Human Allopregnanolone (AP) ELISA Kit

Catalog No.: abx252027

Size: 96T

Range: 1.563 ng/ml - 100 ng/ml

Sensitivity: < 0.938 ng/ml

Storage: Store at 4°C for up to 6 months.

Application: For quantitative detection of AP in Human Serum, Plasma, Tissue Homogenates and other biological fluids.

Introduction: Allopregnanolone, also known as 5α-pregnan-3α-ol-20-one or 3α,5α-tetrahydroprogesterone (3α,5α-THP), as well as brexanolone (USAN), is an endogenous inhibitory pregnane neurosteroid. It is synthesized from progesterone, and is a potent positive allosteric modulator of the action of γ-amininobutyric acid (GABA) at GABAA receptor. Allopregnanolone has effects similar to those of other positive allosteric modulators of the GABA action at GABAA receptor such as the benzodiazepines, including anxiolytic, sedative, and anticonvulsant activity. Endogenously produced allopregnanolone exerts a pivotal neurophysiological role by fine-tuning of GABAA receptor and modulating the action of several positive allosteric modulators and agonists at GABAA receptor. The 21-hydroxylated derivative of this compound, tetrahydrodeoxycorticosterone (THDOC), is an endogenous inhibitory neurosteroid with similar properties to those of allopregnanolone, and the 3β-methyl analogue of allopregnanolone, ganaxolone, is under development to treat epilepsy and other conditions, including post-traumatic stress disorder (PTSD)

Principle of the Assay

This kit is based on a competitive enzyme-linked immuno-sorbent assay technology. AP is pre-coated onto a 96-well plate. The standards, samples and a biotin conjugated antibody specific to AP are added to the wells and incubated. After washing away the unbound conjugates, Streptavidin-HRP (SABC) is added to each microplate well and incubated. After TMB substrate solution is added only wells that contain AP will produce a blue colour product that changes into yellow after adding stop solution. The intensity of the color yellow is inverse proportional to the AP amount bound on the plate. The O.D. absorbance is measured spectrophotometrically at 450 nm in a microplate reader, and then the concentration of AP can be calculated.

Kit components

1. One pre-coated 96 well plate
2. Standard: 2 tubes
3. Sample/Standard Diluent Buffer: 20 ml
4. Biotin conjugated antibody (Dilution 1:100): 60 µl
5. Antibody diluent buffer: 10 ml
6. Streptavidin-HRP conjugate (SABC) (Dilution 1:100): 120 µl
7. SABC diluent buffer: 10 ml
8. TMB substrate: 10 ml
9. Stop solution: 10 ml
10. Wash buffer (25X): 30 ml

Material Required But Not Provided

1. 37°C incubator
2. Microplate reader (wavelength: 450 nm)
3. High-precision pipette and sterile pipette tips
4. Automated plate washer
5. ELISA shaker
6. 1.5 ml tubes to prepare standard/sample dilutions
7. Deionized or distilled water
8. Absorbent filter papers
9. 100 ml and 1 liter graduated cylinders

Protocol

A. Preparation of sample and reagents

1. Sample

Store samples to be assayed within 24 hours at 2-8°C. Alternatively, aliquot and store at -20°C or -80°C for long term. Avoid repeated freeze-thaw cycles.

- **Serum:** Samples should be collected into a serum separator tube. Coagulate the serum by leaving the tube undisturbed in a vertical position overnight at 4°C or at room temperature for up to 60 minutes. Centrifuge at approximately 1000 × g for 20 min. Analyze the serum immediately or aliquot and store at -20°C or -80°C.
- **Plasma:** Collect plasma using EDTA as an anticoagulant. Centrifuge for 15 minutes at 1000 x g within 30 minutes of collection. Assay immediately or aliquot and store at -20°C. Avoid hemolysis and high cholesterol samples.
- **Tissue homogenates:** The preparation of tissue homogenates will vary depending upon tissue type – this is just an example. Rinse tissues with ice-cold PBS to remove the excess of blood. Weigh before homogenization. Finely mince tissues and homogenize with a tissue homogenizer on ice in PBS and sonicate the cell suspension. Centrifuge the homogenates at 5000 × g for 5 min and collect the supernatant. Assay immediately or aliquot and store at -20°C.
- **Other biological fluids:** Centrifuge at approximately 1000 × g for 20 min to remove precipitant. Analyze immediately or aliquot and store at -20°C or -80°C.

Note:

» Fresh samples or recently obtained samples are recommended to prevent degradation and denaturalization that may lead to erroneous results. It is recommended to store samples to be used within 5 days at 4°C, within 1 month at -20°C and within 2 months at -80°C.

» Samples should be clear and transparent. Samples must be diluted so that the expected concentration falls within the kit's range.

» Please bring sample slowly to room temperature. Sample hemolysis will influence the result. Hemolyzed specimen should not be used. Samples that contain NaN_3 cannot be detected as it interferes with HRP.

» Always use non-pyrogenic, endotoxin-free tubes for blood collection.

Sample dilution guideline:

Estimate the concentration of the target in the sample and select the correct dilution factor to make the diluted target concentration fall near the middle of the kit's range. Generally, for high concentration (1000 ng/ml - 10000 ng/ml), dilute 1:100, for medium concentration (100 ng/ml - 1000 ng/ml), dilute 1:10 and for low concentration (1.563 ng/ml - 100 ng/ml), dilute 1:2. Very low concentrations (≤ 1.563 ng/ml) do not need dilution. Dilute the sample with the provided Sample Diluent Buffer and mix thoroughly. Several trials may be necessary to determine the optimal dilution factor.

2. Wash buffer

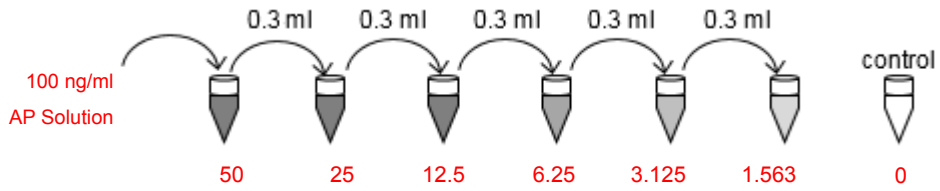
Dilute the concentrated Wash buffer 25-fold (1/25) with distilled water (i.e. add 30 ml of concentrated wash buffer into 720 ml of distilled water).

3. Standard

Preparation of the AP standard: standard solution should be prepared no more than 15 min prior to the experiment. Centrifuge at 10,000×g for 1 minute as the powder may drop off from the cap when opening if you do not spin down. **(Note: Do not dilute the standard directly in the plate).**

a.) 100 ng/ml standard solution. Add 1 ml of Sample/Standard diluent buffer into one Standard tube,Allow the reconstituted standard to sit for 15 minutes with gentle agitation prior to carrying out the serial dilutions; avoiding foaming or bubbles.

b.) 50 ng/ml → 1.5625 ng/ml standard solutions: Label 6 tubes with 50 ng/ml, 25 ng/ml, 12.5 ng/ml, 6.25 ng/ml, 3.125 ng/ml and 1.5625 ng/ml. Aliquot 0.3 ml of the Sample / Standard diluent buffer into each tube. Add 0.3 ml of the above 100 ng/ml standard solution into 1st tube and mix thoroughly. Transfer 0.3 ml from 1st tube to 2nd tube and mix thoroughly. Transfer 0.3 ml from 2nd tube to 3rd tube and mix thoroughly, and so on.



Note: Do not vortex the standard during reconstitution, as this will destabilize the protein. Once your standard has been reconstituted, it should be used right away. We do not recommend reusing the reconstituted standard. Please use the diluted Standards for a single assay procedure and discard after use.

4. Preparation of Biotin conjugated antibody working solution: prepare no more than 1 hour before the experiment.
a.) Calculate the total volume of the working solution: 0.05 ml / well × quantity of wells. (Allow 0.1-0.2 ml more than the total volume).
b.) Dilute the Biotin conjugated antibody with Antibody diluent buffer at 1/100 and mix thoroughly. i.e. Add 1 µl of Biotin conjugated antibody into 99 µl of Antibody diluent buffer.

5. Preparation of Streptavidin-HRP Conjugate (SABC) working solution: prepare no more than 30 min before the experiment.
a.) Calculate the total volume of the working solution: 0.1 ml / well × quantity of wells. (Allow 0.1-0.2 ml more than the total volume).
b.) Dilute the SABC with SABC diluent buffer at 1/100 and mix thoroughly. i.e. Add 1 µl of SABC into 99 µl of SABC diluent buffer.

B. Assay Procedure
Equilibrate the SABC working solution to room temperature and TMB substrate at 37°C for 30 minutes prior to use. It is recommended to plot a standard curve for each test.

1. **Wash the plate two times before adding standard, samples and buffers.** Any strips that are not being used should be kept dry and stored at 4°C. 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.
2. Add 50 µl of the prepared standards solutions into the standard wells.
3. Add 50 µl of Sample / Standard diluent buffer into the control (zero) well.
4. Add 50 µl of appropriately diluted sample into test sample wells.
5. Immediately add 50 µl of Biotin conjugated antibody working solution into each well. (Please add the solution at the bottom of each well without touching the side wall).
6. Cover the plate with the plate sealer. Gently tap the plate to mix thoroughly. Incubate at 37°C for 45 minutes.
7. Remove the cover and wash the plate 3 times. Discard the solution without touching the side walls. Blot the plate on an absorbent material. Fill each well completely (approximately 400 µl) with Wash buffer and incubate on an ELISA shaker for 2 min. Invert the plate each time and decant the contents; tap it 4-5 times on absorbent paper towel to completely remove the liquid. Repeat this procedure for a total of three times.

Please note: For automated washing, discard the solution and wash the plate three times overfilling the wells with Wash buffer. After the final wash invert the plate, decant the contents; tap it 4-5 times on absorbent paper towel to completely remove the liquid. It is recommended that the washer be set for a soaking time of 1-2 min.

8. Add 100 µl of Streptavidin-HRP Conjugate (SABC) working solution into each well, cover the plate with a new sealer and incubate at 37°C for 30 minutes.
9. Remove the cover and wash the plate 5 times with Wash buffer. Allow the wash buffer to remain in the wells 1-2 min for each wash. Discard the washing buffer and blot the plate onto absorbent filter papers or other absorbent material.

10. Add 90 µl of TMB substrate into each well. Cover the plate and incubate at 37°C in dark conditions for 15-20 minutes (incubation time is for reference only, do not exceed 30 minutes). When an apparent gradient appears in the standard wells the reaction can be terminated.
11. Add 50 µl of Stop solution into each well (including the blank well). There should be a color change to yellow. Gently tap the plate to ensure thorough mixing.
12. Ensure that there are no fingerprints or water on the bottom of the plate, and that the fluid in the wells is free of bubbles. Measure the absorbance at 450 nm immediately.

This assay is competitive, therefore there is an inverse correlation between AP concentration in the sample and the absorbance measured. Create a graph with the log of the standard concentration (y-axis) and absorbance measured (x-axis). Apply a best fit trendline through the standard points. Use this graph calculate sample concentrations based on their OD values. If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

Note: If the samples measured were diluted, multiply the dilution factor by the concentrations from interpolation to obtain the concentration before dilution.

C. Precautions

1.Before using the kit, centrifuge the tubes briefly to bring down the contents trapped in the lid. For each step in the procedure, total dispensing time for addition of reagents to the assay plate should not exceed 10 minutes.

2.Avoid foaming or bubbles when mixing or reconstituting components. Prepare the Standard solutions within 15 min of starting the experiment. Please use the diluted Standard for a single assay procedure and discard after use.

3.It is recommended to assay all standards, controls and sample in duplicate. Do NOT let the plate dry out completely as this will inactivate the biological material on the plate.

4.Ensure plates are properly sealed or covered during incubation steps.

5.Complete removal of all solutions and buffers during wash steps is necessary for accurate measurement readings.

6.To avoid cross contamination do not reuse pipette tips and tubes.

7.Wash buffer may crystallize and separate. If this happens, please warm the tube and mix gently to dissolve.

8.The TMB Substrate solution is easily contaminated; work under sterile conditions when handling the TMB substrate solution. The TMB Substrate solution should also be protected from light. Unreacted substrate should be colorless or very light yellow in appearance. Aspirate the dosage needed with sterilized tips and do not dump the residual solution back into the vial.

D. Precision

Intra-assay Precision (Precision within an assay): 3 samples with low, medium and high levels of AP were tested 20 times on one plate, respectively.

Inter-assay Precision (Precision between assays): 3 samples with low, medium and high levels of AP were tested on 3 different plates, 8 replicates in each plate.

CV (%) = (Standard Deviation / mean) × 100

Intra-Assay: CV<8%

Inter-Assay: CV<10%

E. Typical Data & Standard Curve

Typical Standard Curve Data provided for demonstration purposes only. A new standard curve must be generated for each assay performed.

Concentration ng/ml	0	1.5625	3.125	6.25	12.5	25	50	100
OD450	2.396	1.488	0.904	0.548	0.299	0.211	0.142	0.093