ARBOR ASSAYS™
Interactive Assay Solutions™

MULTI SPECIES

MOST SENSITIVE

DetectX®

Allopregnanolone
Enzyme Immunoassay Kit

1 Plate Kit Catalog Number K061-H1
5 Plate Kit Catalog Number K061-H5

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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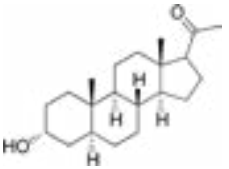
BACKGROUND

Allopregnanolone (3α-hydroxy-5α-pregnan-20-one) is a neurosteroid present in the blood and 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. 3α-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, g-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. It may be involved in neuronal plasticity, learning and memory processes, aggression, epilepsy, in addition to the modulation of stress responses, 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] and 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 Immunoassay 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 clear microtiter plate coated with an antibody to capture mouse 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 monoclonal antibody to allopregnanolone to each well.

The kit has two format options: A primary incubation of 2 hours at room temperature with shaking, or overnight at 4°C. At the end of the incubation period the plate is washed and substrate is added. The substrate reacts with the bound allopregnanolone-peroxidase conjugate. After a short incubation, the reaction is stopped and the intensity of the generated color is detected in a microtiter plate reader capable of measuring 450 nm wavelength. 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
Arg8-Vasopressin (AVP) Chemiluminescent ELISA Kits	K049-C1/C5
Atrial Natriuretic Peptide (ANP) ELISA Kits	K071-H1/H5
Butyrylcholinesterase Fluorescent Activity Kit	K016-F1
Corticosterone ELISA Kits	K014-H1/H5
Corticosterone Chemiluminescent ELISA Kits	K014-C1/C5
Cortisol ELISA Kits	K003-H1/H5
Cortisone ELISA Kits	K017-H1/H5
Cortisone Chemiluminescent ELISA Kits	K017-C1/C5
Endothelin-1 (ET-1) ELISA Kit	K045-H1
Progesterone ELISA Kits	K025-H1/H5
Progesterone Metabolites ELISA Kits	K068-H1/H5
Prostaglandin E_2 (PGE_2) Multi-Format ELISA Kits	K051-H1/H5

SUPPLIED COMPONENTS

Coated Clear 96 Well Plates
Clear plastic microtiter plate(s) coated with goat anti-mouse IgG.

Kit K061-H1 **or** -H5	1 **or** 5 Each	Catalog Number X012-1EA

Allopregnanolone Standard
Allopregnanolone at 100,000 pg/mL in a special stabilizing solution.

Kit K061-H1 **or** -H5	125 µL **or** 625 µL	Catalog Number C195-125UL **or** -625UL

DetectX® Allopregnanolone Antibody
A mouse monoclonal antibody specific for allopregnanolone.

Kit K061-H1 **or** -H5	3 mL **or** 13 mL	Catalog Number C226-3ML **or** -13ML

DetectX® Allopregnanolone Conjugate **Must be stored at -20°C.**
Allopregnanolone-peroxidase conjugate in a special stabilizing solution.

Kit K061-H1 **or** -H5	3 mL **or** 13 mL	Catalog Number C154-3ML **or** -13ML

Assay Buffer Concentrate
The kit uses a 5X concentrate that should be diluted with deionized or distilled water.

Kit K061-H1 **or** -H5	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 K061-H1 **or** -H5	30 mL **or** 125 mL	Catalog Number X007-30ML **or** -125ML

TMB Substrate

Kit K061-H1 **or** -H5	11 mL **or** 55 mL	Catalog Number X019-11ML **or** -55ML

Stop Solution
A 1M solution of hydrochloric acid. **CAUSTIC**.

Kit K061-H1 **or** -H5	5 mL **or** 25 mL	Catalog Number X020-5ML **or** -25ML

Plate Sealer

Kit K061-H1 **or** -H5	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 Conjugate which must be stored at -20°C.** The frozen Allopregnanolone 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 µL, 50 µL and 100 µL.

A microplate shaker.

Colorimetric 96 well microplate reader capable of reading optical density at 450 nm.

Software for converting raw relative optical density 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.

Laboratory temperature is important. Please make sure that the kit incubates at a temperature between 22°C and 24°C.

The Stop Solution is acid. The solution should not come in contact with skin or eyes. Take appropriate precautions when handling this reagent.

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. We expect this kit will 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 the Assay Buffer. A minimum of 125 µL of Assay Buffer should be used.

Urine Samples

Urine samples should be diluted ≥ 1:4 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 in Assay Buffer. The end-user should determine the appropriate dilution for the media samples prior to running their samples.

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

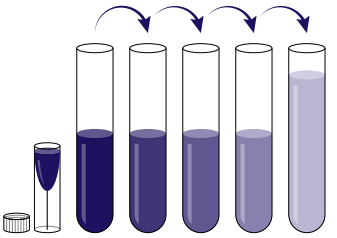
Dilute Assay Buffer Concentrate 1:5 by adding one part of the concentrate to four parts of deionized water. Once diluted this is stable at 4°C for 3 months.

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 room temperature for 3 months.

Standard Preparation

Label test tubes as #1 through #7. Pipet 450 µL of 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 50 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 10,000, 5,000, 2,500, 1,250, 625, 312.5, and 156.25 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)	**450**	250	250	250	250	250	250
Addition	Stock	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6
Vol of Addition (µL)	**50**	250	250	250	250	250	250
Final Conc (pg/mL)	10,000	5,000	2,500	1,250	625	312.5	156.25

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 50 µL of samples or standards into wells in the plate.
3. Pipet 75 µL of Assay Buffer into the non-specific binding (NSB) wells.
4. Pipet 50 µL of Assay Buffer into the maximum binding (B0 or Zero standard) wells.
5. Add 25 µL of the DetectX® Allopregnanolone Conjugate to each well using a repeater pipet.
6. Add 25 µL of the DetectX® Allopregnanolone Antibody to each well, **except the NSB wells**, using a repeater pipet.
7. Gently tap the sides of the plate to ensure adequate mixing of the reagents. Cover the plate with the plate sealer.
8. Incubation Options

   EITHER:

   a. Shake at room temperature for 2 hours. We recommend shaking at around 700–900 rpm. If the plate is not shaken, signals bound will be approximately 45% lower.

   OR:

   b. 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. Then incubate at 4°C for 16-18 hours.
9. If using Option 8b., the following day remove the TMB Substrate from the refrigerator and allow to come to room temperature for at least 30 minutes before use. **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 TMB Substrate to each well, using a repeater pipet.
12. Incubate the plate at room temperature for 30 minutes without shaking.
13. Add 50 µL of the Stop Solution to each well, using a repeater pipet.
14. Read the optical density generated from each well in a plate reader capable of reading at 450 nm.
15. 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 OD 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 OD's 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 online tool from MyAssays to calculate the data:
www.myassays.com/arbor-assays-allopregnanolone-eia-kit-monoclonal.assay

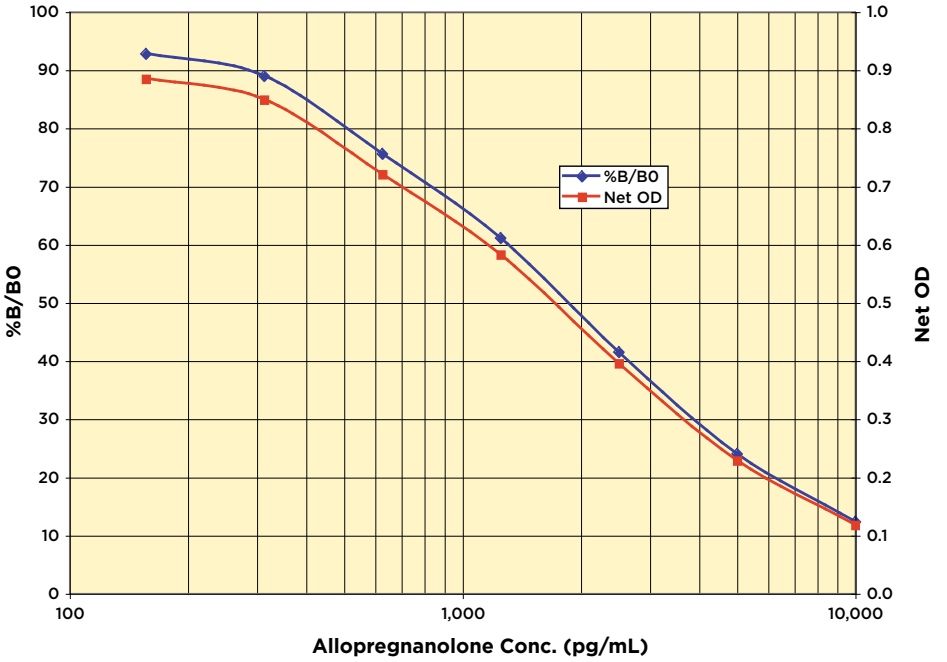
2 HOUR ASSAY TYPICAL DATA

Sample	Mean OD	Net OD	% B/B0	Allopregnanolone Conc. (pg/mL)
NSB	0.063	0.000	-	-
Standard 1	0.181	0.118	12.37	10,000
Standard 2	0.292	0.229	24.00	5,000
Standard 3	0.459	0.396	41.51	2,500
Standard 4	0.646	0.583	61.11	1,250
Standard 5	0.784	0.721	75.58	625
Standard 6	0.912	0.849	88.99	312.5
Standard 7	0.948	0.885	92.77	156.25
B0	1.017	0.954	100	0
Sample 1	0.367	0.304	31.81	3,594.6
Sample 2	0.527	0.464	48.58	1,932.9
Sample 3	0.818	0.755	79.14	552.7

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

Conversion Factor: 1 pg/mL of allopregnanolone is equivalent to 3.14 pM.

Typical 2-Hour 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 nineteen 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 129.7 pg/mL.**

The Limit of Detection for the assay was determined in a similar manner by comparing the OD's for twenty runs for each of the zero standard and a low concentration mammalian sample. **Limit of Detection was determined as 65.9 pg/mL**

Linearity

Linearity was determined for fecal extracts and diluted urine by taking two samples for each, 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.

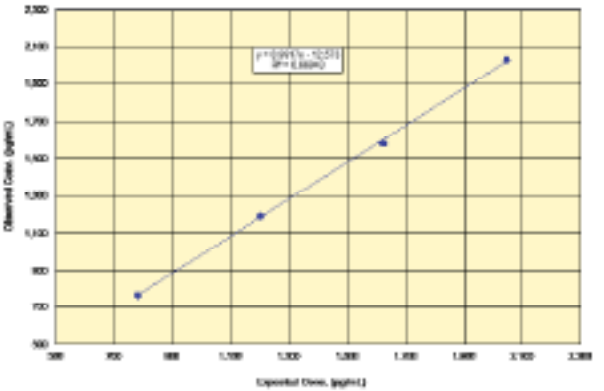
Fecal Extract

High Fecal Extract	Low Fecal Extract	Expected Conc. (pg/mL)	Observed Conc. (pg/mL)	% Recovery
80%	20%	2,046.9	2,027.5	99.1%
60%	40%	1,625.1	1,580.4	97.2%
40%	60%	1,203.4	1,187.6	98.7%
20%	80%	781.7	764.3	97.8%
			Mean Recovery	**98.2%**

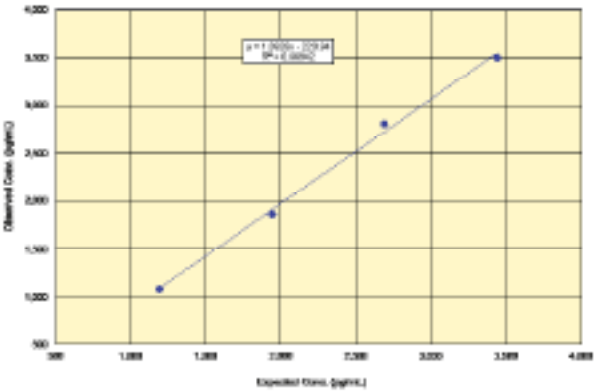
Urine

High Urine	Low Urine	Expected Conc. (pg/mL)	Observed Conc. (pg/mL)	% Recovery
80%	20%	3,441.7	3,493.3	101.5%
60%	40%	2,691.7	2,811.0	104.4%
40%	60%	1,941.7	1,860.0	95.8%
20%	80%	1,191.6	1,078.3	90.5%
			Mean Recovery	**98.1%**

Fecal Extract Linearity



Urine Linearity



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	3,576.5	3.7
2	1,768.8	4.6
3	545.7	12.9

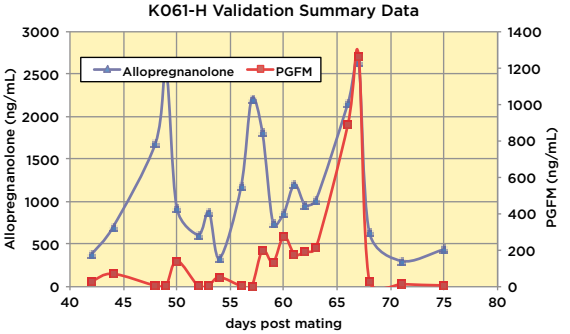
Inter Assay Precision

Three mammalian samples were diluted with Assay Buffer and run in duplicates in twenty 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	3,538.8	6.2
2	1,710.6	7.0
3	499.2	11.2

SAMPLE VALUES

A number of diethyl ether extracted serum samples from pregnant humans were tested in the assay. Adjusted neat concentrations of allopregnanolone read over 2,900 pg/mL. A number of serum samples from non-pregnant human samples were extracted and tested in the assay. Adjusted neat concentrations of allopregnanolone ranged from 738 to over 1,698 pg/mL. A number of urine samples, diluted 1:4 to 1:16 from pregnant and non-pregnant human and other mammalian species were tested in the assay. Adjusted concentration of allopregnanolone varied from 1,500 to over 46,000 pg/mL for non-pregnant and 7,700 to over 57,000 pg/mL for pregnant samples. Timed dried fecal extracts from a pregnant Iberian Lynx were tested in this kit and in our PGFM EIA kit, K022-H1/H5.



Iberian lynx fecal extracts were the kind gift from Professor Martin Dehnhard, Leibniz Institute for Zoo & Wildlife Research, Berlin.

CROSS REACTIVITY

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

Steroid	Cross Reactivity (%)	Steroid	Cross Reactivity (%)
Allopregnanolone	100%	Estrone	< 0.08%
Tetrahydrodeoxycorticosterone (THDOC)	3.08%	Dihydrodeoxycorticosterone (DHDOC)	< 0.08%
Pregnanolone	2.19%	11α-hydroxyprogesterone	< 0.08%
Progesterone	0.12%	20α-hydroxyprogesterone	< 0.08%
Dihydrotestosterone	0.095%	Cortisone	< 0.08%
Tetrahydrocorticosterone	< 0.08%	Cortisol	< 0.08%
5α-dihydroprogesterone	< 0.08%	Estradiol	< 0.08%
Corticosterone	< 0.08%	Testosterone	< 0.08%

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

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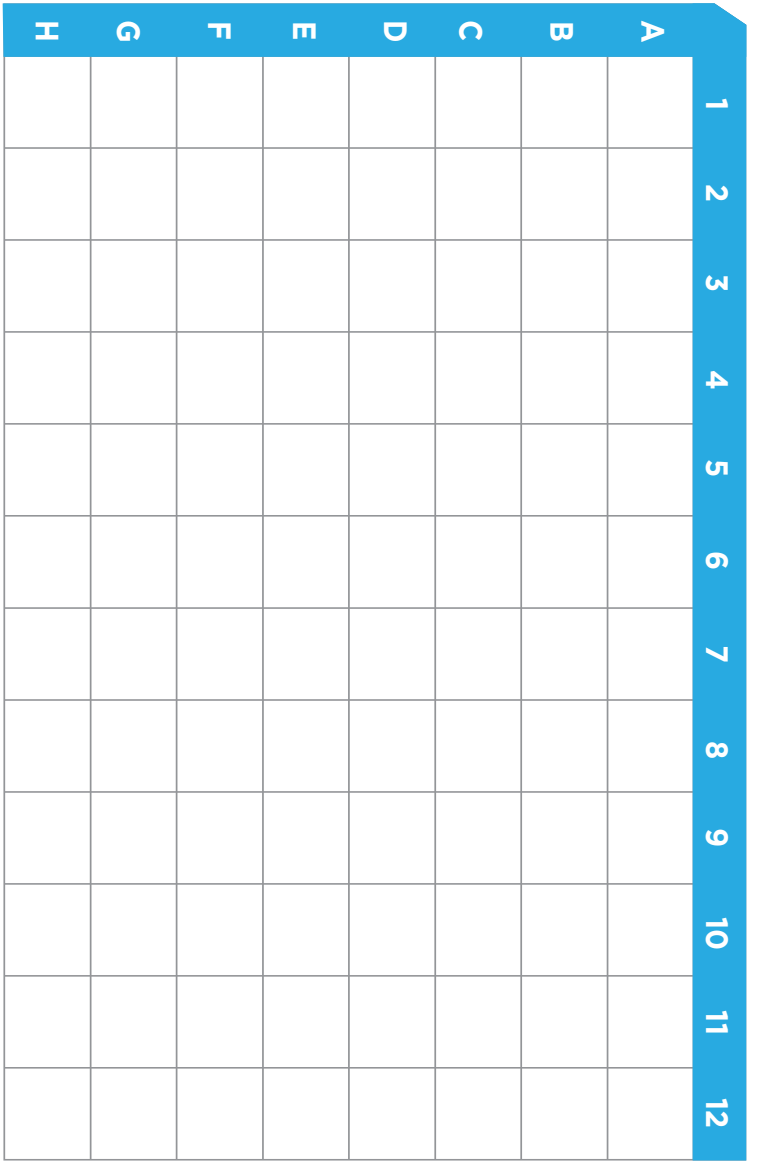
OFFICIAL SUPPLIER TO ISWE

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

ASSAYS™
FOR SERIOUS SCIENCE



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