

# **ab108825 – Complement C4 Human ELISA Kit**

## Instructions for Use

For the quantitative measurement of Human Complement C4 in milk, urine, saliva and CSF samples.

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

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## 1. BACKGROUND

Abcam's Complement C4 Human *in vitro* ELISA (Enzyme-Linked Immunosorbent Assay) kit is designed for the quantitative measurement of Complement C4 levels in milk, urine, saliva and CSF samples.

A Complement C4 specific antibody has been precoated onto 96-well plates and blocked. Standards or test samples are added to the wells and subsequently a Complement C4 specific biotinylated detection antibody is added and then followed by washing with wash buffer. Streptavidin-Peroxidase Conjugate is added and unbound conjugates are washed away with wash buffer. TMB is then used to visualize Streptavidin-Peroxidase enzymatic reaction. TMB is catalyzed by Streptavidin-Peroxidase to produce a blue color product that changes into yellow after adding acidic stop solution. The density of yellow coloration is directly proportional to the amount of Complement C4 captured in plate.

Complement component 4 (C4) plays a key role in the activation of the classical complement pathway. C4 is synthesized as a single-chain precursor molecule (200 kDa) but processed to the three-chain disulphide-linked structure with alpha (93 kDa), beta (78 kDa) and gamma (33 kDa) chains prior to secretion. After activation by C1s, C4 is processed to C4a and C4b. C4a anaphylatoxin is a mediator of local inflammation and induces smooth muscle contraction. C4b, the major activation product, is an essential subunit of the C3 and C5 convertases of the classical complement pathway. C4 deficiency is associated with systemic lupus erythematosus. The C4b degradation product C4d is a marker for humoral rejection in allografts.

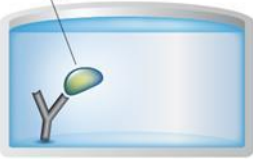
## 2. ASSAY SUMMARY

### Primary capture antibody



Prepare all reagents, samples and standards as instructed.

### Sample



Add standard or sample to each well used. Incubate at room temperature.

### Primary detector antibody



Wash and add prepared biotin antibody to each well. Incubate at room temperature.

### Streptavidin Label



Wash and add prepared Streptavidin-Peroxidase Conjugate. Incubate at room temperature.

### Substrate **Colored product**



Add Chromogen Substrate to each well. Incubate at room temperature. Add Stop Solution to each well. Read immediately.

## 3. PRECAUTIONS

Please read these instructions carefully prior to beginning the assay.

Modifications to the kit components or procedures may result in loss of performance.

## 4. STORAGE AND STABILITY

Store kit at 4°C immediately upon receipt, apart from the SP Conjugate & Biotinylated Antibody, which should be stored at -20°C.

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in sections 9 & 10.

## 5. MATERIALS SUPPLIED

Item	Amount	Storage Condition (Before Preparation)
Complement C4 Microplate (12 x 8 well strips)	96 wells	4°C
Complement C4 Standard	1 vial	4°C
10X Diluent N Concentrate	30 mL	4°C
Biotinylated Human Complement C4 Antibody	1 vial	-20°C
100X Streptavidin-Peroxidase Conjugate (SP Conjugate)	80 µL	-20°C
Chromogen Substrate	7 mL	4°C
Stop Solution	11 mL	4°C
20X Wash Buffer Concentrate	2 x 30 mL	4°C
Sealing Tapes	3	N/A

### **6. MATERIALS REQUIRED, NOT SUPPLIED**

These materials are not included in the kit, but will be required to successfully utilize this assay:

- 1 Microplate reader capable of measuring absorbance at 450 nm.
- Precision pipettes to deliver 1  $\mu$ L to 1 mL volumes.
- Adjustable 1-25 mL pipettes for reagent preparation.
- 100 mL and 1 liter graduated cylinders.
- Absorbent paper.
- Distilled or deionized water.
- Log-log graph paper or computer and software for ELISA data analysis.
- 6 tubes to prepare standard or sample dilutions.

### **7. LIMITATIONS**

- Do not mix or substitute reagents or materials from other kit lots or vendors.

### 8. TECHNICAL HINTS

- Samples generating values higher than the highest standard should be further diluted in the appropriate sample dilution buffers.
- Avoid foaming or bubbles when mixing or reconstituting components.
- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.
- Ensure plates are properly sealed or covered during incubation steps.
- Complete removal of all solutions and buffers during wash steps.
- **This kit is sold based on number of tests. A 'test' simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.**

## 9. REAGENT PREPARATION

Equilibrate all reagents to room temperature (18-25°C) prior to use. Prepare fresh reagents immediately prior to use. If crystals have formed in the concentrate, mix gently until the crystals have completely dissolved.

### 9.1 1X Diluent N

Dilute the 10X Diluent N Concentrate 1:10 with reagent grade water. Mix gently and thoroughly. *Store for up to 1 month at 4°C.*

### 9.2 1X Wash Buffer

Dilute the 20X Wash Buffer Concentrate 1:20 with reagent grade water. Mix gently and thoroughly.

### 9.3 1X Biotinylated Complement C4 Detector Antibody

9.3.1 The stock Biotinylated Complement C4 Antibody must be diluted with 1X Diluent N according to the label concentration to prepare 1X Biotinylated Complement C4 Antibody for use in the assay procedure. Observe the label for the “X” concentration on the vial of Biotinylated Complement C4 Antibody.

9.3.2 Calculate the necessary amount of 1X Diluent N to dilute the Biotinylated Complement C4 Antibody to prepare a 1X Biotinylated Complement C4 Antibody solution for use in the assay procedure according to how many wells you wish to use and the following calculation:

Number of Wells Strips	Number of Wells	(V <sub>T</sub> ) Total Volume of 1X Biotinylated Antibody (μL)
4	32	1760
6	48	2640
8	64	3520
10	80	4400
12	96	5280

*Any remaining solution should be frozen at -20°C.*

## ASSAY PREPARATION

### Where:

$C_S$  = Starting concentration (X) of stock Biotinylated Complement C4 Antibody (variable)

$C_F$  = Final concentration (always = 1X) of 1X Biotinylated Complement C4 Antibody solution for the assay procedure

$V_T$  = Total required volume of 1X Biotinylated Complement C4 Antibody solution for the assay procedure

$V_A$  = Total volume of (X) stock Biotinylated Complement C4 Antibody

$V_D$  = Total volume of 1X Diluent N required to dilute (X) stock Biotinylated Complement C4 Antibody to prepare 1X Biotinylated Complement C4 solution for assay procedures

Calculate the volume of (X) stock Biotinylated Antibody required for the given number of desired wells:

$$(C_F / C_S) \times V_T = V_A$$

Calculate the final volume of 1X Diluent N required to prepare the 1X Biotinylated Complement C4 Antibody:

$$V_T - V_A = V_D$$

### Example:

**NOTE: This example is for demonstration purposes only. Please remember to check your antibody vial for the actual concentration of antibody provided.**

$C_S$  = 50X Biotinylated Complement C4 Antibody stock

$C_F$  = 1X Biotinylated Complement C4 Antibody solution for use in the assay procedure

$V_T$  = 3520  $\mu$ L (8 well strips or 64 wells)

$$(1X/50X) \times 3520 \mu\text{L} = 70.4 \mu\text{L}$$

$$3520 \mu\text{L} - 70.4 \mu\text{L} = 3449.6 \mu\text{L}$$

$V_A$  = 70.4  $\mu$ L total volume of (X) stock Biotinylated Complement C4 Antibody required

$V_D$  = 3449.6  $\mu$ L total volume of 1X Diluent N required to dilute the 50X stock Biotinylated Antibody to prepare 1X Biotinylated Complement C4 Antibody solution for assay procedures

9.3.3 First spin the Biotinylated Complement C4 Antibody vial to collect the contents at the bottom.

9.3.4 Add calculated amount  $V_A$  of stock Biotinylated Complement C4 Antibody to the calculated amount  $V_D$  of 1X Diluent N. Mix gently and thoroughly.

### 9.4 **1X SP Conjugate**

Spin down the 100X Streptavidin-Peroxidase Conjugate (SP Conjugate) briefly and dilute the desired amount of the conjugate 1:100 with 1X Diluent N.

*Any remaining solution should be frozen at  $-20^{\circ}\text{C}$ .*

## 10. STANDARD PREPARATIONS

- Prepare serially diluted standards immediately prior to use. Always prepare a fresh set of standards for every use.
- Any remaining standard should be stored at -20°C after reconstitution and used within 30 days.
- This procedure prepares sufficient standard dilutions for duplicate wells.

10.1 Reconstitution of the Complement C4 Standard vial to prepare a 20 ng/mL Complement C4 **Standard #1**:

10.1.1 First consult the Complement C4 Standard vial to determine the mass of protein in the vial.

10.1.2 Calculate the appropriate volume of 1X Diluent N to add when resuspending the Complement C4 Standard vial to produce a 20 ng/mL Complement C4 **Standard #1** by using the following equation:

$C_S$  = Starting mass of Complement C4 Standard (see vial label)  
( $\mu\text{g}$ )

$C_F$  = 20 ng/mL Complement C4 **Standard #1** final required concentration

$V_D$  = Required volume of 1X Diluent N for reconstitution ( $\mu\text{L}$ )

Calculate total required volume 1X Diluent N for resuspension:

$$(C_S / C_F) \times 1000 = V_D$$

### Example:

**NOTE: This example is for demonstration purposes only. Please remember to check your standard vial for the actual amount of standard provided.**

$C_S$  = 36 ng of Complement C4 Standard in vial

$C_F$  = 20 ng/mL Complement C4 **Stock Standard** final concentration

$V_D$  = Required volume of 1X Diluent N for reconstitution

$$(36 \text{ ng} / 20 \text{ ng/mL}) \times 1000 = 1800 \text{ } \mu\text{L}$$

- 10.1.3 First briefly spin the Complement C4 Standard Vial to collect the contents on the bottom of the tube.
- 10.1.4 Reconstitute the Complement C4 Standard vial by adding the appropriate calculated amount  $V_D$  of 1X Diluent N to the vial to generate the 20 ng/mL Complement C4 **Standard #1**. Mix gently and thoroughly.
- 10.2 Allow the reconstituted 20 ng/mL Complement C4 **Standard #1** to sit for 10 minutes with gentle agitation prior to making subsequent dilutions
- 10.3 Label six tubes #2-6.
- 10.4 Add 360  $\mu\text{L}$  of 1X Diluent N to tubes #2 – 6.
- 10.5 To prepare **Standard #2**, add 120  $\mu\text{L}$  of the **Standard #1** into tube #2 and mix gently.
- 10.6 To prepare **Standard #3**, add 120  $\mu\text{L}$  of the **Standard #2** into tube #3 and mix gently.
- 10.7 Using the table below as a guide, prepare subsequent serial dilutions.
- 10.8 1X Diluent N serves as the zero standard, 0 ng/mL (tube #6).

# ASSAY PREPARATION

## Standard Dilution Preparation Table

Standard #	Volume to Dilute ( $\mu\text{L}$ )	Volume Diluent N ( $\mu\text{L}$ )	Total Volume ( $\mu\text{L}$ )	Starting Conc. (ng/mL)	Final Conc. (ng/mL)
1	Step 10.1				20.000
2	120	360	480	20.000	5.000
3	120	360	480	5.000	1.250
4	120	360	480	1.250	0.313
5	120	360	480	0.313	0.078
6	-	360	360	-	0



## 11. SAMPLE PREPARATION

### 11.1 **Milk**

Collect milk using sample tube. Centrifuge samples at 800 x g for 10 minutes. Milk dilution is suggested at 1:8000 into 1X Diluent N or within the range of 500X – 50000X; however, the user should determine the optimal dilution factor. The undiluted samples can be stored at -20°C or below for up to 3 months. Avoid repeated freeze-thaw cycles.

### 11.2 **Saliva**

Collect saliva using sample pot. Centrifuge samples at 600 x g for 10 minutes. Dilute samples 1:200 into 1X Diluent N or within the range of 50X – 1000X and assay. The undiluted samples can be stored at -20°C or below for up to 3 months. Avoid repeated freeze-thaw cycles.

### 11.3 **CSF**

Collect cerebrospinal fluid (CSF) using sample pot. Centrifuge samples at 3000 x g for 10 minutes. Dilute samples 1:500 into Diluent N or within the range of 50X – 10000X and assay. If necessary, further dilute samples. The undiluted samples can be stored at -80°C for up to 3 months. Avoid repeated freeze-thaw cycles.

### 11.4 **Urine**

Collect urine using sample pot. Centrifuge samples at 600 x g for 10 minutes. Dilute samples 1:2 into 1X Diluent N or within the range of 1X – 10X and assay. The undiluted samples can be stored at -20°C or below for up to 3 months. Avoid repeated freeze-thaw cycles.

## 12. PLATE PREPARATION

- The 96 well plate strips included with this kit are supplied ready to use. It is not necessary to rinse the plate prior to adding reagents.
- Unused well plate strips should be returned to the plate packet and stored at 4°C.
- For statistical reasons, we recommend each sample should be assayed with a minimum of two replicates (duplicates).
- Well effects have not been observed with this assay. Contents of each well can be recorded on the template sheet included in the Resources section.

## **13. ASSAY PROCEDURE**

- **Equilibrate all materials and prepared reagents to room temperature (18 - 25°C) prior to use.**
  - **It is recommended to assay all standards, controls and samples in duplicate.**
- 13.1 Prepare all reagents, working standards and samples as instructed. Equilibrate reagents to room temperature before use. The assay is performed at room temperature (18-25°C).
  - 13.2 Remove excess microplate strips from the plate frame and return them immediately to the foil pouch with desiccant inside. Reseal the pouch securely to minimize exposure to water vapor and store in a vacuum desiccator.
  - 13.3 Add 50 µL of Complement C4 Standard or sample per well. Cover wells with a sealing tape and incubate for two hours. Start the timer after the last sample addition.
  - 13.4 Wash five times with 200 µL of 1X Wash Buffer manually. Invert the plate each time and decant the contents; tap it 4-5 times on absorbent paper towel to completely remove the liquid. If using a machine wash six times with 300 µL of 1X Wash Buffer and then invert the plate, decant the contents; tap it 4-5 times on absorbent paper towel to completely remove the liquid.
  - 13.5 Add 50 µL of 1X Biotinylated Complement C4 Antibody to each well and incubate for one hour.
  - 13.6 Wash microplate as described above.
  - 13.7 Add 50 µL of 1X SP Conjugate to each well and incubate for 30 minutes. Turn on the microplate reader and set up the program in advance.
  - 13.8 Wash microplate as described above.
  - 13.9 Add 50 µL of Chromogen Substrate per well and incubate for 20 minutes until the optimal blue colour density develops.

Gently tap plate to ensure thorough mixing and break the bubbles in the well with pipette tip.

- 13.10 Add 50  $\mu\text{L}$  of Stop Solution to each well. The color will change from blue to yellow.
- 13.11 Read the absorbance on a microplate reader at a wavelength of 450 nm immediately. If wavelength correction is available, subtract readings at 570 nm from those at 450 nm to correct optical imperfections. Otherwise, read the plate at 450 nm only. Please note that some unstable black particles may be generated at high concentration points after stopping the reaction for about 10 minutes, which will reduce the readings.

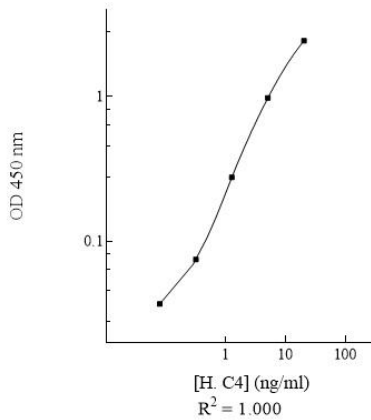
### **14. CALCULATIONS**

Calculate the mean value of the triplicate readings for each standard and sample. To generate a Standard Curve, plot the graph using the standard concentrations on the x-axis and the corresponding mean 450 nm absorbance on the y-axis. The best-fit line can be determined by regression analysis using log-log or four-parameter logistic curve-fit. Determine the unknown sample concentration from the Standard Curve and multiply the value by the dilution factor.

## 15. TYPICAL DATA

**TYPICAL STANDARD CURVE** – Data provided for demonstration purposes only. A new standard curve must be generated for each assay performed.

Human Complement C4 Standard Curve



## 16. TYPICAL SAMPLE VALUES

### SENSITIVITY –

The minimum detectable dose of Complement C4 is typically 32 pg/mL.

### SPIKING RECOVERY –

Sample	Unspiked Sample (ng/ml)	Spiking Value (ng/ml)	Recovery (%)
Milk	0.404	2.253	99
		0.545	105
		0.179	99
CSF	2.003	2.253	107
		0.545	107
		0.179	110
<b>Average Recovery (%)</b>			105

### LINEARITY OF DILUTION –

Milk Dilution	Average % Expected Value
1:4000	92
1:8000	99
1:16000	109

### PRECISION –

	Intra-Assay	Inter-Assay
% CV	3.9	8.0

**17. ASSAY SPECIFICITY**

<b>Species</b>	<b>% Cross Reactivity</b>
Canine	<7
Bovine	None
Equine	<1
Monkey	40
Mouse	None
Rat	<2
Swine	None
Rabbit	None
<b>Protein</b>	<b>% Cross Reactivity</b>
Human Complement C1	10

- No significant cross-reactivity observed with complement C1q, C1r, C1s, C2, C3, C4BP, C5, C6, C7, C8, C8G, C9, Factor B, Factor D, Factor H, Factor I, and Factor P.

## 18. TROUBLESHOOTING

<b>Problem</b>	<b>Cause</b>	<b>Solution</b>
Poor standard curve	Improper standard dilution	Confirm dilutions made correctly
	Standard improperly reconstituted (if applicable)	Briefly spin vial before opening; thoroughly resuspend powder (if applicable)
	Standard degraded	Store sample as recommended
	Curve doesn't fit scale	Try plotting using different scale
Low signal	Incubation time too short	Try overnight incubation at 4°C
	Target present below detection limits of assay	Decrease dilution factor; concentrate samples
	Precipitate can form in wells upon substrate addition when concentration of target is too high	Increase dilution factor of sample
	Using incompatible sample type (e.g. serum vs. cell extract)	Detection may be reduced or absent in untested sample types
	Sample prepared incorrectly	Ensure proper sample preparation/dilution
Large CV	Bubbles in wells	Ensure no bubbles present prior to reading plate
	All wells not washed equally/thoroughly	Check that all ports of plate washer are unobstructed wash wells as recommended
	Incomplete reagent mixing	Ensure all reagents/master mixes are mixed thoroughly
	Inconsistent pipetting	Use calibrated pipettes and ensure accurate pipetting
	Inconsistent sample preparation or storage	Ensure consistent sample preparation and optimal sample storage conditions (eg. minimize freeze/thaws cycles)

## RESOURCES

<b>Problem</b>	<b>Cause</b>	<b>Solution</b>
High background/ Low sensitivity	Wells are insufficiently washed	Wash wells as per protocol recommendations
	Contaminated wash buffer	Make fresh wash buffer
	Waiting too long to read plate after adding STOP solution	Read plate immediately after adding STOP solution
	Improper storage of ELISA kit	Store all reagents as recommended. Please note all reagents may not have identical storage requirements.
	Using incompatible sample type (e.g. Serum vs. cell extract)	Detection may be reduced or absent in untested sample types

19. NOTES









## **Technical Support**

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