

ARBOR ASSAYS™  
Interactive Assay Solutions™



**DetectX®**

## **ALLOPREGNANOLONE** **Enzyme Immunoassay Kit**

1 Plate Kit Catalog Number K044-H1

5 Plate Kit Catalog Number K044-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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**WEB INSERT 180301**

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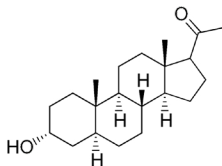
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## BACKGROUND

Allopregnanolone (3 $\alpha$ -hydroxy-5 $\alpha$ -pregnan-20-one) is a neurosteroid present in the blood and also the brain. Allopregnanolone is made from progesterone which is converted into 5 $\alpha$ -dihydroprogesterone by 5 $\alpha$ -reductase type I. 3 $\alpha$ -hydroxysteroid oxidoreductase isoenzymes convert this intermediate into allopregnanolone. 3 $\alpha$ -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,  $\gamma$ -amino-butyric acid (GABA)<sup>1</sup>. While allopregnanolone, like other GABA<sub>A</sub> receptor active neurosteroids, such as allotetrahydrodeoxycorticosterone, positively modulates all GABA<sub>A</sub> receptor isoforms, those isoforms containing  $\delta$ -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<sub>A</sub> receptors, including anxiolytic and anticonvulsant activity<sup>2</sup>. Anxiety and depression are common side effects of 5 $\alpha$ -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 mouse model of Alzheimer's disease<sup>3</sup>. Allopregnanolone has also been shown to restore functionality in a mouse model of Parkinson's Disease<sup>4</sup>. It has also been shown to improve behavioral problems in post-traumatic stress disorder<sup>5,6</sup>.

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, 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 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 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.
<b>Acetylcholinesterase Fluorescent Activity Kit</b>	K015-F1
<b>Allopregnanolone CLIA Kits</b>	K044-C1/C5
<b>Allopregnanolone EIA Kits, Monoclonal Antibody Based</b>	K061-H1/H5
<b>Butyrylcholinesterase Fluorescent Activity Kit</b>	K016-F1
<b>Corticosterone EIA &amp; CLIA Kits</b>	K014-H1/H5, K014-C1/C5
<b>Cortisol EIA Kits</b>	K003-H1/H5
<b>Cortisone EIA &amp; CLIA Kits</b>	K017-H1/H5, K017-C1/C5
<b>Progesterone EIA Kits</b>	K025-H1/H5



## SUPPLIED COMPONENTS

### Coated Clear 96 Well Plates

Clear plastic microtiter plate(s) coated with goat anti-rabbit IgG.

Kit K044-H1 **or** -H5 1 **or** 5 Each Catalog Number X016-1EA

### Allopregnanolone Standard

Allopregnanolone at 1,000 ng/mL in a special stabilizing solution.

Kit K044-H1 **or** -H5 125  $\mu$ L **or** 625  $\mu$ L Catalog Number C155-125UL **or** -625UL

### DetectX<sup>®</sup> Allopregnanolone Antibody

A rabbit polyclonal antibody specific for allopregnanolone.

Kit K044-H1 **or** -H5 3 mL **or** 13 mL Catalog Number C153-3ML **or** -13ML

### DetectX<sup>®</sup> Allopregnanolone Conjugate **Must be stored at -20°C.**

Allopregnanolone-peroxidase conjugate in a special stabilizing solution.

Kit K044-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 K044-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 K044-H1 **or** -H5 30 mL **or** 125 mL Catalog Number X007-30ML **or** -125ML

### TMB Substrate

Kit K044-H1 **or** -H5 11 mL **or** 55 mL Catalog Number X019-11ML **or** -55ML

### Stop Solution

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

Kit K044-H1 **or** -H5 5 mL **or** 25 mL Catalog Number X020-5ML **or** -25ML

### Plate Sealer

Kit K044-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, 50, and 100  $\mu\text{L}$ .

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](http://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](http://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  $\geq$  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  $\leq$  -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

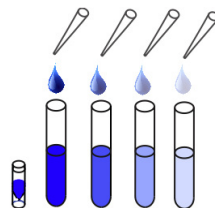
Dilute Assay Buffer Concentrate 1:5 by adding one part of the concentrate to four parts of deionized water. Once diluted this is stable at 4°C for 3 months.

### 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 #8. Pipet 475  $\mu\text{L}$  of Assay Buffer into tube #1 and 250  $\mu\text{L}$  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  $\mu\text{L}$  of the allopregnanolone stock solution to tube #1 and vortex completely. Take 250  $\mu\text{L}$  of the allopregnanolone solution in tube #1 and add it to tube #2 and vortex completely. Repeat the serial dilutions for tubes #3 through #8. The concentration of allopregnanolone in tubes 1 through 8 will be 50, 25, 12.5, 6.25, 3.125, 1.563, 0.781 and 0.391 ng/mL.



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

	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6	Std 7	Std 8
<b>Assay Buffer (<math>\mu\text{L}</math>)</b>	<b>475</b>	250	250	250	250	250	250	250
<b>Addition</b>	Stock	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6	Std 7
<b>Vol of Addition (<math>\mu\text{L}</math>)</b>	<b>25</b>	250	250	250	250	250	250	250
<b>Final Conc (ng/mL)</b>	50	25	12.5	6.25	3.125	1.563	0.781	0.391





## 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 (BO or Zero standard) wells.
5. Add 25 µL of the DetectX<sup>®</sup> Allopregnanolone-Conjugate to each well using a repeater pipet.
6. Add 25 µL of the DetectX<sup>®</sup> 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. 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. Incubate at 4°C for 16-18 hours.

9. If using Option 8. b., the following day remove the TMB 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 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/BO 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.assay](http://www.myassays.com/arbor-assays-allopregnanolone-eia-kit.assay)



### 2 HOUR ASSAY TYPICAL DATA

Sample	Mean OD	Net OD	% B/BO	Allopregnanolone Conc. (ng/mL)
NSB	0.084	0.000	-	-
Standard 1	0.158	0.074	15.1	50
Standard 2	0.192	0.108	22.0	25
Standard 3	0.232	0.148	30.1	12.5
Standard 4	0.288	0.204	41.5	6.25
Standard 5	0.354	0.270	55.0	3.125
Standard 6	0.425	0.341	69.5	1.5625
Standard 7	0.496	0.412	83.9	0.781
Standard 8	0.522	0.438	89.2	0.391
BO	0.575	0.491	100	0
Sample 1	0.304	0.220	44.7	5.475
Sample 2	0.497	0.413	84.0	0.660

**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.**



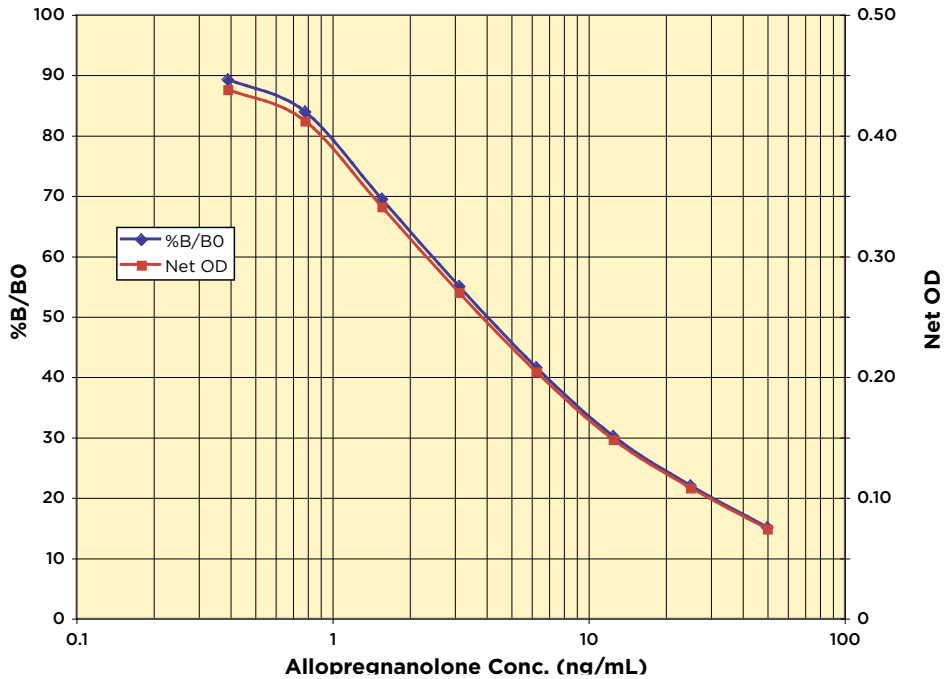
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## Typical Standard Curves



**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 BO and standard #8. The detection limit was determined at two (2) standard deviations from the BO along the standard curve.

**Sensitivity was determined as 0.13 ng/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 0.24 ng/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. (ng/mL)	Observed Conc. (ng/mL)	% Recovery
80%	20%	22.77	23.08	101.4
60%	40%	17.23	17.90	103.9
40%	60%	11.68	10.74	92.0
20%	80%	6.14	6.13	99.8
<b>Mean Recovery</b>				<b>99.3%</b>

### Urine

High Urine	Low Urine	Expected Conc. (ng/mL)	Observed Conc. (ng/mL)	% Recovery
80%	20%	11.31	10.88	96.3
60%	40%	8.81	7.74	87.8
40%	60%	6.31	5.83	92.4
20%	80%	3.81	3.78	99.1
<b>Mean Recovery</b>				<b>93.9%</b>



### 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. (ng/mL)	%CV
1	12.5	5.6
2	6.0	5.0
3	0.7	11.1

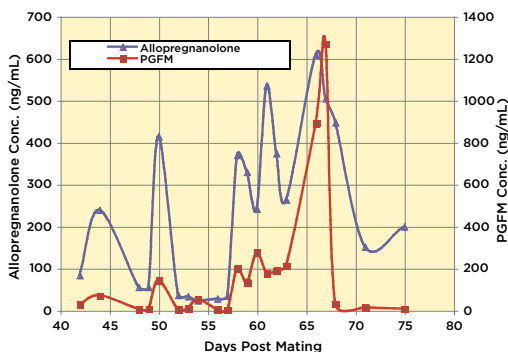
### Inter Assay Precision

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

Sample	Allopregnanolone Conc. (ng/mL)	%CV
1	10.9	10.3
2	5.0	8.4
3	0.6	11.5

## 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 10.5 to over 35.7 ng/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 0.35 to over 1.36 ng/mL. A number of urine samples, diluted 1:2 to 1:20 from pregnant and non-pregnant human and other mammalian species were tested in the assay. Adjusted concentration of allopregnanolone varied from 1.6 to 1.92 ng/mL for non-pregnant to 21.06 to 27.61 ng/mL for pregnant samples. Timed dried fecal extracts from a pregnant Iberian Lynx were tested in the allopregnanolone assay 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	0.10%
Pregnanolone	2.26%	Progesterone	0.062%
Tetrahydrodeoxycorticosterone (THDOC)	1.00%	11 $\alpha$ -hydroxyprogesterone	< 0.04%
Dihydrodeoxycorticosterone (DHDOC)	0.38%	20 $\alpha$ -hydroxyprogesterone	< 0.04%
Dihydrotestosterone	0.24%	Cortisone	< 0.04%
Tetrahydrocorticosterone	0.20%	Cortisol	< 0.04%
5 $\alpha$ -dihydroprogesterone	0.13%	Estradiol	< 0.04%
Corticosterone	0.12%	Testosterone	< 0.04%



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## 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:

### Arbor Assays

1514 Eisenhower Place  
Ann Arbor, Michigan 48108 USA

Phone: 734-677-1774

Fax: 734-677-6860

Web: [www.ArborAssays.com](http://www.ArborAssays.com)

### E Mail Addresses:

[Info@ArborAssays.com](mailto:Info@ArborAssays.com)

[Orders@ArborAssays.com](mailto:Orders@ArborAssays.com)

[Technical@ArborAssays.com](mailto:Technical@ArborAssays.com)

[Contracts@ArborAssays.com](mailto:Contracts@ArborAssays.com)



## 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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