplied

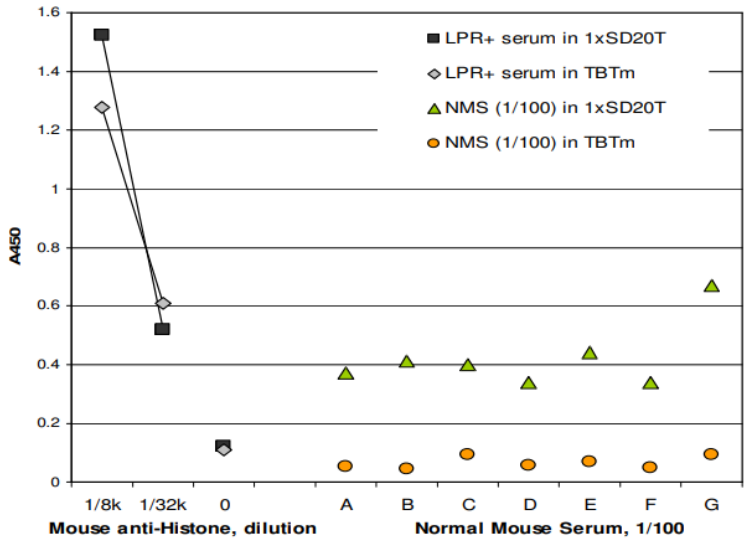
1. Pipettors and pipettes that deliver 100ul and 1-10ml. A multi-channel pipettor is recommended. 2. Disposable glass or plastic 5-15ml tubes for diluting samples and Anti-Mouse Ig HRP Concentrate. 3. Graduated cylinder to dilute Wash Concentrate; 0.2 to 1L. 4. Stock bottle to store diluted Wash Solution; 200ml to 1L. 5. Distilled or deionized water to dilute reagent concentrates. 6. Microwell plate reader at 450 nm wavelength.

Storage

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.

Specimen Collection And Preparation

The Low NSB Sample Diluent, TBTm, lowers NSB even more than does the 1×SD20TG Diluent, without diminishing true positive antibody signals, thus offering a greater discrimination between positives and non-



immune samples. **Sample Collection and Handling** Culture medium, 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. For other samples, including tissue culture media, clarify the sample by centrifugation and/or filtration prior to dilution in Sample Diluent. **Antibody Stability** 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): 10µl serum +40µl WSD [or 0.1ml+0.4ml] Further(1/100):10µl initial(1/5) +190µl LNSD **Assay Design** Review Calculation of Results and Limits of the Assay before proceeding: 1. Select the proper sample dilutions. Account for expected potency of positives and minimize non-specific binding (NSB) and other matrix effects; for example, non-immune samples should give net signal <0.5 OD. This is usually 1/100 or greater dilution for mouse sera with normal levels of IgG and IgM. Dilute samples in Working Sample Diluent (1×SD20T) or in Low NSB Sample Diluent (TBTm) (see above). **Note: all samples must be diluted in the same diluent for proper comparison – either TBTm or 1×SD20T.** 2. 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. See Method A and B. 3. Run a set of Calibrators. Calibrators validate that the assay was performed to specifications, and can be used to normalize between assay variation for enhanced precision. Reading values off a Calibrator curve, Method C, has limitations. See Limitations. 4. 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. 5. 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 Preparation

1. 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. 2. Remove the appropriate number of microwell strips from the pouch and return unused strips to the pouch. Reseal the pouch and store refrigerated. 3. Add 200-300µl 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.

Reagent Preparation

1. Wash Solution Concentrate (100×): 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. 2. Sample Diluent Concentrate (20×): 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. 3. Anti-Mouse Ig-HRP Conjugate Concentrate (100×): Peroxidase conjugated anti-Mouse Ig in buffer with protein, detergents and antimicrobial as stabilizers. Dilute fresh as needed; 10µl of concentrate to 1ml of Working Sample Diluent is sufficient for one 8-well strip. Use within the working day and discard. Return 100× to 2-8°C storage.

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** Add 100µl of calibrators, samples and controls each to predetermined 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** Add 100µl of diluted Anti-Mouse Ig HRP to each well. Incubate for 30 minutes. Wash wells 5 times as in step 2. **3. Substrate Incubation** Add 100µl 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** Add 100µl 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.

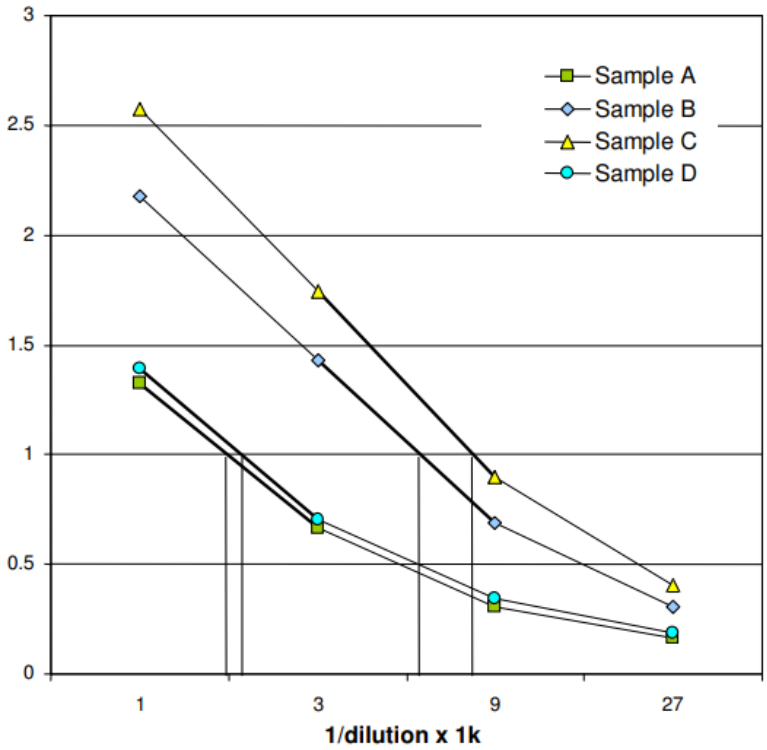
Calculation

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., 250 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/Nonimmune 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

	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**		

Activity level. Example: **Method C. Use of a Calibrator Curve** When the dilution curves of samples are parallel to the Calibrator curve (see Limits of the Assay), the Anti-histone activity units may be determined by interpolation from the Calibrator curve. The results may be calculated using any immunoassay software package. If software is not available, Anti-histone activity concentrations may be determined as follows: 1. Calculate the mean OD of duplicate samples. 2. On graph paper plot the mean OD of the calibrators (y-axis) against the concentration (U/ml) of Anti-histone (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. 3. The Anti-histone activity concentrations in unknown samples and controls can be determined by interpolation from the calibrator curve. 4. Multiply the values obtained for the samples by the dilution factor of each sample. 5. Samples producing signals higher than the 1000 U/ml calibrator should be further diluted and re-assayed. **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., 250 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 = Total Ig Antibody Activity Units Example: **1.0 OD** Index was used to determine titer of 4 antibodies.

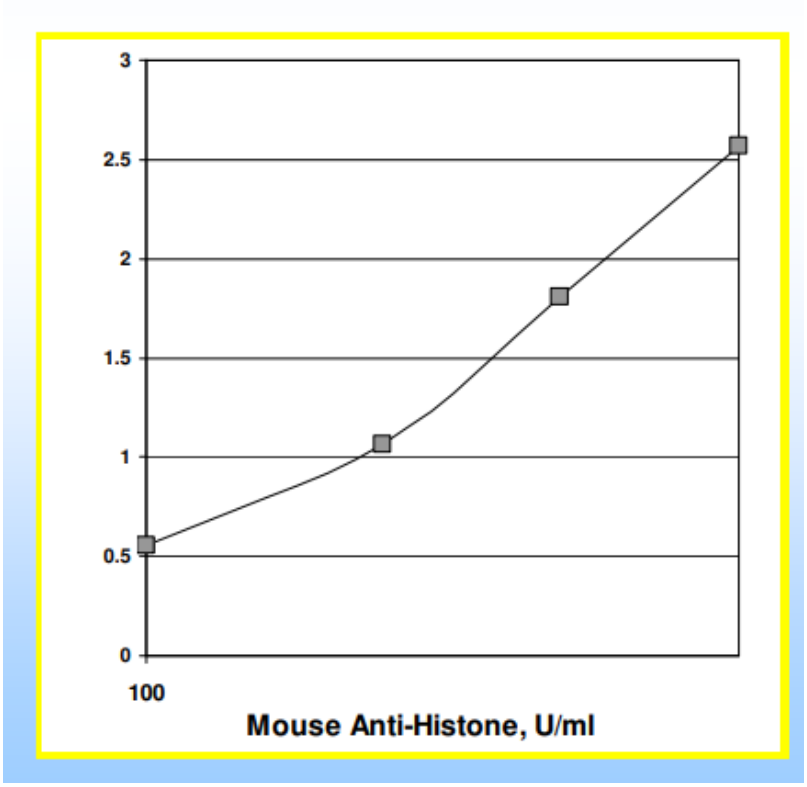


Titer Values
Sample A = 1.72 kU
Sample B = 5.70 kU
Sample C = 1.85 kU
Sample D = 7.90 kU

Typical Standard Curve

Wells	Calibrators		A450 nm
A1,2	**Negative Diluent Blank**		0.21
B1,2	100 U/ml	**Calibrator**	0.55
C1,2	250 U/ml	**Calibrator**	1.06
D1,2	500 U/ml	**Calibrator**	1.81
E1,2	1000 U/ml	**Calibrator**	2.56
F1,2	Sample	**1:100**	1.36

Sample Result: **295** U/ml x 100 dilution = **29.5** kU/ml



Specificity

Purified bovine histones are used to coat the microwells; thus the assay is specific for antibodies directed to histones. The anti-Mouse IgG+IgA+IgM (H+L) HRP conjugate reacts with mouse IgG, IgA and IgM class antibodies that bind to histone on the plate. IgE antibody would not be measured above background signals.

Limitations

Quantitation of Antibody in a Sample The ELISA measures Anti-SSA activity, a combination of antibody concentration and avidity for the SSA antigens. Antibodies with substantially different IgG concentrations may display similar Anti-SSA activities, due to differences in avidity. The quantitation or activity of the samples is, therefore, appropriately expressed in activity Units (titer), rather than mass units of IgG (e.g., ug/ml).
Calibrator Curve Quantitation To quantitate antibody activity from a calibrator curve (such as provided with the kit, Method C, the dilution curve of the samples must be parallel to the calibrator curve, to avoid different values being obtained from different regions of the curve. Antibodies that are not matched in Anti-SSA avidity will often have non-parallel dilution curves. In these cases, antibody activity is best expressed as Method A: ELISA value & dilution; Method B: a multiple of a Positive Index titer; or Method D: Anti-SSA titer from a sample dilution curve (see Calculation).