ARBOR ASSAYS™
Interactive Assay Solutions™

MULTI SPECIES

DetectX®

20-Hydroxyecdysone
Enzyme Immunoassay Kit

1 Plate Kit	Catalog Number K066-H1
5 Plate Kit	Catalog Number K066-H5

Species Independent

Sample Types Validated:

Tissue Extracts

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

www.ArborAssays.com

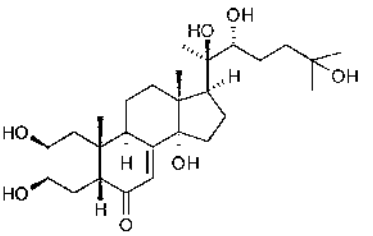
WEB 180702

TABLE OF CONTENTS

BACKGROUND

The first insect molting hormone was isolated from silkworm pupae and determined to be a steroid, so named ecdysone, in 1954[1]. Later, 20-hydroxyecdysone was identified in crayfish and recognized as a derivative of ecdysone[2]. These molecules and related forms are a family of steroid hormones that regulate metamorphosis, cell death, reproduction in arthropods, and are widely distributed in plant species (phytoecdysteroids)[3]. Of the many ecdysteroids, 20-hydroxyecdysone is the most functionally active and widely distributed in arthropods[4]. To accommodate growth during all immature stages of insects and other arthropods, 20-hydroxyecdysone levels change and activate signaling through an ecdysone receptor that results in the synthesis of a new exoskeleton and ecdysis of the old cuticle[4,5]. In female mosquitoes and flies, 20-hydroxyecdysone regulates egg development[5]. In plants, 20-hydroxyecdysone facilitates the defense mechanisms against insects[3,4]. Recent studies of vertebrate animals have discovered the ability of 20-hydroxyecdysone to increase osteogenesis and bone mass by reducing cartilage degradation and increasing protein synthesis in humans[6-9]. There is also medical research and marketing interest in the use of 20-hydroxyecdysone as a bodybuilding supplement to increase muscle mass[9].

20-Hydroxyecdysone



1. Über die Isolierung eines Metamorphose-Hormons der Insekten in kristallisierter Form. Butenandt, A., and Karlson, PZ., Z. Naturforsch. 1954, 9p, 389-391.
2. Structure of crust-ecdysone, a crustacean molting hormone. Hampshire, F. and Horn, D. H. S. 1966, J. Chem. Soc. Chem. Commun., 37-38.
3. Distribution and biosynthesis of 20-hydroxyecdysone in plants of Achyranthes japonica Nakai., Bok, KH, et. al., Biosci. Biotechnol. Biochem. 2010, 74(11): 2226-31.
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5. Evaluation of ecdysteroid antisera for a competitive enzyme immunoassay and extraction procedures for the measurement of mosquito ecdysteroids. McKinney, D. A., Strand, M. R., and Brown, M. R. 2017, Gen. Comp. Endocrinol. 253:60-69.
6. ß-Ecdysone augments peak bone mass in mice of both sexes., Dai, W., et. al., Clin. Orthop. Relat. Res. 2015, 473(8): 2495-504
7. Arthropod steroid hormone (20-Hydroxyecdysone) suppresses IL-1ß-induced catabolic gene expression in cartilage., Sheu SY, Ho SR, Sun JS, Chen CY, Ke CJ., 2015, BMC Complement. Altern. Med 24; 15:1
8. Prevention of glucocorticoid induced bone changes with beta-ecdysone. Dai, W., et. al., 2015, Bone, 74, 48-57.
9. The Karlson Lecture. Phytoecdysteroids: what use are they? Dinan L., 2009, Arch Insect Biochem Physiol. 72(3):126-41.

ASSAY PRINCIPLE

The DetectX® 20-Hydroxyecdysone (20E) Immunoassay kit is designed to quantitatively measure 20E present in extracted tissue samples from hemolymph, plants, or anthropods. Please read the complete kit insert before performing this assay. A 20-hydroxyecdysone 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. A 20-hydroxyecdysone-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 20-hydroxyecdysone to each well. After a 2 hour incubation the plate is washed and substrate is added. The substrate reacts with the bound 20-hydroxyecdysone-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 20-hydroxyecdysone in the sample is calculated, after making suitable correction for the dilution of the sample, using software available with most plate readers

RELATED PRODUCTS

DetectX® Kits	Catalog No.
Corticosterone Enzyme Immunoassay Kits	K014-H1/H5
Corticosterone Chemiluminescent Immunoassay Kits	K014-C1/C5
Cortisol Enzyme Immunoassay Kits (Strip Wells)	K003-H1/H5
Cortisol Enzyme Immunoassay Kits (Whole Plate)	K003-H1W/H5W
Cortisone Enzyme Immunoassay Kits	K017-H1/H5
Cortisone Chemiluminescent Immunoassay Kits	K017-C1/C5
Estradiol Enzyme Immunoassay Kits	K030-H1/H5
Progesterone Enzyme Immunoassay Kits	K025-H1/H5
Testosterone Enzyme Immunoassay kits	K032-H1/H5

SUPPLIED COMPONENTS

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

Kit K066-H1 **or** -H5	1 **or** 5 Each	Catalog Number X016-1EA

20-Hydroxyecdysone Standard
20-Hydroxyecdysone at 2,500 ng/mL in a special stabilizing solution.

Kit K066-H1 **or** -H5	40 µL **or** 200 µL	Catalog Number C240-40UL **or** -200UL

DetectX® 20-Hydroxyecdysone Antibody
A rabbit polyclonal antibody specific for 20-Hydroxyecdysone.

Kit K066-H1 **or** -H5	3 mL **or** 13 mL	Catalog Number C238-3ML **or** -13ML

DetectX® 20-Hydroxyecdysone Conjugate
A 20-Hydroxyecdysone-peroxidase conjugate in a special stabilizing solution.

Kit K066-H1 **or** -H5	3 mL **or** 13 mL	Catalog Number C239-3ML **or** -13ML

Assay Buffer Concentrate
A 5X concentrate that must be diluted with deionized or distilled water.

Kit K066-H1 **or** -H5	28 mL **or** 55 mL	Catalog Number X053-28ML **or** -55ML

Wash Buffer Concentrate
A 20X concentrate that must be diluted with deionized or distilled water.

Kit K066-H1 **or** -H5	30 mL **or** 125 mL	Catalog Number X007-30ML **or** -125ML

TMB Substrate

Kit K066-H1 **or** -H5	11 mL **or** 55 mL	Catalog Number X019-11ML **or** -55ML

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

Kit K066-H1 **or** -H5	5 mL **or** 25 mL	Catalog Number X020-5ML **or** -25ML

Plate Sealer

Kit K066-H1 **or** -H5	1 **or** 5 Each	Catalog Number X002-1EA

STORAGE INSTRUCTIONS

All components of this kit should be stored at 4°C until the expiration date of the kit.

OTHER MATERIALS REQUIRED

Distilled or deionized water.

Repeater pipet, such as an Eppendorf M4, with disposable tips capable of dispensing 25 µL, 50 µL and 100 µL is recommended to obtain acceptable CVs.

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.

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 tested and validated with extracted and dried Dungeness crab hemolymph samples. 20-Hydroxyecdysone is identical across millions of species including arthropods and variety of plant species. Therefore, we expect this kit to measure 20-hydroxyecdysone in all sources including animal and plant extracts. The end user should evaluate the extraction recoveries of 20-hydroxyecdysone in other samples being tested. To evaluate the extraction efficiency run each sample with and without a known amount of 20-hydroxyecdysone standard added (spiked) and an assay buffer control (similarly spiked) for comparison.

SAMPLE PREPARATION

Samples may need to be extracted depending on source. We suggest a study of the literature to determine suitable methods of isolating 20-Hydroxyecdysone from organisms and plants.

Hemolymph Samples

It is recommended to perform an extraction of hemolymph samples with methanol prior to testing to remove matrix effects. Hemolymph samples are extracted by taking one part of aqueous sample and adding 3 volumes of chilled methanol (75% of total volume) and vortexing for 30 seconds. Centrifuge the solution at 10,000 rpm for 10 min at 4°C. Supernatant is carefully drawn out without disturbing the pellet and dried down completely using a centrifugal concentrator at 30°C for 2-3 hrs. The dried powder/pellet can be stored at -20°C until assayed, or can be dissolved in a minimum of 125µL assay buffer to run immediately[10,11].

Extraction Efficiency Determination

We suggest checking the efficiency of extraction by preparing a 20-hydroxyecdysone solution of known concentration in the kit Assay Buffer (AB). Spike one aliquot of your sample with a volume of the steroid solution in AB (Control Spike) and one aliquot of sample with the same volume of AB (Control Sample). Extract samples and Controls with chilled methanol as described above. Efficiency is calculated as below:

Determine the extraction efficiency by comparing the concentration of the steroid measured in the extracted Control (Control Spike-Control Sample) with the concentration of the steroid measured before extraction (steroid solution of known concentration). Further details on extractions, assays and extraction efficiency can be found at https://www.scribd.com/document/57985925/Brown-2005

10. Circulating Ecdysteroid Concentrations in Alaskan Dungeness Crab (*Cancer Magister*). 2006, Thomton, JD, Tamone, SL and Aktinson, S., J. Crustacean Biol.26(2), 176-181
11. Effect of Eyestalk-Ablation on Circulating Ecdysteroids in Hemolymph of Snow Crabs, *Chionoecetes opilio*: Physiological Evidence for a Terminal Molt, 2005, Tamone SL, Adams MM, and Dutton JM. Integ. Curr. Biol. 45(1)): 166-171

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

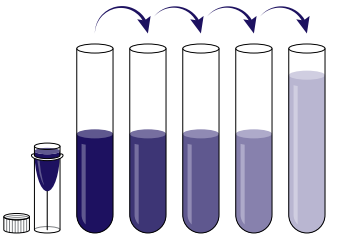
Dilute Assay Buffer Concentrate 1:5 by adding one part of the concentrate to four parts of deionized water. Once diluted this is stable for 3 months at 4°C.

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 990 µL of Assay Buffer into tube #1 and 300 µL into tubes #2 to #7. **The 20-hydroxyecdysone stock solution contains an organic solvent. Prerinse the pipet tip several times to ensure accurate delivery.** Carefully add 10 µL of the 20-hydroxyecdysone stock solution to tube #1 and vortex completely. Take 200 µL of the 20-hydroxyecdysone 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 20-hydroxyecdysone in the tubes will be 25,000, 10,000, 4,000, 1,600, 640, 256 and 102.4 pg/mL.



Use all Standards within 2 hour of preparation.

	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6	Std 7
Assay Buffer Volume (µL)	**990**	300	300	300	300	300	300
Addition	Stock	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6
Volume of Addition (µL)	**10**	200	200	200	200	200	200
Final Conc (pg/mL)	25,000	10,000	4,000	1,600	640	256	102.4

ASSAY PROTOCOL

We recommend that all standards and samples be run in duplicate to allow the end user to accurately determine 20-Hydroxyecdysone concentrations.

1. Use the plate layout sheet on the back page to aid in proper sample and standard identification.
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 into the maximum binding (B0 or Zero standard) wells.
5. Add 25 µL of the DetectX® 20-Hydroxyecdysone Conjugate to each well using a repeater pipet.
6. Add 25 µL of the DetectX® 20-Hydroxyecdysone 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 and shake at room temperature for 2 hours. If the plate is not shaken signals bound will be approximately 20% lower.
8. Aspirate the plate and wash each well 4 times with 300 µL wash buffer. Tap the plate dry on clean absorbent towels.
9. Add 100 µL of the TMB Substrate to each well, using a repeater pipet.
10. Incubate the plate at room temperature for 30 minutes without shaking.
11. Add 50 µL of the Stop Solution to each well, using a repeater pipet.
12. Read the optical density generated from each well in a plate reader capable of reading at 450 nm.
13. Use the plate reader's built-in 4PLC software capabilities to calculate 20-Hydroxyecdysone 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. 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-detectx-20-hydroxyecdysone-eia-kit.assay

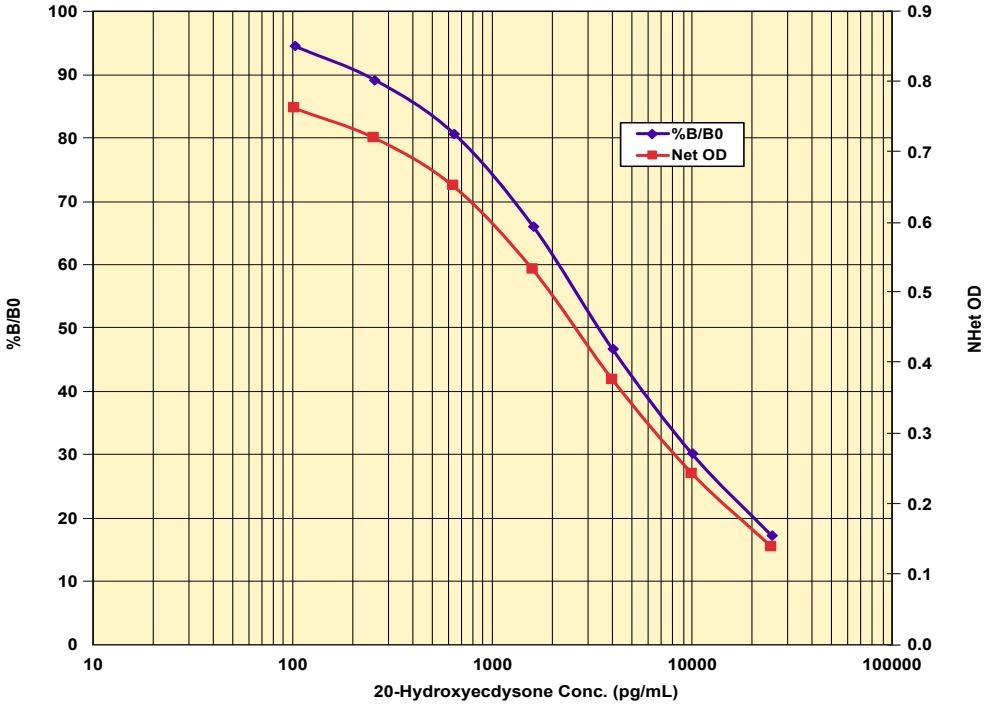
TYPICAL DATA

Sample	Mean OD	Net OD	% B/B0	20-Hydroxyecdysone Conc. (pg/mL)
NSB	0.081	0	-	-
Standard 1	0.220	0.139	17.2	25,000
Standard 2	0.324	0.243	30.1	10,000
Standard 3	0.456	0.376	46.6	4,000
Standard 4	0.613	0.532	66	1,600
Standard 5	0.731	0.651	80.7	640
Standard 6	0.800	0.720	89.2	256
Standard 7	0.843	0.762	94.5	102.4
B0	0.887	0.807	100	0
Sample 1	0.447	0.366	30.13	4,347
Sample 2	0.58	0.5	65.964	1,948

Always run your own standard curve for calculation of results. Do not use this data.
Conversion Factor: 100 pg/mL of 20-hydroxyecdysone is equivalent to 208.06 pM.

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

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

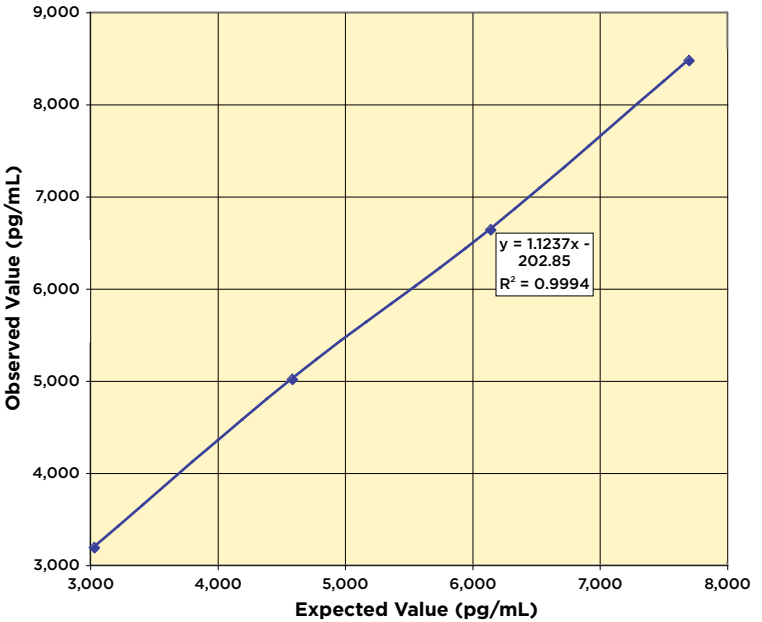
Sensitivity was calculated by comparing the OD's for twenty wells run for each of the B0 and standard #7. The detection limit was determined at two (2) standard deviations from the B0 along the standard curve. **Sensitivity was determined as 197.8 pg/mL.**

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 sample. **Limit of Detection was determined as 139.8 pg/mL.**

Linearity

Linearity was determined by taking two spiked hemolymph samples diluted 1:20, one with a low 20-hydroxyecdysone level of 1,478 pg/mL and one with a higher level of 9,256 pg/mL and mixing them in the ratios given below. The measured concentrations were compared to the expected values based on the ratios used.

High Hemolymph	Low Hemolymph	Observed Conc. (pg/mL)	Expected Conc. (pg/mL)	% Recovery
80%	20%	8,473	7,701	110%
60%	40%	6,639	6,145	108%
40%	60%	5,014	4,589	109%
20%	80%	3,187	3,034	105%
			Mean Recovery	**108%**



Intra Assay Precision

Three spiked hemolymph samples were diluted with Assay Buffer and run in replicates of 20 in an assay. The mean and precision of the calculated 20-Hydroxyecdysone concentrations were:

Sample	20-Hydroxyecdysone Conc. (pg/mL)	%CV
1	4,623	8.9
2	2,707	10.6
3	1,997	10.2

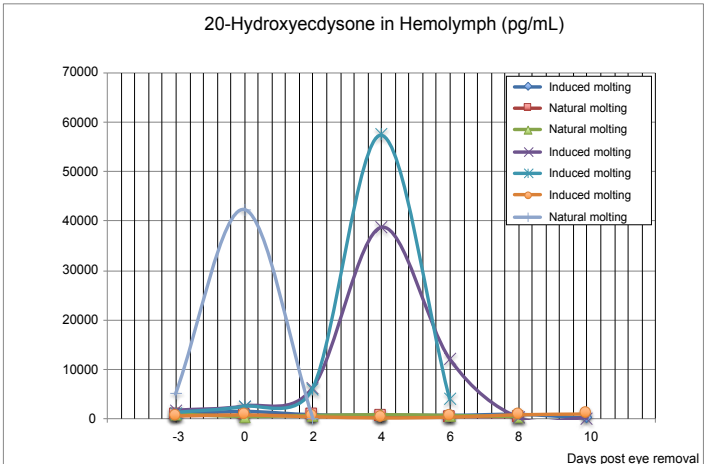
Inter Assay Precision

Three spiked hemolymph samples were diluted with Assay Buffer and run in duplicates in eighteen assays run over multiple days by multiple operators. The mean and precision of the calculated 20-Hydroxyecdysone concentrations were:

Sample	20-Hydroxyecdysone Conc. (pg/mL)	%CV
1	4,522	9.1
2	2,706	9.6
3	1,867	7.8

SAMPLE VALUES

This assay has been tested and validated with extracted Dungeness crab and crayfish hemolymph samples. In Dungeness crab, the concentrations of ecdysones are low during intermolt (~20 ng/mL) and maximal during premolt (~2000 ng/mL)[10]. In crayfish, the ecdysone level can increase to ~60 ng/mL during natural molts and ~80 ng/mL during induced molts[12]. Three Dungeness crab hemolymph during intermolt phase were assayed to yield concentrations ranging from 30.7 to 34.0 ng/mL with an average of 31.9 ng/mL. Four crayfish hemolymph during induced molts and three during natural molts were assayed as represented in the graph below. Induced molting sample concentrations measured 38.8 and 57.5 pg/mL and one natural molting sample was measured at 42.4 pg/mL. Results were comparable to published literature[12].



Samples were generously provided by Professor Sherry Tamone, University of Alaska Southeast for Dungeness crab hemolymph and Dr. Elizabeth Addis, Gonzaga University for Crayfish hemolymph.

12. Hemolymph Ecdysone and Electrolytes during the Molting Cycle of Crayfish: A Comparison of Natural Molts with Those Induced by Eyestalk Removal or Multiple Limb Autotomy. Michele G. Wheatly & Mary K. Hart. Physiological Zoology. 68(4): 583-607

CROSS REACTIVITY

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

Steroid	Cross Reactivity (%)	Steroid	Cross Reactivity (%)
20-Hydroxyecdysone	100%	Testosterone	0.05%
Makisterone A (MAK A)	5.90%	Corticosterone	0.04%
Ecdysone	0.71%	7-Dehydrocholesterol	0.02%
Ponasterone A (PON A)	0.61%	Cortisol	0.04%
ß-Estradiol	0.09%		

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

INTERNATIONAL SOCIETY of WILDLIFE ENDOCRINOLOGY

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.

The antiserum used in this kit was produced for the laboratories of Mark R. Brown and Michael R. Strand (University of Georgia, Athens, GA U.S.A) and used to develop specific immunoassays to measure ecdysteroids in mosquitoes[5].

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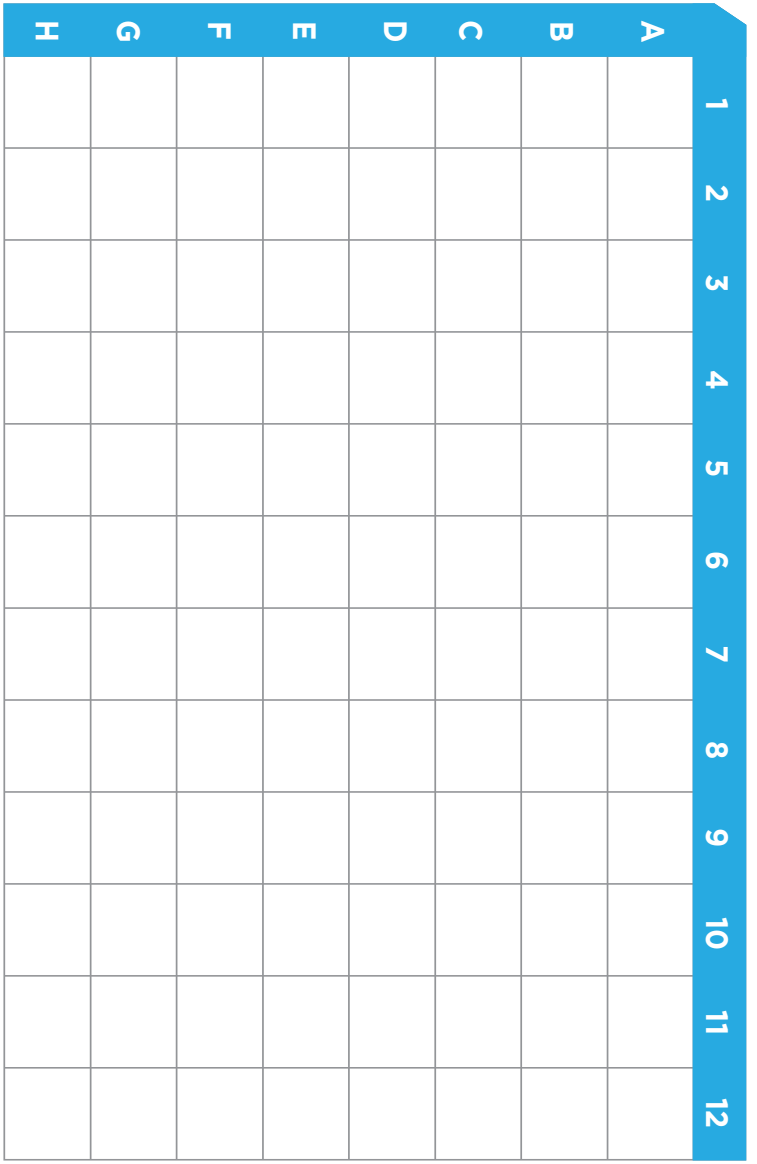
FOR SERIOUS SCIENCE



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