For Research Use Only

December 17, 2012 Ver. 3

[Mouse IgG Rheumatoid Factor ELISA KIT]

(Code No.:AKRRG-101)

Please, read this instruction carefully before use.

This kit is manufactured by Shibayagi Co., Ltd.
Use only the current version of Instruction Manual enclosed with the kit! For the detailed assay procedure, refer to Key points for ELISA by movie on our website:
http://www.shibayagi.co.jp/index-E.htm

1. Intended use

Mouse IgG Rheumatoid factor ELISA Kit is a sandwich ELISA system for quantitative measurement of mouse IgG Rheumatoid factor antibody titer. This is intended for research use only.

2. Storage and expiration

When the intact kit is stored at 2-8°C, the kit is stable until the expiration date shown on the label on the box. Reagents, once opened, should be used as soon as possible to avoid losing its optimal assay performance by storage environment.

3. Introduction

Rheumatoid factors are autoantibodies against Fc region of of IgG and is found in 70-80% of patients suffering from chronic rheumatoid arthritis, and are considered to be closely related to its pathological syndrome.

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.

This kit enables quantification and comparison of IgG type rheumatoid factor with a calibration curve using standard antibody preparation.

4. Assay principle

In Shibayagi's Mouse IgG Rheumatoid factor ELISA Kit, standards or samples are incubated in monoclonal mouse rheumatoid factor antigen-coated wells to capture IgG Rheumatoid factor. After 2 hours incubation and washing, HRP (horse radish peroxidase)-labeled anti-mouse IgG is added and incubated for 2 hours together with captured anti-mouse IgG. After washing, HRP-complex remaining in wells is reacted with a chromogenic substrate (TMB) for 20 minutes, and 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 IgG Rheumatoid factor concentration. The standard curve is prepared by plotting absorbance against standard IgG Rheumatoid factor concentrations. IgG Rheumatoid bfactor 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.
- Wear gloves and laboratory coats when handling assay materials.
- 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 solution, which contains hydrogen peroxide and tetramethylbenzidine (TMB). Wear gloves and eye and clothing protection when handling these reagents.
- The materials must not be pipetted by mouth.
- Residual 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.
- 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 seal supplied, during incubation.
- ELISA can be easily affected by your laboratory environment. Room temperature should be at 20-25°C strictly. Avoid airstream velocity over 0.4 m/sec. ① (including wind from air conditioner), and humidity less than 30%. ①For airstream, refer to [Assay circumstance] on our web site.
- Do not use heat-inactivated samples.

6. Reagents supplied

Components	State	Amount
(A) Mouse Rheumatoid factor antigen-coated 96 well-plate	Use after washing	96 wells/1 plate
(B) Standard Mouse antibody (10,000 mU/ml)* (derived from mouse) *The value is different depending on the lot.	Concentrated. Use after dilution.	100 µl/1 vial
(C) Buffer solution	Ready for use.	60 ml/1 bottle
(D) HRP-labeled anti-mouse IgG	Concentrated. Use after dilution.	20 µl/1 vial
(E) Chromogenic substrate (TMB) solution	Ready for use.	12 ml/1 bottle
(F) Reaction stopper (1M H_2SO_4) Be careful!	Ready for use.	12 ml/1 bottle
(G) Concentrated washing buffer (10x)	Concentrated. Use after dilution.	100 ml/1 bottle
Plate seal	—	3 sheets
Instruction Manual	—	1 copy

7. Equipments required but not supplied ☐Use as a check box

☐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 5 µl precisely, and another for 50-500 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 (600-1200rpm)
☐An automatic washer for 96 well-plate (if available), or a wash bottle with a jet nozzle. (refer to our web movie [Washing of microplate])
☐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).

8. Preparation of reagents

◆Bring all reagents of the kit to room temperature (20-25 °C) before use.

◆Prepare reagent solutions in appropriate volume for your assay. Do not store the diluted reagents.

【Concentrated reagents】

[(B) Standard Mouse antibody (10,000 mU/ml)]

Make a serial dilution of original standard solution to prepare each standard solution. Example is shown below.

Volume of standard solution	Buffer solution	Concentration(mU/ml)
Original solution : 50 µl	450 µl	1000
1000 mU/ml solution : 250 µl	250 µl	500
500 mU/ml solution : 250 µl	250 µl	250
250 mU/ml solution : 250 µl	250 µl	125
125 mU/ml solution : 250 µl	250 µl	62.5
62.5 mU/ml solution : 250 µl	250 µl	31.3
31.3 mU/ml solution : 250 µl	250 µl	15.6
0 (Blank)	250 µl	0

[(D) HRP-labeled anti-mouse IgG]

Prepare working solution by dilution of (D) with the buffer solution (C) to 1:2000.
2 steps-dilution is recommended.

[(G) 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】

[(A) Mouse Rheumatoid factor antigen-coated 96 well-plate]

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.

[(B) Standard Mouse antibody (10,000 mU/ml)]

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

[(C) Buffer solution] and [(E) Chromogenic substrate solution]

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

[(D) HRP-labeled anti-mouse IgG]

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

[(F) Reaction stopper (1 M H_2SO_4)]

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

[(G) Concentrated washing buffer (10x)]

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

9. Technical tips

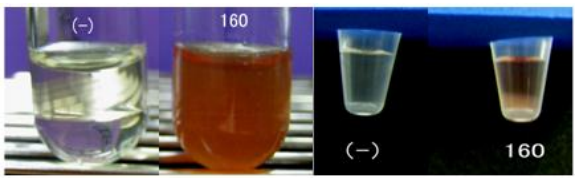
- In manual operation, proficiency in pipetting technique is recommended.
- The reagents are prepared to give accurate results only when used in combination within the same box. Therefore, do not combine the reagents from kits with different lot numbers. Even if the lot number is the same, it is best 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, and 1 tip for 1 well.
- 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 substrate (TMB) solution 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 96 well-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 over 0.4 m/sec. and the humidity less than 30%, seal the well plate with a plate seal and place the well plate in an incubator or a styrofoam box in each step of incubation. For more details, watch our web movie [Assay circumstance].

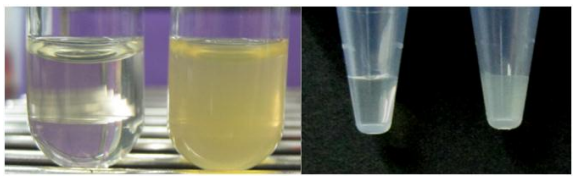
10. Preparation of samples

This kit is intended to measure anti-IgG Rheumatoid factor in mouse serum or plasma. The necessary sample volume for the standard procedure is 5 µl. Do not use heat-inactivated samples. Dilute samples using the kit's buffer so that to be within the assay range (15.6-1,000 mU/ml). **Recommended dilution rates are 51x, 101x and 201x.**

Hemolytic and hyperlipemic samples are not suitable.

* To avoid influence of blood (high lipid or hemolysis, etc.), if your original samples have heavy chyle or hemolysis as the pictures below, do not use them for assay. Abnormal value might be obtained with hemolysis above 160mg/dL with this kit.





Normal	Hemolysis 160mg/dL	normal	Hemolysis 160mg/dL	Normal	Chyle Highly lipid sample	Normal	Chyle Highly lipid sample

If presence of interfering substance is suspected, dilute the sample more than 100x for assay, and examine by dilution test at more than 2 points. Turbid samples or those containing insoluble materials should be centrifuged before testing to remove any particulate matter. Sample dilution should be carried out with the buffer solution of the kit using small test tubes such as PP, PE or glass, before assay.

Storage and stability

Sample is stable at 2-8°C within a week. If you have to store assay samples for a longer period, snap-freeze samples and keep them below –35°C. Avoid repeated freezing and thawing cycles.

11. Assay procedure

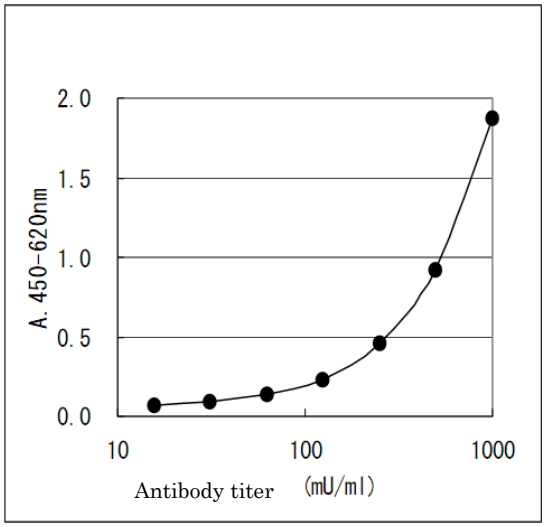
Remove the cover sheet of the 96 well-plate after bringing up to room temperature.

(1) Wash the Mouse Rheumatoid factor antigen-coated 96 well-plate (A) by filling the well with washing buffer and discard 3 times(*②), then strike the plate upside-down onto several layers of paper towels to remove residual buffer in the wells.
(2) Pipette 100 µl of standards or dilutes samples to the wells designated for each.
(3) Shake the plate gently on a plate shaker (*③).
(4) Stick a plate seal (*④) on the plate and incubate for 2 hours at 20-25°C.
(5) Discard the reaction mixture and rinse wells as step (1).
(6) Pipette 100 µl of HRP-labeled anti-mouse IgG solution (D) to all wells, and shake as step (3).
(7) Stick a plate seal (*④) on the plate and incubate the plate for 2 hours at 20-25°C.
(8) Discard the reaction mixture. Rinse wells as step (1).
(9) Pipette 100 µl of chromogenic substrate solution (E) to wells, and shake as step (3).
(10) Stick a plate seal (*④) on the plate and incubate the plate for 20 minutes at 20-25°C.
(11) Add 100 µl of the reaction stopper (F) to all wells and shake as step (3).
(12) Measure the absorbance of each well at 450 nm (reference wavelength, 620*nm) using a plate reader within 30 minutes.

*Refer to the page 7 for notes of *②, *③ and *④.

12. Calculations

(1) Prepare a standard curve using semi-logarithmic or two-way logarithmic section paper by plotting absorbance* (Y-axis) against IgG Rheumatoid factor concentration (mU/ml) on X-axis. Physiological or pathological situation of animals should be judged comprehensively taking other examination results into consideration.
(2) Using the standard curve, read the IgG Rheumatoid factor concentration of a sample at its absorbance*, and multiply the assay value by dilution factor. 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.



Mouse IgG Rheumatoid factor assay standard curve (an example)
Absorbance may change due to assay situation.

13. Performance characteristics

- Assay range

The assay range of the kit is 15.6 ~ 1,000 mU/ml.

- Specificity

The HRP-labeled anti-mouse IgG of this kit is specific to anti-mouse IgG Rheumatoid factor. The cross-reactivity with anti-mouse IgM is less than the detection limit.

- Precision of assay

Within assay variation (N=30), the mean CV was 6.9 %.

- Reproducibility

Between assay variation (N=30, 3 days), the mean CV was 8.7 %

14. Reference assay data

A novel assay kits for autoantibodies rate on spontaneous autoimmune model mice.
Kikukawa, T, Kojima, M., and Abe, C.
Jap J Inflammation 20: 697-701, 2000

15. Trouble shooting

- Low absorbance in all wells

Possible explanations:

1) The standard or samples might not be added.
2) Reagents necessary for coloration such as HRP-labeled anti-mouse IgG antibody or TMB might not be added.
3) Wrong reagents related to coloration might have been added. Wrong dilution of HRP-labeled anti-mouse IgG antibody.
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 TMB solution soon after taking out from a refrigerator might cause poor coloration owing to low temperature.

- Blank OD was higher than that of the lowest standard concentration (15.6 mU/ml).

Possible explanations:
Improper or inadequate washing. (Change washing frequency from 4 times to 5-8 times at the constant stroke after the reaction with HRP- labeled anti-mouse IgG 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 ☐ : Use as a check box

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

For more details, watch our web movie [ELISA by MOVIE] on our website.

☐Bring the well-plate and all reagents to 20-25°C for 2 hours.

☐Concentrated washing buffer must be diluted to 10 times by purified water.

☐Standard solution dilution example:

Concentration (mU/ml)		1,000	500	250	125	62.5	31.3	15.6	0
Std. solution (µl)	orig.sol.	50	250*	250*	250*	250*	250*	250*	0
Buffer solution (µl)		450	250	250	250	250	250	250	250

*One rank higher standard.

			Precautions & related info
☐	Mouse Rheumatoid factor antigen-coated 96 well-plate		
☐	↓ Washing 3 times(*②)		*⑥
☐	Diluted Samples / Standards	100 µl	*⑦ [Handling of pipetting]
☐	↓ Shaking(*③), Incubation for 2 hours at 20-25°C (Standing***)		*⑧ [Assay circumstance]
☐	Dilute HRP-labeled anti-mouse IgG (D) to 2,000x with Buffer (C) returned to 20-25°C.		2-step dilution is recommended.
☐	↓ Washing 3 times(*②)		*⑥
☐	HRP-labeled anti-mouse IgG	100 µl	*⑦ [Handling of pipetting]
☐	↓ Shaking(*③), Incubation for 2 hours at 20-25°C (Standing(*④))		*⑧ [Assay circumstance]
☐	↓ Washing 3 times(*②)		*⑥
☐	Chromogenic substrate (TMB)	100 µl	After dispense, the color turns to blue depending on the concentration.
☐	↓ Shaking(*③), Incubation for 20 min at 20-25°C (Standing(*④))		*⑧ [Assay circumstance]
☐	Reaction stopper (1M H_2SO_4)	100 µl	After dispense, the color turns to yellow depending on the concentration.
☐	↓ Shaking(*③)		Immediately shake.
☐	Measurement of absorbance (450nm, Ref 620nm(*⑤))		Ref. wave cancels the dirt in the back of plate.

*②After dispensing wash buffer to wells, lightly shake the plate on your palm for 10 sec and remove the buffer. Guideline of washing volume: 300µl/well for an automatic washer and for a pipette if the washing buffer is added by pipette. In case of washing by using 8 channel pipette, sometimes the back ground tends to be high. If so, change washing frequency from 3 times to 4-6 times at the constant stroke after the reaction with HRP conjugated streptavidin.
Standard of plate-washing pressure: 5-25ml/min. (Adjust it depending on the nozzle's diameter.) Refer to our web movie [Washing of microplate].

*③Guideline of shaking: 600-1,200rpm for 10 seconds x 3 times.

*④Seal the plate during the reaction after shaking. Peel off the protective paper from the seal and stick the seal on the plate. Do not reuse the plate seal used once.

*⑤600-650 nm can be used as reference wavelength.

*⑥After removal of wash buffer, immediately dispense the next reagent.

*⑦Refer to our web movie [Handling of pipetting].

*⑧Refer to our web movie [Assay circumstance].

Worksheet example

	Strip 1&2	Strip 3&4	Strip 5&6	Strip 7&8	Strip 9&10	Strip 11&12
A	1000 mU/ml	Sample 1	Sample 9	Sample 17	Sample 25	Sample 33
B	500 mU/ml	Sample 2	Sample 10	Sample 18	Sample 26	Sample 34
C	250 mU/ml	Sample 3	Sample 11	Sample 19	Sample 27	Sample 35
D	125 mU/ml	Sample 4	Sample 12	Sample 20	Sample 28	Sample 36
E	62.5 mU/ml	Sample 5	Sample 13	Sample 21	Sample 29	Sample 37
F	31.3 mU/ml	Sample 6	Sample 14	Sample 22	Sample 30	Sample 38
G	15.6 mU/ml	Sample 7	Sample 15	Sample 23	Sample 31	Sample 39
H	0	Sample 8	Sample 16	Sample 24	Sample 32	Sample 40

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 2-8°C (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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