ab204530 - 24(S)-Hydroxycholesterol ELISA Kit

Instructions for Use

For the quantitative determination of 24(S)-Hydroxychlolesterol in tissue culture media, cerebral spinal fluid and tissue homogenate samples.

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

Version 5c Last Updated 11 October 2021

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1. BACKGROUND

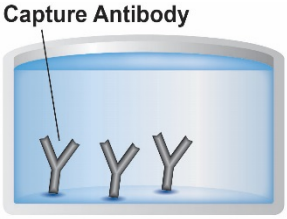
Abcam's 24(S)-Hydroxycholesterol (24-OHC) kit is an competitive in-vitro enzyme-linked immunosorbent assay (ELISA) for the quantitative measurement of 24-OHC levels in tissue culture media, cerebral spinal fluid and tissue homogenate samples.

The homeostasis and trafficking of cholesterol is an essential component of both the central and peripheral nervous system in the maintenance of neuronal tissues, and disturbances in this homeostasis may be due to the onset of various neurological diseases such as Alzheimer's disease, Huntington's disease and multiple sclerosis.

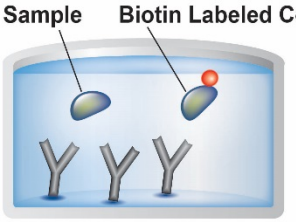
Apolipoprotein E and Cyp46 (also known as 24S-Cholesterol Hydroxylase) are both important in the homeostasis of cerebral cholesterol 6 and thus are of clinical interest in understanding the relation of these molecules with the pathogenesis of these, and potentially other, neurodegenerative diseases.

24-OHC, an enzymatically-generated side chain-hydroxylated derivative of cholesterol, is a pivotal marker in the study of cerebral cholesterol homeostasis. Cholesterol is unable to cross the blood-brain barrier however, Cyp46 converts cholesterol to the more soluble 24-OHC, and this hydroxylated form of cholesterol is able to cross the blood-brain barrier. This conversion allows for the reduction of cholesterol in the brain and the efflux of 24-OHC from the brain into cerebral spinal fluid and blood. The flux of 24-OHC has been observed in patients with a variety of neurodegenerative diseases. In the instance of Alzheimer's disease, the change in 24S-hydroxycholesterol concentrations may be indicative of different pathogenic mechanisms and/or the progression of the disease. As in the case of multiple sclerosis, concentrations of 24-OHC have been shown to decrease, likely due to the loss of neuronal cells responsible for the synthesis.

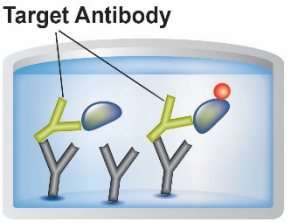
2. ASSAY SUMMARY



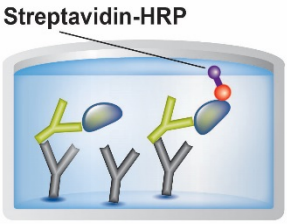
Standards and samples are added to wells pre-coated with a goat anti-rabbit IgG antibody.



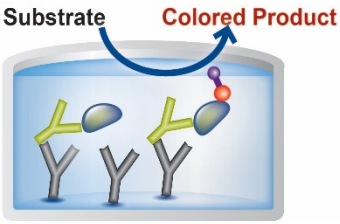
During a simultaneous incubation at room temperature the antibody binds either 24(S)-Hydroxycholesterol (24-OHC) in the sample or the labelled conjugate. The plate is washed, leaving only bound 24-OHC or labelled conjugate.



A solution of streptavidin conjugated to horseradish peroxidase is added to each well, to bind the biotinylated 24-OHC. The plate is again incubated.



The plate is washed to remove excess HRP conjugate. TMB substrate solution is added. An HRP-catalyzed reaction generates a blue color in the solution.



Stop solution is added to stop the substrate reaction. The resulting yellow color is read at 450 nm. The amount of signal is inversely proportional to the level of 24-OHC in the sample.

3. PRECAUTIONS

Please read these instructions carefully prior to beginning the assay.

- Some kit components contain azide, which may react with lead or copper plumbing. When disposing of reagents always flush with large volumes of water to prevent azide build-up
- Stop Solution is a solution of 1 N HCl in water. This solution is caustic; use with appropriate personal protective equipment.
- This kit has been tested with a variety of samples, however it is possible that high levels of interfering substances may cause variation in assay results

4. STORAGE AND STABILITY

Store kit at +2-8°C immediately upon receipt.

Refer to list of materials supplied for storage conditions of individual components.

5. MATERIALS SUPPLIED

Item	Amount	Storage Condition (Before Preparation)
Assay Buffer 40	50 mL	+2-8ºC
24(S)-Hydroxycholesterol Standard (10µg/mL)	70 µL	+2-8ºC
Goat anti-Rabbit IgG Microplate (96 wells)	1 Unit	+2-8ºC
24(S)-Hydroxycholesterol Antibody (1X Soln.)	5 mL	+2-8ºC
24(S)-Hydroxycholesterol Conjugate (100X Conc.)	70 µL	+2-8ºC
Streptavidin-HRP (1X Soln.)	20 mL	+2-8ºC
Wash Buffer Concentrate (20X Conc.)	27 mL	+2-8ºC
TMB Substrate	2X 10 mL	+2-8ºC
Stop Solution (1N Soln.)	10 mL	+2-8ºC
Plate Sealer	3 Units	+2-8ºC

6. MATERIALS REQUIRED, NOT SUPPLIED

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

- Deionized or distilled water.
- Precision pipets for volumes between 5 µL and 1,000 µL.
- Repeater pipet for dispensing 50 µL and 200 µL.
- Disposable beakers for diluting buffer concentrates.
- Graduated cylinders.
- A microplate shaker.
- Mechanical homogenizer or manual dounce homogenizer for tissue sample preparation.
- 95% Ethanol (tissue homogenization only).
- Dichloromethane (tissue homogenization only).
- Rotary evaporator or argon gas (tissue homogenization only).
- Adsorbent paper for blotting.
- Microplate reader capable of reading a 450 nm.
- Software for extrapolating sample values from absorbance measurements utilizing a four parameter logistic curve fit.

7. LIMITATIONS

- The assay is suitable for the measurement of 24(S)-Hydroxycholesterol in tissue culture media, cerebral spinal fluid and tissue homogenate samples. This kit is not species specific; however, samples containing rabbit IgG cannot be used as the goat anti-rabbit IgG coated plate will bind indiscriminately to rabbit IgG. Prior to performing the assay, frozen samples should be brought to 4°C and centrifuged, if necessary, to isolate residual debris.
- Assay kit intended for research use only. Not for use in diagnostic procedures
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.

8. TECHNICAL HINTS

- For accurate measurement of 24(S)-Hydroxycholesterol in cultured media, standards should be diluted directly into uncultured media for preparation of the standard curve. All sample dilutions should also be performed using the same uncultured media. Be sure to use the same media as was used during the preparation of the cultured media samples.
- A minimum dilution of 1:2 in assay buffer is required for analysis of cerebral spinal fluid and tissue homogenates. Tissue culture samples can be run neat.
- Due to differences in samples, users must determine the optimal sample dilution for their particular experiments.
- Pre-rinse the pipette tip with the reagent, use fresh pipette tips for each sample, standard and reagent.
- Pipette standards and samples to the bottom of the wells.
- Add the reagents to the side of the well to avoid contamination.

- Once reagents have been added to the plate, DO NOT let the plate dry at any time during the assay.
- It is recommended that all standards and samples be assayed in duplicate.
- Avoid microbial contamination of reagents and equipment. Automated plate washers can become contaminated thereby causing assay variability. Buffers containing a large quantity of protein should be made under sterile conditions and stored at 2-8°C or be prepared fresh daily.
- Prior to addition of substrate, ensure that there is no residual wash buffer in the wells. Any remaining wash buffer may cause variation in assay results.
- **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 and samples to room temperature (18 - 25°C) prior to use.

Note: Glass or polypropylene tubes may be used for reagent and standard preparation. Avoid polystyrene.

9.1 Wash Buffer

Prepare Wash buffer by diluting 25 mL of the supplied Wash Buffer concentrate with 475 mL of deionized water. Store the diluted wash buffer at room temperature.

9.2 24(S)-Hydroxycholesterol Conjugate

Prepare conjugate by diluting 60 µL of the 100X conjugate stock with 5.94 mL of Assay Buffer 40, vortex thoroughly. Do not store diluted conjugate.

10. STANDARD PREPARATIONS

Prepare serially diluted standards immediately prior to use. Always prepare a fresh set of standards for every use Diluted standards should be used immediately. For analysis of tissue culture samples use uncultured media for all standard and sample dilutions.

10.1 Label nine 12 x 75 mm tubes #1 through #9.

10.2 Pipet 990 µL of Assay Buffer 40 or uncultured media into tube #1.

10.3 Pipet 500 µL of Assay Buffer 40 or uncultured media into tubes #2 through #9.

10.4 Add 10 µL of the supplied 100X standard to tube #1 and vortex thoroughly.

10.5 Remove 500 µL from tube #1 and add to tube #2. Vortex thoroughly.

10.6 Repeat step 10.5 for tubes #3 through #9.

Standard #	Sample to Dilute	Volume to Dilute (µL)	Volume of Diluent (µL)	Starting Conc. (ng/mL)	Final Conc. (ng/mL)
1	Stock	10	990	10,000	100
2	Standard #1	500	500	100	50
3	Standard #2	500	500	50	25
4	Standard #3	500	500	25	12.5
5	Standard #4	500	500	12.5	6.25
6	Standard #5	500	500	6.25	3.13
7	Standard #6	500	500	3.13	1.56
8	Standard #7	500	500	1.56	0.78
9	Standard #8	500	500	0.78	0.39

11. SAMPLE COLLECTION AND STORAGE

Preparing tissue extract requires 95% Ethanol, Dicholoromethane a Tissue Homogenizer and a Rotary evaporator or argon gas.

11.1 In a 12 x 75 mm tube, homogenize 100 mg of tissue in 1 mL of 95% Ethanol.

11.2 Centrifuge extract at 7000 x g at room temperature for 5 minutes.

11.3 Collect and retain the supernatant.

11.4 Add 1 mL of Ethanol: Dichloromethane (1:1; v/v) to the pellet and sonicate for 10 minutes.

11.5 Centrifuge this extract as in Step 11.2.

11.6 Collect supernatant and combine with supernatant from Step 11.3.

11.7 Evaporate the pooled supernatant sample to dryness under a stream of argon or with a rotary evaporator.

11.8 Rehydrate samples at room temperature by adding 16 µL of 95% ethanol followed by 484 µL of Assay Buffer 40, this is required to fully solubilize the 24(S)-Hydroxycholesterol present following this sample preparation procedure.

11.9 Subsequent sample dilutions in Assay Buffer A must be determined empirically by the individual investigator.

12. PLATE MAP

24(S)-Hydroxycholesterol Plate Map:

A1 Blank	A2 Std 3	A3 Std 7	A4	A5	A6	A7	A8	A9	A10	A11	A12
B1 Blank	B2 Std 3	B3 Std 7	B4	B5	B6	B7	B8	B9	B10	B11	B12
C1 NSB	C2 Std 4	C3 Std 8	C4	C5	C6	C7	C8	C9	C10	C11	C12
D1 NSB	D2 Std 4	D3 Std 8	D4	D5	D6	D7	D8	D9	D10	D11	D12
E1 Std 1	E2 Std 5	E3 Std 9	E4	E5	E6	E7	E8	E9	E10	E11	E12
F1 Std 1	F2 Std 5	F3 Std 9	F4	F5	F6	F7	F8	F9	F10	F11	F12
G1 Std 2	G2 Std 6	G3 Bo	G4	G5	G6	G7	G8	G9	G10	G11	G12
H1 Std 2	H2 Std 6	H3 Bo	H4	H5	H6	H7	H8	H9	H10	H11	H12

13. ASSAY PROCEDURE

- **Equilibrate all materials and prepared reagents to room temperature prior to use.**
- **It is recommended to assay all standards, controls and samples in duplicate.**
- **Refer to the Plate Map (12) determine the number of wells to be used.**
- **Remove the wells not needed for the assay and return them, with the desiccant, to the bag and seal. Store unused wells at +2-8°C.**

13.1 Pipet 150 µL of the Assay Buffer 40 into the NSB (nonspecific binding) wells.
13.2 Pipet 100 µL of Assay Buffer 40 into the Bo (0 ng/mL standard) wells.
13.3 Pipet 100 µL of standards and samples, prepared in Assay Buffer 40, to the bottom of the appropriate wells.
13.4 Pipet 50 µL of the diluted conjugate to each well except the blank.
13.5 Pipet 50 µL of the detection antibody into each well, except the blank and the NSB.
13.6 Seal the plate. Incubate for 1 hour on a plate shaker at room temperature.
13.7 Empty the contents of the wells and wash by adding 400 µL of Wash Buffer to each well. Repeat 3 times for a total of 4 washes. After the final wash, empty or aspirate the wells and firmly tap the plate on a lint free paper towel to remove any remaining liquid.
13.8 Pipet 200 µL of 1X streptavidin-HRP to each well, except the blank.
13.9 Seal the plate. Incubate for 30 minutes on a plate shaker at room temperature.
13.10 Wash as above (Step 13.7).
13.11 Pipet 200 µL of TMB solution into each well.

13.12 Seal the plate. Incubate for 30 minutes at room temperature without shaking.

13.13 Pipet 50 µL Stop Solution into each well.

13.14 After blanking the plate reader against the substrate, read optical density at 450 nm. If the plate reader is not capable of adjusting for the blank, manually subtract the mean OD of the substrate blank from all readings.

Note: The optimal speed for each shaker will vary and may range from 120-700 rpm. The speed must be set to ensure adequate mixing of the wells, but not so vigorously that the contents of the wells splash out and contaminate other wells.

14. CALCULATIONS

14.1 Calculate the average net absorbance measurement (Average Net OD) for each standard and sample by subtracting the average NSB absorbance measurement from the average absorbance measurement (Average OD) for each standard and sample.

Average Net OD = Average OD - Average NSB OD

14.2 Calculate the binding of each pair of standard wells as a percentage of the maximum binding wells (Bo), using the following formula:

$$\text{Percent Bound} = \frac{\text{Average Net OD}}{\text{Average Net Bo OD}} \times 100$$

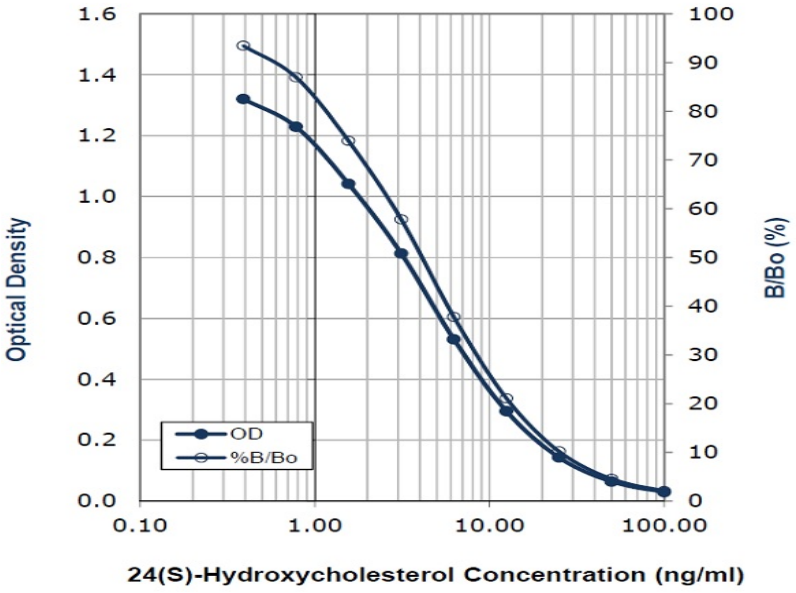
14.3 Plot the Percent Bound (B/Bo) versus concentration of 24(S)-Hydroxycholesterol for the standards. Fit a line through the data. The concentration of 24(S)-Hydroxycholesterol in the unknowns can be determined by interpolation.

Note: A four parameter algorithm (4PL) provides the best fit, though other algorithms can be examined to see which provides the most accurate fit of the data (e.g. linear, semi-log, log/log, 4 parameter logistic). Interpolate protein concentrations for unknown samples from the standard curve plotted.

Samples producing signals greater than that of the highest standard should be further diluted and reanalyzed, then multiplying the concentration found by the appropriate 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. The following data are obtained using the different concentrations of standard as described in this protocol:



Sample	Average Net OD (-NSB)	Percent Bound	24-OHC ng/mL
Blank	(0.037)	N/A	N/A
NSB	(0.051)	N/A	N/A
Bo	1.414	100%	0
Standard #1	0.029	2.07	100
Standard #2	0.064	4.57	50
Standard #3	0.143	10.2	25
Standard #4	0.295	21.1	12.5
Standard #5	0.531	37.8	6.25
Standard #6	0.813	57.8	3.13
Standard #7	1.042	73.9	1.56
Standard #8	1.229	87.0	0.78
Standard #9	1.320	93.5	0.39

16. TYPICAL SAMPLE VALUES

The sensitivity, defined as 2 standard deviations from the mean signal at zero, was determined from 10 independent standard curves. The standard deviation was determined from 24 zero standard replicates. The sensitivity was found to be 0.78 ng/mL.

INTRA-ASSAY PRECISION was determined by assaying 24 replicates of two buffer controls containing 24(S)-Hydroxycholesterol in a single assay.

	1	2
n=	24	24
Mean (ng/mL)	28.2	9.0
%CV	9.3	5.8

INTER-ASSAY PRECISION was determined by measuring two buffer controls containing 24(S)-Hydroxycholesterol in multiple assays (n=10) over several days.

	1	2
n=	10	10
Mean (ng/mL)	28.6	11.1
%CV	18.4	18.5

RECOVERY

Synthetic 24(S)-Hydroxycholesterol was spiked into the following matrices. Matrix background was subtracted from the spiked values and the average recovery was compared to the recovery of identical spikes in assay buffer. The average percent recovery for each matrix is indicated below.

Sample Type	Recommended Dilution	Recovery of Spike %
Cerebral Spinal Fluid	1:2	99.0
Brain Tissue Homogenate	1:2	98.6
TCM (serum free)	Neat	95.5
TCM (1% FBS)	Neat	107.9
TCM + 10% FBS	Neat	101.6

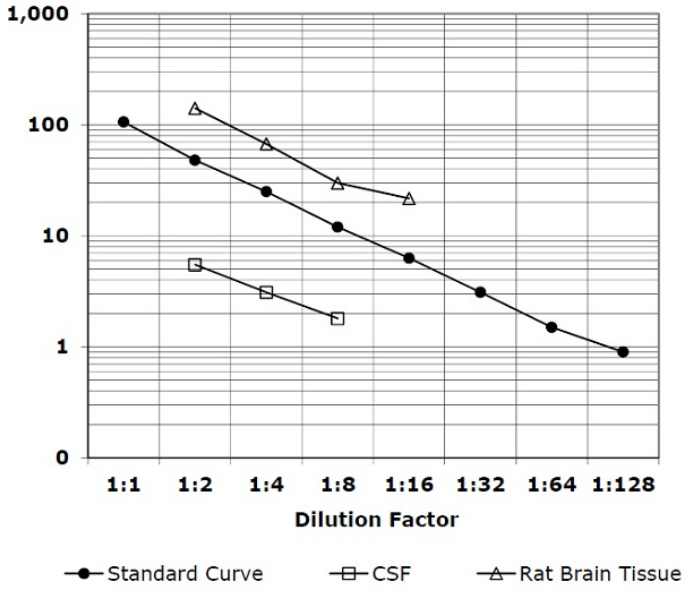
LINEARITY

Human cerebral spinal fluid and rat brain tissue samples were serially diluted 1:2 in assay buffer and measured in the assay alongside standards prepared in assay buffer. Tissue culture media (TCM) (RPMI1640+/- FBS*), was spiked with synthetic 24(S)-Hydroxycholesterol then serially diluted into uncultured media and compared to standards prepared in the corresponding uncultured media. The minimum required dilution was determined by identifying the dilution at which linearity was observed.

Dilution	Average % of expected				
	TCM (serum free)	TCM (1% FBS)	TCM (10% FBS)	CSF	Tissue homogenate
Neat	97	90	103	-	-
1:2	91	108	97	87	118
1:2	91	103	97	97	112
1:2	86	100	118	100	100

PARALLELISM

Dose-response curves from human cerebral spinal fluid and rat brain tissue homogenate were diluted into assay buffer and compared to the 24(S)-Hydroxycholesterol standard curve. The parallel response indicates the standard effectively mimics the native molecule.



17. ASSAY SPECIFICITY

THE CROSS REACTIVITIES for related compounds were determined by diluting the cross reactants in the kit assay buffer at a concentration of one hundred times the high standard. These samples were then measured in the assay.

Analyte	Cross Reactivity %
Cholesterol	0.004
22-Hydroxycholesterol	0.042
25-Hydroxycholesterol	0.09
27-Hydroxycholesterol	0.018
Dehydroepiandrosterone	0.006

18. TROUBLESHOOTING

Problem	Cause	Solution
Poor standard curve	Inaccurate pipetting	Check pipettes
	Improper standards dilution	Prior to opening, briefly spin the stock standard tube and dissolve the powder thoroughly by gentle mixing
Low Signal	Incubation times too brief	Ensure sufficient incubation times; change to overnight standard/sample incubation
	Inadequate reagent volumes or improper dilution	Check pipettes and ensure correct preparation
Samples give higher value than the highest standard	Starting sample concentration is too high.	Dilute the specimens and repeat the assay
Large CV	Plate is insufficiently washed	Review manual for proper wash technique. If using a plate washer, check all ports for obstructions
	Contaminated wash buffer	Prepare fresh wash buffer
Low sensitivity	Improper storage of the kit	Store the all components as directed.

19. NOTES

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