ARBOR ASSAYS™
Interactive Assay Solutions™

MULTI SPECIES
N-CAL™ KIT

NCal™ International Standard Kit

DetectX®

Cortisol
Enzyme Immunoassay Kit

1 or 5 Strip Plates	Catalog Number K003-H1/H5
1 or 5 Whole Plates	Catalog Number K003-H1W/H5W

Species Independent

New Extended Assay Range

Sample Types Validated:

Dried Fecal Extracts, Saliva, Urine, Serum, EDTA and Heparin Plasma and Tissue Culture Media

Calibrated to NIST Standard Reference Material Lot No. 921

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

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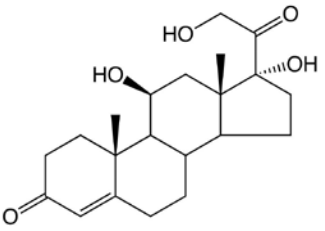
BACKGROUND

Cortisol, $C_{21}H_{30}O_5$, (hydrocortisone, compound F) is the primary glucocorticoid produced and secreted by the adrenal cortex. It is often referred to as the "stress hormone" as it is involved in the response to stress and it affects blood pressure, blood sugar levels, and other actions of stress adaptation. Immunologically, cortisol functions as an important anti-inflammatory and plays a role in hypersensitivity, immunosuppression, and disease resistance[1]. In the metabolic aspect, cortisol promotes gluconeogenesis, liver glycogen deposition, and the reduction of glucose utilization[2]. Production of cortisol follows an ACTH-dependent circadian rhythm, with a peak level in the morning and decreasing levels throughout the day. Most serum cortisol, all but about 4%, is bound to proteins including corticosteroid binding globulin and serum albumin[1,3]. Only free cortisol is available to most receptors and it is through these receptors that physiological processes are modulated. Abnormal cortisol levels are being evaluated for correlation with a variety of different conditions, such as prostate cancer[4], depression[5], and schizophrenia[6]. It is already known that abnormal levels of cortisol are involved in Cushing's Syndrome and Addision's disease[7].



1. E. Friess, et al., Eur J Clin Invest, 2000, 30, Suppl 3:46-50.
2. Freeman, Scott, 2002. Biological Science. Prentice Hall; 2nd Pkg edition (December 30, 2004).
3. C. Longscope., J. Endocrinology, 1996, , Suppl S125-S127.
4. J. Herbert, Lancet, 1995 345, 1193-1194.
5. A. Michael, et al., Biol. Psychiatry, 2000, 48, 989-95.
6. C.R. Dequet and D.J. Wallace, Current Opin. Ivest. Drugs, 2001, 8, 1045-53.
7. W.M. Jeffries, Med. Hypotheses, 1998, 51, 114-4.

ASSAY PRINCIPLE

The DetectX® Cortisol Immunoassay Kit is designed to quantitatively measure cortisol present in dried fecal extracts, saliva, urine, serum, plasma and tissue culture media samples. Please read the complete kit insert before performing this assay. Total cortisol is measured in extracted samples and in serum and plasma and free cortisol in saliva and urine. A cortisol standard is provided to generate a standard curve for the assay and all samples must be read off a user-generated standard curve. Standards or diluted samples are pipetted into a clear microtiter plate coated with an antibody to capture mouse antibodies. A cortisol-peroxidase conjugate is added to the wells. The binding reaction is initiated by the addition of a monoclonal antibody to cortisol.

The immunological reaction occurs between the limiting amount of added anti-cortisol monoclonal antibody, the cortisol antigen in the sample or standard, and the limiting amount of added cortisol-peroxidase conjugate. As the concentration of cortisol in the sample increases, the amount of cortisol-peroxidase conjugate bound decreases causing an decrease in signal, and *vice versa*. The signal is generated from the cortisol-peroxidase bound to the anti-cortisol antibody which itself is bound to the goat anti-mouse IgG coated plates. Excess cortisol-peroxidase does not bind to the plates and is washed out of the well prior to the addition of substrate. For a further explanation go to: www.ncbi.nlm.nih.gov/books/NBK92434/pdf/immunometh.pdf, page 28.

After an hour incubation the plate is washed and substrate is added. The substrate reacts with the bound cortisol-peroxidase conjugate. After a short incubation, the reaction is stopped and the intensity of the generated color is detected in a microtiter plate reader at 450 nm wavelength. The concentration of the cortisol 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
Cortisone Chemiluminescent Immunoassay Kits	K017-C1/C5
Cortisone Enzyme Immunoassay Kits	K017-H1/H5
Urinary Creatinine Detection Kits	K002-H1/H5

SUPPLIED COMPONENTS

Clear Coated 96 Well Plate
Each are coated with goat anti-mouse IgG.

Kit K003-H1 **or** -H5	1 **or** 5 Each	Catalog Number X012-1EA, 1 x 8 Strip Well
Kit K003-H1W **or** -H5W	1 **or** 5 Each	Catalog Number X011-1EA, Whole Well

Cortisol Standard
Cortisol at 32,000 pg/mL in a special stabilizing solution.

Kit K003-H1/H1W **or** -H5/H5W	125 µL **or** 625 µL	Catalog Number C040-125UL **or** -625UL

Calibrated to NIST Standard Reference Material Lot Number 921.

DetectX® Cortisol Antibody
A mouse monoclonal antibody specific for cortisol.

Kit K003-H1/H1W **or** -H5/H5W	3 mL **or** 13 mL	Catalog Number C041-3ML **or** -13ML

DetectX® Cortisol Conjugate
A cortisol-peroxidase conjugate in a special stabilizing solution.

Kit K003-H1/H1W or -H5/H5W	3 mL **or** 13 mL	Catalog Number C039-3ML **or** -13ML

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

Kit K003-H1/H1W or -H5/H5W	28 mL **or** 55 mL	Catalog Number X053-28ML **or** -55ML

Dissociation Reagent
Allow to warm completely to **Room Temperature** prior to use. **Dissociation Reagent is to be used only with Serum and Plasma samples.**

Kit K003-H1/H1W **or** -H5/H5W	1 mL **or** 5 mL	Catalog Number X017-1ML **or** -5ML

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

Kit K003-H1/H1W **or** -H5/H5W	30 mL **or** 125 mL	Catalog Number X007-30ML **or** -125ML

TMB Substrate

Kit K003-H1/H1W **or** -H5/H5W	11 mL **or** 55 mL	Catalog Number X019-11ML **or** -55ML

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

Kit K003-H1/H1W **or** -H5/H5W	5 mL **or** 25 mL	Catalog Number X020-5ML **or** -25ML

Plate Sealer

Kit K003-H1/H1W **or** -H5/H5W	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 with disposable tips capable of dispensing 25 µL, 50 µL and 100 µL.

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

Cortisol is identical across all species and this kit will measure cortisol from sources other than human. The end user should evaluate recoveries of cortisol in other samples. This assay has been validated for saliva, urine, serum and EDTA and heparin plasma and for tissue culture samples. It has been validated for dried fecal extract samples. Samples containing particulates should be centrifuged prior to using. Moderate to severely hemolyzed samples should not be used in this kit.

SAMPLE PREPARATION

Serum and plasma samples need to be treated with the supplied Dissociation Reagent. Addition of this reagent will yield the total cortisol concentration in serum or plasma. **Dissociation Reagent is to be used only with Serum and Plasma samples.** Free cortisol can be measured in saliva and urine samples as directed below.

Dried Fecal Samples

We have a detailed Extraction Protocol available on our website at: www.ArborAssays.com/assets/steroid-solid-extraction-protocol.pdf. The ethanol concentration in the final Assay Buffer dilution added to the well should be < 5%.

Serum and Plasma Samples

The normal reference range for human serum cortisol is 2-25 µg/dL (20-250 ng/mL)[8]. Allow the Dissociation Reagent (DR) to warm completely to **Room Temperature** before use. We suggest pipeting 5 µL of DR into 1 mL Eppendorf tubes. Add 5 µL of serum or plasma to the DR in the tube, vortex gently and incubate at room temperature for 5 minutes or longer. Dilute by adding 490 µL of supplied Assay Buffer. This 1:100 dilution can be diluted further with Assay Buffer for higher cortisol sample concentrations. Final serum and plasma dilutions must be ≥ 1:100.

NOTE: Dissociation Reagent is to be used only with Serum and Plasma samples.

Saliva Samples

Saliva samples should be diluted ≥ 1:4 or greater with the supplied Assay Buffer prior running in the assay. See our Saliva Sample Handling Instructions at www.ArborAssays.com/assets/saliva-sample-protocol.pdf.

Urine Samples

Urine samples should be diluted ≥ 1:8 with the supplied Assay Buffer prior running in the assay. Urinary cortisol normally ranges from 0.7-119 µg/gram[9] of creatinine or approximately 100,000 to 1,000,000 pg/mL[9] in 24 hour urine samples. Samples may need to be diluted substantially to read within the standard curve range.

Tissue Culture Media

Cortisol in tissue culture media (TCM), samples should be read off a standard curve generated in TCM. Samples may need to be diluted further in TCM. This assay has been validated using RPMI-1640.

Use all Samples within 2 Hours of preparation, or stored at ≤ -20°C until assaying.

8. Tietz, NW, In "Textbook of Clinical Chemistry", WB Saunders, 1986.
9. www.mayomedicallaboratories.com/test-catalog/. Mayo Medical Laboratories: Reference Laboratory.

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

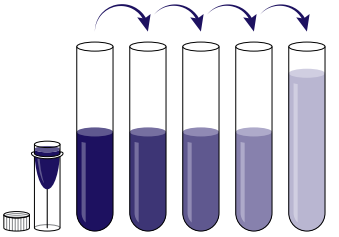
Dilute Assay Buffer Concentrate 1:5 by adding one part of the concentrate to four parts of deionized water. Once diluted this is stable for 3 months at 4°C.

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 450 µL of Assay Buffer into tube #1 and 250 µL into tubes #2 to #7. **The cortisol stock solution contains an organic solvent. Prerinse the pipet tip several times to ensure accurate delivery.** Carefully add 50 µL of the cortisol stock solution to tube #1 and vortex completely. Take 250 µL of the cortisol 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 cortisol in the tubes will be 3,200, 1,600, 800, 400, 200, 100 and 50 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)	**450**	250	250	250	250	250	250
Addition	Stock	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6
Volume of Addition (µL)	**50**	250	250	250	250	250	250
Final Conc (pg/mL)	3,200	1,600	800	400	200	100	50

ASSAY PROTOCOL

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

1. Use the plate layout sheet on the back page to aid in proper sample and standard identification.
2. If you are using the 1 by 8 well strip plate version of the kit, K003-H1 or -H5, determine the number of wells to be used and return unused wells to foil pouch with desiccant. Seal the ziploc plate bag and store at 4˚C.

   Pipet standards or samples down the plate strip columns (A to H) to ensure maximum use of the strip wells.

   The use of any wells in the whole plate versions of the kit, K003-H1W and K003-H5W will not allow use of unused parts of that plate in a later assay.
3. Pipet 50 µL of samples or standards into wells in the plate.
4. Pipet 75 µL of Assay Buffer into the non-specific binding (NSB) wells.
5. Pipet 50 µL of Assay Buffer into into the maximum binding (B0 or Zero standard) wells.
6. Add 25 µL of the DetectX® Cortisol Conjugate to each well using a repeater pipet.
7. Add 25 µL of the DetectX® Cortisol Antibody to each well, **except the NSB wells**, using a repeater pipet.
8. 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 1 hour.
9. Aspirate the plate and wash each well 4 times with 300 µL wash buffer. Tap the plate dry on clean absorbent towels.
10. Add 100 µL of the TMB Substrate to each well, using a repeater pipet.
11. Incubate the plate at room temperature for 30 minutes without shaking.
12. Add 50 µL of the Stop Solution to each well, using a repeater pipet.
13. Read the optical density generated from each well in a plate reader capable of reading at 450 nm.
14. Use the plate reader's built-in 4PLC software capabilities to calculate cortisol 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.

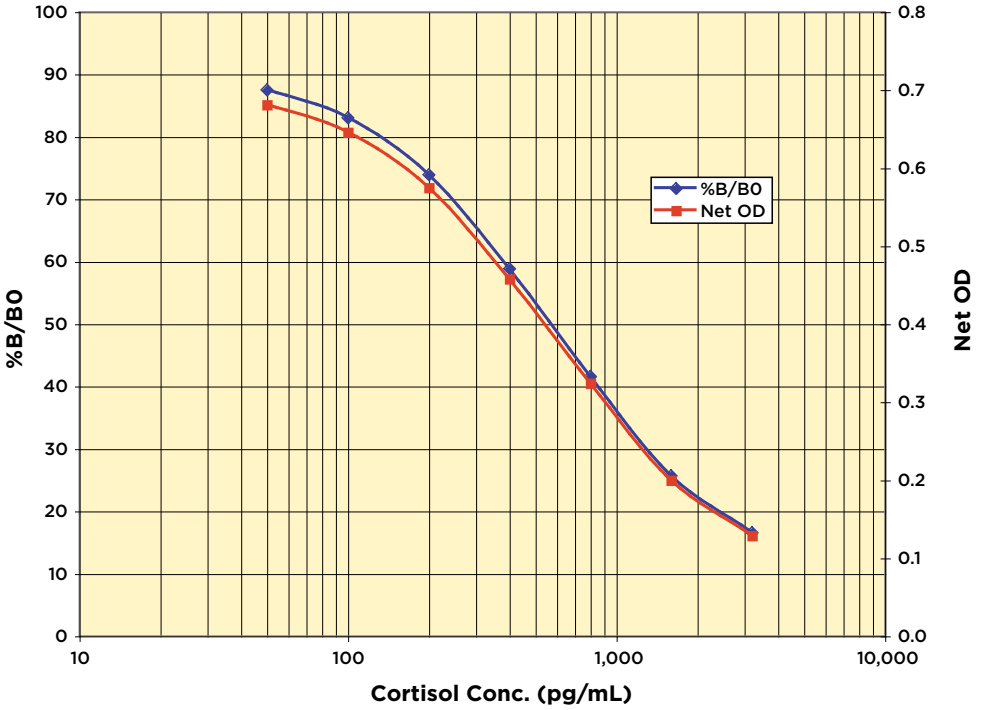
Or use the online tool from MyAssays to calculate the data:
www.myassays.com/arbor-assays-detectx-cortisol-(extended-range).assay

TYPICAL DATA

Sample	Mean OD	Net OD	% B/B0	Cortisol Conc. (pg/mL)
NSB	0.080	0	-	-
Standard 1	0.209	0.129	16.58	3,200
Standard 2	0.280	0.200	25.71	1,600
Standard 3	0.404	0.324	41.65	800
Standard 4	0.538	0.458	58.87	400
Standard 5	0.655	0.575	73.91	200
Standard 6	0.726	0.646	83.03	100
Standard 7	0.761	0.681	87.53	50
Standard				
B0	0.858	0.778	100	0
Sample 1	0.318	0.238	30.53	1,265.8
Sample 2	0.639	0.559	71.79	224.3

Always run your own standard curve for calculation of results. Do not use this data.
Conversion Factor: 100 pg/mL of cortisol is equivalent to 275.9 pM.

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

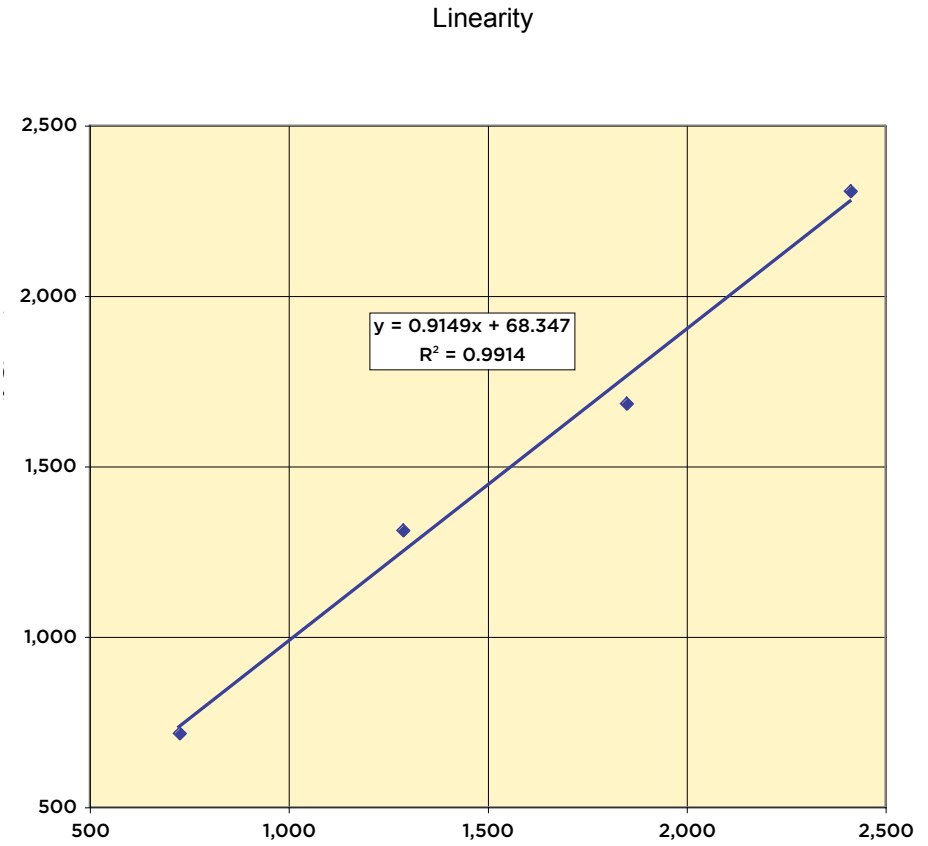
Sensitivity was calculated by comparing the OD's for twenty wells run for each of the B0 and standard #7. The detection limit was determined at two (2) standard deviations from the B0 along the standard curve. **Sensitivity was determined as 27.6 pg/mL.**

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 human sample. **Limit of Detection was determined as 45.4 pg/mL.**

Linearity

Linearity was determined by taking two human urine samples diluted 1:140, one with a low diluted cortisol level of 163.9 pg/mL and one with a higher diluted level of 2,974.9 pg/mL and mixing them in the ratios given below. The measured concentrations were compared to the expected values based on the ratios used.

Low Urine	High Urine	Observed Conc. (pg/mL)	Expected Conc. (pg/mL)	% Recovery
80%	20%	715.7	726.1	98.6
60%	40%	1,311.5	1,288.3	101.8
40%	60%	1,683.3	1,850.5	91.0
20%	80%	2,306.3	2,412.7	95.6
			Mean Recovery	**96.7%**



Intra Assay Precision

Three human samples were diluted with Assay Buffer and run in replicates of 20 in an assay. The mean and precision of the calculated Cortisol concentrations were:

Sample	Cortisol Conc. (pg/mL)	%CV
1	1,174.3	6.0
2	475.9	5.6
3	177.4	14.7

Inter Assay Precision

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

Sample	Cortisol Conc. (pg/mL)	%CV
1	1,188.1	7.2
2	508.7	6.3
3	199.7	10.9

SAMPLE VALUES

Six random human serum and plasma samples were tested in the assay. Neat sample values ranged from 8.5 to 23.8 µg/dL with an average of 12.2 µg/dL. The normal reference range for serum cortisol is 3-23 µg/dL[9]. Four random human urine samples were tested in the assay. Neat sample values ranged from 98.1 to 304.9 µg/g creatinine with an average of 159.8 µg/g creatinine. Creatinine levels were determined using the DetectX® Creatinine kits, K002-H1 and K002-H5.

Dried fecal samples were processed as described on page 7 and run in the assay. Samples kindly donated by Dr. J. Williams at the Indianapolis Zoo, which included Amur Tiger, Giraffe, Kudu, Lion, Reeves Muntjac, White Handed Gibbon, White Rhino, and Zebra, were tested and cortisol values obtained ranged from 2.48 to 27.22 pg/mg dried fecal material.

Palme and Möestl and colleagues have shown that radiolabeled administered cortisol is excreted in differing amounts in urine and feces[10] across species, with fecal excretion ranging from 7% of administered cortisol in the pig to 82% in the cat[11,12, 13]. Palme has also shown that the peak of fecal cortisol concentrations occur at 12 hours for sheep, but takes 48 hours to peak in pigs. It is therefore necessary to evaluate the timing and relative fecal or urine excretion of glucocorticoids for each species.

10. Möstl, E., et al, Vet. Res. Commun. “Measurement of Cortisol Metabolites in Faeces or Ruminants.” 2002, 26:127-139.
11. Palme, R., et al, Animal Reprod. Sci., “Excretion of infused ^{14}C-steroid hormones via faeces and urine in domestic livestock.” 1996, 43:43-63.
12. Teskey-Gerstl, A., et al, J. Comp. Physiol. B, “Excretion of corticosteroids in urine and faeces of hares (Lepus europaeus).” 2000, 170: 163-168.
13. Schatz, S. and Palme, R., Vet. Res. Commun., Measurement of Faecal Cortisol Metabolites in Cats and Dogs: A Non-Invasive Method for Evaluating Adrenocortical Function.”, 2001, 25:271-287.

CROSS REACTIVITY

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

Steroid	Cross Reactivity (%)
Cortisol	100%
Dexamethasone	18.8%
Prednisolone (1-Dehydrocortisol)	7.8%
Corticosterone	1.2%
Cortisone	1.2%
Progesterone	< 0.1%
Estradiol	< 0.1%
Cortisol 21-Glucuronide	< 0.1%
1α-hydroxycorticosterone	< 0.1%
Testosterone	< 0.1%

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 assay kits and reagents for wildlife conservation research.

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FOR SERIOUS SCIENCE

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