For in vitro laboratory use only March 3, 2010 Ver. 2

# [Mouse anti-dsDNA-IgG2a ELISA Kit]

(Code No.:AKD2A-011)

**Please, read this instruction carefully before use.**

Contents



This kit is manufactured by Shibayagi Co., Ltd.
Use only the current version of Instruction Manual enclosed with the kit!

## 1. Intended use

Mouse anti-dsDNA-IgG2a ELISA Kit is a sandwich ELISA system for quantitative measurement of mouse anti-dsDNA-IgG2a.

### Features

(1) This is intended for research use only.

(2) Rapid assay (total reaction time: 2 h 30min.).

(3) This kit is for dsDNA-IgG2a in mouse serum or plasma (Do not use heparin).

(4) A small sample volume of serum or plasma (10 μl) is needed.

(5) Assay format is 96 wells.

(6) Standard dsDNA-IgG2a is derived from mouse.

(7) All reagents are provided in liquid form.

## 2. Storage and expiration

When the complete kit is stored at 2-8°C, the kit is stable until the expiration date shown on the label on the box.
Opened reagents should be used as soon as possible to avoid loss in optimal assay performance caused by storage environment.

## 3. Introduction

Autoantibodies against DNA are IgG type or IgM type reacting with natural double stranded DNA (dsDNA), single-stranded DNA (ssDNA), or both types. In human SLE (Systemic Lupus Erythematosus), anti-dsDNA-IgG are found with high incidence. In SLE blood anti-dsDNA titer is closely related to DNA-anti-DNA complex and low complement level, and serves as an important marker for the activity of the disease.

Experimental animal models with spontaneous autoimmune diseases similar to those in humans, and animals with artificially-induced inflammation have been used to elucidate the mechanism of autoimmune diseases and to search for potential new remedies. A representative model animal of spontaneous autoimmune diseases is MRL/lpr mouse. As MRL/lpr shows high incidence of lymph node tumor, nephritis, angitis, and arthritis, this animal strain is useful for studies of the mechanism of human autoimmune diseases including rheumatoid arthritis. Autoantibodies found in MRL/lpr serum are IgG type rheumatoid factor, IgM type rheumatoid factor, anti-ssDNA antibodies, anti-dsDNA antibodies, and anti-Sm antibody, etc. Anti-dsDNA-IgG2a has been reported to be the most abundant isotype in MRL/lpr mouse serum.

This kit enables quantification and comparison of IgG2a type anti-dsDNA autoantibody with a calibration curve using standard antibody preparation.

## 4. Assay principle

In Shibayagi's Mouse anti-dsDNA-IgG2a ELISA Kit, standards or diluted samples are incubated in antigen-coated plate to capture dsDNA-IgG2a. After a 1 hour incubation and washing, peroxidase-conjugated anti-dsDNA-IgG2s antibody is added and incubated for another 1 hour to bind with captured dsDNA-IgG2a. After washing, bound peroxidase-conjugated dsDNA-IgG2a is reacted with a chromogenic substrate reagent (TMB) for 30 minutes, and the reaction is stopped by addition of acidic solution, and absorbance of yellow product is measured spectrophotometrically at 450 nm. The absorbance is nearly proportional to dsDNA-IgG2a concentration. The standard curve is prepared by plotting absorbance against standard dsDNA-IgG2a concentrations. DsDNA-IgG2a concentrations in unknown samples are determined using this standard curve.

## 5. Precautions

- For professional use only. Beginners are advised to use this kit under the guidance of experienced person.
- Do not drink, eat or smoke in the areas where assays are carried out.
- In treating assay samples of animal origin, be careful for possible biohazards.
- This kit contains components of animal origin. These materials should be handled as potentially infectious.
- Be careful not to allow the reagent solutions of the kit to touch the skin, eyes and mucus membranes. Especially be careful for the reaction stopper because it is 1 M sulfuric acid. The reaction stopper and the substrate solution may cause skin/eyes irritation. In case of contact with these wash skin/eyes thoroughly with water and seek medical attention, when necessary.
- Avoid contact with the acidic Reaction stopper solution and Chromogenic substrate reagent containing hydrogen peroxide and tetramethylbenzidine (TMB). Wear gloves and eye and clothing protection when handling these reagents.
- The materials must not be pipetted by mouth.
- Unused samples and used tips should be rinsed in 1% formalin, 2% glutal aldehyde, or more than 0.1% sodium hypochlorite solution for more than 1 hour, or be treated by an autoclave before disposal.
- Dispose consumable materials and unused contents in accordance with applicable regional/national regulatory requirements.
- Use clean laboratory glassware.
- Samples with heated-decomplementation should not be used.
- Do not use heparin as anticoagulant.
- In order to avoid dryness of wells, contamination of foreign substances and evaporation of dispensed reagents, never forget to cover the well plate with a plate cover supplied, during incubation.
- ELISA can be easily affected by your laboratory environment. Room temperature should be at 20-25℃ strictly. Avoid airstream velocity over 0.4 m/sec. (including wind from air conditioner), and humidity less than 30%.

## 6. Technical tips

- In manual operation, proficiency in pipetting technique is recommended.
- The reagents are prepared to give accurate results only when used in combination within the same box. Therefore, do not combine the reagents from kits with different lot numbers. Even if the lot number is the same, it is best not to mix the reagents with those that have been preserved for some period.
- Be careful to avoid any contamination of assay samples and reagents. We recommend the use of disposal pipette tips.
- Optimally, the reagent solutions of the kit should be used immediately after reconstitution. Otherwise, store them in a dark place at 2-8 °C.
- Time the reaction from the pipetting of the reagent to the first well.
- Prepare a standard curve for each assay.
- Dilution of the assay sample must be carried out using the buffer solution provided in the kit.
- The chromogenic sucstrate reagent (TMB) should be almost colorless before use. It turns blue during reaction, and gives yellowish color after addition of reaction stopper. Greenish color means incomplete mixing.
- To avoid denaturation of the coated antibody, do not let the plate go dry.
- As the antibody-coated plate is module type of 8wells x 12 strips, each strip can be separated by cutting the cover sheet with a knife and used independently.
- When ELISA has to be done under the airstream velocity of over 0.4 m/sec. and the humidity of less than 30%,

completely close each well in addition to cover the well plate with a plate cover in each step of incubation.
Ex.) Cover the well plate with parafilm, and put the plate cover on it. Or place the well plate with the plate cover in an incubator, or in a styrofoam box. Take the best way depending on situation of each laboratory.

## 7. Reagents supplied

| Components | State | Amount |
|---|---|---|
| (A) Antigen-coated plate | Use after washing | 96 wells/1 plate |
| (B) anti-dsDNA-IgG2a Standard solution (10000 mU/ml) | Concentrated. Use after dilution | 600 µl/1 vial |
| (C) Buffer solution | Ready for use. | 60 ml/1 bottle |
| (D) Peroxitase-conjugated anti-IgG2a antibody | Concentrated. Use after dilution. | 200 µl/1 vial |
| (E) Chromogenic substrate reagent (TMB) | Ready for use. | 12 ml/1 bottle |
| (F) Reaction stopper (1M $H_2SO_4$) | Ready for use. | 12 ml/1 bottle |
| (G) Concentrated washing buffer (10x) | Concentrated. Use after dilution. | 100 ml/1 bottle |
| Plate cover | − | 1 plate |
| Instruction Manual | − | 1 copy |

## 8. Equipments or supplies required but not supplied

- Purified water (distilled water)
- Test tubes for preparation of standard solution series.
- Glassware for dilution of washing buffer (a graduated cylinder, a bottle)
- Pipettes (disposable tip type). One should be able to deliver 10 µl precisely, and another for 50-250 µl.
- Syringe-type repeating dispenser like Eppendorf multipette plus which can dispense 100 µl.
- Paper towel to remove washing buffer remaining in wells.
- A vortex-type mixer.
- A shaker for 96 well-plate (~800rpm)
- An automatic washer for 96 well-plate (if available), or a wash bottle with a jet nozzle.
- A 96 well-plate reader (450nm ±10nm, 620nm: 600-650nm)
- Software for data analysis, if available. Shibayagi is proposing the use of assay results calculation template for EXCEL. Please check our website (http://www.shibayagi.co.jp/en/tech_003.html).

## 9. Preparation of reagents

- ✧ Bring all reagents of the kit to room temperature before use.
- ✧ Prepare reagent solutions in appropriate volume for your assay. Do not store the diluted reagents.
- ✧ Do not use the reagent after expiration date.

### ● Reagents ready for use after return to room temperature

[Antigen-coated plate]

Storage and stability

If seal is not removed, put the strip back in a plastic bag with zip-seal originally used for well-plate container and store at 2-8 °C. The strip will be stable until expiration date.

[Buffer solution]

[Chromogenic substrate reagent]

Storage and stability

If not opened, store at 2-8 °C. It maintains stability until expiration date. Once opened, we recommend using as soon as possible to avoid influence by environmental condition.

[Reaction stopper (1 M $H_2SO_4$)]

Storage and stability

Close the stopper tightly and store at 2-8 ° C. It maintains stability until expiration date.

### ● Concentrated reagents

[Concentrated washing buffer (10x)]

Dilute 1 volume of the concentrated washing buffer (10x) to 10 volume with deionized water to prepare working solution. Example: 100 ml of concentrated washing buffer (10x) and 900ml of deionized water.

Storage and stability

The rest of undiluted buffer: if stored tightly closed at 2-8 °C, it is stable until expiration date.

Dispose any unused diluted buffer.

[Anti-dsDNA-IgG2a Standard solution (10000 mU/ml)]

Make a serial dilution of master standard solution to prepare each standard solution.

| Volume of standard solution | Buffer solution | Concentration (mU/ml) |
|---|---|---|
| Original solution | 0 µl | 10000 |
| Original solution 250 µl | 250 µl | 5000 |
| 5000mU/ml solution 250 µl | 250 µl | 2500 |
| 2500mU/ml solution 250 µl | 250 µl | 1250 |
| 1250mU/ml solution 250 µl | 250 µl | 625 |
| 625mU/ml solution 250 µl | 250 µl | 313 |
| 313mU/ml solution 250 µl | 250 µl | 156 |
| 0 (Blank) | 250 µl | 0 |

Storage and stability

Standard solutions prepared above should be used as soon as possible, and should not be stored.

The rest of original standard: if stored tightly closed at 2-8 °C, it is stable until expiration date.

[Peroxidase-conjugated anti-dsDNA-IgG2a antibody]

Prepare working solution by dilution of (D) with the buffer solution (C) to 1:100.

Storage and stability

Unused working solution (already diluted) should be disposed.

The rest of the undiluted solution: if stored tightly closed at 2-8 °C, it is stable until expiration date.

## 10. Preparation of samples

This kit is intended to measure dsDNA-IgG2a in mouse serum or plasma. Samples should be serum or plasma collected by established rule. Do not use heparin as anticoagulant. Heated-decomplementation samples are not appropriate. Dilute samples by buffer contained kit components so that they will be within the assay range (156-10000mU/ml). Recommended dilution for normal mouse samples are 10x. Samples should be immediately assayed or stored below –35 °C until assay. Dilution of a sample should be made in a test tube using buffer solution prior to adding them to wells. If there seem to be influence of interfering substances, dilute the same samples over 20x. Turbid samples or those containing insoluble materials should be centrifuged before testing to remove any particulate matter. Hemolytic and hyperlipemic serum samples are not suitable. If presence of interfering substance is suspected, examine by dilution test at more than 2 points.

Storage and stability

Use samples soon after collected. Collected samples can be stable within 1 week after collection if stored at 2-8°C. If you have to store assay samples for a longer period, snap-freeze samples and keep them below –35°C. Defrosted samples should be mixed thoroughly for best results. Avoid repeated freeze-thaw cycles.

## 11. Assay procedure

Remove the cover sheet of the antigen-coated plate after bringing up to room temperature.

(1) Wash the antigen-coated plate (A) by filling the wells with washing buffer and discard 3 times, then strike the plate upside-down onto several sheets of paper towel to remove residual buffer in the wells.
(2) Pipette 100 µl of properly diluted samples to the designated sample wells.
(3) Pipette 100µl of standard solution to the wells designated for standards.
(4) Shake the plate gently on a plate shaker (800rpm for 10 seconds x 3 times).
(5) Put a plate cover on the plate and incubate for 1 hour at room temperature (20-25°C).
(6) Discard the reaction mixture. Rinse wells by filling the wells with washing buffer and discard 3 times, then strike the plate upside-down onto several sheets of paper towel to remove residual buffer in the wells.
(7) Pipette 100µl of Peroxidase-conjugated anti-dsDNA-IgG2a antibody to all wells, and shake as step (4).
(8) Put a plate cover on the plate and incubate the plate for 1 hour at room temperature.
(9) Discard the reaction mixture. Rinse wells by filling the wells with washing buffer and discard 3 times, then strike the plate upside-down onto several sheets of paper towel to remove residual buffer in the wells.
(10) Pipette 100µl of Chromogenic substrate reagent to wells, and shake as step (4).
(11) Put a plate cover on the plate and incubate the plate for 30 minutes at room temperature.
(12) Add 100 µl of the reaction stopper to all wells and shake as step (4).
(13) Measure the absorbance of each well at 450 nm (reference wavelength, 620*nm) using a plate reader within 30 minutes.

Note: For manual washing procedure see “Kit operation (Power point)” or “Shibayagi’s Manual Operation” at http://www.shibayagi.co.jp/

*600-650nm can be used as reference wavelength. For washing of the plate, an automatic plate washer is

preferable, however, a washing bottle with a jet nozzle can be used. A syringe type repeating dispenser like Eppendorf's multipet plus set at 300 µl is also useful.
Standard of plate-washer pressure: 5-25ml/min. (Adjust it depending on the nozzle's diameter.) Be careful not to make the well-plate dry.

### Worksheet example

| | Strip 1&2 | Strip 3&4 | Strip 5&6 | Strip 7&8 | Strip 9&10 | Strip 11&12 |
|---|---|---|---|---|---|---|
| **A** | **10000 mU/ml** | Pos. Control | Sample 8 | Sample 16 | Sample 24 | Sample 32 |
| **B** | **5000 mU/ml** | Sample 1 | Sample 9 | Sample 17 | Sample 25 | Sample 33 |
| **C** | **2500 mU/ml** | Sample 2 | Sample 10 | Sample 18 | Sample 26 | Sample 34 |
| **D** | **1250 mU/ml** | Sample 3 | Sample 11 | Sample 19 | Sample 27 | Sample 35 |
| **E** | **625 mU/ml** | Sample 4 | Sample 12 | Sample 20 | Sample 28 | Sample 36 |
| **F** | **313 mU/ml** | Sample 5 | Sample 13 | Sample 21 | Sample 29 | Sample 37 |
| **G** | **156 mU/ml** | Sample 6 | Sample 14 | Sample 22 | Sample 30 | Sample 38 |
| **H** | **0** | Sample 7 | Sample 15 | Sample 23 | Sample 31 | Sample 39 |

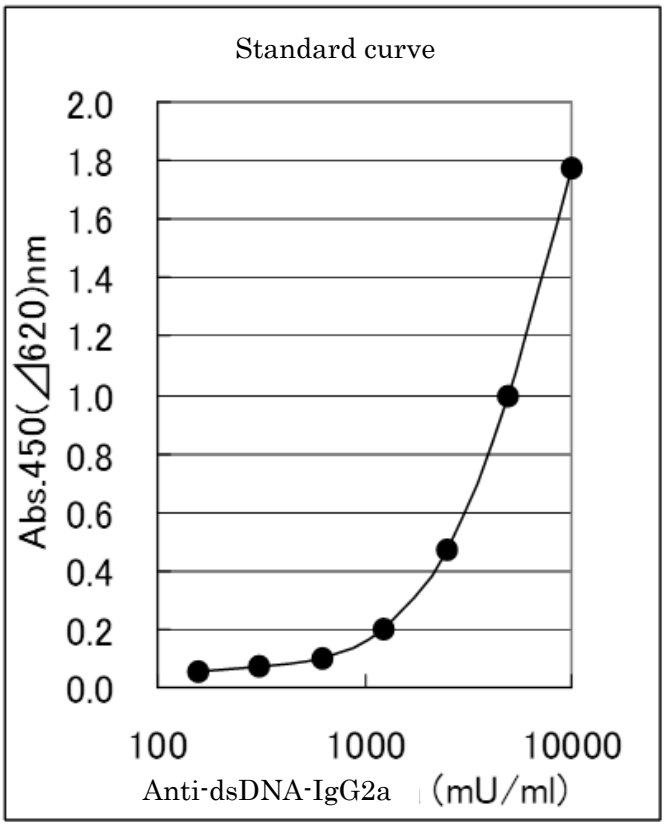
## 12. Calculations

(1) Prepare a standard curve by plotting standard concentration on X-axis and absorbance on Y-axis.
(Refer to our web site for more detailed explanation about standard curve. Shibayagi is offering a convenient Excel template. http://www.shibayagi.co.jp/en/tech_003.html)

(2) Using the standard curve, read the dsDNA-IgG2a concentration of a sample at its absorbance*, and multiply the assay value by dilution factor if the sample has been diluted. Though the assay range is wide enough, in case the absorbance of some samples is higher than that of the highest standard, please repeat the assay after proper dilution of samples with the buffer solution.

* We recommend the use of 3rd order regression curve for log-log plot, or 4 parameters method for log-normal plot in computer calculation.

Physiological or pathological situation of animals should be judged comprehensively taking other examination results into consideration.




**Mouse anti-dsDNA-IgG2a assay standard curve (an example)**
Absorbance may change due to assay environment.

## 13. Performance characteristics

- Assay range

The assay range of the kit is 156 mU/ml ~ 10000 mU/ml.

- Specificity

The antibodies used in this kit are specific to anti-dsDNA-igG2a.
This kit dose not cross-react with mouse IgG1, IgG2b, IgG3, and IgM.

- Precision of assay

Within assay variation (2 samples, 5 replicates assay,)
Mean CV is less than 5%.

- Reproducibility

Between assay variation (3 samples, 4 days, 2 replicates assay)
Mean CV is less than 5%

## 14. Trouble shooting

- Low absorbance in all wells

Possible explanations:

1) The standard or samples might not be added.
2) Reagents necessary for coloration might not be added.
3) Wrong reagents related to coloration might have been added or wrong dilution of the reagents.
4) Contamination of enzyme inhibitor(s).
5) Influence of the temperature under which the kits had been stored.
6) Excessive hard washing of the well plate.
7) Addition of chromogenic substrate reagent soon after taking out from a refrigerator might cause poor coloration owing to low temperature.

- Blank OD was higher that that of the lowest standard concentration (156 mU/ml).

Possible explanations:

Improper or inadequate washing. (Change washing frequency from 3 times to 4-6 times at the constant stroke after the reaction with Peroxidase-conjugated antibody.)

- High coefficient of variation (CV)

Possible explanation:

1) Improper or inadequate washing.
2) Improper mixing of standard or samples.
3) Pipetting at irregular intervals.

- Q-1: Can I divide the plate to use it for the other testing?

A-1: Yes, cut off the clear seal on the plate with cutter along strip. Put the residual plate, which is still the seal on, in a refrigerator soon

- Q-2: I found there contains liquid in 96 well-plate when I opened the box. What is it?

A-2: When we manufacture 96 well-plate, we insert preservation stabilizer in wells.

For detailed FAQS and explanations, refer to **"Trouble shooting and Important Points in Shibayagi's ELISA kits"** on our website (http://www.shibayagi.co.jp/en/tech_004.html).

## Summary of assay procedure

***First, read this instruction manual carefully and start your assay after confirmation of details.**

☐Bring the well-plate and all reagents back to room temperature.

☐ Concentrated washing buffer must be diluted to 10 times by purified water that returned to room temperature.

☐dsDNA-IgG2a Standard solution dilution example:

| Concentration (mU/ml) | 10000 | 5000 | 2500 | 1250 | 625 | 313 | 156 | 0 |
|---|---|---|---|---|---|---|---|---|
| Std. solution (µl)→ | Ori.Sol. | Ori.Sol.: 250* | 250* | 250* | 250* | 250* | 250* | 0 |
| Buffer solution (µl) | | 250 | 250 | 250 | 250 | 250 | 250 | 250 |

*One rank higher standard.

☐Prepare the positive sample.

| | |
|---|---|
| ☐ | **Antigen-coated plate** |
| ☐ | **↓Washing 3 times*** |
| ☐ | **Diluted samples, or Standards 100 µl** |
| ☐ | **↓ Shaking**, Incubation for 1 hour at room temp. (Standing***)** |
| ☐ | **Dilute peroxidase-conjugated anti-dsDNA-IgG2a antibody** to 100x with buffer returned to room temp. (This should be prepared during incubation.) |
| ☐ | **↓Washing 3 times*** |
| ☐ | **Peroxidase-conjugated anti-dsDNA-IgG2a antibody solution 100 µl** |
| ☐ | **↓ Shaking**, Incubation for 1 hour at room temp. (Standing***)** |
| ☐ | **↓Washing 3 times*** |
| ☐ | **Chromogenic substrate reagent (TMB) 100 µl** |
| ☐ | **↓ Shaking**, Incubation for 30 minutes at room temp. (Standing***)** |
| ☐ | **Reaction stopper (1M $H_2SO_4$) 100 µl** |
| ☐ | **↓Shaking**** |
| ☐ | **Measurement of absorbance (450nm, Ref 620nm) Use the value (abs.450nm－abs.620nm)** |

☐ : Use as a check box **Room temp: 20~25℃**

* Guideline of washing volume: 300µl/well for an automatic washer
Guideline of auto-washer's pressure: 5-25ml/min.
Be careful not to make the well-plate dry.

** Guideline of shaking: 800rpm for 10 seconds x 3 times. 600-650 nm can be used as reference wavelength.

***Put a plate cover on the plate while the reaction after shaking.

Assay worksheet

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | | | | | | | | | | | | |
| B | | | | | | | | | | | | |
| C | | | | | | | | | | | | |
| D | | | | | | | | | | | | |
| E | | | | | | | | | | | | |
| F | | | | | | | | | | | | |
| G | | | | | | | | | | | | |
| H | | | | | | | | | | | | |

[Storage condition] Store the kit at 2-8C (Do not freeze).
[Term of validity] 6 months from production (Expiration date is indicated on the container.)

This kit is manufactured by **Shibayagi Co., Ltd.**
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