ELISA PRODUCT INFORMATION & MANUAL

24(S)-Hydroxycholesterol

NBP2-62130

Enzyme-linked Immunosorbent Assay for quantitative detection of Non-species specific 24(S)-Hydroxycholesterol.

For research use only.

Not for diagnostic or therapeutic procedures.

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Several Novus Biologicals products and product applications are covered by US and foreign patents and patents pending.

FOR RESEARCH USE ONLY.
NOT FOR USE IN DIAGNOSTIC PROCEDURES.

Please read entire booklet before proceeding with the assay.

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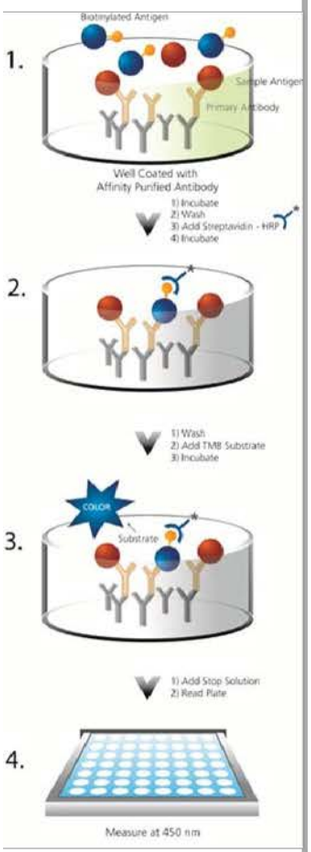
BACKGROUND

The Novus Biologicals 24(S)-Hydroxycholesterol (24-OHC) Enzyme-Linked Immunosorbent Assay (ELISA) kit is a complete kit for the quantitative determination of 24-OHC 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,[1,2] 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.[3,4,5] 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 enzyme converts cholesterol to the more soluble 24-OHC, and this hydroxylated form of cholesterol is able to cross the blood-brain barrier.[7,8] 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.[3,4,5] In the instance of Alzheimer's disease, the change in 24S-hydroxycholesterol concentrations may be indicative of different pathogenetic mechanisms and/or the progression of the disease.[3] 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.[5]



PRINCIPLE

1. Standards and Samples are added to wells coated with a goat anti-rabbit IgG antibody. A solution of biotinylated 24(S)-Hydroxycholesterol is then added, followed by a solution of rabbit polyclonal antibody to 24(S)-Hydroxycholesterol.
2. During a simultaneous incubation at room temperature the antibody binds, in a competitive manner, the 24(S)-Hydroxycholesterol in the sample or conjugate. The plate is washed, leaving only bound 24(S)-Hydroxycholesterol.
3. A solution of streptavidin conjugated to horseradish peroxidase is added to each well, to bind the biotinylated 24(S)-Hydroxycholesterol. The plate is again incubated.
4. 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.
5. 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(S)-Hydroxycholesterol in the sample.

Do not mix components from different kit lots or use beyond the expiration date of the kit.

MATERIALS SUPPLIED

1. **Assay Buffer 40:**
   50ml
   A mixture of phosphate buffered saline and ethanol
2. **24(S)-Hydroxycholesterol Standard:**
   70µl
   One vial containing 10µg/ml of 24(S)-Hydroxycholesterol
3. **Goat anti-Rabbit IgG Microtiter Plate:**
   One plate of 96 wells
   A clear plate of break-apart strips coated with a goat anti-rabbit polyclonal antibody
4. **24(S)-Hydroxycholesterol Antibody:**
   5ml
   1X solution of polyclonal antibody to 24(S)-Hydroxycholesterol
5. **24(S)-Hydroxycholesterol Conjugate:**
   70µl
   One vial containing 100X concentrate of biotinylated 24(S)-Hydroxycholesterol
6. **Streptavidin-HRP:**
   20ml
   1X solution of streptavidin conjugated to horseradish peroxidase
7. **Wash Buffer Concentrate:**
   27ml
   20X Tris buffered saline containing detergents
8. **TMB Substrate:**
   2 x 10ml each
   A solution of 3,3’5,5’ tetramethylbenzidine (TMB) and hydrogen peroxide
9. **Stop Solution 2:**
   10ml
   A 1N solution of hydrochloric acid in water
10. **24(S)-Hydroxycholesterol Assay Layout Sheet:**
    1 each
11. **Plate Sealer:**
    3 each

Protect substrate from prolonged exposure to light.

Stop solution is caustic. Keep tightly capped.

STORAGE

All kit components should be stored at 4°C upon receipt.
Shipping conditions may not reflect storage conditions.

OTHER MATERIALS NEEDED

1. Deionized or distilled water
2. Precision pipets for volumes between 5µl and 1,000µl
3. Repeater pipet for dispensing 50µl and 200µl
4. Disposable beakers for diluting buffer concentrates
5. Graduated cylinders
6. A microplate shaker
7. Mechanical homogenizer or manual dounce homogenizer for tissue sample preparation
8. 95% Ethanol (tissue homogenization only)
9. Dichloromethane (tissue homogenization only)
10. Rotary evaporator or argon gas (tissue homogenization only)
11. Adsorbent paper for blotting
12. Microplate reader capable of reading a 450nm
13. Software for extrapolating sample values from optical density readings utilizing a four parameter logistic curve fit.

If buffers other than those provided are used, the end-user must determine the appropriate dilution and assay variation.

Sample handling procedures should be completed prior to reagent preparation.

SAMPLE HANDLING

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 will interfere in the assay due to the goat anti-rabbit IgG coated plate. Prior to assay, frozen samples should be brought to 4°C and centrifuged, if necessary, to isolate residual debris.

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; whereas, tissue culture samples can be run neat.

Due to differences in samples, users must determine the optimal sample dilution for their particular experiments.

Linearity

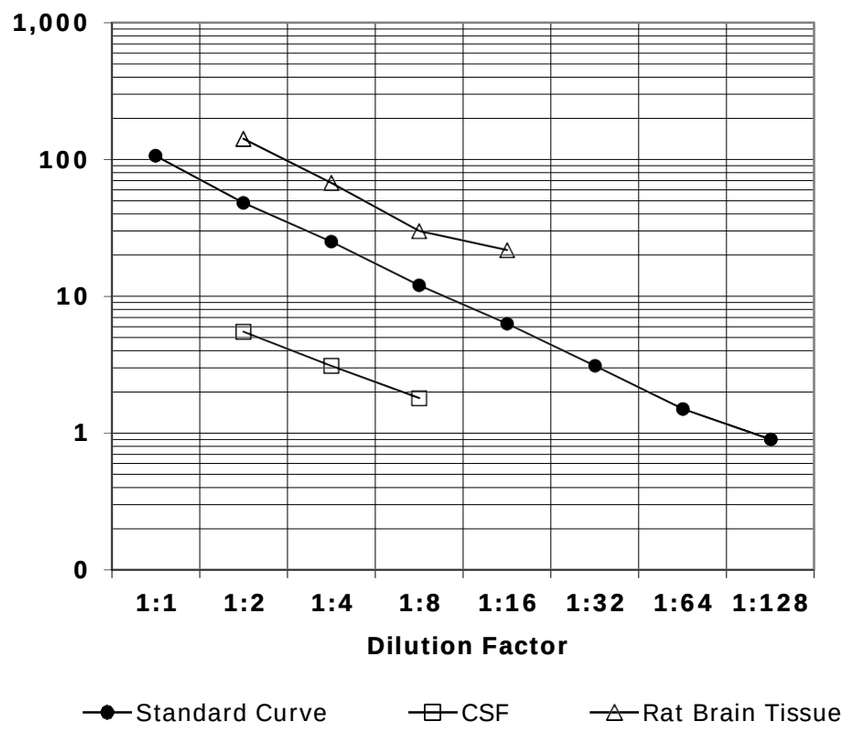
Human cerebral spinal fluid and rat brain tissue samples were serially diluted 1:2 in assay buffer and measured in the assay alongside standards prepared in assay buffer. Tissue culture media (TCM) (RPMI1640+/- FBS*), was spiked with synthetic 24(S)-Hydroxycholesterol then serially diluted into uncultured 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.

	Average % of expected				
Dilution	TCM (serum free)	TCM (1% FBS)	TCM (10% FBS)	CSF	Tissue homogenate
Neat	97	90	103	---	---
1:2	91	108	97	87	118
1:4	91	103	97	97	112
1:8	86	100	118	100	100

*DMEM (Gibco, Cat.#10569-044) was also tested and showed comparable results to RPMI 1640 (Gibco, Cat.#21870-092). Data not shown.

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.



Spike and 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	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%

TISSUE EXTRACTION PROTOCOL

MATERIALS NEEDED

1. 95% Ethanol
2. Dicholoromethane
3. Tissue Homogenizer
4. Rotary evaporator or argon gas

PROCEDURE

1. In a 12 x 75mm tube, homogenize 100mg of tissue in 1ml of 95% Ethanol.
2. Centrifuge extract at 7000 x *g* at room temperature for 5 minutes.
3. Collect and retain the supernatant.
4. Add 1ml of Ethanol:Dichloromethane (1:1; v/v) to the pellet and sonicate for 10 minutes.
5. Centrifuge this extract as in Step 2.
6. Collect supernatant and combine with supernatant from Step 3.
7. Evaporate the pooled supernatant sample to dryness under a stream of argon or with a rotary evaporator.
8. Rehydrate samples at room temperature by adding 16µl of 95% ethanol followed by 484 µl of Assay Buffer, this is required to fully solubilize the 24(S)-Hydroxycholesterol present following this sample preparation procedure.
9. Subsequent sample dilutions in assay buffer must be determined empirically by the individual investigator.

REAGENT PREPARATION

1. **Wash Buffer**

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

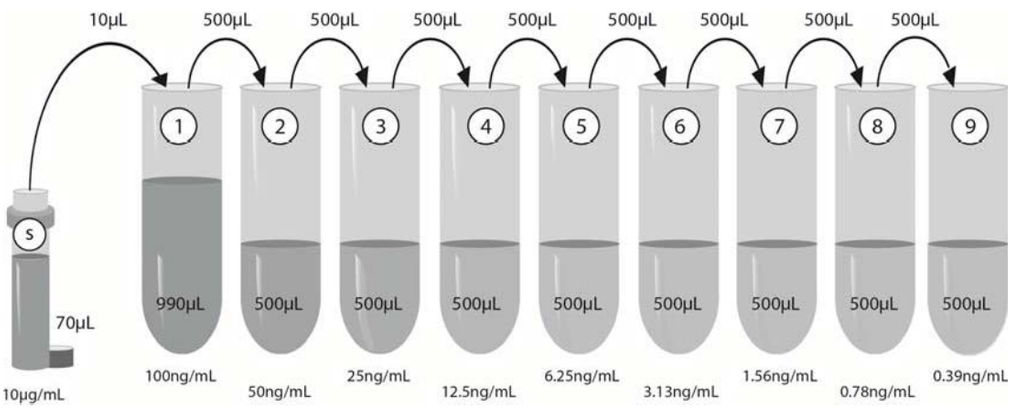
2. **24(S)-Hydroxycholesterol Conjugate**

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

3. **24(S)-Hydroxycholesterol Standard**

   Label nine 12 x 75mm tubes #1 through #9. Pipet 990µl of assay buffer or uncultured media (see Sample Handling) into tube #1. Pipet 500µl of assay buffer or uncultured media into tubes #2 through #9. Add 10µl of the supplied 100X standard to tube #1 and vortex thoroughly. Remove 500µl from tube #1 and add to tube #2. Vortex thoroughly. Continue this for tubes #3 through #9.

   Do not store diluted standard.



   Diluted standards should be used immediately. The concentrations of 24(S)-Hydroxycholesterol in the tubes are labeled above.

   For analysis of tissue culture samples use uncultured media for all standard and sample dilutions.

Glass or polypropylene tubes may be used for standard preparation. Avoid polystyrene.

ASSAY PROCEDURE

Bring all reagents to room temperature for at least 30 minutes prior to opening.

Refer to the Assay Layout Sheet to 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 4°C.

All standards and samples should be run in duplicate.

1. Pipet 150µl of the assay buffer into the NSB (non-specific binding) wells.
2. Pipet 100µl of Assay Buffer into the Bo (0 ng/ml standard) wells.
3. Pipet 100µl of standards and samples, prepared in Assay Buffer, to the bottom of the appropriate wells.
4. Pipet 50µl of the diluted conjugate to each well except the blank.
5. Pipet 50µl of the detection antibody into each well, except the blank and the NSB.
6. Seal the plate. Incubate for 1 hour on a plate shaker at room temperature.
7. Empty the contents of the wells and wash by adding 400µl of Wash Buffer to every well. Repeat 3 more 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 wash buffer.
8. Pipet 200µl of the diluted streptavidin-HRP to each well, except the blank.
9. Seal the plate. Incubate for 30 minutes on a plate shaker at room temperature.
10. Wash as above (Step 7).
11. Pipet 200µl of TMB solution into each well.
12. Seal the plate. Incubate for 30 minutes at room temperature without shaking.
13. Pipet 50µl 1N HCl into each well.
14. After blanking the plate reader against the substrate, read optical density at 450nm. If the plate reader is not capable of adjusting for the blank, manually subtract the mean OD of the substrate blank from all readings.

Pipet the reagents to the sides of the wells to avoid possible contamination.

Prior to the addition of the substrate, ensure there is no residual wash buffer in the wells. Remaining wash buffer may cause variation in results.

Note: The optimal speed for each shaker will vary and may range from 120-700rpm. 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.

CALCULATION OF RESULTS

Be sure to multiply sample concentrations by the dilution factor used during sample preparation.

Several options are available for the calculation of the concentration of 24(S)-Hydroxycholesterol in the samples. We recommend that the data be handled by an immunoassay software package utilizing a 4 parameter logistic (4PL) curve fitting program. The concentration of 24(S)-Hydroxycholesterol can be calculated as follows:

1. Calculate the average net OD for each standard and sample by subtracting the average NSB OD from the average OD for each standard and sample.

   Average Net OD = Average OD - Average NSB OD

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

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

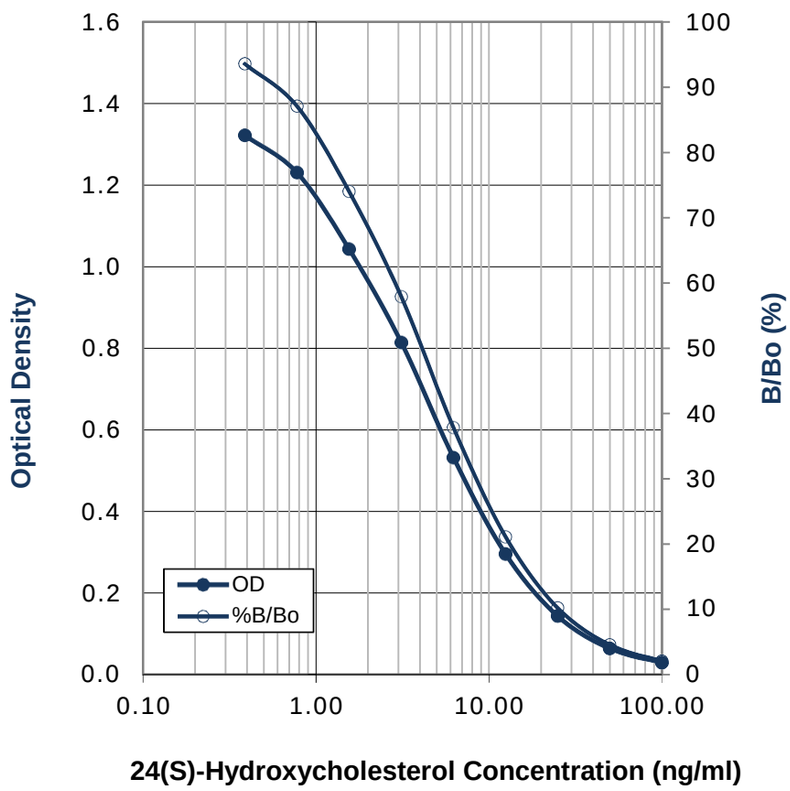
3. Plot the Percent Bound (B/Bo) versus concentration of 24(S)-Hydroxycholesterol for the standards. Approximate a straight line through the points. The concentration of 24(S)-Hydroxycholesterol in the unknowns can be determined by interpolation.

Samples with concentrations outside of the standard curve range will need to be re-analyzed using a different dilution.

TYPICAL RESULTS

The results shown below are for illustration only and should not be used to calculate results from another assay.

Sample	Average Net OD	Percent Bound	24(S)-Hydroxycholesterol (ng/ml)
Blank	(0.037)	---	---
NSB	(0.051)	---	---
Bo	1.414	100%	0
S1	0.029	2.07	100
S2	0.064	4.57	50
S6	0.143	10.2	25
S4	0.295	21.1	12.5
S5	0.531	37.8	6.25
S6	0.813	57.8	3.13
S7	1.042	73.9	1.56
S8	1.229	87.0	0.78
S9	1.320	93.5	0.39



PERFORMANCE CHARACTERISTICS

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%

Sensitivity

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.

Precision

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

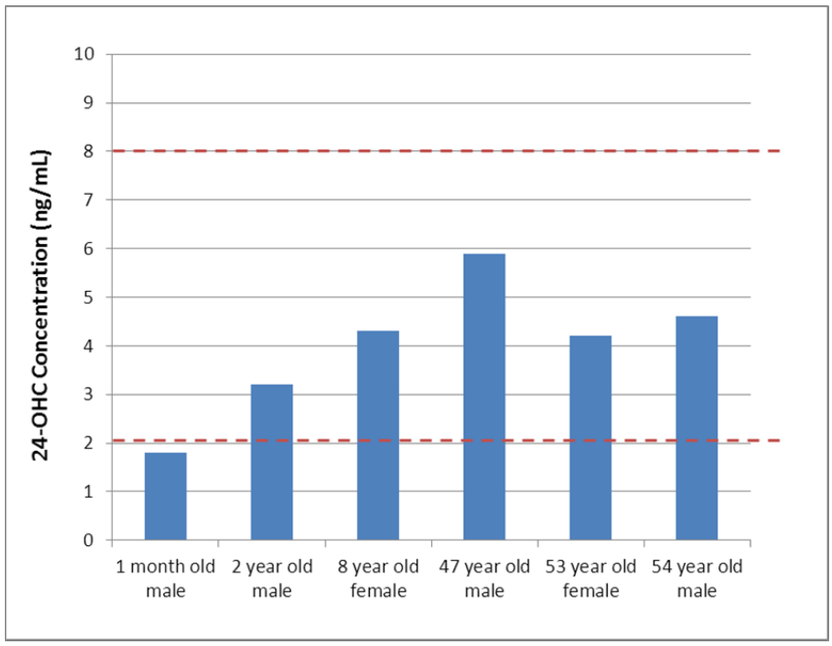
ng/ml	%CV
28.2	9.3
9.0	5.8

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

ng/ml	%CV
28.6	18.4
11.1	18.5

Experimental Evaluation

Normal (non-diseased) human cerebral spinal fluid samples were diluted 1:2 in assay buffer and analyzed in the assay for 24(S)-Hydroxycholesterol levels. The blue bars represent the levels of 24(S)-Hydroxycholesterol in each sample as determined by ELISA. The dashed lines represent the maximum and minimum range of endogenous CSF 24(S)-Hydroxycholesterol levels as reported in literature.[9,10]



REFERENCES

1. Poirier, J. (1994). Apolipoprotein E in animal models of CNS injury and in Alzheimer's disease. Trends in Neuroscience, 17(12):525- 530.
2. Beffert, U., Danik, M., Krzywkowski, P., Ramassamy, C., Berrada, F., Poirier, J. (1998). The neurobiology of apolipoproteins and their receptors in the CNS and Alzheimer's disease. Brain Research Reviews, 27(2): 119-142.
3. Zuliani, G., Donnorso, M. P., Bosi, C., Passaro, A., Nora, E. D., Zurlo, A., Bonetti, F., Mozzi, A. F., Cortese, C. (2011). Plasma 24S-hydroxycholesterol levels in elderly subjects with late onset Alzheimer's disease or vascular dementia: a case-control study. BMC Neurology. 11: 121.
4. Bjorkhem I., Leoni, V., Meaney, S. (2010). Thematic Review Series: Genetics of Human Lipid Diseases. Genetic connections between neurological disorders and cholesterol metabolism. Journal of Lipid Research, 51: 2489-2503.
5. Leoni, V., Masterman, T., Diczfalusy, U., Luca, G. D., Hillert, J., Bjorkhem, I. (2002). Changes in human plasma levels of the brain specific oxysterol 24S-hydroxycholesterol during progression of multiple sclerosis. Neuroscience Letters. 331: 163-166.
6. Martins, I. J., Hone, E., Foster, J. K., Sunram-Lea, S. I., Gnjec A., Fuller, S. J., Nolan, D., Gandy, S. E., Martins, R. N. (2006). Apolipoprotein E, cholesterol metabolism, diabetes, and the convergence of risk factors for Alzheimer's disease and cardiovascular disease. Molecular Psychiatry. 11: 721-736.
7. Dietschy, J. M., and S. D. Turley. (2004). Thematic review series: brain Lipids. Cholesterol metabolism in the central nervous system during early development and in the mature animal. J. Lipid Research. 45: 1375-1397.
8. Bjorkhem I., and S. Meaney. (2004). Brain cholesterol: long secret life behind a barrier. Arterioscler. Thromb. Vasc. Biol. 24: 806-815.
9. Papassotiropoulos, A., Lütjohann, D., Bagli, M.,Locatelli, S., Jessen, F., Buschfort, R., Ptok, U., Björkhem, I., von Bergmann, K., Heun, R. (2002). 24S-hydroxycholesterol in cerebrospinal fluid is elevated in early stages of dementia. Journal of Psychiatric Research. 36: 27-32.
10. Lütjohann, D., Breuer, O., Ahlborg, G., Nennesmo, I., Sidén, A., Diczfalusy, U., Björkhem, I. (1996). Cholesterol homeostasis in human brain: evidence for an age-dependent flux of 24S-hydroxycholesterol from the brain into the circulation. Proc. Natl. Acad. Sci. 93: 9799-9804.

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