

## INTENDED USE

The Monkey ANA (Anti-Nuclear Antibodies) IgM ELISA Kit is an immunoassay suitable for quantifying or titrating IgM antibody activity specific for extractable nuclear antigens (ENA) in serum or plasma. Other biological fluids, including tissue culture medium, may be validated for use. For research use only (RUO), not for diagnosis, cure or prevention of the disease.

## GENERAL INFORMATION

Antibodies reactive with autologous nuclear components, such as DNA and histones, can represent an autoimmune basis for pathological conditions such as systemic lupus erythematosus (SLE) in humans, and in mice homozygous for the lymphoproliferation spontaneous mutation (Fas<sup>lpr</sup>), a systemic autoimmunity with massive lymphadenopathy associated with proliferation of aberrant T cells, arthritis and immune complex glomerulonephritis. These conditions include elevated levels of anti-dsDNA and other anti-nuclear antigen (ANA) antibodies which often increase as the animal ages. Also, 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, monkeys and rats, 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 as underlying cause of the ANA type autoimmunity; these may be induced by drugs, including vaccines and adjuvants, with aging, or with other health conditions.

## PRINCIPLE OF THE TEST

The Monkey ANA IgM ELISA kit is based on the binding of monkey ANA IgM in samples to ANAs immobilized on the microwells, and ANA IgM antibody is detected by anti-monkey IgM-HRP conjugate. After a washing step, chromogenic substrate (TMB) is added and color (blue) is developed, which is directly proportional to the amount of ANA IgM present in the sample. Stopping Solution is added to terminate the reaction (converts blue to yellow), and A450nm is measured using an ELISA reader. The activity of monkey IgM antibody in samples is calculated relative to ANA Calibrators.

## PRODUCT SPECIFICATIONS

### Specificity

ENA used in the kit is a mixture of several nuclear autoantigens (dsDNA, SSA/Ro, SSB/La, Scl70, Sm, RNP, and Jo-1). The Monkey ANA test may detect antibodies directed to any of these. The anti-Monkey IgM HRP conjugate reacts with monkey IgM antibodies that bind to ENA on the plate; IgA, IgG and IgE class antibodies would not be measured above background signals. The anti-monkey IgM conjugate will react with IgM from rhesus, cynomolgous, and other primates. Assay kits are also available for quantitating ANA in mice, rats and humans.

### Positive Control

The Positive Control, monkey serum that produces a mid-level signal in the assay, is an indicator of proper assay performance and can be used to normalized interassay results.

## 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 ambient temperature until kit is used entirely.
<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 Diluent</b> and store at 2-8°C until the kit lot expires or is used up.
<b>Anti-Monkey IgM - HRP Conjugate Concentrate (100x)</b> Part H-MkG.181, 0.15ml	Peroxidase conjugated anti-Monkey IgM in buffer with protein, detergents and antimicrobial as stabilizers. Dilute fresh as needed; 10ul of concentrate to 1ml of <b>Working Sample Diluent</b> is sufficient for 1 8-well strip. Use within the working day and discard. Return 100X to 4°C storage.

Ready For Use: Store as indicated on labels.

Component	Part	Amt	Contents
<b>ENA Microwell Strip Plate</b>	5211	8-well strips (12)	Coated with ENA, and post-coated with stabilizers.
<b>ANA Calibrators</b>			
50 U/ml	5212B	0.65 ml	Four (4) vials, each containing ANA IgM levels in arbitrary activity Units; diluted in buffer with protein, detergents and antimicrobial as stabilizers.
200 U/ml	5212C	0.65 ml	
500 U/ml	5212D	0.65 ml	
1000 U/ml	5212E	0.65 ml	
<b>Low NSB Sample Diluent</b>	TBTm	30 ml	Buffer with protein, detergents and antimicrobial. Use as is for sample dilution. See <b>Assay Design</b> , page 3. <b>Not</b> for HRP Conjugate dilution.
Reduces non-specific binding			
<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. A multi-channel pipettor is recommended.
- Graduated cylinder to dilute Wash Concentrate; 0.2-1L.
- Distilled or deionized water to dilute reagent concentrates.
- Microwell plate reader at 450 nm wavelength.

## ASSAY DESIGN AND SET-UP

### Sample Collection and Handling

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. If samples will not be assayed immediately, store refrigerated for up to a few weeks, or frozen for long-term storage. Avoid freeze-thaw cycles.

### Sample Dilution & 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): 10ul serum + 40ul WSD [or 0.1ml + 0.4ml]  
Further (1/50): 10ul initial (1/5) + 90ul LNSD (1/50)

### Assay Design

Review Calculation of Results (p5-7) and Limits of the Assay (above) 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 <0.5 OD. This is usually 1/100 or greater dilution for monkey sera 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 (**See Method A,B**). Blank OD should be <0.3.
- Run a set of **Calibrators**. Calibrators validate that the assay was performed to specifications; results can be used to normalize between-assay variation for enhanced precision. Reading values off a Calibrator curve, **Method C**, has limitations. See Limits of the Assay (above).
- 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**.
- 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 and 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.

- 1st Incubation [100ul – 60 min; 4 washes]**
  - Add 100ul of sample diluent (blank), 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.
- 2nd Incubation [100ul – 30 min; 5 washes]**
  - Add 100ul of diluted Anti-Monkey IgM HRP to each well.
  - Incubate for 30 minutes.
  - Wash wells 5 times as in step 2.
- 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).
- 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.
- 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.

## LIMITATIONS OF THE ASSAY

### 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 ANA 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 100 U/ml Calibrator, or another Calibrator in the kit (see Calculation of Results).

## INTERPRETATION OF RESULTS

### Calculation of Results

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., 200 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/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 **quantifies** the positive Antibody Activity level.

### Example:

Sample	Assay Net OD		Calculated Antibody Activity	
	Control	Exptl	Control	Exptl
1	0.243	2.358	0.49	<b>4.79</b>
2	0.351	0.597	0.71	<b>1.21</b>
3	0.286	1.421	0.58	<b>2.89</b>
4	0.357	1.268	0.73	<b>2.58</b>
5	0.512	0.857	<b>1.04</b>	<b>1.74</b>
6	0.342	1.296	0.70	<b>2.63</b>
7	0.298	0.608	0.61	<b>1.24</b>
8	0.285	0.369	0.58	0.75
9	0.157	0.864	0.32	<b>1.76</b>
10	0.187	0.543	0.38	<b>1.10</b>
Mean	0.302			
SD	0.095			
Mean +2 SD	<b>0.492</b>			<b>= Positive Index</b>

## 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&category\\_id=2430&visit=10](http://4adi.com/commerce/info/showpage.jsp?page_id=1060&category_id=2430&visit=10)

## CALCULATION OF RESULTS (continued)

### Method C. Use of a Calibrator Curve

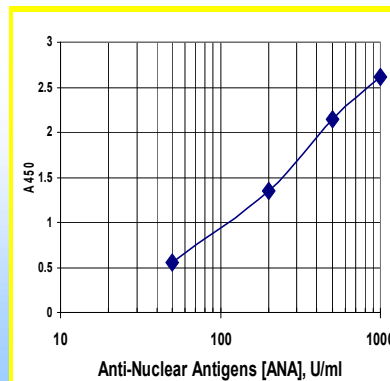
When the dilution curves of samples are parallel to the Calibrator curve (see Limits of the Assay), the ANA activity units may be determined by interpolation from the Calibrator curve. The results may be calculated using any immunoassay software package. If software is not available, ANA activity concentrations 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 ANA (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 ANA 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.

### Typical Results:

Wells	Calibrators & Samples	A450 nm
A1,2	Negative Diluent Blank	0.32
B1,2	50 U/ml Calibrator	0.55
C1,2	200 U/ml Calibrator	1.35
D1,2	500 U/ml Calibrator	2.14
E1,2	1000 U/ml Calibrator	2.62
F1,2	Positive Control	1.86

Positive IgM Control: 390 U/ml



### Calibrator Values

The calibrators are dilutions of antibody reactive to ENA. Values are assigned as arbitrary ANA activity units.

## CALCULATION OF RESULTS (continued)

### 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:

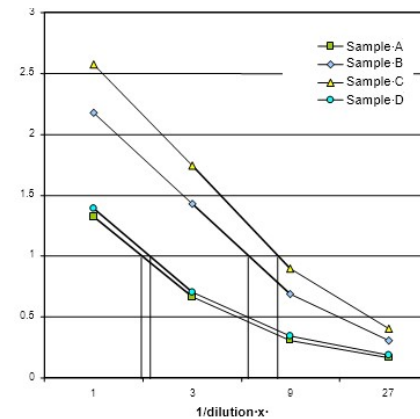
1. Use an OD value Index in the mid-range of the assay (2.0 – 0.5 OD); this provides the best sensitivity and reproducibility for comparing experimental groups and replicates. An arbitrary 1.0 OD is commonly used.
2. Prepare serial dilutions of each sample to provide a series that will produce signals 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., 200 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  
**= IgM Antibody Activity Units**

### Example:

II. A 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

### Assay Sensitivity

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

# Monkey Anti-Nuclear Antibodies (ANA) IgM ELISA Kit

Cat # 670-115-ANM, 96 Tests

For Quantitation of ANA IgM in Serum or plasma or other biological fluids

For research use only (RUO), not for diagnosis, cure or prevention of the disease.



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ELISA Kit Components	Amount	Part
ENA Coated Microwell Strip Plate	8-well strips (12)	5211
ANA Calibrator	50 U/ml	0.65 ml 5212B
ANA Calibrator	200 U/ml	0.65 ml 5212C
ANA Calibrator	500 U/ml	0.65 ml 5212D
ANA Calibrator	1000 U/ml	0.65 ml 5212E
Anti-Monkey IgM HRP Conjugate (100X)	0.15 ml	H-MkG.181
Sample Diluent Concentrate (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-670-110-ANM