ARBOR ASSAYS™
Interactive Assay Solutions™

MULTI SPECIES

MOST SENSITIVE

DetectX®

Allopregnanolone
Chemiluminescent Immunoassay Kit

1 Plate Kit Catalog Number K044-C1
5 Plate Kit Catalog Number K044-C5

Species Independent

Sample Types Validated:

Extracted Serum, Plasma, and Dried Fecal Samples, or Urine and Tissue Culture Media

Please read this insert completely prior to using the product.
For research use only. Not for use in diagnostic procedures.

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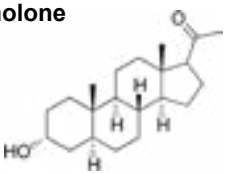
BACKGROUND

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



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].

1. Paul SM, Purdy RH., "Neuroactive Steroids", FASEB J., 1992, 6:2311-2322.
2. Kokate TG, Svensson BE, and Rogawski MA, "Anticonvulsant activity of neurosteroids: correlation with gamma-aminobutyric acid-evoked chloride current potentiation." J. Pharmacol. Exp. Ther. 1994, 270:1223-1229.
3. Wang JM., et al., "Allopregnanolone reverses neurogenic and cognitive deficits in mouse model of Alzheimer's disease." PNAS. 2010, 107:6498–6503.
4. Adeosun, SO, et al., "Allopregnanolone Reinstates Tyrosine Hydroxylase Immunoreactive Neurons and Motor Performance in an MPTP-Lesioned Mouse Model of Parkinson's Disease", 2012, PLOS One, 7(11):e50040.
5. Brunton, PJ, et al., "Central Opioid Inhibition of Neuroendocrine Stress Responses in Pregnancy in the Rat Is Induced by the Neurosteroid Allopregnanolone". 2009, J. Neurosci., 29:6449-6460.
6. Pinna, G, "In a mouse model relevant for PTSD, selective brain steroidogenic stimulants (SBSSs) improve behavioral deficits by normalizing allopregnanolone biosynthesis" Behav. Pharmacol. 2010, 21: 438-450.

ASSAY PRINCIPLE

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	Catalog No.
Acetylcholinesterase Fluorescent Activity Kit	K015-F1
Allopregnanolone EIA Kits	K044-H1/H5
Butyrylcholinesterase Fluorescent Activity Kit	K016-F1
Corticosterone EIA Kits & CLIA Kits	K014-H1/H5, K014-C1/C5
Cortisol EIA Kits	K003-H1/H5
Cortisone EIA & CLIA Kits	K017-H1/H5, K017-C1/C5
Progesterone EIA Kits	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 Must be stored at -20°C.
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/#general-info. 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.

SAMPLE TYPES

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: www.arborassays.com/resources/#protocols.

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: www.arborassays.com/resources/#protocols. 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 1:4 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.

REAGENT PREPARATION

Allow the kit reagents to come to room temperature for 30 minutes. 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.

	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6	Std 7
Assay Buffer (µL)	**475**	250	250	250	250	250	250
Addition	Stock	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6
Vol of Addition (µL)	**25**	250	250	250	250	250	250
Final Conc (pg/mL)	5,000	2,500	1,250	625	312.5	156.25	78.125

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.

ASSAY PROTOCOLS

We recommend that all standards and samples be run in duplicate to allow the end user to accurately determine allopregnanolone concentrations.

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 the maximum binding (B0 or Zero standard) wells.
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. We recommend shaking at around 700–900 rpm. 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.

NOTE: If you are using only part of a strip well plate, at the end of the assay throw away the used wells and retain the plate frame for use with the remaining unused wells.

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:

www.myassays.com/arbor-assays-allopregnanolone-clia-kit.assay

TYPICAL DATA

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

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.

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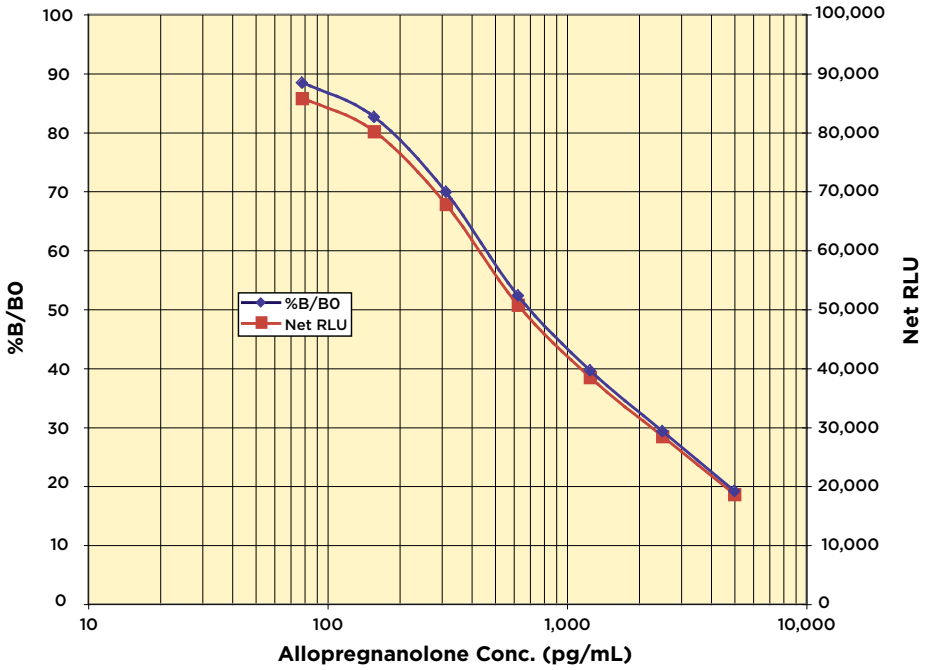
Typical Standard Curves



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

VALIDATION 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

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

High Fecal Extract	Low Fecal Extract	Expected Conc. (pg/mL)	Observed Conc. (pg/mL)	% Recovery
80%	20%	2,041.5	1,826.9	89.5
60%	40%	1,572.3	1,576.2	100.2
40%	60%	1,103.2	928.4	84.2
20%	80%	634.0	661.4	104.3
			Mean Recovery	**94.6%**

Urine

High Urine	Low Urine	Expected Conc. (pg/mL)	Observed Conc. (pg/mL)	% Recovery
80%	20%	930.1	794.9	85.5
60%	40%	716.4	681.4	95.1
40%	60%	502.8	464.4	92.4
20%	80%	289.1	283.0	97.9
			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:

Sample	Allopregnanolone Conc. (pg/mL)	%CV
1	2,713.2	11.2
2	1,199.0	9.1
3	793.7	9.0

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:

Sample	Allopregnanolone Conc. (pg/mL)	%CV
1	2,354.7	11.9
2	1,009.3	8.5
3	685.2	9.1

SAMPLE VALUES

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 concentration 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 Dehnhard, Leibniz Institute for Zoo & Wildlife Research, Berlin) were diluted 1:25 to 1:150 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.

Steroid	Cross Reactivity (%)
Allopregnanolone	100%
Pregnanolone	2.26%
Tetrahydrodeoxycorticosterone (THDOC)	1.00%
Dihydrodeoxycorticosterone (DHDOC)	0.38%
Dihydrotestosterone	0.24%
Tetrahydrocorticosterone	0.20%
5a-dihydroprogesterone	0.13%
Corticosterone	0.12%
Estrone	0.10%
Progesterone	0.062%
11a-hydroxyprogesterone	< 0.04%
20a-hydroxyprogesterone	< 0.04%
Cortisone	< 0.04%
Cortisol	< 0.04%
Estradiol	< 0.04%
Testosterone	< 0.04%

LIMITED WARRANTY

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

For details concerning this kit or to order any of our products please contact us:

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OFFICIAL SUPPLIER TO ISWE

Arbor Assays and the International Society of Wildlife Endocrinology (ISWE) signed an exclusive agreement for Arbor Assays to supply ISWE members with EIA kits for wildlife conservation research.

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ASSAYS™
FOR SERIOUS SCIENCE

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