INTENDED USE

The Mouse anti-nRNP IgM ELISA Kit is an immunoassay for quantifying or titering IgM antibody activity specific for native ribonuclear protein (nRNP) in serum or plasma. Other biological fluids, including tissue culture medium, may be validated for use.

GENERAL INFORMATION

The **nRNP Complex** antigen is a non-histone nuclear protein composed of several polypeptides of differing molecular weights. RNP and Sm are distinct antigenic sites on the same nuclear molecular matrix consisting of RNA and eight polypeptides, including 70 kD, A(33 kD), C(22 kD) and the Sm peptides B (26 kD), B'(27 kD), D(13 kD), E/F (11 kD doublet), and G (9 kD). Autoantibodies against the nRNP antigen precipitate the U1a and U1b small nuclear RNAs. The nRNP antigen is involved in normal post transcriptional, pre-messenger RNA processing to excise introns. nRNP antigen is both RNase and tryptic digestion sensitive. Autoantibodies to the nRNP antigen have been strongly associated with Mixed Connective Tissue Disease (MCTD) in humans. Anti-nRNP antibodies only bind the U1 RNP while the anti-Sm binds with the U1 RNP, U2RNP, U4/6RNP, and U5RNP. Anti-RNP antibodies are often accompanied by anti-Sm antibodies. 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.

PRINCIPLE OF THE TEST

The Mouse anti-nRNP IgM ELISA kit is based on the binding of mouse anti-nRNP IgM in samples to nRNP immobilized on the microwells. Total anti-nRNP antibody is detected by anti-mouse IgM-HRP. After a washing step, HRP substrate (TMB) is added and color is developed which is directly proportional to the amount of anti-nRNP IgM present in the sample. Stop Solution is added to terminate the reaction and A450nm is then measured using an ELISA reader. Sample values are calculated relative to anti-nRNP calibrators.

PRODUCT SPECIFICATIONS

Specificity

RNP antigens used in this kit have been affinity purified using human anti-nRNP. Purified nRNP antigens are complex of Sm/RNPs. Therefore, antibodies to the complex will be detected. Antibodies to SS-A, SS-B, Scl70, and Jo-1 did not bind to the RNP/Sm complex. Thus the assay is specific for antibodies directed to nRNP complex. The anti-Mouse IgM-HRP conjugate reacts with mouse IgM; IgG, IgA, and IgE class antibodies would not be measured above background signals.

Assay Sensitivity

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

Calibrator Values

The calibrators are dilutions of antibody reactive to nRNP. Values are assigned as arbitrary anti-nRNP activity units (see Limits of the Assay).

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 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-Mouse IgM - HRP Conjugate Concentrate (100x) Part: H-MsM.611, 0.15ml	Peroxidase conjugated anti-Mouse IgM in buffer with protein, detergents, and antimicrobial as stabilizers. Dilute fresh as needed; 10ul of concentrate to 1 ml of **Working Sample Diluent** is sufficient for one 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
nRNP Microwell Strip Plate	5411	8-well strips (12)	Coated with nRNP and post-coated with stabilizers.
Anti-nRNP Calibrators			
50 U/ml 150 U/ml 400 U/ml 1000 U/ml	5412B 5412C 5412D 5412E	0.65 ml 0.65 ml 0.65 ml 0.65 ml	Four (4) vials, each containing anti-nRNP levels in arbitrary activity Units; diluted in buffer with protein, detergents, and antimicrobial as stabilizers.
Low NSB Sample Diluent Reduces non-specific binding	TBTm **Not** for HRP Conjugate dilution.	30 ml	Buffer with protein, detergents, and antimicrobial as stabilizers. Use as is for sample dilution. See **Assay Design**, page 3. **Not** for HRP Conjugate dilution.
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.
- Graduated cylinder to dilute Wash Concentrate; 0.2 to 1L.
- 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.

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. For other samples, clarify the sample by centrifugation and/or filtration prior to dilution in Sample Diluent. If samples will not be assayed immediately, store refrigerated for up to a few weeks or frozen for long-term storage.

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 (LSNB)**, which provides the lowest assay background, should be at least 5 times the initial dilution and performed the same day as the assay.

Example: Initial (1/5): 10ul serum + 40ul WSD
Further (1/50): 10ul initial (1/5) + 90ul LNSD (1/50)

Assay Design

Review Interpretation of Results and Limits of the Assay (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 **50 U/ml Calibrator**. This is usually 1/100 or greater dilution for mouse serum/plasma 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. Blank OD should be <0.3.
- Run a set of **Calibrators**, which validate that the assay was performed to specifications: **1000 U/ml** should give a high signal (>1.5 OD); **50 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 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, 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.

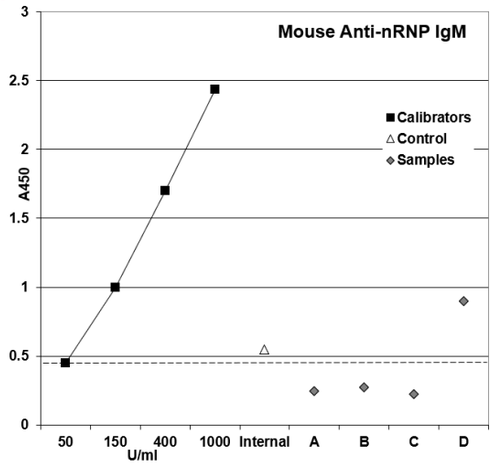
INTERPRETATION OF RESULTS

Method A. Antibody Activity Threshold Index

Compare Samples to **50 U/ml Calibrator** or **Internal Control**

= Positive/Negative Cut-off.

Example:



Results

The **sensitivity** of the assay to detect anti-nRNP IgM from either natural infection or vaccination is controlled so that the **50 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-nRNP antibody shows the OD range of the assay; high value indicates optimal sensitivity of the assay.

50 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.

Internal Control – a low level positive from an immunized animal that represents the investigator's experience in distinguishing low positive from negative samples. This should be run in each assay to supplement the **50 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 **50 U/ml Calibrator** can be used to calculate a **Threshold Index** that numerically discriminates Positive/Negative:

- Divide each Sample net OD by the **50 U/ml Calibrator** net OD. Values above 1.0 are a measure of **Positive** Antibody Activity; below 1.0 are **Negative** for antibody.

CALCULATION OF RESULTS (continued)

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

Example:

	Assay Net OD		Calculated Antibody Activity	
Sample	Control	Exptl	Control	Exptl
1	0.244	**C** 2.293	0.57	**5.34**
2	0.204	**C** 1.490	0.48	**3.47**
3	0.237	**C** 0.833	0.55	**1.94**
4	0.26	**C** 0.326	0.61	0.76
5	0.388	**P** 1.106	0.90	**2.58**
6	0.407	**I** 0.310	0.95	0.72
7	0.288	**E** 0.672	0.67	**1.56**
8	0.263	**E** 0.363	0.61	0.85
9	0.322	**E** 0.560	0.75	**1.31**
10	0.343	**E** 0.490	0.80	1.14
Mean	0.295			
SD	0.067			
Mean +2 SD	**0.429**	= **Positive Index**		

Results

Experimental Samples are represented as follows:

C – Calibrator
P – Positive Control
I – Internal Control; lab's threshold positive serum
E – Experimental sample

CALCULATION OF RESULTS (continued)

Method C. 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. 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**

Limits of the Assay

- Anti-nRNP antibody levels of an autoimmune-susceptible animal may be below detection threshold related to the time course of the condition, e.g., too early for positive titer development.

- **Calibrator Curve Quantitation** To quantitate antibody activity from a calibrator curve (such as provided with the kit), 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 nRNP avidity will often have non-parallel dilution curves. In these cases, antibody activity is best expressed as a titer relative to a reference positive such as the **400 U/ml Calibrator**, or another Calibrator in the kit (see Calculation of Results).

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 No. M-5420

Mouse Anti-nRNP IgM Antibodies

Cat. #5420, 96 tests

For Quantitation of Anti-Nuclear Ribonuclear Protein (nRNP) IgM in Serum

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ELISA Kit Components	Amount	Part
nRNP Coated Microwell Strip Plate	8-well strips (12)	5411
Mouse Anti-nRNP Calibrator 50 U/ml	0.65 ml	5412B
Mouse Anti-nRNP Calibrator 150 U/ml	0.65 ml	5412C
Mouse Anti-nRNP Calibrator 400 U/ml	0.65 ml	5412D
Mouse Anti-nRNP Calibrator 1000 U/ml	0.65 ml	5412E
Anti-Mouse IgM HRP Conjugate (100X)	0.15 ml	H-MsM.611
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-5420