Technical Manual

Human C4a ELISA Kit

- **Catalogue Code: HUES01456**
- **Sandwich ELISA Kit**
- **Research Use Only**

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1. Description and Principle

The Assay Genie Complement Component 4a ELISA Kit can assay for the specific analyte in the following samples: serum, blood, plasma, cell culture supernatant and other related supernatants and tissues.

How Do Our ELISA Kit Assays Work?

This kit uses the Sandwich ELISA principle. The ELISA plate provided in this kit has been pre-coated with an antibody specific to Complement Component 4a. Standards or samples are added to the ELISA plate wells and combined with the pre-coated antibodies. Then a biotinylated detection antibody specific for Complement Component 4a and Avidin-Horseradish Peroxidase (HRP) conjugate are added successively to each plate well and incubated. Free components are washed away, and the substrate solution is then added to each well. Only wells that contain Complement Component 4a, 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 Complement Component 4a. The concentration of Complement Component 4a can be calculated in the samples by comparing the OD of the samples to the standard curve.

2. Key features and Sample Types

Sensitivity: 0.94 ng/mL

Detection Range: 1.56 - 100 ng/mL

ELISA Type: Sandwich

Specificity: This kit recognizes Complement Component 4a in samples. No significant cross-reactivity or interference between Complement Component 4a and analogues was observed.

SUMMARY

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

3. Kit Contents

Product	Size	Cat. Code
Human C4a ELISA Kit	24/96 assays	HUES01456

Each kit contains reagents for 24/96 assays in a 24/96 well plate including:

Item	24T	96T	Storage
Micro ELISA Plate (Dismountable)	8 wells x 3 strips	8 wells x 12 strips	-20°C, 6 months
Reference Standard	1 vial	2 vials	
Concentrated Biotinylated Detection Ab (100×)	1 vial, 60 μL	1 vial, 120 μL	
Concentrated HRP Conjugate (100×)	1 vial, 60 μL	1 vial, 120 μL	-20°C (shading light), 6 months
Reference Standard & Sample Diluent	1 vial, 20 mL	1 vial, 20 mL	4°C, 6 months
Biotinylated Detection Ab Diluent	1 vial, 14 mL	1 vial, 14 mL	
HRP Conjugate Diluent	1 vial, 14 mL	1 vial, 14 mL	
Concentrated Wash Buffer (25×)	1 vial, 30 mL	1 vial, 30 mL	
Substrate Reagent	1 vial, 10 mL	1 vial, 10 mL	4°C (shading light)
Stop Solution	1 vial, 10 mL	1 vial, 10 mL	4°C
Plate Sealer	5 pieces	5 pieces	
Product Description	1 copy	1 copy	

Additional Materials 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°C
4. Deionized or distilled water
5. Absorbent paper

4. Shipping and Storage

An unopened kit can be stored at 4°C for 1 month. If the kit is not used within 1 month, store the items separately according to the vial labels.

5. Sample Preparation

Serum: Allow samples to clot for 2 hours at room temperature or overnight at 4°C before centrifugation for 15 min at 1000×g at 2~8°C. Collect the supernatant to carry out the assay. Blood collection tubes should be disposable and be endotoxin-free.

Plasma: Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge samples for 15 min at 1000×g at 2~8°C within 30 min of collection. Collect the supernatant to carry out the assay. Hemolysed samples are not suitable for ELISA assay.

Cell lysates: For adherent cells, gently wash the cells with pre-cooled PBS and dissociate the cells using trypsin. Collect the cell suspension in a tube and centrifuge for 5 min at 1000×g. Discard the medium and wash the cells 3 times with precooled PBS. For each 1×10^6 cells, add 150-250μL of pre-cooled PBS to keep the cells suspended. Optimal cell concentration is 1 million/ml. To release cellular components, dilute the cell pellet in PBS and use 3-4 freeze-thaw cycles in liquid Nitrogen (commercial lyses buffers can be used according to manufacturer's instructions). Centrifuge at 4°C for 20 mins at 2000-3000 rpm to pellet debris and remove clear supernatant to clean microcentrifuge tube for ELISA or storage.

Tissue homogenates: It is recommended to get detailed references from the literature before analyzing different tissue types. For general information, hemolysed 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 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°C. Collect the supernatant to carry out the assay.

Notes:

1. Samples should be assayed within 7 days when stored at 4°C. Otherwise samples must be aliquoted and stored at -20°C (≤1 month) or -80°C (≤3 months). Avoid repeated freeze-thaw cycles.

2. Determine the protein 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.

5. Some recombinant proteins may not be detected due to a mismatch with the coated antibody or detection antibody.

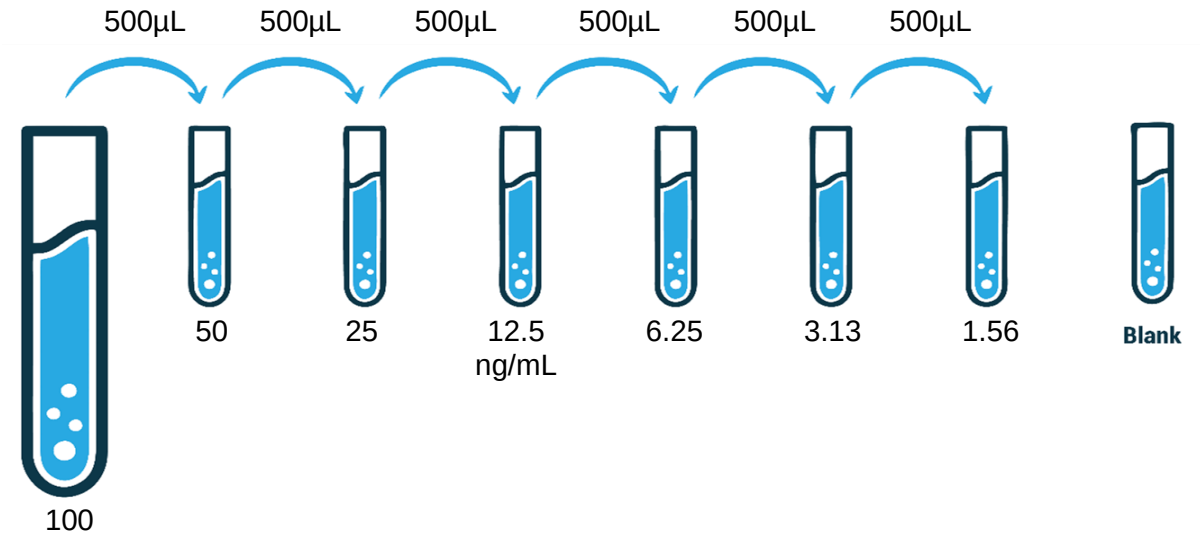
6. Protocol

1. Bring all reagents to room temperature (18~25°C) before use. Follow the microplate reader manual for set-up and preheat it for 15 min before OD measurement.

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°C 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 1 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. This reconstitution produces a working solution of 100 ng/mL. 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. Note: the last tube is regarded as a blank. Don't pipette solution into it from the former tube.

Dilution method: Take 7 EP tubes, add 500μL of Reference Standard & Sample Diluent to each tube. Pipette 500μL of the 100 ng/mL working solution to the first tube and mix up to produce a 50 ng/mL working solution. Pipette 500μL of the solution from the former tube into the latter one according to these steps. The illustration below is for reference.



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 stock tube before use, dilute the 100× Concentrated Biotinylated Detection Ab to 1×working solution with Biotinylated Detection Ab Diluent.

5. Concentrated HRP Conjugate working solution: Calculate the required amount before the experiment (100μL/well). In preparation, slightly more than calculated should be prepared. Dilute the 100× Concentrated HRP Conjugate to 1× working solution with Concentrated HRP Conjugate Diluent.

7. Assay procedure

1. Set standard, test sample and control (zero) wells on the pre-coated plate and record their positions. It is recommended to measure each standard and sample in duplicate. Note: add all solutions to the bottom of the plate wells while avoiding contact with the well walls. Ensure solutions do not foam when adding to the wells.

2. Aliquot 100µl of standard solutions into the standard wells.

3. Add 100µl of Sample / Standard dilution buffer into the control (zero) well.

4. Add 100µl of properly diluted sample (serum, plasma, tissue homogenates and other biological fluids) into test sample wells.

5. Cover the plate with the sealer provided in the kit and incubate for 90 min at 37°C.

6. Aspirate the liquid from each well, do not wash. Immediately add 100µL of Biotinylated Detection Ab working solution to each well. Cover the plate with a plate seal and gently mix. Incubate for 1 hour at 37°C.

7. Aspirate or decant the solution from the plate and add 350µL of wash buffer to each well and incubate for 1-2 minutes at room temperature. Aspirate the solution from each well and clap the plate on absorbent filter paper to dry. Repeat this process 3 times. Note: a microplate washer can be used in this step and other wash steps.

8. Add 100µL of HRP Conjugate working solution to each well. Cover with a plate seal and incubate for 30 min at 37°C.

9. Aspirate or decant the solution from each well. Repeat the wash process for five times as conducted in step 7.

10. Add 90µL of Substrate Reagent to each well. Cover with a new plate seal and incubate for approximately 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 by more than 30min.

11. 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.

12. Determine the optical density (OD value) of each well immediately with a microplate reader set at 450 nm.

8. Data analysis

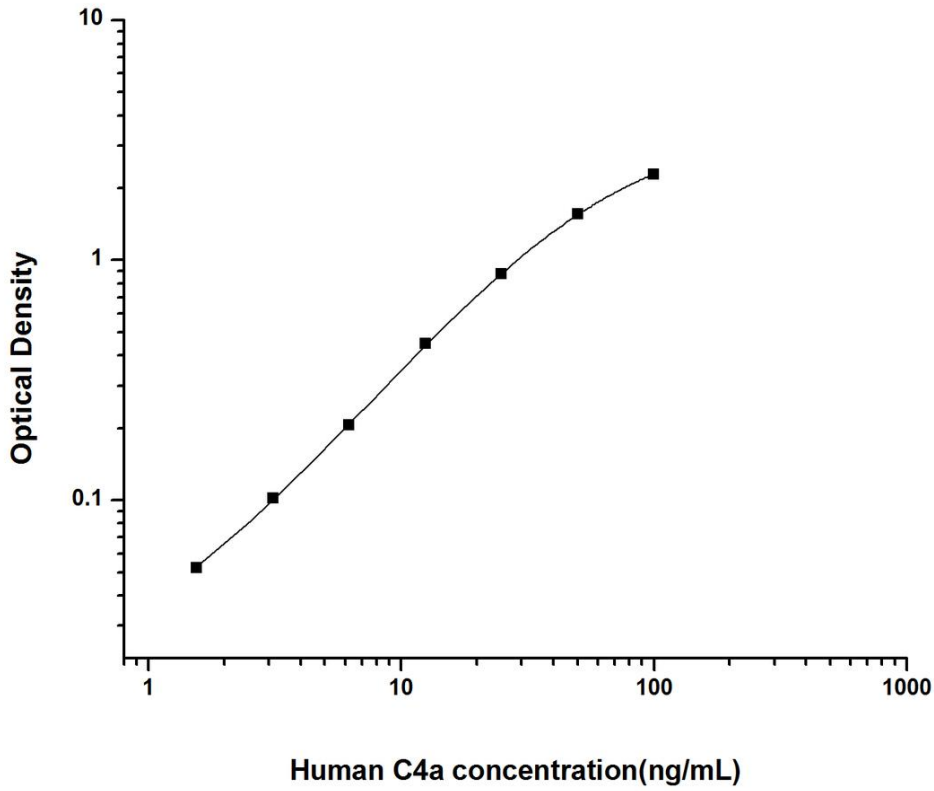
Average the duplicate readings for each standard and samples, then subtract the average zero standard optical density. Plot a four-parameter logistic curve on log-log graph paper, with standard concentration on the x-axis and OD values on the y-axis.

If the samples have been diluted, the concentration calculated from the standard curve must be multiplied by the dilution factor. 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.

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 generate a standard curve for each experiment. Typical standard curve and data is provided below (for reference only).

Concentration (ng/mL)	O.D	Average	Corrected
100	2.332 2.35	2.341	2.28
50	1.61 1.614	1.612	1.551
25	0.95 0.922	0.936	0.875
12.5	0.492 0.526	0.509	0.448
6.25	0.269 0.263	0.266	0.205
3.13	0.17 0.156	0.163	0.102
1.56	0.106 0.12	0.113	0.052
0	0.057 0.065	0.061	--



Precision

Intra-assay Precision (Precision within an assay): 3 samples with low, mid-range and high level Complement Component 4a were tested 20 times on one plate.
Inter-assay Precision (Precision between assays): 3 samples with low, mid-range and high level Complement Component 4a 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	39.00
Standard deviation	0.27	0.70	1.77	0.23	0.75	1.56
C V (%)	5.58	5.31	4.12	5.02	5.50	4.00

Recovery

The recovery of Complement Component 4a 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 Complement Component 4a 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

9. Important General Notes:

Problem	Causes	Solutions
Poor standard curve	Inaccurate pipetting	Check pipettes.
	Improper standard dilution	Centrifuge the standard vial and ensure contents are dissolved thoroughly.
	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 color change.
Deep color but low value	Plate reader setting is not optimal	Verify the wavelength and filter setting on the Microplate reader.
		Pre-heat 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 washing procedure. 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.

Additional Notes:

1. Please wear lab coats, eye goggles and latex gloves for protection. Perform the experiment following the national safety guidelines for biological laboratories, especially when using blood samples or other bodily fluids.

2. A freshly opened ELISA Plate may appear to have a water-like substance, this is normal and will not have any impact on the experimental results.

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(±10 nm) filter installed and a detector that can detect this wavelength. The optical density should be within 0~3.5.

5. Do not mix or use components from other lots.

6. Change pipette tips in between adding standards, sample additions and reagent additions. Also, use separate reservoirs for each reagent.

Declaration

1. Limited by current scientific technology, we can't conduct comprehensive identification and analysis on all the raw materials provided [So, there might be some qualitative and technical risks for users using the kit].

2. The final experimental results will be closely related to the validity of products, operational skills and the experimental environment. Please make sure that sufficient samples are available.

3. To get the best results, please only use the reagents supplied with this kit and strictly comply with the instructions.

4. Incorrect results may occur from incorrect reagent 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.

5. Each kit passes a strict QC procedure. 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 also arise.

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