# Human C4d(Complement Fragment 4d) ELISA Kit

Catalog NO.: RE2615H

This manual must be read attentively and completely before using this product. If you have any problems, please contact our Technical Service Center for help.

Phone: +86-27-65316809
Email: service@reedbiotech.com
Website: www.reedbiotech.com

Please refer to specific expiry date on the label outside of box.

Please kindly provide us with the lot number (on the outside of the box) of the kit for more efficient service.

## Contents

Intended use.....3
Character.....3
Test principle.....4
Kit components & Storage.....5
Other supplies required.....6
Sample collection.....6
Note.....8
❊Note for kit.....8
❊Note for sample.....9
Dilution Method.....10
Reagent preparation.....10
Assay procedure.....13
Calculation of results.....14
Assay Procedure Summary.....15
Typical data.....16
Performance.....16
❊Precision.....16
❊Recovery.....17
❊Linearity.....17
Troubleshooting & Solutions.....18
Declaration.....19

## Intended use

This ELISA kit applies to the in vitro quantitative determination of Human C4d concentrations in serum, plasma and other biological fluids.

## Character

| | |
|---|---|
| Sensitivity | 0.38 ng/mL |
| Detection Range | 0.63-40ng/mL |
| Specificity | This kit recognizes Human C4d in samples. No significant cross-reactivity or interference between Human C4d and analogs was observed |
| Repeatability | Coefficient of variation is < 10% |

## Test principle

This ELISA kit uses the Sandwich-ELISA principle. The micro ELISA plate provided in this kit has been pre-coated with an antibody specific to Human C4d. Samples (or Standards) are added to the micro ELISA plate wells and combined with the specific antibody. Then a biotinylated detection antibody specific for Human C4d and Avidin-Horseradish Peroxidase (HRP) conjugate are added successively to each micro plate well and incubated. Free components are washed away. The substrate solution is added to each well. Only those wells that contain Human C4d, biotinylated detection antibody and Avidin-HRP conjugate will appear blue in color. The enzyme-substrate reaction is terminated by the addition of stop solution and the color turns yellow. The optical density (OD) is measured spectrophotometrically at a wavelength of 450 nm. The OD value is proportional to the concentration of Human C4d. You can calculate the concentration of Human C4d in the samples by comparing the OD of the samples to the standard curve.

## Kit components & Storage

An unopened kit can be stored at 2-8℃ for 6 months. If the opened kit is not used up, store the items separately according to the following conditions.

| Item | Specifications | Storage |
|---|---|---|
| Micro ELISA Plate (Dismountable) | 96T: 8 wells ×12 strips<br>48T: 8 wells ×6 strips<br>24T: 8 wells ×3 strips | -20℃, 6 months |
| Reference Standard | 96T: 2 vials<br>48T: 1 vial<br>24T: 1 vial | |
| Concentrated Biotinylated Detection Ab (100×) | 96T:1 vial, 120μL<br>48T:1 vial, 60μL<br>24T:1 vial, 60μL | |
| Concentrated HRP Conjugate (100×) | 96T:1 vial, 120μL<br>48T:1 vial, 60μL<br>24T:1 vial, 60μL | -20℃, 6 months |
| Reference Standard & Sample Diluent | 1 vial, 20 mL | 2-8℃, 6 months |
| Biotinylated Detection Ab Diluent | 1 vial, 13 mL | |
| HRP Conjugate Diluent | 1 vial, 13mL | |
| Concentrated Wash Buffer (25×) | 1 vial, 30 mL | |
| Substrate Reagent | 1 vial, 10 mL | 2-8℃ (Protect from light) |
| Stop Solution | 1 vial, 10 mL | 2-8℃, 6 months |

| | | |
|---|---|---|
| Plate Sealer | 5 pieces | RT |
| Product Description | 1 copy | |
| Certificate of Analysis | 1 copy | |

Note: All reagent bottle caps must be tightened to prevent evaporation and microbial pollution. The volume of reagents in partial shipments is a little more than the volume marked on the label, please use accurate measuring equipment instead of directly pouring into the vial(s).

## Other supplies required

1. Microplate reader with 450 nm wavelength filter
2. High-precision transfer pipette, EP tubes and disposable pipette tips
3. Incubator capable of maintaining 37℃
4. Deionized or distilled water
5. Absorbent paper
6. Loading slot

## Sample collection

(More detailed information please view our website: www.reedbiotech.com)

**1. Serum:** Allow samples to clot for 1 hour at room temperature or overnight at 2-8℃ before centrifugation for 20 min at 1000×g at 2-8℃. Collect the supernatant to carry out the assay.

**2. Plasma:** Collect plasma using EDTA-$Na_2$ as an anticoagulant. Centrifuge samples for 15 min at 1000×g at 2-8℃ within 30 min of collection. Collect the supernatant to carry out the assay.

**3. Tissue homogenates:** It is recommended to get detailed references from the literature before analyzing different tissue types. For general information, hemolyzed blood may affect the results, so the tissues should

be minced into small pieces and rinsed in ice-cold PBS (0.01M, pH=7.4) to remove excess blood thoroughly. Tissue pieces should be weighed and then homogenized in PBS (tissue weight (g): PBS (mL) volume=1:9) with a glass homogenizer on ice. To further break down the cells, you can sonicate the suspension with an ultrasonic cell disrupter or subject it to freeze-thaw cycles. The homogenates are then centrifuged for 5-10 min at 5000×g at 2-8℃ to get the supernatant.

**4. Cell lysates:** For adherent cells, gently wash the cells with moderate amount of pre-cooled PBS and dissociate the cells using trypsin. Collect the cell suspension into a centrifuge tube and centrifuge for 5 min at 1000×g. Discard the medium and wash the cells 3 times with pre-cooled PBS. For each $1\times10^6$ cells, add 150-250μL of pre-cooled PBS to keep the cells suspended. Repeat the freeze-thaw process several times or use an ultrasonic cell disrupter until the cells are fully lysed. Centrifuge for 10 min at 1500×g at 2-8℃. Remove the cell fragments, collect the supernatant to carry out the assay.

**5. Cell culture supernatant or other biological fluids:** Centrifuge samples for 20 min at 1000×g at 2-8℃. Collect the supernatant to carry out the assay.

**6. Recommended reagents for sample preparation:** 10×EDTA Anticoagulant, PMSF Protease Inhibitor, 0.25% Trypsin Solution.

# Note

## ■ Note for kit

1) For research use only. Not for therapeutic or diagnostic purposes.
2) Please wear lab coats, goggles and latex gloves for protection. Please perform the experiment following the national security protocols of biological laboratories, especially when detecting blood samples or other bodily fluids.
3) A freshly opened ELISA plate may appear a water like substance, which is normal and will not have any impact on the experimental results. Return the unused wells to the foil pouch and store according to the conditions suggested in the above table.
4) Do not reuse the reconstituted standard, biotinylated detection Ab working solution, concentrated HRP conjugate working solution. The unspent undiluted concentrated biotinylated detection Ab (100×) and other stock solutions should be stored according to the storage conditions in the above table.
5) The microplate reader should be able to be installed with a filter that can detect the wave length at 450 ±2 nm. The optical density should be within 0-3.5. Follow the Instructions of the Microplate Reader for set up and preheat it for 15 min before OD measurement.
6) Do not mix or substitute reagents with those from other lots or sources.
7) Change pipette tips in between adding of each standard level, between sample adding and between reagent adding. Also, use separate reservoirs for each reagent.
8) Do not use expired reagents.

## ■ Note for sample

1) Tubes for blood collection should be disposable and be non-endotoxin. Samples with high hemolysis or much lipid are not suitable for ELISA assay.

2) Samples should be assayed within 7 days when stored at 2- 8℃, otherwise samples must be divided up and stored at -20℃ (≤1 month) or -80℃ (≤3 months). Avoid repeated freeze thaw cycles. Prior to assay, the frozen samples should be slowly thawed and centrifuged to remove precipitates.

3) The detection range of the kit is not the same as the concentration range of the tested substance in the sample. If the concentration of tested substance is too high or too low, dilute or concentrate the sample appropriately.

4) If the sample type is not included in the manual, a preliminary experiment is suggested to verify the validity.

5) If a lysis buffer is used to prepare tissue homogenates or cell lysates, there is a possibility of causing a deviation due to the introduced chemical substance.

6) Some recombinant protein may not be detected due to a mismatching with the coated antibody or detection antibody.

## Dilution Method

Please predict the concentration range of the sample in advance. If your samples need to be diluted, please refer to the following dilution instructions:

For 100 folds dilution: One step dilution. Add 5μL sample to 495μL sample diluent to yield 100 folds dilution.

For 1000 folds dilution: Two step dilution. Add 5μL sample to 95μL

sample diluent to yield 20 folds dilution, then add 5μL 20 folds diluted sample to 245μL sample diluent, after this, the neat sample has been diluted at 1000 folds successfully.

For 100,000 folds dilution: Three step dilution. Add 5μL sample to 195μL sample diluent to yield 40 folds dilution, then add 5μL 40 folds diluted sample to 245μL sample diluent to yield 50 folds dilution, and finally add 5μL 2000 folds diluted sample to 245μL sample diluent, after this, the neat sample has been diluted at 100,000 folds successfully.

## Reagent preparation

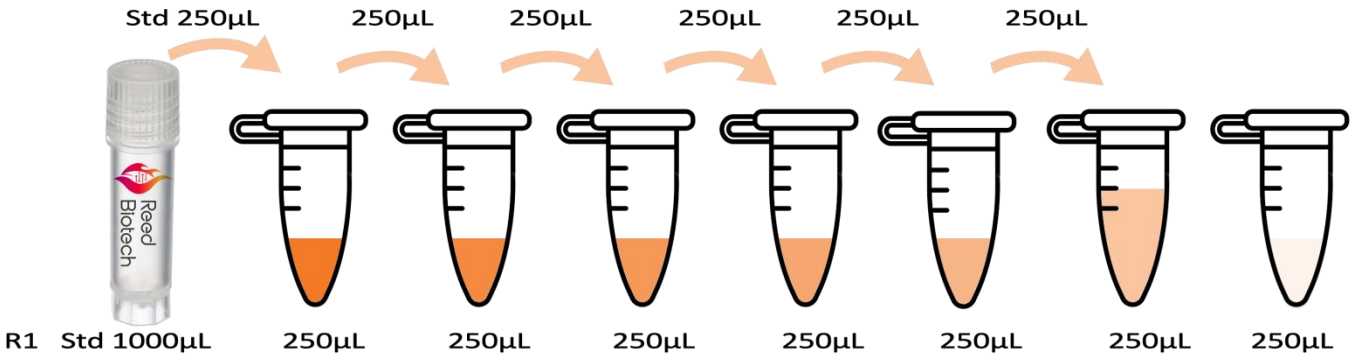
1. Bring all reagents to room temperature (18-25℃) before If the kit will not be used up in one assay, please only take out the necessary strips and reagents for present experiment, and store the remaining strips and reagents at required condition.

2. Wash Buffer: Dilute 30 mL of Concentrated Wash Buffer with 720 mL of deionized or distilled water to prepare 750 mL of Wash Buffer. Note: if crystals have formed in the concentrate, warm it in a 40 ℃ water bath and mix it gently until the crystals have completely dissolved.

3. Standard working solution: Centrifuge the standard at 10, 000×g for 1 min. Add 1mL of Reference Standard &Sample Diluent, let it stand for 10 min and invert it gently several times. After it dissolves fully, mix it thoroughly with a pipette. This reconstitution produces a working solution of 40ng/mL (or add 1mL of Reference Standard &Sample Diluent, let it stand for 1-2 min and then mix it thoroughly with a vortex meter of low speed. Bubbles generated during vortex could be removed by centrifuging at a relatively low speed). Then make serial dilutions as needed. The

recommended dilution gradient is as follows: 40, 20, 10, 5, 2.5, 1.25, 0.63ng/mL.

Dilution method: Take 7 EP tubes, add 250μL of Reference Standard & Sample Diluent to each tube. Pipette 250μL of the 40ng/mL working solution to the first tube and mix up to produce a 20ng/mL working solution. Pipette 250μL of the solution from the former tube into the latter one according to this step. The illustration below is for reference. Note: the last tube is regarded as a blank. Don't pipette solution into it from the former tube. (The operation diagram is shown on the next page)




| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 40 | 20 | 10 | 5 | 2.5 | 1.25 | 0.63 | 0 |
| ng/mL | ng/mL | ng/mL | ng/mL | ng/mL | ng/mL | ng/mL | ng/mL |

4. Biotinylated Detection Ab working solution: Calculate the required amount before the experiment (100μL/well). In preparation, slightly more than calculated should be prepared. Centrifuge the Concentrated Biotinylated Detection Ab at 800×g for 1 min, then dilute the 100× Concentrated Biotinylated Detection Ab to 1× working solution with Biotinylated Detection Ab Diluent (Concentrated Biotinylated Detection Ab: Biotinylated Detection Ab Diluent= 1: 99).

5. HRP Conjugate working solution: Calculate the required amount before the experiment (100μL/well). In preparation, slightly more than calculated should be prepared. Centrifuge the Concentrated HRP Conjugate at 800×g for 1 min, then dilute the 100× Concentrated HRP Conjugate to 1× working solution with HRP Conjugate Diluent (Concentrated HRP Conjugate: HRP Conjugate Diluent= 1: 99).

## Assay procedure

1. The Micro ELISA Plate slats to be used were removed from the plate frame and the remaining slats were returned to the aluminum foil bag containing the desiccants and then resealed for storage.
2. Determine wells for diluted standard, blank and sample. Add 100µL each dilution of standard, blank and sample into the appropriate wells (It is recommended that all samples and standards be assayed in duplicate). Cover the plate with the sealer provided in the kit. Incubate for 90 min at 37°C. Note: solutions should be added to the bottom of the micro ELISA plate well, avoid touching the inside wall and causing foaming as much as possible.
3. Aspirate and wash plate for 3 times. Immediately add 100µL of Biotinylated Detection Ab working solution to each well. Cover the plate with a new sealer. Incubate for 1 hour at 37°C.
4. Decant the solution from each well add 300µL of wash buffer to each well. Soak for 0.5 min and aspirate or decant the solution from each well and pat it dry against clean absorbent paper. Repeat this wash step 3 times. Note: a microplate washer can be used in this step and other wash steps. Make the tested strips in use immediately after the wash step. Do not allow wells to be dry.
5. Add 100µL of HRP Conjugate working solution to each well. Cover the plate with a new sealer. Incubate for 30 min at 37°C.
6. Decant the solution from each well, repeat the wash process for 3 times as conducted in step 4.
7. Add 100µL of Substrate Reagent to each well. Cover the plate with a new sealer. Incubate for about 15 min at 37°C. Protect the plate from light. Note: the reaction time can be shortened or extended according to the actual color change, but not more than 30 min. Preheat the Microplate

Reader for about 15 min before OD measurement.

8. Add 50μL of Stop Solution to each well. Note: adding the stop solution should be done in the same order as the substrate solution.

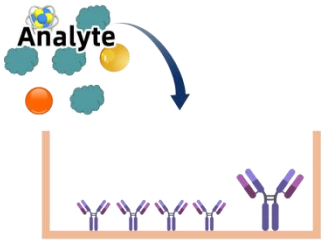
9. Determine the optical density (OD value) of each well at once with a micro plate reader set to 450 nm.

**Calculation of results**

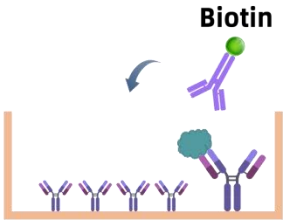
1. Average the duplicate readings for each standard and samples, then subtract the average zero standard optical density. Plot a four parameters logistic curve on log-log graph paper, with standard concentration on the x-axis and OD values on the y-axis.

2. If the OD of the sample surpasses the upper limit of the standard curve, you should re-test it with an appropriate dilution. The actual concentration is the calculated concentration multiplied by the dilution factor.

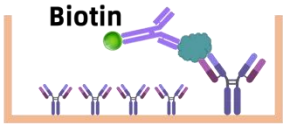
# Assay Procedure Summary



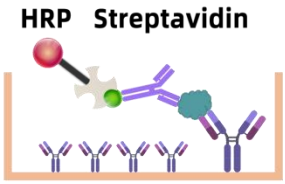

1、Add 100μL standard or sample to the wells. Incubate for 90 min at 37℃. Aspirate and wash plate for 3 times



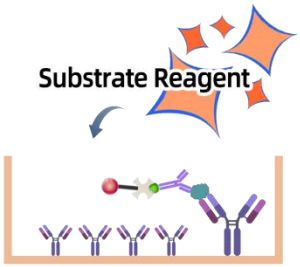

2、Immediately add 100μL Biotinylated Detecting Ab working solution to each well. Incubate for 60 min at 37℃



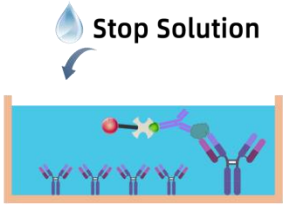

3、Aspirate and wash the plate for 3 times



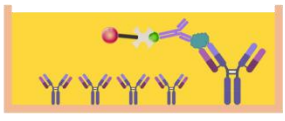

4、Add 100μL HRP conjugate working solution. Incubate for 30 min at 37℃




5、Aspire and wash the plate for 3 times. Add 100μL Substrate Reagent. Incubate for 15 min at 37℃



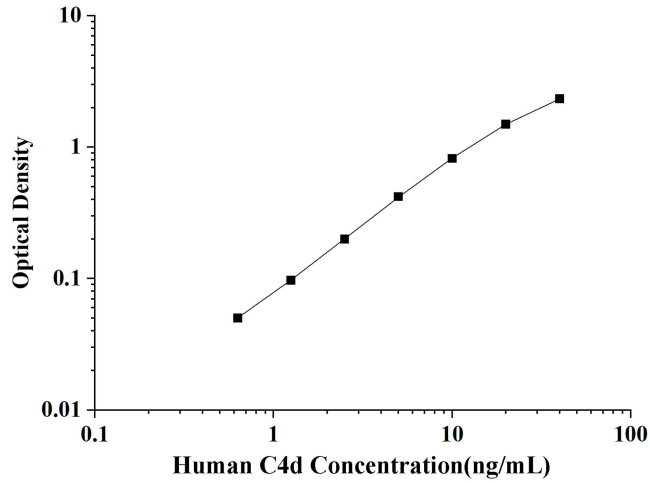

6、Add 50μL Stop Solution



7、Read the plate at 450nm immediately. Calculation of the results

## Typical data

The operator should establish a standard curve for each test. Typical standard curve and data is provided below for reference only.

| ng/mL | OD | Corrected OD | Standard Curve |
|---|---|---|---|
| 40 | 2.386 | 2.334 |  |
| 20 | 1.546 | 1.494 | |
| 10 | 0.872 | 0.82 | |
| 5 | 0.472 | 0.42 | |
| 2.5 | 0.252 | 0.2 | |
| 1.25 | 0.149 | 0.097 | |
| 0.63 | 0.102 | 0.05 | |
| 0 | 0.052 | 0 | |



## Performance

### ■Precision

Intra-assay Precision (Precision within an assay): 3 samples with low, mid and high level were tested 20 times on one plate, respectively.

Inter-assay Precision (Precision between assays): 3 samples with low, mid and high level were tested 20 times on each plate, respectively.

| | Intra-assay Precision | | | Inter-assay Precision | | |
|---|---|---|---|---|---|---|
| Sample | 1 | 2 | 3 | 1 | 2 | 3 |
| n | 20 | 20 | 20 | 20 | 20 | 20 |
| Mean(ng/mL) | 0.93 | 5.94 | 11.34 | 0.93 | 8.33 | 14.1 |

| | | | | | | |
|---|---|---|---|---|---|---|
| Standard deviation | 0.06 | 0.46 | 0.8 | 0.04 | 0.41 | 0.75 |
| CV(%) | 2.9 | 3.07 | 6.75 | 3.35 | 3.41 | 3.17 |

## ■Recovery

The recovery of Human C4d spiked at three different levels in samples throughout the range of the assay was evaluated in various matrices.

| Sample Type | Range(%) | Average Recovery(%) |
|---|---|---|
| Serum(n=8) | 87-99 | 93 |
| EDTA Plasma(n=8) | 80-92 | 85 |
| Cell culture media(n=8) | 92-107 | 99 |

## ■Linearity

Samples were spiked with high concentrations of Human C4d and diluted with Reference Standard & Sample Diluent to produce samples with values within the range of the assay.

| | | Serum (n=8) | EDTA Plasma (n=8) | Cell culture media (n=8) |
|---|---|---|---|---|
| 1:2 | Range(%) | 81-93 | 80-102 | 88-97 |
| | Average(%) | 81 | 81 | 92 |
| 1:4 | Range(%) | 80-91 | 80-102 | 88-103 |
| | Average(%) | 82 | 99 | 89 |
| 1:8 | Range(%) | 87-98 | 83-96 | 86-92 |
| | Average(%) | 95 | 90 | 88 |
| 1:16 | Range(%) | 95-107 | 80-93 | 91-99 |
| | Average(%) | 104 | 86 | 94 |

## Troubleshooting & Solutions

| Problems | Causes | Solutions |
|---|---|---|
| High background | Plate is insufficiently washed | Review the manual for proper wash and ensure the right way to wash. |
| | Wrong incubation procedure | Ensure recommended incubation temperature and time. |
| | Contaminated reagent | Prepare fresh reagent. |
| Low signal | Incorrect use of reagents | Check reagent concentrations and dilution factor. Ensure reagents are used in the correct order. |
| | Plate reader setting is not optimal | Open the Microplate Reader ahead to preheat. Verify the wavelength and filter setting on the Microplate reader. |
| | Insufficient incubation time | Ensure sufficient incubation time. |
| | Inadequate reagent volumes and Improper dilution | Check pipettes and ensure correct preparation. |
| | Matrix effect | Use positive control. |
| High signal | TMB improper storage | Check the color of TMB. |
| | Plate Sealer used repeatedly | Use fresh Plate Sealer. |
| | Concentration of target protein is too high | Use recommended dilution factor. |

| | | |
|---|---|---|
| Low repeatability | Inaccurate pipetting | Check pipettes. |
| | There are impurities and precipitates in the sample | Sample centrifuged before using it. |
| | Inadequate mixing of reagents | All reagents and samples mixed thoroughly before load. |

## Declaration

1. Limited by current conditions and scientific technology, we can't conduct comprehensive identification and analysis of all the raw material provided. There might be some qualitative and technical risks for users using the kit.
2. This assay is designed to eliminate interference by factors present in biological samples. Until all factors have been tested in the ELISA immunoassay, the possibility of interference cannot be excluded.
3. The final experimental results will be closely related to the validity of products, operational skills of the operators, the experimental environments and so on We are only responsible for the kit itself, but not for the samples consumed during the assay. The users should calculate the possible amount of the samples used in the whole test. Please reserve sufficient samples in advance.
4. To get the best results, please only use the reagents supplied by the manufacturer and strictly comply with the instructions.
5. Incorrect results may occur because of incorrect operations during the reagents preparation and loading, as well as incorrect parameter settings of the Micro plate reader. Please read the instructions carefully and adjust the instrument prior to the experiment.
6. Even the same operator might get different results in two separate

experiments. In order to get reproducible results, the operation of every step in the assay should be controlled.

7. Every kit has strictly passed QC test. However, results from end users might be inconsistent with our data due to some variables such as transportation conditions, different lab equipment, and so on. Intra assay variance among kits from different batches might arise from the above reasons too.

8. Kits from different manufacturers or other methods for testing the same analyte could bring out inconsistent results, since we haven't compared our products with those from other manufacturers.

9. The kit is designed for research use only, we will not be responsible for any issues if the kit is applied in clinical diagnosis or any other related procedures.