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# User Manual

The manual may be updated as a result of continuous improvements.

Please refer to the hard copy manual included in the kit for your experiment.

**Human C4a(Complement Component 4a) ELISA Kit**

Catalog No: **EKE60162**

96T/48T/24T

**This kit is for scientific research use only. Not for use in diagnostic procedures.**

## Intended use

This ELISA kit applies to the in vitro quantitative determination of Human C4a concentrations in serum, plasma and other biological fluids.

## Specification

- Sensitivity: 0.94 ng/mL
- Detection Range: 1.56-100 ng/mL
- Specificity: This kit recognizes Human C4a in samples. No significant cross-reactivity or interference between Human C4a and analogues 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 C4a. Standards or samples are added to the micro ELISA plate wells and combined with the specific antibody. Then a biotinylated detection antibody specific for Human C4a 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 C4a, 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 ± 2 nm. The OD value is proportional to the concentration of Human C4a. You can calculate the concentration of Human C4a 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 12 months. After opening, store the items separately according to the following conditions.

| Item | Specifications | Storage |
|---|---|---|
| Micro ELISA Plate | 96T: 8 wells ×12 strips<br>48T: 8 wells ×6 strips<br>24T: 8 wells ×3 strips | -20℃, 12 months |
| Reference Standard | 96T: 2 vials<br>48T: 1 vial<br>24T: 1 vial | |
| Concentrated Biotinylated Detection Ab(100×) | 96T: 1 vial, 120 μL<br>48T: 1 vial, 60 μL<br>24T: 1 vial, 60 μL | |
| Concentrated HRP Conjugate(100×) | 96T: 1 vial, 120 μL<br>48T: 1 vial, 60 μL<br>24T: 1 vial, 60 μL | -20℃(protect from light), 12 months |
| Reference Standard & Sample Diluent | 2vials 20mL | 2-8℃, 12 months |
| Biotinylated Detection Ab Diluent | 1vial 14mL | |
| HRP Conjugate Diluent | 1vial 14mL | |
| Concentrated Wash Buffer (25×) | 1vial 30mL | |
| Substrate Reagent | 1vial 10mL | 2-8℃(protect from light) |
| Stop Solution | 1vial 10mL | 2-8℃ |
| Plate Sealer | 5pieces | |
| 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

Microplate reader with 450 nm wavelength filter
High-precision transfer pipette, EP tubes and disposable pipette tips
Incubator capable of maintaining 37 ℃
Deionized or distilled water
Absorbent paper
Loading slot for Wash Buffer

## Note

1. Please wear lab coats, eye protection and latex gloves for protection. Please perform the experiment following the national security protocols of biological laboratories, especially when analyzing blood samples or other bodily fluids.
2. 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 provided in the kit, store it according to the conditions suggested in the above table.
3. 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.
4. The microplate reader should have a 450 (±2 nm) filter installed and a detector that can detect the wavelength. 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.
5. Do not mix or use components from other lots.
6. Change pipette tips in between adding standards, in between sample additions, and in between reagent additions. Also, use separate reservoirs for each reagent.

## Sample collection

**Serum:** Allow samples to clot for 1 hour at room temperature or overnight at 4 ℃ before centrifugation for 15 min at 1000×g at 2~8 ℃. Collect the supernatant to carry out the assay. Blood collection tubes should be disposable, and be non-endotoxin.

**Plasma:** Collect plasma using EDTA or heparin 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. Hemolyzed samples are not suitable for ELISA assay!

**Cell lysates:** For adherent cells, gently wash the cells with a 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 150-250 μL 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 4℃. Remove the cell fragments, collect the supernatant to carry out the assay. Avoid repeated freeze-thaw cycles.

**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 (0.01 M, 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 min at 5000×g to get the supernatant.

**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.

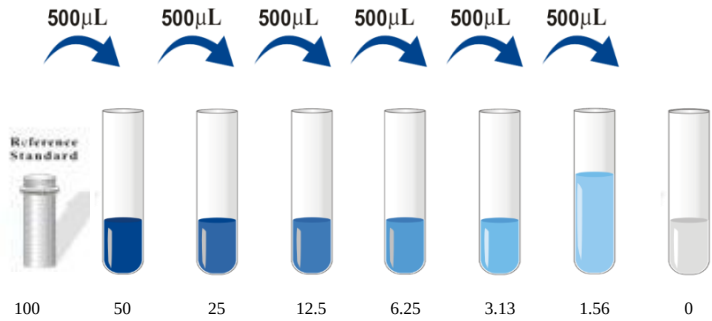
## Note for sample

1. Samples should be assayed within 7 days when stored at 4℃, 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.
2. Please predict the concentration before assaying. If the sample concentration is not within the range of the standard curve, users must determine the optimal sample dilutions for their particular experiments.
3. If the sample type is not included in the manual, a preliminary experiment is suggested to verify the validity.
4. If a lysis buffer is used to prepare tissue homogenates or cell culture supernatant there is a possibility of causing a deviation, due to the introduced chemical substance.
5. Some recombinant protein may not be detected due to a mismatching with the coated antibody or detection antibody.

## Reagent preparation

Bring all reagents to room temperature (18~25℃) before use. Follow the Microplate reader manual for set-up before OD measurement.

1. **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.
2. **Standard working solution:** Centrifuge the standard at 10,000×g for 1 min. Add 1.0 mL 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 tip.
   This reconstitution produces a working solution of 100 ng/mL(or add 1.0mL 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: 100、50、25、12.5、6.25、3.13、1.56、0 ng/mL.
   Dilution method: Take 7 EP tubes, add 500μL of Reference Standard & Sample Diluent to each tube. Pipette 500 μL of the100 ng/mL working solution to the first tube and mix up to produce a 50 ng/mL working solution. Pipette 500 μL of the solution from the former tube into the latter one according to these steps. 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.




3. **Biotinylated Detection Ab working solution**: Calculate the required amount before the experiment (100 μL/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).

4. **Concentrated HRP Conjugate working solution**: Calculate the required amount before the experiment (100 μL/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. 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℃. 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.
2. Decant the solution from each well, do not wash. Immediately add 100 μL of Biotinylated Detection Ab working solution to each well. Cover with the Plate sealer. Gently mix. Incubate for 1 hour at 37 ℃.
3. Decant the solution from each well，add 350 μL of wash buffer to each well. Soak for 1~2 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 and other wash steps. Make the tested strips in use immediately after the wash step. Do not allow wells to be dry.
4. Add 100 μL of HRP Conjugate working solution to each well. Cover with the Plate sealer. Incubate for 30 min at 37 ℃.
5. Decant the solution from each well，repeat the wash process five times as conducted in step 3.
6. Add 90 μL of Substrate Reagent to each well. Cover with a new plate sealer. Incubate for about 15 min at 37 ℃. Protect the plate from light. Note: the reaction time can be shortened or extended according to the actual color change, but no more than 30 min. Preheat the Microplate Reader for about 15 min before OD measurement.
7. Add 50 μL of Stop Solution to each well. Note: Adding the stop solution should be done in the same order as the substrate solution.
8. Determine the optical density (OD value) of each well at once with a micro-plate reader set to 450 nm.

## Calculation of results

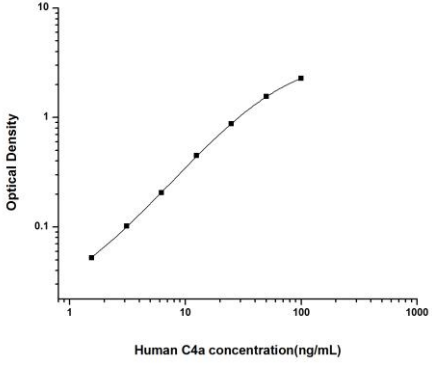
Average the duplicate readings for each standard and sample, then subtract the average zero standard optical density reading. Plot a four-parameter logistic curve on log-log graph paper, with standard concentration on the x-axis and OD values on the y-axis.

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 factors including the amounts used in the sample preparation procedure.

## Typical data

As the OD values of the standard curve may vary according to the conditions of the actual assay performance (e.g. operator, pipetting technique, washing technique or temperature effects), the operator should establish a standard curve for each test. Typical standard curve data is provided below for reference only.

| Concentration (ng/mL) | 100 | 50 | 25 | 12.5 | 6.25 | 3.13 | 1.56 | 0 |
|---|---|---|---|---|---|---|---|---|
| OD | 2.341 | 1.612 | 0.936 | 0.509 | 0.266 | 0.163 | 0.113 | 0.061 |
| Corrected OD | 2.28 | 1.551 | 0.875 | 0.448 | 0.205 | 0.102 | 0.052 | - |



## Precision

Intra-assay Precision (Precision within an assay): 3 samples with low, mid range and high level Human C4a were tested 20 times on one plate, respectively.
Inter-assay Precision (Precision between assays): 3 samples with low, mid range and high level Human C4a were tested on 3 different plates, 20 replicates in each plate.

| | Intra-assay Precision | | | Inter-assay Precision | | |
|---|---|---|---|---|---|---|
| Sample | 1 | 2 | 3 | 1 | 2 | 3 |
| n | 20 | 20 | 20 | 20 | 20 | 20 |
| Mean(ng/mL) | 4.84 | 13.18 | 42.91 | 4.58 | 13.63 | 39 |
| Standard deviation | 0.27 | 0.7 | 1.77 | 0.23 | 0.75 | 1.56 |
| CV (%) | 5.58 | 5.31 | 4.12 | 5.02 | 5.5 | 4 |

## Recovery

The recovery of Human C4a 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=5) | 88-100 | 95 |
| EDTA plasma (n=5) | 86-101 | 92 |
| Cell culture media (n=5) | 91-106 | 97 |

## Linearity

Samples were spiked with high concentrations of Human C4a and diluted with Reference Standard & Sample Diluent to produce samples with values within the range of the assay.

| | | Serum (n=5) | EDTA plasma (n=5) | Cell culture media (n=5) |
|---|---|---|---|---|
| 1:2 | Range (%) | 91-103 | 98-113 | 87-99 |
| | Average (%) | 98 | 104 | 94 |
| 1:4 | Range (%) | 91-102 | 87-101 | 85-98 |
| | Average (%) | 96 | 93 | 91 |
| 1:8 | Range (%) | 93-105 | 84-96 | 86-96 |
| | Average (%) | 98 | 88 | 91 |
| 1:16 | Range (%) | 89-104 | 82-92 | 87-99 |
| | Average (%) | 95 | 87 | 94 |

## Troubleshooting

If the results are not good enough, please take pictures and save the experimental data in time. Keep the used plate and remaining reagents. Then contact our technical support to solve the problem. Meanwhile, you could also refer to the following materials:

| **Problem** | **causes** | **solutions** |
|---|---|---|
| Poor standard curve | Inaccurate pipetting | Check pipettes. |
| | Improper standard dilution | Ensure briefly spin the vial of standard and dissolve the powder thoroughly by gentle mixing. |
| | Wells are not completely aspirated | Completely aspirate wells in between steps. |
| Low signal | Insufficient incubation time | Ensure sufficient incubation time. |
| | Incorrect assay temperature | Use recommended incubation temperature. Bring substrate to room temperature before use. |
| | Inadequate reagent volumes | Check pipettes and ensure correct preparation. |
| | Improper dilution | |
| | HRP conjugate inactive or TMB failure | Mix HRP conjugate and TMB, rapid coloring. |
| Deep color but low value | Plate reader setting is not optimal | Verify the wavelength and filter setting on the Microplate reader. |
| Large CV | Inaccurate pipetting | Check pipettes |
| High background | Concentration of target protein is too high | Use recommended dilution factor. |
| | Plate is insufficiently washed | Review the manual for proper wash. If using a plate washer, check that all ports are unobstructed. |
| | Contaminated wash buffer | Prepare fresh wash buffer. |
| Low sensitivity | Improper storage of the ELISA kit | All the reagents should be stored according to the instructions. |
| | Stop solution is not added | Stop solution should be added to each well before measurement. |

## SUMMARY

1. Add 100μL standard or sample to each well. Incubate for 90mintues at 37℃
2. Remove the liquid. Add 100μL Biotinylated Detection Ab. Incubate for 1 hour at 37℃
3. Aspirate and wash 3 times
4. Add 100μL HRP Conjugate. Incubate for 30 minutes at 37℃
5. Aspirate and wash 5 times
6. Add 90μL Substrate Reagent. Incubate for 15 minutes at 37℃
7. Add 50μL Stop Solution. Read at 450nm immediately
8. Calculation of results

## Declaration

1. Limited by current conditions and scientific technology, we can't conduct comprehensive identification and analysis on all the raw material provided. So 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.
10. Valid period: 12 months.