Please read this insert completely prior to using the product.

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

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WEB INSERT

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Allopregnanolone (3α-hydroxy-5α-pregnan-20-one) is a neurosteroid present in the blood and also the brain. Allopregnanolone is made from progesterone which is converted into 5α-dihydroprogesterone by 5α-reductase type I. 3α-hydroxysteroid oxidoreductase isoenzymes convert this intermediate into allopregnanolone. 3cr-hydroxysteroids do not interact with classical intracellular steroid receptors but bind stereoselectively and with high affinity to receptors for the major inhibitory neurotransmitter in brain, γ-amino-butyric acid (GABA) (1). While allopregnanolone, like other GABA_A receptor active neurosteroids, such as allotetrahydrodeoxycorticosterone, positively modulates all GABA_A receptor isoforms, those isoforms containing δ-subunits exhibit greater magnitude potentiation. As physiologic consequences, it may be involved in neuronal plasticity, learning and memory processes, aggression and epilepsy, and the modulation of the responses to stress, anxiety and depression. Allopregnanolone has pharmacological properties similar to other positive modulators of GABA_A receptors, including anxiolytic and anticonvulsant activity (2). Anxiety and depression are common side effects of 5α-reductase inhibitors such as finasteride and dutasteride, and they are believed to be caused, in part, by the prevention of the endogenous production of allopregnanolone.

Allopregnanolone aids neurogenesis and has been found to reverse neuron proliferative deficit and cognitive deficits in a mouse model of Alzheimer’s disease (3). Allopregnanolone has also been shown to restore functionality in a mouse model of Parkinson’s Disease (4). It has also been shown to improve behavioral problems in post-traumatic stress disorder (5,6).

The DetectX® Allopregnanolone Chemiluminescent Immunoassay (CLIA) kit is designed to quantitatively measure Allopregnanolone present in extracted serum, plasma, or dried fecal samples, or in diluted urine, and tissue culture media samples. Please read the complete kit insert before performing this assay. An allopregnanolone standard is provided to generate a standard curve for the assay and all samples should be read off the standard curve.

Standards or diluted samples are pipetted into a white microtiter plate coated with an antibody to capture rabbit antibodies. An allopregnanolone-peroxidase conjugate is added to the standards and samples in the wells. The binding reaction is initiated by the addition of a polyclonal antibody to allopregnanolone to each well.

The assay is incubated overnight at 4°C. At the end of the incubation period the plate is washed and chemiluminescent substrate is added. The chemiluminescent substrate reacts with the bound Allopregnanolone-peroxidase conjugate to generate light. The generated luminescent signal is detected in a microtiter plate luminometer or multimode reader capable of measuring luminescence. The concentration of the allopregnanolone in the sample is calculated, after making suitable correction for the dilution of the sample, using software available with most plate readers.

### Related Products

#### KITS

- **Allopregnanolone EIA Kits**  
  Catalog Number K044-H1/H5
- **Corticosterone EIA Kits**  
  Catalog Number K014-H1/H5
- **Corticosterone CLIA Kits**  
  Catalog Number K014-C1/C5
- **Cortisol EIA Kits**  
  Catalog Number K003-H1/H5
- **Cortisone CLIA Kits**  
  Catalog Number K017-C1/C5
- **Acetylcholinesterase Fluorescent Activity Kit**  
  Catalog Number K015-F1
- **Butyrylcholinesterase Fluorescent Activity Kit**  
  Catalog Number K016-F1
- **Progesterone Enzyme Immunoassay Kits**  
  Catalog Number K025-H1/H5
Supplied Components

**Coated White 96 Well Plates**
White plastic microtiter plate(s) coated with goat anti-rabbit IgG.
- Kit K044-C1 OR -C5 1 OR 5 Each  Catalog Number X014-1EA

**Allopregnanolone Standard**
Allopregnanolone at 100 ng/mL in a special stabilizing solution.
- Kit K044-C1 OR -C5 125 µL OR 625 µL  Catalog Number C195-125UL OR -625UL

**DetectX® Allopregnanolone CLIA Antibody**
A rabbit polyclonal antibody specific for allopregnanolone.
- Kit K044-C1 OR -C5 3 mL OR 13 mL  Catalog Number C193-3ML OR -13ML

**DetectX® Allopregnanolone CLIA Conjugate**
Allopregnanolone-peroxidase conjugate in a special stabilizing solution.
- Kit K044-C1 OR -C5 3 mL OR 13 mL  Catalog Number C194-3ML OR -13ML

**Assay Buffer Concentrate**
The kit uses a 5X concentrate that should be diluted with deionized or distilled water.
- Kit K044-C1 OR -C5 28 mL OR 55 mL  Catalog Number X067-28ML OR -55ML

**Wash Buffer Concentrate**
A 20X concentrate that should be diluted with deionized or distilled water.
- Kit K044-C1 OR -C5 30 mL OR 125 mL  Catalog Number X007-30ML OR -125ML

**Substrate Solution A**
- Kit K044-C1 OR -C5 6 mL OR 28 mL  Catalog Number X077-6ML OR -28ML

**Substrate Solution B**
- Kit K044-C1 OR -C5 6 mL OR 28 mL  Catalog Number X078-6ML OR -28ML

**Plate Sealer**
- Kit K044-C1 OR -C5 1 OR 5 Each  Catalog Number X002-1EA

Storage Instructions

*The unopened kit must be stored at -20°C.*
Once opened the kit can be stored at 4°C up to the expiration date on the kit label, except for the **Allopregnanolone CLIA Conjugate which must be stored at -20°C.** The frozen Allopregnanolone CLIA Conjugate can be freeze-thawed multiple times.
**OTHER MATERIALS REQUIRED**

Distilled or deionized water.

Repeater pipet, such as an Eppendorf repeater, with disposable tips to accurately dispense 25, 50 and 100 µL.

A microplate shaker.

96 well microplate reader capable of reading glow chemiluminescence. A list of some models of suitable readers can be found on our website at [www.ArborAssays.com/resources/](http://www.ArborAssays.com/resources/). All luminometers read Relative Light Units (RLU). These RLU readings will vary with make or model of plate reader. The number of RLUs obtained is dependant on the sensitivity and gain of the reader used. If you are unsure of how to properly configure your reader contact your plate reader manufacturer or carry out the following protocol:

Dilute 5 µL of the Allopregnanolone Conjugate Concentrate into 345 µL of deionized water. Pipet 5 µL of this dilution into an uncoated white well and add 100 µL of prepared CLIA substrate (see page 8 for details). This well will give you an intensity of about 1-2 times the maximum binding for the assay. Adjust the gain or sensitivity so that your reader is giving close to the readers maximum signal.

To properly analyze the data, software will be required for converting raw RLU readings from the plate reader and carrying out four parameter logistic curve (4PLC) fitting. Contact your plate reader manufacturer for details.

**PRECAUTIONS**

As with all such products, this kit should only be used by qualified personnel who have had laboratory safety instruction. The complete insert should be read and understood before attempting to use the product.

The antibody coated plate needs to be stored desiccated. The silica gel pack included in the foil ziploc bag will keep the plate dry. The silica gel pack will turn from blue to pink if the ziploc has not been closed properly.

This kit utilizes a peroxidase-based readout system. Buffers, including other manufacturers Wash Buffers, containing sodium azide will inhibit color production from the enzyme. Make sure all buffers used for samples are **azide free**. Ensure that any plate washing system is rinsed well with deionized water prior to using the supplied Wash Buffer as prepared on Page 8.
This assay has been validated for extracted serum, EDTA or heparin plasma, and dried fecal samples. It will also measure allopregnanolone in diluted urine and tissue culture samples. Samples containing visible particulate should be centrifuged prior to using. Moderate to severely hemolyzed samples should not be used in this kit. Allopregnanolone can be assayed in other sample types by using the extraction protocol below or the protocols available on our website at: http://www.ArborAssays.com/resources/.

Allopregnanolone is identical across all species and we expect this kit may measure allopregnanolone from sources other than human. The end user should evaluate recoveries of allopregnanolone in other samples being tested.

**Sample Preparation**

**Dried Fecal Samples**
We have a detailed Extraction Protocol available on our website at: http://www.ArborAssays.com/resources/. The ethanol concentration in the final Assay Buffer dilution added to the well should be <5%.

**Serum and Plasma Samples**
Serum and plasma samples must be extracted prior to being run in the kit.

1. Add diethyl ether or ethyl acetate to samples at a 5:1 (v/v) solvent:sample ratio.
2. Mix solutions by vortexing for 2 minutes. Allow solvent layer to separate for 5 minutes.
3. Freeze samples in a dry ice/ethanol bath and pour solvent solution from the top of the sample into a clean tube. Repeat steps 1-3 for maximum extraction efficiency, combining top layer of ether solutions.
4. Dry pooled solvent samples down in a Speedvac for 2-3 hrs, or under a nitrogen stream until dry. If samples need to be stored they should be kept desiccated at -20°C.
5. Redissolve samples at room temperature in prediluted Assay Buffer. A minimum of 250 µL of Assay Buffer should be used.

**Urine Samples**
Urine samples should be diluted ≥ 1:2 with the provided Assay Buffer. For comparison to creatinine as a urine volume marker please see our NIST-calibrated 2 plate and 10 plate Urinary Creatinine Detection kits, K002-H1 and K002-H5.

**Tissue Culture Media**
For measuring allopregnanolone in tissue culture media (TCM), samples must be diluted at least 4-fold in Assay Buffer. Samples may need to be diluted further in Assay Buffer.

**Use all Samples within 2 Hours of preparation, or stored at ≤ -20°C until assaying.**
Allow the kit reagents to come to room temperature for 30 minutes. We recommend that all standards and samples be run in duplicate to allow the end user to accurately determine allopregnanolone concentrations. Ensure that all samples have reached room temperature and have been diluted as appropriate prior to running them in the kit.

**Assay Buffer**
Dilute Assay Buffer Concentrate 1:5 by adding one part of the concentrate to four parts of deionized water. Once diluted this is stable at 4°C for 3 months.

**Wash Buffer**
Dilute Wash Buffer Concentrate 1:20 by adding one part of the concentrate to nineteen parts of deionized water. Once diluted this is stable at 4°C for 3 months.

**Standard Preparation**
Label test tubes as #1 through #7. Pipet 475 µL of diluted Assay Buffer into tube #1 and 250 µL into the remaining tubes. The allopregnanolone stock solution contains an organic solvent. Prerinse the pipet tip several times to ensure accurate delivery. Carefully add 25 µL of the allopregnanolone stock solution to tube #1 and vortex completely. Take 250 µL of the allopregnanolone solution in tube #1 and add it to tube #2 and vortex completely. Repeat the serial dilutions for tubes #3 through #7. The concentration of allopregnanolone in tubes 1 through 7 will be 5,000, 2,500, 1,250, 625, 312.5, 156.25, and 78.125 pg/mL.

**Use all Standards within 2 hours of preparation.**

<table>
<thead>
<tr>
<th>Std 1</th>
<th>Std 2</th>
<th>Std 3</th>
<th>Std 4</th>
<th>Std 5</th>
<th>Std 6</th>
<th>Std 7</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Assay Buffer (µL)</strong></td>
<td>475</td>
<td>250</td>
<td>250</td>
<td>250</td>
<td>250</td>
<td>250</td>
</tr>
<tr>
<td><strong>Addition</strong></td>
<td>Stock</td>
<td>Std 1</td>
<td>Std 2</td>
<td>Std 3</td>
<td>Std 4</td>
<td>Std 5</td>
</tr>
<tr>
<td><strong>Vol of Addition (µL)</strong></td>
<td>25</td>
<td>250</td>
<td>250</td>
<td>250</td>
<td>250</td>
<td>250</td>
</tr>
<tr>
<td><strong>Final Conc (pg/mL)</strong></td>
<td>5,000</td>
<td>2,500</td>
<td>1,250</td>
<td>625</td>
<td>312.5</td>
<td>156.25</td>
</tr>
</tbody>
</table>

**Chemiluminescent Substrate**
Mix one part of the Substrate Solution A with one part of Substrate Solution B in a brown bottle. Once mixed the substrate is stable for one month when stored at 4°C.
1. Use the plate layout sheet on the back page to aid in proper sample and standard identification. Determine the number of wells to be used and return unused wells to the foil pouch with desiccant. Seal the ziploc plate bag and store at 4°C.

2. Pipet 100 µL of samples or standards into wells in the plate.

3. Pipet 125 µL of Assay Buffer into the non-specific binding (NSB) wells.

4. Pipet 100 µL of Assay Buffer into wells to act as maximum binding wells (B0 or 0 ng/mL).

5. Add 25 µL of the DetectX® Allopregnanolone CLIA Conjugate to each well using a repeater pipet.

6. Add 25 µL of the DetectX® Allopregnanolone CLIA Antibody to each well, except the NSB wells, using a repeater pipet.

7. Cover the plate with the plate sealer.

8. Shake the plate in a plate shaker at room temperature for 15 minutes to ensure adequate mixing of the reagents. Incubate at 4°C for 16-18 hours.

9. The following day remove the Chemiluminescent Substrate from the refrigerator and allow to come to room temperature for at least 30 minutes. **Addition of cold Substrate will cause depressed signal.**

10. At the end of the incubation time aspirate the plate and wash each well 4 times with 300 µL wash buffer. Tap the plate dry on clean absorbent towels.

11. Add 100 µL of the prepared CLIA Substrate to each well, using a repeater pipet.

12. Incubate the plate at room temperature for 5 minutes without shaking.

13. Read the luminescence generated from each well in a mutimode or chemiluminescent plate reader using a 0.1 second read time per well. The chemiluminescent signal will decrease about 40% over 60 minutes.

14. Use the plate reader’s built-in 4PLC software capabilities to calculate allopregnanolone concentration for each sample.
**CALCULATION OF RESULTS**

Average the duplicate RLU readings for each standard and sample. Create a standard curve by reducing the data using the 4PLC fitting routine on the plate reader, after subtracting the mean RLUs for the NSB. **To obtain accurate sample concentrations a 4- or 5-PLC program must be used.** The sample concentrations obtained, calculated from the %B/B0 curve, should be multiplied by the dilution factor to obtain neat sample values.

Or use the MyAssays online tool:


to calculate the data.

*The MyAssays logo is a registered trademark of MyAssays Ltd.*

**Typical Data**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean RLU</th>
<th>Net RLU</th>
<th>% B/B0</th>
<th>Allopregnanolone Conc. (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSB</td>
<td>3,350</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Standard 1</td>
<td>21,970</td>
<td>18,620</td>
<td>19.2</td>
<td>5,000</td>
</tr>
<tr>
<td>Standard 2</td>
<td>31,770</td>
<td>28,420</td>
<td>29.3</td>
<td>2,500</td>
</tr>
<tr>
<td>Standard 3</td>
<td>41,790</td>
<td>38,440</td>
<td>39.6</td>
<td>1,250</td>
</tr>
<tr>
<td>Standard 4</td>
<td>54,100</td>
<td>50,750</td>
<td>52.3</td>
<td>625</td>
</tr>
<tr>
<td>Standard 5</td>
<td>71,190</td>
<td>67,840</td>
<td>69.9</td>
<td>312.5</td>
</tr>
<tr>
<td>Standard 6</td>
<td>83,520</td>
<td>80,170</td>
<td>82.6</td>
<td>156.25</td>
</tr>
<tr>
<td>Standard 7</td>
<td>89,125</td>
<td>85,775</td>
<td>88.4</td>
<td>78.125</td>
</tr>
<tr>
<td>B0</td>
<td>100,405</td>
<td>97,055</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Sample 1</td>
<td>45,430</td>
<td>42,080</td>
<td>43.4</td>
<td>1,022.2</td>
</tr>
<tr>
<td>Sample 2</td>
<td>54,260</td>
<td>50,910</td>
<td>52.5</td>
<td>674.6</td>
</tr>
</tbody>
</table>

Always run your own standard curve for calculation of results. Do not use this data.

**Conversion Factor:** 1 ng/mL of allopregnanolone is equivalent to 3.14 nM.
Typical Standard Curves

Always run your own standard curves for calculation of results. Do not use this data.

**Validaton Data**

**Sensitivity and Limit of Detection**
Sensitivity was calculated by comparing the OD’s for twenty wells run for each of the B0 and standard #7. The detection limit was determined at two (2) standard deviations from the B0 along the standard curve.

Sensitivity was determined as 20.9 pg/mL.

The Limit of Detection for the assay was determined in a similar manner by comparing the RLU’s for twenty runs for each of the zero standard and a low concentration mammalian sample.

Limit of Detection was determined as 50.6 pg/mL.

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**Linearity**

Linearity was determined for fecal extracts and diluted urine by taking two samples, one with a low level and one with a higher level of allopregnanolone, and mixing them in the ratios given below. The measured concentrations were compared to the expected values based on the ratios used.

### Fecal Extract

<table>
<thead>
<tr>
<th>High Fecal Extract</th>
<th>Low Fecal Extract</th>
<th>Observed Conc. (pg/mL)</th>
<th>Expected Conc. (pg/mL)</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>80%</td>
<td>20%</td>
<td>1,826.9</td>
<td>2,041.5</td>
<td>89.5</td>
</tr>
<tr>
<td>60%</td>
<td>40%</td>
<td>1,576.2</td>
<td>1,572.3</td>
<td>100.2</td>
</tr>
<tr>
<td>40%</td>
<td>60%</td>
<td>928.4</td>
<td>1,103.2</td>
<td>84.2</td>
</tr>
<tr>
<td>20%</td>
<td>80%</td>
<td>661.4</td>
<td>634.0</td>
<td>104.3</td>
</tr>
</tbody>
</table>

**Mean Recovery** 94.6%

### Urine

<table>
<thead>
<tr>
<th>High Urine</th>
<th>Low Urine</th>
<th>Observed Conc. (pg/mL)</th>
<th>Expected Conc. (pg/mL)</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>80%</td>
<td>20%</td>
<td>794.9</td>
<td>930.1</td>
<td>85.5</td>
</tr>
<tr>
<td>60%</td>
<td>40%</td>
<td>681.4</td>
<td>716.4</td>
<td>95.1</td>
</tr>
<tr>
<td>40%</td>
<td>60%</td>
<td>464.4</td>
<td>502.8</td>
<td>92.4</td>
</tr>
<tr>
<td>20%</td>
<td>80%</td>
<td>283.0</td>
<td>289.1</td>
<td>97.9</td>
</tr>
</tbody>
</table>

**Mean Recovery** 92.7%
Intra Assay Precision
Three mammalian samples were diluted with Assay Buffer and run in replicates of 20 in an assay. The mean and precision of the calculated Allopregnanolone concentrations were:

<table>
<thead>
<tr>
<th>Sample</th>
<th>Allopregnanolone Conc. (pg/mL)</th>
<th>%CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2,713.2</td>
<td>11.2</td>
</tr>
<tr>
<td>2</td>
<td>1,199.0</td>
<td>9.1</td>
</tr>
<tr>
<td>3</td>
<td>793.7</td>
<td>9.0</td>
</tr>
</tbody>
</table>

Inter Assay Precision
Three mammalian samples were diluted with Assay Buffer and run in duplicates in seventeen assays run over multiple days by four operators. The mean and precision of the calculated Allopregnanolone concentrations were:

<table>
<thead>
<tr>
<th>Sample</th>
<th>Allopregnanolone Conc. (pg/mL)</th>
<th>%CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2,354.7</td>
<td>11.9</td>
</tr>
<tr>
<td>2</td>
<td>1,009.3</td>
<td>8.5</td>
</tr>
<tr>
<td>3</td>
<td>685.2</td>
<td>9.1</td>
</tr>
</tbody>
</table>
A number of diethyl ether extracted serum samples from pregnant humans were tested in the assay. Adjusted neat concentrations of allopregnanolone ranged from 1,380 to over 5,300 pg/mL with a mean at 2,492 pg/mL. The 5,300 pg/mL sample was from a woman in labor. A number of serum samples from non-pregnant male and female human samples were extracted and tested in the assay. Adjusted neat concentrations of allopregnanolone ranged from 170 to over 1,459 pg/mL with a mean of 603.2 pg/mL. A number of urine samples, diluted 1:5 to 1:30 from pregnant and non-pregnant human and other mammalian species were tested in the assay. Adjusted concentrations of allopregnanolone varied from 235 to 1,177 pg/mL for non-pregnant to 2,453 to over 20,000 pg/mL for pregnant samples.

Timed dried fecal extracts from a pregnant Iberian Lynx (the kind gift from Professor Martin Dehnard, Leibniz Institute for Zoo & Wildlife Research, Berlin) were diluted in buffer 25-150 fold and tested in the allopregnanolone assay. Adjusted allopregnanolone concentrations ranged from 24,745 to over 310,000 pg/mL.

**CROSS REACTIVITY**

The following cross reactants were tested in the assay and calculated at the 50% binding point.

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Cross Reactivity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Allopregnanolone</td>
<td>100%</td>
</tr>
<tr>
<td>Pregnanolone</td>
<td>2.26%</td>
</tr>
<tr>
<td>Tetrahydrodeoxycorticosterone (THDOC)</td>
<td>1.00%</td>
</tr>
<tr>
<td>Dihydrodeoxycorticosterone (DHDOC)</td>
<td>0.38%</td>
</tr>
<tr>
<td>Dihydrotestosterone</td>
<td>0.24%</td>
</tr>
<tr>
<td>Tetrahydrocorticosterone</td>
<td>0.20%</td>
</tr>
<tr>
<td>5α-dihydroprogesterone</td>
<td>0.13%</td>
</tr>
<tr>
<td>Corticosterone</td>
<td>0.12%</td>
</tr>
<tr>
<td>Estrone</td>
<td>0.10%</td>
</tr>
<tr>
<td>Progesterone</td>
<td>0.062%</td>
</tr>
<tr>
<td>11α-hydroxyprogesterone</td>
<td>&lt; 0.04%</td>
</tr>
<tr>
<td>20α-hydroxyprogesterone</td>
<td>&lt; 0.04%</td>
</tr>
<tr>
<td>Cortisone</td>
<td>&lt; 0.04%</td>
</tr>
<tr>
<td>Cortisol</td>
<td>&lt; 0.04%</td>
</tr>
<tr>
<td>Estradiol</td>
<td>&lt; 0.04%</td>
</tr>
<tr>
<td>Testosterone</td>
<td>&lt; 0.04%</td>
</tr>
</tbody>
</table>
Arbor Assays warrants that at the time of shipment this product is free from defects in materials and workmanship. This warranty is in lieu of any other warranty expressed or implied, including but not limited to, any implied warranty of merchantability or fitness for a particular purpose.

We must be notified of any breach of this warranty within 48 hours of receipt of the product. No claim shall be honored if we are not notified within this time period, or if the product has been stored in any way other than outlined in this publication. The sole and exclusive remedy of the customer for any liability based upon this warranty is limited to the replacement of the product, or refund of the invoice price of the goods.

CONTACT INFORMATION

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