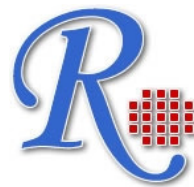


RayBio® Human IgG ELISA Kit

**User Manual
(Revised Mar 1, 2012)**

**RayBio® Human IgG ELISA
Kit Protocol**

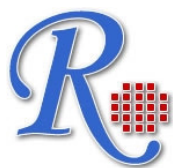
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**RayBio® Human IgG
ELISA Kit Protocol**

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I. INTRODUCTION

The human immune system consists of two functional components classified as the innate system (the physical, biochemical and cellular barriers), and the adaptive immune system (including lymphocytes and immunoglobulins). Immunoglobulins are the key elements of the humoral immune response in vertebrate against parasitic invasion. The polypeptide chains of immunoglobulins composed of two identical heavy (H) chains and two identical light (L) chains linked together by inter-chain disulfide bonds. While the amino-terminal portions that exhibits highly variable amino-acid composition are involved in antigen binding, the C terminal constant parts are involved in complement binding, placental passage and binding to cell membranes. Based upon the variation of the constant region of the heavy chain, nine immunoglobulin heavy chain isotypes are found in humans: IgA (with subclasses IgA1 and IgA2), IgD, IgE, IgM, and IgG (with subclasses IgG1, IgG2, IgG3, and IgG4). IgG is the predominant immunoglobulin in the serum, which accounts for 75% of the total serum antibody of healthy individuals. IgG has a molecular weight of about 150 kDa. Four distinct subgroups of human IgG (IgG1, IgG2, IgG3, and IgG4) were first demonstrated in the 1960's by using polyclonal antisera prepared in animals immunized with human myeloma proteins.

The RayBio® Human IgG ELISA (Enzyme-Linked Immunosorbent Assay) kit is an *in vitro* enzyme-linked immunosorbent assay for the quantitative measurement of human IgG in serum and plasma. This assay employs an antibody specific for human IgG coated on a 96-well plate. Standards and samples are pipetted into the wells and IgG present in a sample is bound to the wells by the immobilized antibody. The wells are washed and biotinylated anti-human IgG antibody is added. After washing away unbound biotinylated antibody, HRP-conjugated streptavidin is pipetted to the wells. The wells are again washed, a TMB substrate solution is added to the wells and color develops in proportion to the amount of IgG bound. The Stop Solution changes the color from blue to yellow, and the intensity of the color is measured at 450 nm.

II. REAGENTS

1. IgG Microplate (Item A): 96 wells (12 strips x 8 wells) coated with anti-human IgG.
2. Wash Buffer Concentrate (20x) (Item B): 25 ml of 20x concentrated solution.
3. Standards (Item C): 2 vials of human IgG.
4. Assay Diluent (Item E): 2 bottles of 15 ml 5x concentrated buffer. For Sample and reagent diluent.
5. Detection Antibody IgG (Item F): 2 vial of biotinylated anti-human IgG (each vial is enough to assay half microplate).
6. HRP-Streptavidin Concentrate (Item G): 200 μ l 4,000x concentrated HRP-conjugated streptavidin.
7. TMB One-Step Substrate Reagent (Item H): 12 ml of 3,3',5,5'-tetramethylbenzidine (TMB) in buffer solution.
8. Stop Solution (Item I): 8 ml of 0.2 M sulfuric acid.

III. STORAGE

May be stored for up to 6 months at 2° to 8°C from the date of shipment. Standard should be stored at -20°C or -80°C (recommended at -80°C) after reconstitution. Opened Microplate Wells or reagents may be store for up to 1 month at 2° to 8°C. Return unused wells to the pouch containing desiccant pack, reseal along entire edge.

Note: the kit can be used within one year if the whole kit is stored at -20°C. Avoid repeated freeze-thaw cycles.

IV. ADDITIONAL MATERIALS REQUIRED

- 1 Microplate reader capable of measuring absorbance at 450 nm.
- 2 Precision pipettes to deliver 2 μ l to 1 ml volumes.
- 3 Adjustable 1-25 ml pipettes for reagent preparation.
- 4 100 ml and 1 liter graduated cylinders.

- 5 Absorbent paper.
- 6 Distilled or deionized water.
- 7 Log-log graph paper or computer and software for ELISA data analysis.
- 8 Tubes to prepare standard or sample dilutions.

V. REAGENT PREPARATION

1. Bring all reagents and samples to room temperature (18 - 25°C) before use.
2. Sample dilution: 1x Assay Diluent (Item E) should be used for dilution of serum/plasma samples.

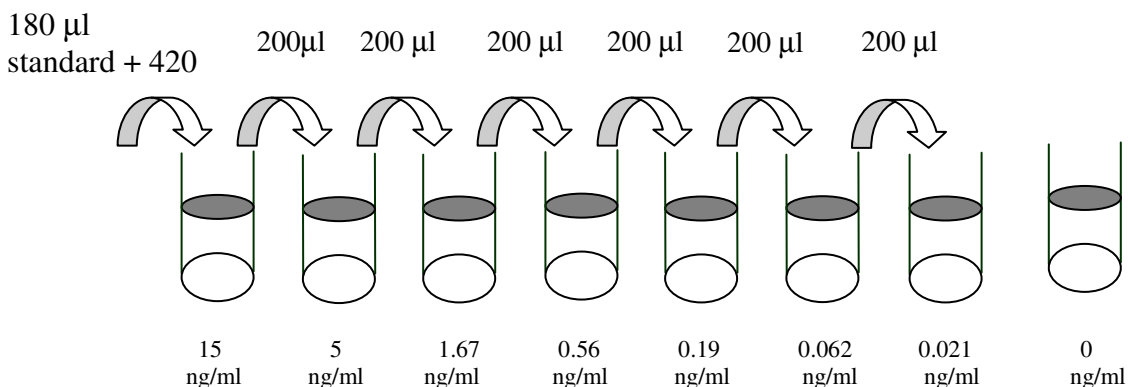
Suggested dilution for normal serum/plasma: 10,000,000 fold*.

For example, add 1 µl of serum/plasma into a tube with 99 µl 1x Assay Diluent B to prepare a 100-fold diluted sample. Mix through and then pipette 1 µl of prepared 100-fold diluted sample into a tube with 99 µl 1x Assay Diluent to prepare a 10,000 fold diluted sample . Mix through and then pipette 1 µl of prepared 10,000-fold diluted sample into a tube with 999 µl 1x Assay Diluent to prepare a final 10,000,000 fold diluted sample.

*Please note that levels of the target protein may vary between different specimens. Optimal dilution factors for each sample must be determined by the investigator.

3. 5x Assay Diluent (Item E) should be diluted 5-fold with deionized or distilled water before use.
4. Preparation of standard: **Briefly spin the vial of Item C** and then add 400 µl 1x Assay Diluent into Item C vial to prepare a 50 ng/ml standard solution. **Dissolve the powder thoroughly by a gentle mix.** Add 180 µl IgG standard (50 ng/ml) from the vial of Item C, into a tube with 420

μl 1x Assay Diluent to prepare a 15 ng/ml standard solution. Pipette 400 μl 1x Assay Diluent into each tube. Use the 15 ng/ml standard solution to produce a dilution series (shown below). Mix each tube thoroughly before the next transfer. 1x Assay Diluent serves as the zero standard (0 ng/ml).



5. If the Wash Concentrate (20x) (Item B) contains visible crystals, warm to room temperature and mix gently until dissolved. Dilute 20 ml of Wash Buffer Concentrate into deionized or distilled water to yield 400 ml of 1x Wash Buffer.
6. Briefly spin the Detection Antibody vial (Item F) before use. Add 100 μl of 1x Assay Diluent into the vial to prepare a detection antibody concentrate. Pipette up and down to mix gently (the concentrate can be stored at 4°C for 5 days). The detection antibody concentrate should be diluted 80-fold with 1x Assay Diluent and used in step 4 of Part VI Assay Procedure.
7. Briefly spin the HRP-Streptavidin concentrate vial (Item G) and pipette up and down to mix gently before use. HRP-Streptavidin concentrate should be diluted 4,000-fold with 1x Assay Diluent.

For example: Briefly spin the vial (Item G) and pipette up and down to mix gently . Add 3 μl of HRP-Streptavidin concentrate into a tube

with 12 ml 1x Assay Diluent to prepare a final 4,000 fold diluted HRP-Streptavidin solution (don't store the diluted solution for next day use). Mix well.

VI. ASSAY PROCEDURE:

1. Bring all reagents and samples to room temperature (18 - 25°C) before use. It is recommended that all standards and samples be run at least in duplicate.
2. Add 100 µl of each standard (see Reagent Preparation step 2) and sample into appropriate wells. Cover well and incubate for 2.5 hours at room temperature or over night at 4°C with gentle shaking.
3. Discard the solution and wash 4 times with 1x Wash Solution. Wash by filling each well with Wash Buffer (300 µl) using a multi-channel Pipette or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
4. Add 100 µl of 1x prepared biotinylated antibody (Reagent Preparation step 6) to each well. Incubate for 1 hour at room temperature with gentle shaking.
5. Discard the solution. Repeat the wash as in step 3.
6. Add 100 µl of prepared Streptavidin solution (see Reagent Preparation step 7) to each well. Incubate for 45 minutes at room temperature with gentle shaking.
7. Discard the solution. Repeat the wash as in step 3.

8. Add 100 μ l of TMB One-Step Substrate Reagent (Item H) to each well. Incubate for 30 minutes at room temperature in the dark with gentle shaking.
9. Add 50 μ l of Stop Solution (Item I) to each well. Read at 450 nm immediately.

VII. ASSAY PROCEDURE SUMMARY

1. Prepare all reagents, samples and standards as instructed.



2. Add 100 μ l standard or sample to each well.
Incubate 2.5 hours at room temperature or over night at 4°C.



3. Add 100 μ l prepared biotin antibody to each well.
Incubate 1 hour at room temperature.



4. Add 100 μ l prepared Streptavidin solution.
Incubate 45 minutes at room temperature.



5. Add 100 μ l TMB One-Step Substrate Reagent to each well.
Incubate 30 minutes at room temperature.



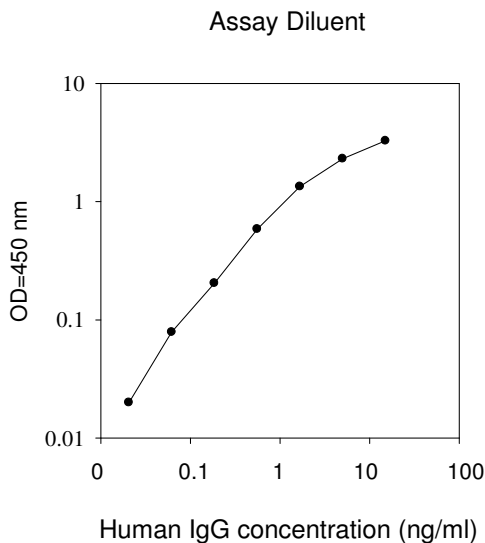
6. Add 50 μ l Stop Solution to each well.
Read at 450 nm immediately.

VIII. CALCULATION OF RESULTS

Calculate the mean absorbance for each set of duplicate standards, controls and samples, and subtract the average zero standard optical density. Plot the standard curve on log-log graph paper or using Sigma plot software, with standard concentration on the x-axis and absorbance on the y-axis. Draw the best-fit straight line through the standard points.

A. TYPICAL DATA

These standard curves are for demonstration only. A standard curve must be run with each assay.



B. SENSITIVITY

The minimum detectable dose of IgG is typically less than 150 pg/ml.

C. RECOVERY

Recovery was determined by spiking various levels of human Ig G into human serum or plasma. Mean recoveries are as follows:

Sample Type	Average % Recovery	Range (%)
Serum	116.1	105-123
Plasma	118.1	99-126

D. LINEARITY

Sample Type		Serum	Plasma
1:2	Average % of Expected	111.3	112.5
	Range (%)	101-122	104-121
1:4	Average % of Expected	113.2	114.4
	Range (%)	103-122	105-120

E. REPRODUCIBILITY

Intra-Assay: CV<10%

Inter-Assay: CV<12%

IX. REFERENCES:

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X. TROUBLESHOOTING GUIDE

Problem	Cause	Solution
1. Poor standard curve	<ol style="list-style-type: none"> 1. Inaccurate pipetting 2. Improper standard dilution 	<ol style="list-style-type: none"> 1. Check pipettes 2. Ensure a brief spin of Item C and dissolve the powder thoroughly by a gentle mix.
2. Low signal	<ol style="list-style-type: none"> 1. Too brief incubation times 2. Inadequate reagent volumes or improper dilution 	<ol style="list-style-type: none"> 1. Ensure sufficient incubation time; assay procedure step 2 may change to over night 2. Check pipettes and ensure correct preparation
3. Large CV	<ol style="list-style-type: none"> 1. Inaccurate pipetting 	<ol style="list-style-type: none"> 1. Check pipettes
4. High background	<ol style="list-style-type: none"> 1. Plate is insufficiently washed 2. Contaminated wash buffer 	<ol style="list-style-type: none"> 1. Review the manual for proper wash. If using a plate washer, check that all ports are unobstructed. 2. Make fresh wash buffer
5. Low sensitivity	<ol style="list-style-type: none"> 1. Improper storage of the ELISA kit 2. Stop solution 	<ol style="list-style-type: none"> 1. Store your standard at $<-20^{\circ}\text{C}$ after reconstitution, others at 4°C. Keep substrate solution protected from light 2. Stop solution should be added to each well before measure

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