Version 22-04/24

User's Manual

Human Therapeutic IgG4 ELISA Kit

REF **DEIA5037**

Σ **96T**

RUO

This product is for research use only and is not intended for diagnostic use.

For illustrative purposes only. To perform the assay the instructions for use provided with the kit have to be used.

Creative Diagnostics

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PRODUCT INFORMATION

Intended Use

CD's Human Therapeutic IgG4 ELISA Kit is an immunometric (i.e. sandwich) assay that can be used for the quantification of human IgG4 in monkey or rodent plasma and serum without prior sample purification.

General Description

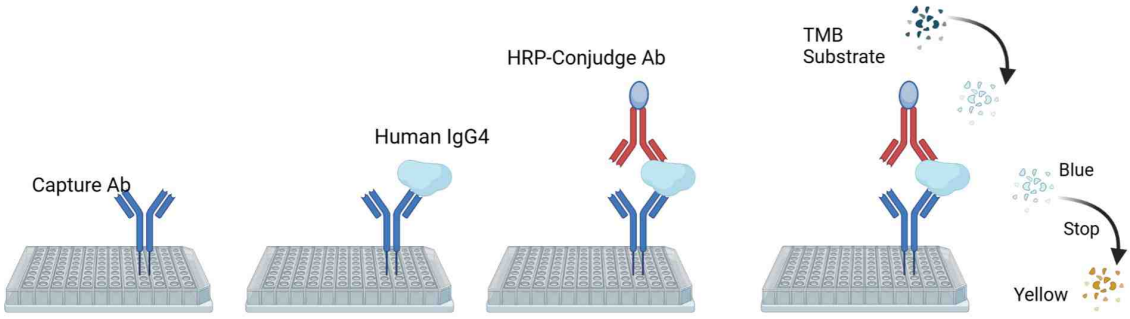
Human therapeutic antibodies have become increasingly common components of early drug discovery and development portfolios in the pharmaceutical and biotech industries. As part of the preclinical toxicology assessment of these agents, they are routinely tested in non-human primates, primarily in rhesus or cynomolgus monkeys. In order to assess the pharmacokinetics of the human antibodies in monkey serum, it is necessary for an assay to be capable of distinguishing the experimentally introduced human IgG from the endogenous monkey IgG. Historically, this has proven difficult due to the high degree of homology between these immunoglobulin species. CD's Human Therapeutic IgG4 ELISA Kit accurately measures human IgG in monkey serum for use in the pharmacokinetic analysis of therapeutic human antibodies.

Most clinically approved therapeutic human antibodies are of the IgG1, IgG2, and IgG4 isotypes. IgG1 is commonly used because of its effector functions: it binds with high affinity to Fc receptors on effector leukocytes and fixes complement. Thus, IgG1 mediates antibody-induced cellular cytotoxicity (ADCC) as well as complement-mediated cellular cytotoxicity. For some therapeutic applications, neither ADCC nor complement activation is desired, in which case the IgG2 or IgG4 isotypes are often employed. CD offers ELISA kits optimized specifically for the detection of human IgG1, human IgG2, or human IgG4.

Principles of Testing

This immunometric assay is based on a double-antibody "sandwich" technique. Each well of the microwell plate supplied with the kit has been coated with an antibody specific for human IgG. This antibody will bind any human IgG introduced into the well. A second antibody conjugated to horseradish peroxidase (HRP), which also recognizes human IgG, is added to the well forming a "sandwich". The "sandwich" is immobilized on the plate and the excess reagents are washed away. The concentration of human IgG is determined by measuring the enzymatic activity of HRP using the chromogenic substrate 3,3',5,5'-tetramethylbenzidine (TMB). After a sufficient period, the reaction is stopped with acid, forming a product with a distinct yellow color that can be measured at 450 nm. The intensity of the color is directly proportional to the amount of bound antibody-HRP conjugate, which is proportional to the concentration of human IgG4

$$\text{Absorbance} \propto [\text{Anti-human IgG HRP}] \propto [\text{IgG4}]$$



Reagents And Materials Provided

1. Anti-Human IgG4 Precoated 96-Well Strip Plate
2. Therapeutic IgG4 Assay HRP-Conjugate: 1 vial/0.75 ml
3. IgG4 (human) ELISA Standard
4. Immunoassay Buffer D Concentrate (5×): 4 vials/10 ml
5. Wash Buffer Concentrate (400×): 1 vial/5 ml
6. Polysorbate 20: 1 vial/3 ml
7. TMB Substrate Solution: 1 vial/12 ml
8. HRP Stop Solution: 1 vial/12 ml
9. Cover Sheet: 3 covers

Materials Required But Not Supplied

1. A plate reader capable of measuring absorbance at 450 nm.
2. An orbital microplate shaker.
3. Adjustable pipettes; multichannel or repeating pipettor recommended.
4. Materials used for Sample Preparation.
5. A source of pure water; glass distilled water or HPLC-grade water is acceptable.

Storage

This kit will perform as specified if stored as directed at 4°C and used before the expiration date indicated on the outside of the box.

Specimen Collection And Preparation

In general, monkey or rodent serum or plasma (prepared using heparin or EDTA as the anticoagulant) can be used directly in the assay following dilution in Assay Buffer (1×).

Plate Preparation

The 96-well plate(s) included with this kit is supplied ready to use. It is not necessary to rinse the plate(s) prior to adding the reagents. NOTE: If you do not need to use all of the strips at once, place the unused strips back in the plate packet and store according to the plate insert at 4°C. Be sure the packet is sealed with the desiccant inside.

Each plate or set of strips must contain an eight point standard curve run in duplicate. Each sample should be assayed at a minimum of two dilutions and each dilution should be assayed in duplicate. For statistical purposes, we recommend assaying samples in triplicate.

Reagent Preparation

Store all diluted buffers at 4°C; they will be stable for approximately two months. **NOTE:** It is normal for the concentrated buffer to contain crystalline salts after thawing. These will completely dissolve upon dilution with pure water.

1. **Assay Buffer Preparation**

Dilute the contents of one vial of Immunoassay Buffer D Concentrate (5×) with 40 ml of pure water. Be certain to rinse the vial to remove any salts that may have precipitated.

2. **Wash Buffer Preparation**

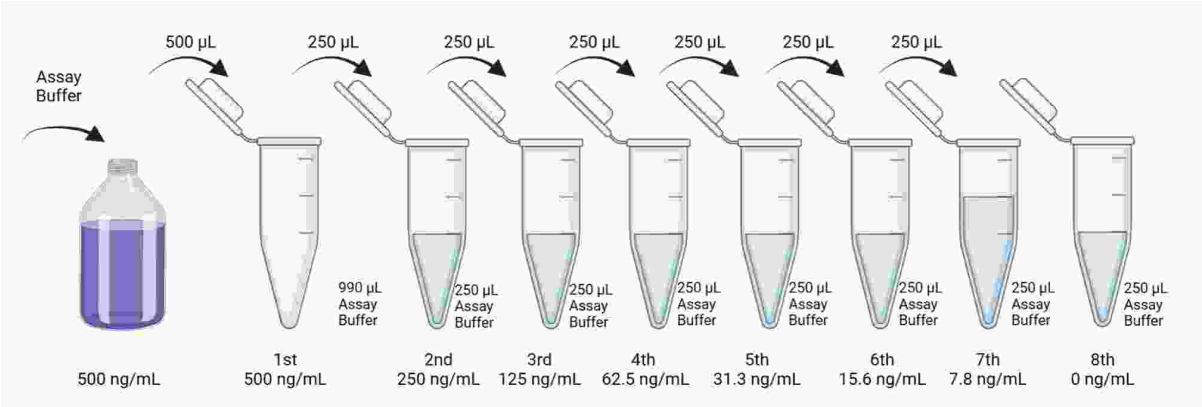
Dilute the contents of one vial of Wash Buffer Concentrate (400×) with pure water to a total volume of 2 L and add 1 ml of Polysorbate 20.

NOTE: Polysorbate 20 is a viscous liquid and cannot be measured by a regular pipette. A positive displacement pipette or a syringe should be used to deliver small quantities accurately.

3. **IgG4 (human) ELISA Standard**

Reconstitute the lyophilized purified IgG4 (human) ELISA Standard with 1.5 ml of Assay Buffer (1×) and mix gently. The concentration of this solution (the bulk standard) will be 500 ng/ml. The reconstituted standard will be stable for approximately two weeks when stored at 4°C.

To prepare the standard for use in the ELISA: Obtain eight clean test tubes and label them #1-8. Aliquot 250 µl of Assay Buffer (1×) to tubes #2-8. Transfer 500 µl of the bulk standard (500 ng/ml) to tube #1. Serially dilute the standard by removing 250 µl from tube #1 and placing into tube #2; mix gently. Next, remove 250 µl from tube #2 and place it into tube #3; mix gently. Repeat this process for tubes #4-7. Do not add any IgG4 to tube #8. This tube is the zero-point vial, the lowest point on the standard curve. These diluted standards should not be stored for more than four hours.



4. **Therapeutic IgG4 Assay HRP-Conjugate**

The Therapeutic IgG4 Assay HRP-Conjugate is supplied as a concentrated (20×) stock solution of goat anti-human IgG polyclonal antibody conjugated to HRP. Immediately before addition to the plate, prepare a 1× working solution by adding 0.6 ml of the concentrated conjugate to 11.4 ml Assay Buffer (1×) for a full plate (12 ml total) or 0.3 ml of the concentrated conjugate to 5.7 ml of Assay Buffer (1×) for a half plate (6 ml total).

Assay Procedure

Pipetting Hints

• Use different tips to pipette each reagent.

• Before pipetting each reagent, equilibrate the pipette tip in that reagent (i.e., slowly fill the tip and gently expel the contents, repeat several times).

• Do not expose the pipette tip to the reagent(s) already in the well.

Addition of Standards and Samples and First Incubation

1. Pipette 100 µl of the IgG4 (human) ELISA Standards or samples into the appropriate wells on the plate. Each sample should be assayed in duplicate, triplicate recommended.
2. Cover the plate with the 96-Well Cover Sheet and incubate for two hours at room temperature on an orbital shaker.

Addition of HRP-Conjugate and Second Incubation

1. Empty the wells and rinse four times with ~300 µl Wash Buffer (1×). After the last wash, gently tap the inverted plate on absorbent paper to remove the residual wash buffer.
2. Prepare a 1X working solution of the Therapeutic IgG4 Assay HRPConjugate as described in the Preparation of Assay-Specific Reagents section.
3. Add 100 µl of the HRP-Conjugate working solution to each well of the plate.
4. Cover the plate with the 96-Well Cover Sheet and incubate for one hour at room temperature on an orbital shaker.

Development of the Plate

1. Empty the wells and rinse four times with ~300 µl Wash Buffer (1×). After the last wash, gently tap the inverted plate on absorbent paper to remove the residual Wash Buffer.
2. Add 100 µl of TMB Substrate Solution to each well of the plate.
3. Cover the plate with the 96-Well Cover Sheet and incubate for 10 minutes at room temperature on an orbital shaker protected from light.
4. DO NOT WASH THE PLATE. Add 100 µl of HRP Stop Solution to each well of the plate. Blue wells should turn yellow and colorless wells should remain colorless. NOTE: The stop solution in this kit contains an acid. Wear appropriate protection and use caution when handling this solution.

Reading the Plate

1. Wipe the bottom of the plate with a clean tissue to remove fingerprints, dirt, etc.
2. Read the plate at a wavelength of 450 nm.

Calculation

Plotting the Standard Curve and Determining the Sample Concentration

Using computer reduction software, plot absorbance (linear y-axis) versus concentration (log x-axis) for standards (S1-S8) and fit the data with a fourparameter logistic equation. Using the equation of the line, calculate the concentration of analyte in each sample, making sure to correct for any sample dilution.

Typical Standard Curve

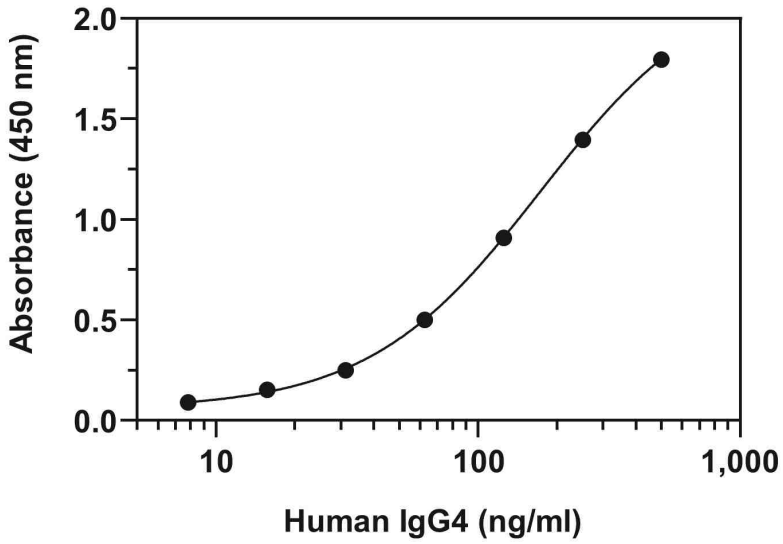
A plot of the absorbance values versus concentration of a series of wells containing various known amounts of analyte.

Representative Data

The standard curve presented here is an example of the data typically produced with this kit; however, your results will not be identical to these. You must run a new standard curve. Do not use the data below to determine the values of your samples. Your results could differ substantially. Development of the plate for 10 minutes typically results in an absorbance of >1.2 optical density (O.D.) units for the 500 ng/ml standard.

IgG4 Standards (ng/ml)	Absorbance	%CV* Intra-Assay Precision (in assay buffer)	%CV* Intra-Assay Precision (in 5% cynomolgus monkey serum)	%CV* Inter-Assay Precision (in assay buffer)	%CV* Inter-Assay Precision (in 5% cynomolgus monkey serum)
500	1.795	8.8	11.6	8.8	8.2
250	1.396	2.5	7.6	4.9	8.8
125	0.909	2.6	5.6	3.7	7.8
62.5	0.5	3.0	4.7	3.3	8.8
31.3	0.251	2.0	4.7	5.6	7.1
15.6	0.152	5.5	4.47	6.2	6.7
7.8	0.089	9.9	10.9	7.5	8.7

*%CV represents the variation in concentration (not absorbance) as determined using a reference standard curve



Precision

Intra-assay precision was determined by analyzing 16 replicates of two serum controls in a single assay.

*In 5% cynomolgus monkey serum

**In 10% cynomolgus monkey serum

Inter-assay precision was determined by analyzing replicates of two serum controls in three separate assays

on different days.

*In 5% cynomolgus monkey serum

**In 10% cynomolgus monkey serum

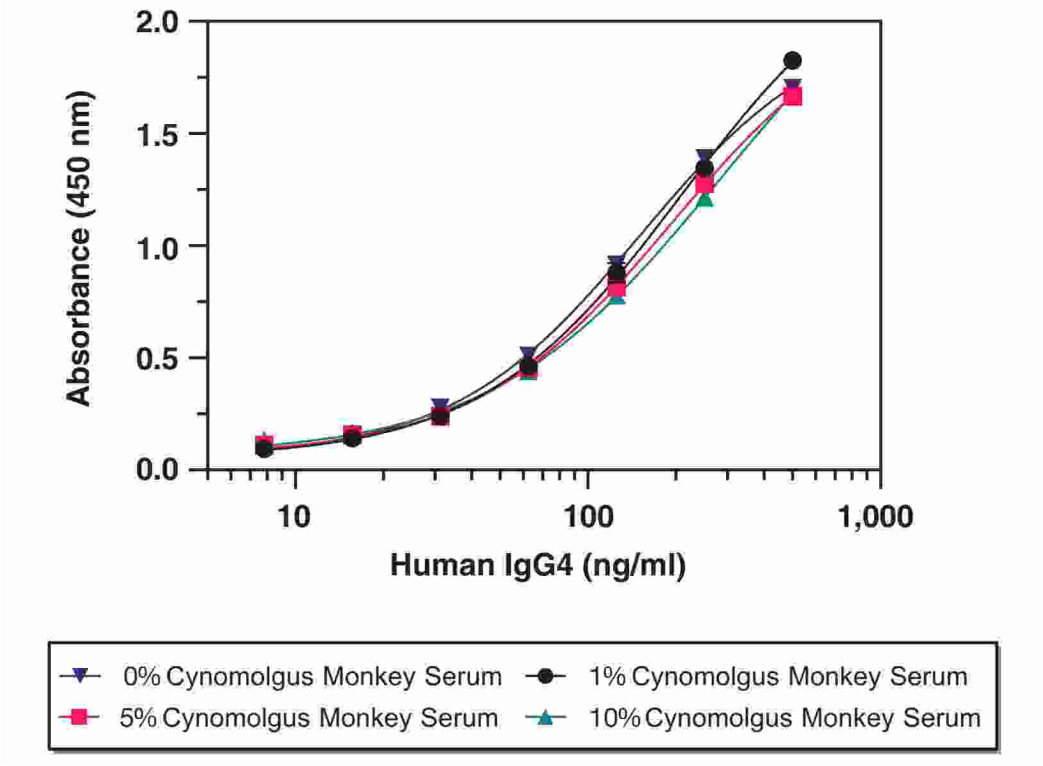
IgG4 (ng/ml)	Mean of O.D.	Standard Deviation (S.D.)	O.D. - (1.64 x S.D.)
500	1.760	0.069	1.648
250	1.381	0.077	1.255
125	0.929	0.046	0.854
62.5	0.536	0.026	0.493
31.3	0.294	0.018	0.264
15.6	0.170	0.011	0.151
7.8	0.107	0.010	0.090
0	0.054	0.007	0.065*

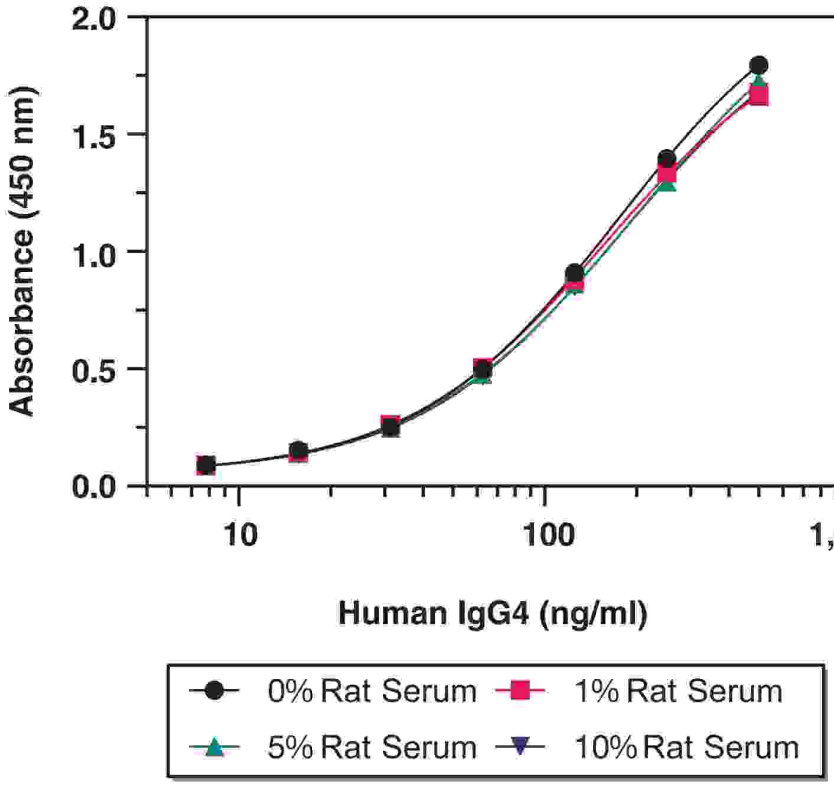
*O.D. + (1.64 x S.D.)

The lower limit of quantitation (LLOQ) is defined as the lowest standard concentration in which O.D. - (1.64 x S.D.) is higher than the blank value of O.D. + (1.64 x S.D.). The LLOQ is 7.8 ng/ml.

Matrix Validation:

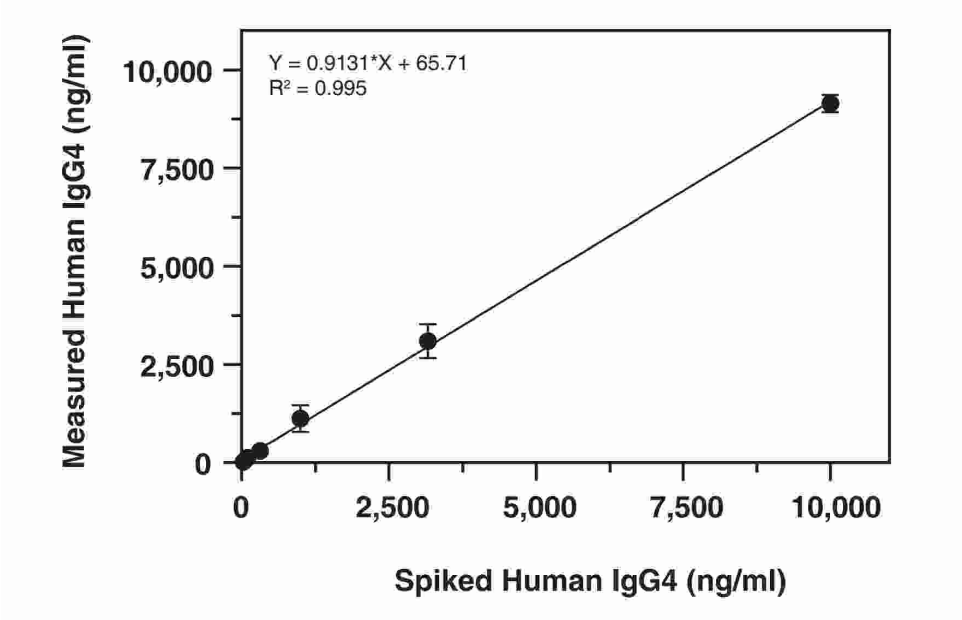
The Therapeutic Human IgG ELISA Kit, including the standard, does not contain serum of any species. The IgG4 standards have been analyzed in this assay in the presence of 1%, 5%, and 10% cynomolgus monkey and rat serum with no significant change in the characteristics of the assay (see Figure 5). When analyzing samples containing greater than 5% serum, we recommend diluting the standards in an equivalent serum concentration.





Spike Recovery:

Cynomolgus monkey serum was spiked with different amounts of human IgG4, serially diluted with Assay Buffer (1X) and evaluated using the Human Therapeutic IgG4 ELISA kit. The error bars represent standard deviations obtained from multiple dilutions of the same sample.



Detection Range

7.8-500 ng/ml

Detection Limit

Lower Limit of Quantification (LLOQ): 7.8 ng/ml

Lower Limit of Detection (LLOD): 1.1 ng/ml

Specificity

The assay has been validated in serum from cynomolgus monkey, rhesus monkey, mouse, and rat.

Linearity

Cynomolgus monkey serum was spiked with 10,000 ng/ml of human IgG4, serially diluted with Assay Buffer (1×), and evaluated for linearity using the Human Therapeutic IgG4 ELISA Kit. The results are shown in Table below.

Dilution	Measured Concentration (ng/ml)	Linearity (%)
Cynomologus Monkey Serum		
25	9302	100
50	8869	95
100	9042	97
200	9350	101

Precautions

1. Please read these instructions carefully before beginning this assay.
2. The reagents in this kit have been tested and formulated to work exclusively with CD's Human Therapeutic IgG4 ELISA Kit. This kit may not perform as described if any reagent or procedure is replaced or modified. The Stop Solution provided with this kit is an acid solution. Please wear appropriate personal protection equipment (e.g., safety glasses, gloves, and lab-coat) when using this material.

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