ARBOR ASSAYS™

DetectX® Progesterone Metabolites
ELISA Kit

1 Plate Kit – Catalog No. K068-H1
5 Plate Kit – Catalog No. K068-H5

Species Independent

Sample Types Tested:
Urine and Dried Fecal Extract

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

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SUPPLIED COMPONENTS & STORAGE

		K068-H1	K068-H5	Description
Goat anti-Rabbit Clear Coated 96-well Plate	Quantity	1	5	Strip well plates coated with goat anti-rabbit IgG
	Catalog No.	X016-1EA	X016-1EA	
Progesterone Standard	Volume	40 µL	200 µL	Progesterone at 1,000 ng/mL in stabilizing solution
	Catalog No.	C252-40UL	C252-200UL	
DetectX® Progesterone Antibody	Volume	3 mL	13 mL	Rabbit polyclonal antibody specific for Progesterone Metabolites
	Catalog No.	C250-3ML	C250-13ML	
DetectX® Progesterone Conjugate	Volume	3 mL	13 mL	Progesterone-peroxidase conjugate in stabilizing solution
	Catalog No.	C251-3ML	C251-13ML	
Assay Buffer Concentrate 5X	Volume	28 mL	55 mL	5X concentrate that must be diluted
	Catalog No.	X065-28ML	X065-55ML	
Wash Buffer Concentrate 20X	Volume	30 mL	125 mL	20X concentrate that must be diluted
	Catalog No.	X007-30ML	X007-125ML	
TMB Substrate	Volume	11 mL	55 mL	3,3',5,5'-Tetramethylbenzidine, a peroxidase substrate
	Catalog No.	X019-11ML	X019-55ML	
Stop Solution	Volume	5 mL	25 mL	1M solution of hydrochloric acid **CAUSTIC**
	Catalog No.	X020-5ML	X020-25ML	
Plate Sealer	Quantity	1	5	-
	Catalog No.	X002-1EA	X002-1EA	

Once opened, the kit can be stored at 4˚C up to the expiration date on the kit label.

OTHER MATERIALS REQUIRED

- Distilled or deionized water
- Adjustable pipettes with disposable tips capable of dispensing 10 µL, 25 µL, 50 µL, and 100 µL. Repeater pipette or multichannel pipettes with corresponding tips are also recommended.
- Glass or high-quality polypropylene test tubes for standard and sample preparation.
- An orbital microplate shaker
- A plate reader capable of measuring absorbance at 450 nm
- Software for converting optical density (OD) readings from the plate reader and carrying out four parameter logistic curve (4PL) fitting. Contact your plate reader manufacturer for details.
- Optional: Automated plate washer. Refer to Plate Washing Instructions for more details.
    - https://bit.ly/3tBT7N4

PRECAUTIONS

- Read this insert completely prior to using the product.
- This kit may not perform as described if any reagent or procedure is replaced or modified. Do not interchange reagents from different kit lots.
- Take appropriate safety precautions, such as: avoid breathing fumes, wear personal protective equipment (gloves, clothing, eye, and face protection), and familiarize yourself with SDS documents.
    - https://www.ArborAssays.com/documentation/msds/K068-H_MSDS.pdf
- Ensure all buffers used for samples are azide free and that any plate washing system is rinsed well with deionized water prior to using the supplied Wash Buffer. Buffers, including other manufacturers' wash buffers, that contain sodium azide will inhibit color production from the enzyme.
- **Take appropriate precautions when handling the Stop Solution, which is a caustic acid.**

BACKGROUND

Progesterone, $C_{21}H_{30}O_2$, also known as P4 (pregn-4-ene-3,20-dione) is a C-21 steroid hormone involved in the female menstrual cycle, gestation, and embryogenesis of humans and other species[1].

Progesterone belongs to a class of hormones called progestogens and is the major naturally occurring human progestogen[2]. Progesterone is an essential regulator of human female reproductive function in the uterus, ovary, mammary gland and brain, and plays an important role in non-reproductive tissues such as the cardiovascular system, bone and the central nervous system[3]. In different animal species, progesterone can be metabolized and excreted as a variety of general progesterone molecules[4]. A few examples would be fecal 5-reduced progesterone (pregnane) metabolites, pregnanolones, and hydroprogesterones. Measurement of these general progesterone molecules can provide vital data about endangered species to aid reproductive strategies. A group specific antibody with a high cross reactivity to most progesterone metabolites provides strong evidence for an ongoing reproductive cycle[5,6].

ASSAY PRINCIPLE

The DetectX® Progesterone Metabolites ELISA Kit is designed to quantitatively measure Progesterone Metabolites present in dried fecal extracts and urine. The Progesterone Metabolites ELISA Kit is a competitive ELISA with a run time of 1.5 hours. Please read the complete kit insert before performing this assay.

A Progesterone 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 Progesterone-peroxidase conjugate is added to the standards and samples in the wells. The binding reaction is initiated by the addition of an antibody to Progesterone Metabolites to each well. As the Progesterone Metabolites concentration in the sample increases, the bound Progesterone-peroxidase conjugate decreases, resulting in a decrease in signal and vice versa.

After an incubation, the plate is washed and substrate is added. The substrate reacts with the bound Progesterone-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 Progesterone Metabolites in the sample is calculated, after making suitable correction for dilution of the sample, using software available with most plate readers.

REAGENT PREPARATION

Except for the reagents listed below, all kit components are ready for use.

Reagent	Preparation	Stability
1X Assay Buffer	Warm 5X Assay Buffer Concentrate to room temperature and mix thoroughly by inversion. Mix 1 volume 5X Assay Buffer Concentrate with 4 volumes deionized water.	1X Assay Buffer is stable for 3 months at 4°C
1X Wash Buffer	Warm 20X Wash Buffer Concentrate to room temperature and mix thoroughly by inversion. Mix 1 volume 20X Wash Buffer Concentrate with 19 volumes deionized water.	1X Wash Buffer is stable for 3 months at room temperature

SAMPLE PREPARATION

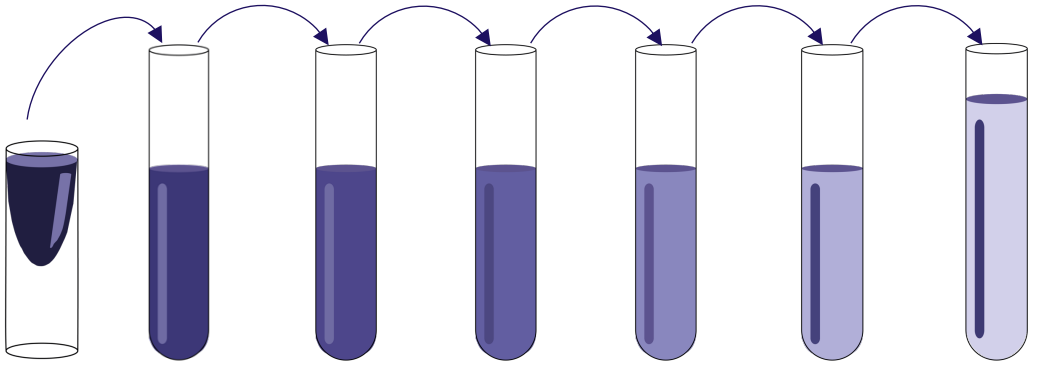
For samples containing particulates, centrifuge prior to use. Upon collection, all samples should be frozen rapidly and stored at -80ºC until testing.

Sample Type	Procedure
Dried Feces	• Please refer to the detailed *Steroid Solid Extraction* Protocol available on our website at www.ArborAssays.com/resources/#protocols. • The ethanol concentration in the final 1X Assay Buffer added to the well must be less than 5%.
Urine	• Prepare a minimum 8-fold dilution of sample by adding 50 µL urine to 350 µL 1X Assay Buffer. • Samples may require further dilution with 1X Assay Buffer to fall within the standard curve range. • Use our Urinary Creatinine Detection Kits (K002-H) to measure urine creatinine for normalization of Progesterone Metabolites in urine specimens.

⚠ **Use all samples within 2 hours of preparation.**

STANDARD PREPARATION

1. Label tubes Standard 1 through Standard 6.
2. Add 990 µL 1X Assay Buffer to Standard 1 tube.
3. Add 300 µL 1X Assay Buffer to Standard 2 – 6 tubes.
4. Add 10 µL of the Progesterone Standard Stock solution to Standard 1 tube. Vortex thoroughly.
   ⚠ **The Progesterone Standard Stock solution contains an organic solvent. Pipet the stock solution up and down several times prior to dispensing to ensure accurate delivery.**
5. Transfer 200 µL of Standard 1 into Standard 2 tube to make a 2.5-fold dilution. Vortex thoroughly.
6. Transfer 200 µL of the mixed solution from Standard 2 into Standard 3 tube to make a 2.5-fold dilution. Vortex thoroughly.
7. Continue serially diluting into the remaining tubes. This process and the final concentrations are summarized in the table below.



	Standard 1	Standard 2	Standard 3	Standard 4	Standard 5	Standard 6
1X Assay Buffer (µL)	990	300	300	300	300	300
Addition	Stock	Std 1	Std 2	Std 3	Std 4	Std 5
Volume of Addition (µL)	10	200	200	200	200	200
Final Concentration (pg/mL)	10,000	4,000	1,600	640	256	102

⚠ **Use all Standards within 2 hours of dilution.**

ASSAY PROTOCOL

Before You Begin:

- **Room Temperature for this assay is defined as 22ºC – 24ºC.**
- **Ensure all reagents have been warmed to room temperature.**
- **Dilute Samples as described in Sample Preparation.**
- **Run all Standards and Samples in duplicate.**
- Use the blank plate template on the back page of this booklet to design your plate layout and aid in proper Sample and Standard identification.
- Be sure to shake the plate as directed. Failing to shake the plate or altering the shaking speed during incubations will result in decreased signal.
- Set plate parameters on the plate reader for a 96-well Corning CoStar 2592 plate. See ArborAssays.com for plate dimension data.
- Determine the number of well strips to be used and return unused well strips to the foil pouch with desiccant. Seal the foil pouch and store at 4°C. Desiccant color will change from blue to pink if the foil pouch is not properly sealed.
- If you are using only part of a strip well plate, at the end of the assay discard the used wells and retain the plate frame for use with the remaining unused wells.

1. Add 50 µL Samples or Standards into duplicate wells.
2. Add 75 µL 1X Assay Buffer into duplicate NSB (non-specific binding) wells.
3. Add 50 µL 1X Assay Buffer into duplicate Zero Standard (maximum binding or B0) wells.
4. Add 25 µL DetectX® Progesterone Conjugate to each well.
5. Add 25 µL DetectX® Progesterone Antibody to each well, **except the NSB wells**.
6. Cover the plate with a plate sealer and shake at room temperature at 700-900 rpm for **1 hour**.
7. Remove the plate sealer, aspirate the plate, and wash each well 4 times with 300 µL 1X Wash Buffer. Tap the plate dry on clean absorbent towels.
8. Add 100 µL TMB Substrate to each well.
    - The substrate solution will begin to turn blue.
9. Incubate at room temperature for **30 minutes** without shaking.
10. Add 50 µL Stop Solution to each well.
    - The substrate solution will begin to turn yellow.
11. Read the optical density at 450 nm within 10 minutes.

CALCULATION OF RESULTS

Follow the instructions below or use this online tool: https://myassays.com/assay.aspx?id=1306

1. Use four-parameter logistic curve (4PL) software to calculate the Progesterone Metabolites concentration for each sample. Gather all raw data OD readings from each Sample and Standard, including the Zero (B0) Standard and NSB.
2. Average the duplicate OD readings for each Sample, Standard, B0, and NSB (Mean OD).

EXAMPLE:

Sample	Replicate 1 OD	Replicate 2 OD	Mean OD
NSB	0.076	0.080	0.078
B0	1.347	1.355	1.351
Sample 1	0.440	0.460	0.450

3. Subtract the NSB from the Mean OD for each Sample, Standard, and the B0 (Net OD).

EXAMPLE:

Sample	Mean OD	NSB Mean OD	Net OD
B0	1.351	0.078	1.273
Sample 1	0.450	0.078	0.372

4. Divide the Net OD for each Sample and Standard by the Net OD for B0 and multiply by 100% (%B/B0).

EXAMPLE:

Sample	Net OD	B0 Net OD	%B/B0
Sample 1	0.372	1.273	29.2

5. Plot the standard curve with %B/B0 for the standards on the y-axis and Progesterone Metabolites concentration (pg/mL) on the x-axis. Perform a 4PL fit.

   Use the Sample %B/B0 readings and the 4PL fit to calculate Progesterone Metabolites concentrations in diluted Samples. If diluted sample Progesterone Metabolites concentrations are outside of the range of the Standards, the Samples should be prepared again at a more appropriate dilution.

EXAMPLE:

Sample	Net OD	%B/B0	Diluted Sample Progesterone Metabolites Concentration (pg/mL)
Sample 1	0.372	29.2	2,373

6. Multiply the diluted Sample Progesterone Metabolites concentration by the sample dilution factor (as done in Sample Preparation) to determine the concentration of Progesterone Metabolites in the original sample.

EXAMPLE:

Sample	Diluted Sample Progesterone Metabolites Concentration (pg/mL)	Sample Dilution Factor	Original Sample Progesterone Metabolites Concentration (pg/mL)
Sample 1	2,373	8x dilution	18,984

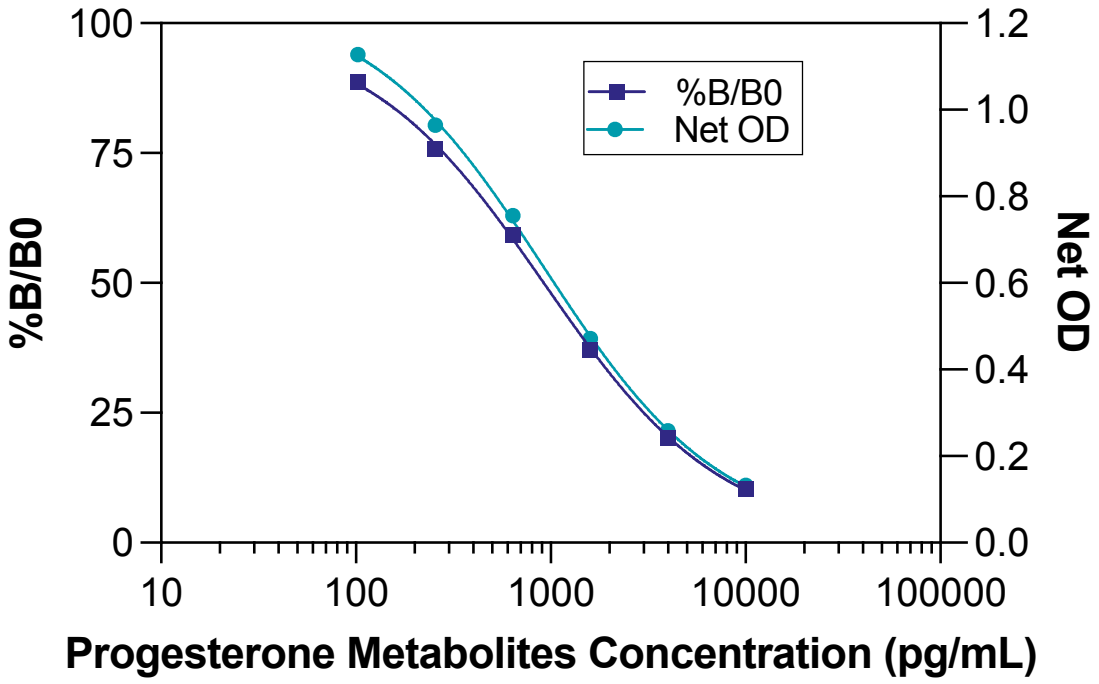
TYPICAL DATA

⚠ **Always run your own standard curve. This data should NOT be used to interpret results.**

Sample	Mean OD	Net OD	%B/B0	Progesterone Metabolites Concentration (pg/mL)
NSB	0.078	-	-	-
Standard 1	0.209	0.132	10.3	10,000
Standard 2	0.335	0.258	20.2	4,000
Standard 3	0.550	0.472	37.1	1,600
Standard 4	0.832	0.755	59.3	640
Standard 5	1.041	0.964	75.7	256
Standard 6	1.206	1.128	88.6	102
B0	1.351	1.273	100.0	0
Sample 1	0.450	0.372	29.2	2,373
Sample 2	0.534	0.456	35.8	1,716

Conversion factor: 100 pg/mL of Progesterone Metabolites is equivalent to 318 pM.

Typical Standard Curve



VALIDATION DATA

Sensitivity and Limit of Detection

Sensitivity was calculated by comparing the ODs for twenty wells run for each of the B0 and Standard 6. The detection limit was determined at two standard deviations from the B0 along the standard curve.

Sensitivity was determined as 44.8 pg/mL

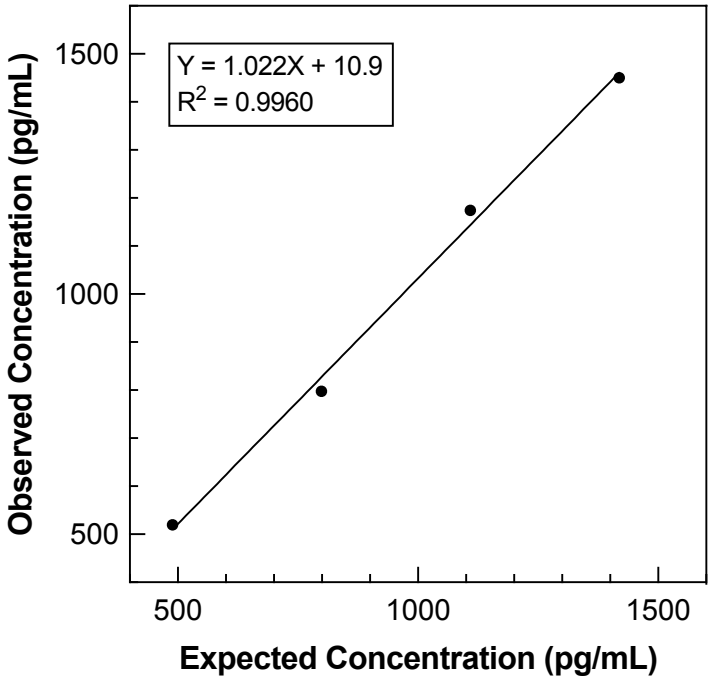
The Limit of Detection was determined in a similar manner by comparing the ODs for twenty wells run for each of the B0 and a low concentration sample.

The Limit of Detection was determined as 67.8 pg/mL

Linearity

Linearity was determined by diluting two human urine samples with known Progesterone Metabolites concentration with 1X Assay Buffer. One sample had a Progesterone Metabolites concentration of 179 pg/mL (Low Sample); a second sample had a Progesterone Metabolites concentration of 1729 pg/mL (High Sample). The two samples were mixed in the ratios given below and the measured concentrations were compared to the expected values for each given ratio.

Low Sample	High Sample	Expected Concentration (pg/mL)	Observed Concentration (pg/mL)	% Recovery
80%	20%	489	520	106.3
60%	40%	799	798	99.8
40%	60%	1,109	1,174	105.9
20%	80%	1,419	1,451	102.2
			Mean Recovery	**103.6%**



Intra Assay and Inter Assay Precision

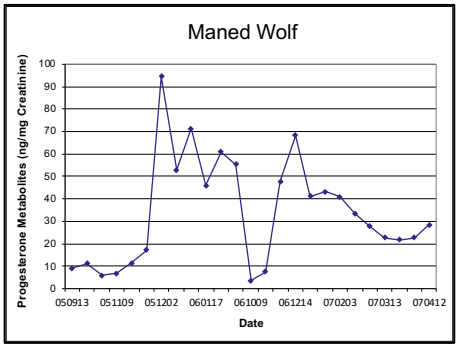
For intra assay precision, three human urine samples were diluted in 1X Assay Buffer, and 20 replicates were run in one assay. For inter assay precision, three human urine samples were diluted in 1X Assay Buffer and duplicates of each sample were run in 19 assays over multiple days by six operators. %CV represents the variation in concentration (not optical density) as determined using a standard curve.

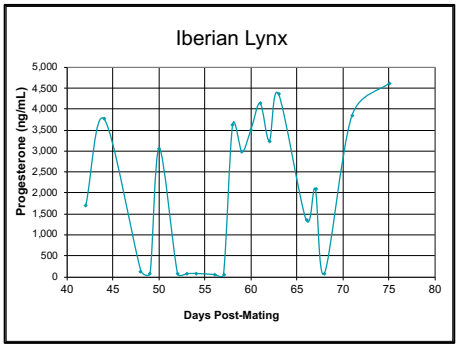
	Intra Assay Precision		Inter Assay Precision	
Sample	Progesterone Metabolites Concentration (pg/mL)	% CV	Progesterone Metabolites Concentration (pg/mL)	% CV
1	2,377	4.9	2,442	6.8
2	1,517	7.2	1,665	6.6
3	710	8.0	845	8.8

SAMPLE VALUES

Four human urine samples were tested in the assay, three came from pregnant women (17, 21, and 26 weeks). Adjusted concentrations of Progesterone Metabolites ranged from 39 to 4,313 ng/mL. When adjusted for urine creatinine using the DetectX® Urinary Creatinine detection kit, K002-H1, the values ranged from 49.7 to 215.9 ng/mg creatinine.

Timed urine samples from a pregnant Maned Wolf over a 12-month period, and dried fecal samples from an Iberian Lynx were tested in the assay; results are depicted in the charts below.





The maned wolf samples were generously gifted by Rachel Santymire from Lincoln Park Zoo, Chicago and the Iberian lynx samples were from Martin Dehnhard, Leibniz Institute for Zoo & Wildlife Research, Berlin.

INTERFERENCE

Potentially interfering substances were evaluated in the assay and the change in signal was calculated.

Interferent	% Added	Effect
DMF	2.5%	6.4% decrease
DMSO	5.0%	4.0% decrease
Ethanol	5.0%	2.0% increase
Methanol	5.0%	4.1% increase

CROSS REACTIVITY

The following cross reactants were tested in the assay at 40x, 4x, 0.4x and 0.04x concentration of the highest standard. Percent cross-reactivity was calculated comparing the observed concentration to the actual concentration of each cross reactant.

Cross Reactant	Cross Reactivity (%)
Progesterone	100
5β-dihydroprogesterone	61.9
5α-dihydroprogesterone	56.7
Pregnanolone (5β-Pregnan-3α-ol-20-one)	41.2
Epiallopregnanolone (5α-pregnan-3β-ol-20-one)	38.3
Allopregnanolone	27.3
Pregnenolone	17.6
Epipregnanolone	10.2
17α-hydroxyprogesterone	5.7
11α-hydroxyprogesterone	4.9
20α-hydroxyprogesterone	0.34
Allopregnandiol	0.29

TROUBLESHOOTING

Issue	Possible Cause & Solution
Reagent Shortage	• Check under the cap for additional reagent. Pulse spin reagent containers to collect contents prior to opening when possible. • When using a multichannel pipette, return unused reagent to container for later use.
Erratic Values	• Ensure the assay plate has been properly blotted after assay washes to remove residual wash buffer. • Prerinse pipet tips with desired reagent prior to aspirating the required volume. • Deliver volume with care to prevent splashing into adjacent wells.
High Background	• Ensure assay plate has been properly washed with the number of washes indicated in the protocol. • Reagent contamination during assay setup. • Verify antibody was not added to the NSB wells.
Low Signal	• Confirm tools, equipment, reagents, and containers used do not contain any trace of sodium azide. • Altering shaking speeds or excluding shaking during incubation steps. • Verify the plate reader wavelength is 450 nm. • Confirm reagents were at room temperature prior to use.

CITATIONS

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2. Pearlman WH, and Cerceo, E. “The isolation of progesterone from human placenta.”, J. Biol. Chem., 1952; 278: 73-89.

3. Li, X and O’Malley, BW., “Unfolding the Action of Progesterone Receptors.”, J. Biol. Chem., 2003; 278: 39261–39264.

4. Schwarzenberger, F., Tomášová, K., Holečková, D., Matern, B., and Möstl, E., “Measurement of Fecal Steroids in the Black Rhinoceros (Diceros bicornis) Using Group-Specific Enzyme Immunoassays for 20-Oxo-Pregnanes.”, Zoo Biology, 1996; 15:159-171.

5. Kancheva, R., Hill, M., Cibula, D., Včeláková, H., Kancheva, L., Vrbíková, J., Fait, T., Pařízek, A., and Stárka, L., “Relationship of circulating pregnanolone isomers and their polar conjugates to the status of sex, menstrual cycle, and pregnancy.”, J. Endocrinology, 2007; 195(1):67-78.

6. Palme, R., Möstl, E., Schellander, K., Bamberg, E., “Faecal Metabolites of Infused 14C-Progesterone in Domestic Livestock.”, Reprod Dom Anim, 1997; 32(4): 199-206.

RELATED PRODUCTS

Kits	Catalog No.
17-Hydroxyprogesterone EIA Kits	K053-H1/H5
Allopregnanolone ELISA Kits	K061-H1/H5
Ceruloplasmin Colorimetric Activity Kit	K035-H1
Dehydroepiandrosterone sulfate (DHEA-S) ELISA Kits	K054-H1/H5
Epiandrosterone ELISA Kits	K063-H1/H5
Estradiol ELISA Kits	K030-H1/H5 KB30-H1/H5
Estriol ELISA Kits	K064-H1/H5
Estrone ELISA Kits	K031-H1/H5
Estrone-3-Glucuronide (E1G) ELISA Kits	K036-H1/H5
Estrone-3-Sulfate (E1S) ELISA Kits	K038-H1/H5
Levonorgestrel (LNG) ELISA Kits	K058-H1/H5
Oxytocin Colorimetric and Chemiluminescent ELISA Kits	K048-H1/H5 K048-C1/C5
PGFM ELISA Kits	K022-H1/H5
Pregnanediol-3-Glucuronide (PDG) ELISA Kits	K037-H1/H5
Prolactin (PRL) ELISA Kits	K040-H1/H5
Testosterone ELISA Kits	K032-H1/H5 K080-H1/H5

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.

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CONTACT INFORMATION

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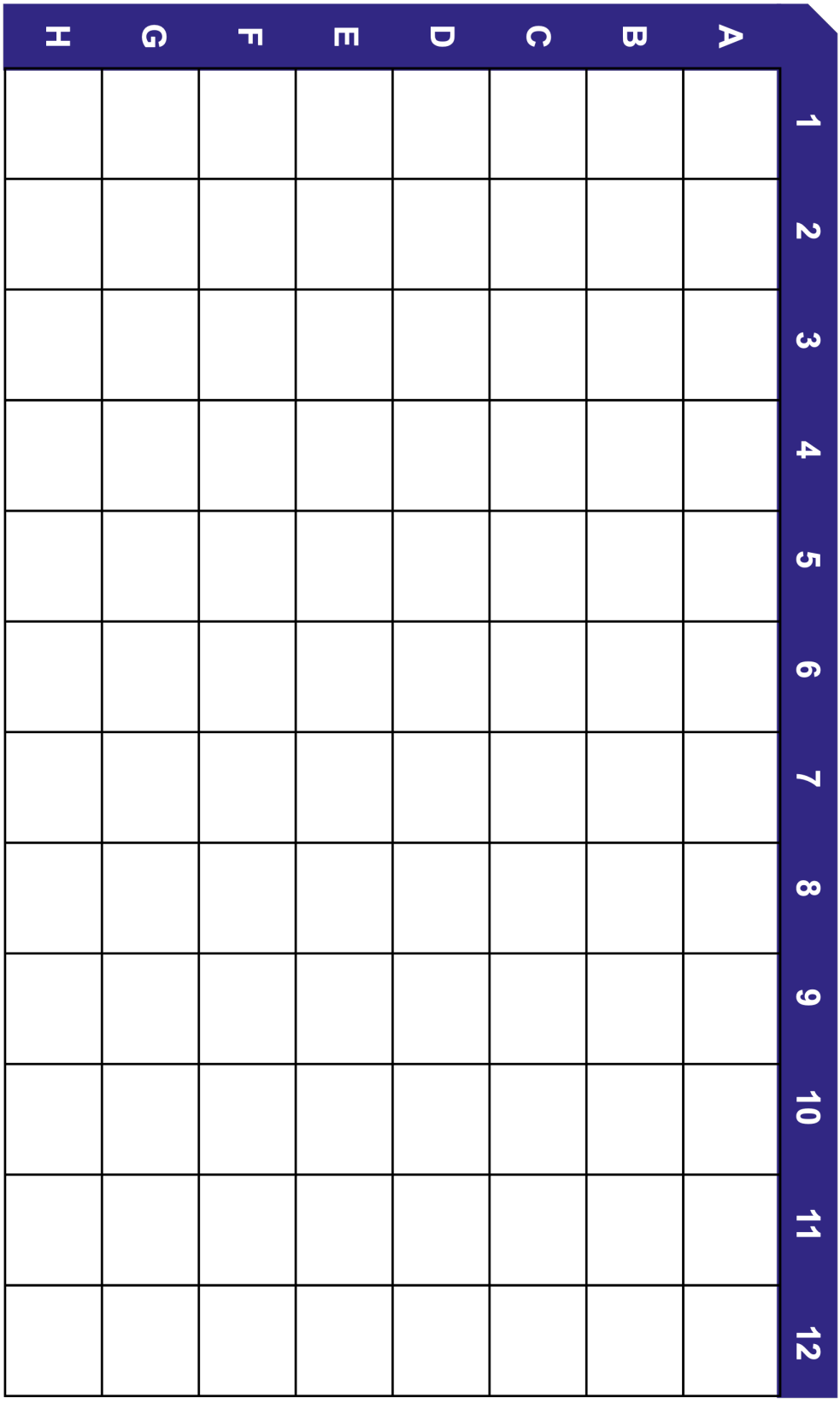
OFFICIAL SUPPLIER TO ISWE

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.

PLATE LAYOUT



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