

Equine/Canine/Rodent Inhibin A ELISA

RUO

AL-161

INTENDED USE

The animal Inhibin A enzyme linked immunosorbent assay (ELISA) kit provides materials for the quantitative measurement of Inhibin A in serum and other biological fluids. This kit is intended for veterinary in-vitro diagnostic use only and is not for use in human diagnostic or therapeutic procedures.

SUMMARY AND EXPLANATION

Inhibins are gonadal glycoproteins produced by the granulosa cells and sertoli cells under the influence of FSH. The fully processed form of the inhibin molecule has a molecular weight of approximately 32 kDa and consists of the two distinct chains (α and β), linked by disulfide bridges. Inhibin- α subunit pairs with β A and β B subunits to form Inhibin A and Inhibin B respectively. Inhibins control androgen secretion and germ cell proliferation. High follicular levels of inhibin A are associated with continued follicular growth and ovulation.¹ Equine fetal testes and fetal ovaries secrete significantly larger amounts of inhibin B than adult testes and ovaries. Plasma concentrations of Inhibin A are significantly higher during the growth of preovulatory follicles than during the transitional anovulatory follicles. Recent findings suggested a decrease in intrafollicular-free insulin-like growth factor I, inhibin A, vascular endothelial growth factor, and prolactin during the final stages of follicular growth. This is the first documented occurrence of dynamic changes among intrafollicular factors and hormones during the stages of follicle dominance and as ovulation approaches.² Granulosa cell tumors (GCTs) are the most common form of ovarian neoplasm in mares, accounting for more than 85% of all tumors of the reproductive tract in female horses.³ Elevated levels of Inhibin B are implicated in granulosa cell tumors of ovary.⁴ Measurement of concentrations of inhibin B and testosterone in serum are useful to support a presumptive diagnosis of GCTs in mares.⁵

In canines, inhibin B can be used as marker for tumors.⁶ Adrenocortical tumors and pituitary-dependent hyperadrenocorticism but not pheochromocytoma are associated with increased serum inhibin concentration. Undetectable inhibin is highly supportive of pheochromocytoma in neutered dogs with adrenal tumors.⁷

In studies of bovine reproduction, results from a study by Kaneko et al, clearly indicate that the bull testis produces inhibin A and B and secretes these hormones in high concentrations into the circulation during postnatal development.⁸ Inhibin B has also been reported as a biomarker of spermatogenesis in toxicological studies.⁹

The expression of inhibin isotypes increases progressively in the testis of mice with increasing postnatal age, suggesting that inhibin is associated with a negative feedback signal for FSH in testicular maturation.¹⁰ Testicular toxicity is an important safety endpoint in drug development and risk assessment, but reliable and translatable biomarkers for predicting injury have eluded researchers. Recently, the Health and Environment Sciences Institute's Developmental Reproductive Toxicity Technical Committee (HESI DART) hosted a consortium of companies to evaluate inhibin B as a potential biomarker for testicular toxicity and summarized the results. Beyond technical performance, the consortium was most interested in the correlation between

decreases in circulating inhibin B levels and the development of testicular pathology in the rat.¹¹

PRINCIPLE OF THE TEST

The Inhibin A ELISA is a quantitative three-step sandwich type immunoassay. In the first step Calibrators, Controls and unknown samples are added to Inhibin A antibody coated microtiter wells and incubated. After the first incubation and washing, the wells are incubated with biotinylated Inhibin A antibody. After the second incubation and washing, the wells are incubated with streptavidin horseradish peroxidase conjugate (SHRP). After the third incubation and washing step, the wells are incubated with substrate solution (TMB). After TMB incubation, an acidic stopping solution is added. In principle, the antibody-biotin conjugate binds to the solid phase antibody-antigen complex which in turn binds to the streptavidin-enzyme conjugate. The antibody-antigen-biotin conjugate-SHRP complex bound to the well is detected by enzyme-substrate reaction. The degree of enzymatic turnover of the substrate is determined by dual wavelength absorbance measurement at 450 nm as primary test filter and 630 nm as reference filter. The absorbance measured is directly proportional to the concentration of Inhibin A in the samples and calibrators.

MATERIALS SUPPLIED

CAL-161A

Inhibin A Calibrators A

One bottle, 10 mL, labeled Inhibin A Calibrator A/Sample Diluent containing 0 pg/mL Inhibin A in protein based buffer and Pro-Clean 400. Store at 2 - 8°C until the expiration date.

CAL-161B - CAL-161F Inhibin A Calibrators B thru F (Lyophilized)

Five vials, labeled B-F, containing concentrations of approximately 6 - 660 pg/mL Inhibin A in protein based buffer and Pro-Clean 400. Refer to **calibration card** for exact concentrations. Store unopened at 2-8°C until the expiration date. Reconstitute calibrators B-F with **1 mL** deionized water. Solubilize for **10 minutes**, mix well and use after reconstitution. Discard after 5 days, if stored at 2-8°C. For longer storage after reconstitution, aliquot and freeze at -20°C or colder for up to one month.

CTR-161-I & CTR-161-II Inhibin A Controls I & II (Lyophilized)

Two vials, labeled Levels I and II containing low and high Inhibin A concentrations in protein based buffer and Pro-Clean 400. Refer to **calibration card** for exact control ranges. Store unopened at 2-8°C until the expiration date. Reconstitute control Levels I and II with **1 mL** deionized water. Solubilize for **10 minutes**, mix well and use after reconstitution. Discard after 5 days, if stored at 2-8°C. For longer storage after reconstitution, aliquot and freeze at -20°C or colder for up to one month.

PLT-123 Inhibin A Coated Microtitration Strips

One strip holder, containing 12 strips and 96 microtitration wells with Inhibin A antibody immobilized to the inside wall of each well. Store at 2-8°C until expiration date in the resealable pouch with a desiccant to protect from moisture.

ASB-123A Inhibin A Assay Buffer A

One bottle, 6 mL, containing a protein-based (BSA)-buffer with a non-mercury preservative. Store at 2-8°C until expiration date.

ASB-123B Inhibin A Assay Buffer B

One bottle, 6 mL, containing a buffer solution with a non-mercury preservative. Store at 2-8°C until expiration date.

BCC-161 Inhibin A Biotin Conjugate Concentrate

One vial, 0.4 mL, containing detection antibody biotin in a protein-based buffer with a non-mercury preservative. Dilute prior to use in Inhibin A Conjugate diluent. Store at 2-8°C until expiration date.

CND-123 Inhibin A Biotin Conjugate Diluent

One bottle, 12 mL, containing a protein based buffer with a non-mercury preservative. Store at 2-8°C until expiration date.

SAR-161 Inhibin A Streptavidin-Enzyme Conjugate—Ready-to-Use (RTU)

One bottle, 12 mL, containing streptavidin-HRP (horseradish peroxidase) in a protein-based buffer and a non-mercury preservative. Store undiluted at 2-8°C until expiration date.

TMB-100 TMB Chromogen Solution

One bottle, 12 mL, containing a solution of tetramethylbenzidine (TMB) in buffer with hydrogen peroxide. Store at 2-8°C until expiration date.

STP-100 Stopping Solution

One bottle, 12 mL, containing 0.2 M sulfuric acid. Store at 2-30°C until expiration date.

WSH-100 Wash Concentrate A

One bottle, 60 mL, containing phosphate buffer saline solution with a nonionic detergent. Store at 2-30°C until expiration date. Dilute 25-fold with deionized water prior to use.

MATERIALS REQUIRED BUT NOT PROVIDED

1. Microplate reader capable of absorbance measurement at 450 nm, 405 nm and 630 nm.
2. Microplate orbital shaker.
3. Microplate washer.
4. Semi-automated/manual precision pipette to deliver 10–250 µL.
5. Vortex mixer.
6. Deionized water.
7. Disposable 12 x 75 mm culture tubes.
8. Tight fitting 12 x 75 mm tube racks.

WARNINGS AND PRECAUTIONS**For veterinary in vitro diagnostic use**

The following precautions should be observed:

- a) Follow good laboratory practice.
- b) Use personal protective equipment. Wear lab coats and disposable gloves when handling immunoassay materials.
- c) Handle and dispose of all reagents and material in compliance with applicable regulations.
- d) If external package is damaged, inspect the components inside for any other damage. Do not use if the components are damaged.

WARNING: Potential Biohazardous Material

Samples and blood-derived products may be routinely processed with minimum risk using the procedure described. However, handle these products as potentially infectious according to universal precautions and good clinical

laboratory practices, regardless of their origin, treatment or prior certification.¹² Use an appropriate disinfectant for decontamination. Store and dispose of these materials and their containers in accordance with local regulations and guidelines.

WARNING: Potential Chemical Hazard

Some reagents in this kit contain Pro-Clean 400 and Sodium azide¹³ as a preservative. Pro-Clean 400 and Sodium azide in concentrated amounts are irritants to skin and mucous membranes.

For further information regarding hazardous substances in the kit, please refer to the MSDS, either at AnshLabs.com or by request.

SAMPLE COLLECTION AND PREPARATION

- a) Serum is the recommended sample type.
- b) Sample handling, processing, and storage requirements depend on the brand of blood collection tube that you use. Please reference the manufacturer's instructions for guidance. Each laboratory should determine the acceptability of its own blood collection tubes and serum separation products.
- c) Samples may be stored at 4°C if assayed within 24 hours; otherwise samples must be stored at -20°C or -80°C to avoid loss of bioactivity and contamination.
- d) Avoid assaying lipemic, hemolyzed or icteric samples.
- e) Avoid repeated freezing and thawing of samples.

For shipping, place specimens in leak proof containers in biohazard specimen bags with appropriate specimen identification and test requisition information in the outside pocket of the biohazard specimen bag. Follow DOT and IATA requirements when shipping specimens.¹⁴

PROCEDURAL NOTES

1. A thorough understanding of this package insert is necessary for successful use of the Inhibin A ELISA assay. It is the user's responsibility to validate the assay for their purpose. Accurate results will only be obtained by using precise laboratory techniques and following the package insert.
2. A calibration curve must be included with each assay.
3. Bring all kit reagents to room temperature (23 ± 2°C) before use. Thoroughly mix the reagents before use by gentle inversion. Do not mix various lots of any kit component and do not use any component beyond the expiration date.
4. Use a clean disposable pipette tip for each reagent, calibrator, control or sample. Avoid microbial contamination of reagents, contamination of the substrate solutions with the HRP conjugates. The enzyme used as the label is inactivated by oxygen, and is highly sensitive to microbial contamination, sodium azide, hypochlorous acid and aromatic chlorohydrocarbons often found in laboratory water supplies. Use deionized water.
5. Incomplete washing will adversely affect the outcome and assay precision. To minimize potential assay drift due to variation in the substrate incubation time, care should be taken to add the substrate solution into the wells. Avoid exposure of the reagents to excessive heat or direct sunlight during storage and incubation.

PREPARATION OF REAGENTS

1. **Inhibin A Calibrators B-F and Inhibin A Controls I & II:** Tap and reconstitute Inhibin A Calibrators B-F and Inhibin A Controls I & II each with 1 mL deionized water. Solubilize for 10 minutes, mix well and use after reconstitution.

2. **Wash Solution:** Dilute wash concentrate 25-fold with deionized water. The wash solution is stable for one month at room temperature ($23 \pm 2^\circ\text{C}$) when stored in a tightly sealed bottle.
3. **Microtitration Wells:** Select the number of coated wells required for the assay. The remaining unused wells should be placed in the resealable pouch with a desiccant. The pouch must be resealed to protect from moisture.
4. **Inhibin A Assay Buffers Premix Solution:** The Inhibin A Assay Buffer A (ASB-123A) and Inhibin A Assay Buffer B (ASB-123B) should be mixed by gentle inversion in equal volumes (1:1 ratio) according to the number of wells used. If an entire plate is to be used mix exactly 3 mL of the ASB-123 B in to 3 mL of the ASB-123 A. The pre-mixture solution is stable for use up to 4 hours. Discard the pre-mix solution after 4 hours.
5. **Inhibin A Antibody-Biotin Conjugate Solution:** The Inhibin A Antibody-Biotin Conjugate Concentrate should be diluted at a ratio of 1 part conjugate to 50 parts of Inhibin A Conjugate Diluent, according to the number of wells used. If an entire plate is to be used pipet exactly 220 μL of the Concentrate in to 11 mL of the diluent.

ASSAY PROCEDURE

Allow all specimens and reagents to reach room temperature ($23 \pm 2^\circ\text{C}$) and mix thoroughly by gentle inversion before use. Calibrators, controls, and unknowns should be assayed in duplicate.

NOTE:

- i. All intact mouse and rat serum samples should be diluted 1 part in 3 parts (1:4) in Calibrator A/Sample diluent prior to assay.
- ii. All serum samples reading higher than the highest calibrator should be mixed and diluted in the 0 pg/mL reconstituted Calibrator A prior to assay.

1. Label the microtitration strips to be used.
2. Pipette 50 μL of the Calibrator, Controls and Unknowns to the appropriate wells.
3. Add 50 μL of Inhibin A Assay Buffer premix (ASB-123A and ASB-123B in 1:1 ratio as described under the Preparation of the Reagents section of this package insert) to each well using a repeater pipette.
4. Incubate the plate, shaking at a fast speed (600-800 rpm) on an orbital microplate shaker, for 2.5 hour at room temperature ($23 \pm 2^\circ\text{C}$).
5. During the last 20-30 minutes of incubation, prepare the Inhibin A Antibody-Biotin Conjugate Solution by diluting the Inhibin A Biotin Conjugate Concentrate in Inhibin A Conjugate Diluent as described under the Preparation of the Reagents section of this package insert.
6. Aspirate and wash each strip 5 times with Washing Solution (350 μL /per well) using an automatic microplate washer.
7. Add 100 μL of the Antibody-Biotin Conjugate Solution to each well using a repeater pipette.
8. Incubate the plate, shaking at a fast speed (600-800 rpm) on an orbital microplate shaker, for 1 hour at room temperature ($23 \pm 2^\circ\text{C}$).
9. Aspirate and wash each strip 5 times with the Wash Solution (350 μL /per well) using an automatic microplate washer.
10. Add 100 μL of the Streptavidin-Enzyme Conjugate-RTU to each well using a repeater pipette.
11. Incubate the plate, shaking at a fast speed (600-800 rpm) on an orbital microplate shaker, for 30 minutes at room temperature ($23 \pm 2^\circ\text{C}$).
12. Aspirate and wash each strip 5 times with the Wash Solution (350 μL /per well) using an automatic microplate washer.
13. Add 100 μL of the TMB chromogen solution to each well using a repeater pipette. Avoid exposure to direct sunlight.
14. Incubate the wells, shaking at 600-800 rpm on an orbital microplate shaker, for 12-15 min at room temperature ($23 \pm 2^\circ\text{C}$).

NOTE: Visually monitor the color development to optimize the incubation time.

15. Add 100 μL of the stopping solution to each well using a repeater pipette. Read the absorbance of the solution in the wells within 20 minutes, using a microplate reader set to 450 nm.

NOTE: Zero calibrator should be programmed as "Blank" while reading the optical density. If instrument has a wavelength correction, set the instrument to dual wavelength measurement at 450 nm with background wavelength correction at 630 nm.

RESULTS

NOTE: The results in this package insert were calculated by plotting the log optical density (OD) data on the y-axis and log Inhibin A concentration on X-axis using a cubic regression curve-fit. Alternatively, log vs. log quadratic regression curve-fit can be used. Other data reduction methods may give slightly different results.

1. Optimum results can be obtained at incubation temperature of $23 \pm 2^\circ\text{C}$.
2. Calculate the mean OD for each calibrator, Control, or Unknown.
3. Plot the log of the mean OD readings for each of the Calibrators along the y-axis versus log of the Inhibin A concentrations in pg/mL along the x-axis, using a linear regression curve-fit.
4. Determine the Inhibin A concentrations of the Controls and unknowns from the calibration curve by matching their mean OD readings with the corresponding Inhibin A concentrations.
5. Any sample reading higher than the highest Calibrator should be appropriately diluted with the 0 pg/mL (CAL A) and re-assayed.
6. Any sample reading lower than the analytical sensitivity should be reported as such.
7. Multiply the value by a dilution factor, if required.

LIMITATIONS

The reagents supplied in this kit are optimized to measure Inhibin A levels in serum. If there is evidence of microbial contamination or excessive turbidity in a reagent, discard the vial. For assays employing antibodies, the possibility exists for interference by heterophile antibodies in the samples.¹⁵

QUALITY CONTROL

- Each laboratory should establish mean values and acceptable ranges to assure proper performance.
- Inhibin A ELISA controls or other commercial controls should fall within established confidence limits.
- The confidence limits for Inhibin A controls are printed on the Calibration card.
- A full calibration curve, low and high level controls, should be included in each assay.
- TMB should be colorless. Development of any color may indicate reagent contamination or instability.

REPRESENTATIVE CALIBRATION CURVE DATA

Well Number	Calibrators	Mean OD	Conc (pg/mL)
A1, A2	A	0.041 (Blank)	0
B1, B2	B	0.027	6.0
C1, C2	C	0.099	23.3
D1, D2	D	0.362	81.1
E1, E2	E	1.068	229.7
F1, F2	F	2.888	667.8

CAUTION: The above data must not be employed in lieu of data obtained by the user in the laboratory.

ANALYTICAL CHARACTERISTICS

All analytical characteristics are stated in pg/mL.

Analytical Sensitivity:

The analytical sensitivity in the Inhibin A ELISA assay, as calculated by the interpolation of mean plus two standard deviations of 18 replicates of calibrator A (0 pg/mL) and calibrator B (6 pg/mL), is **2.3 pg/mL**.

Imprecision:

Reproducibility of the Inhibin A ELISA assay was determined in a study using two kit controls assayed in replicates of 36. Representative data were calculated and are presented in the following table.

Sample	Mean conc.	Within run		Between run		Total	
	(pg/mL)	SD	%CV	SD	%CV	SD	%CV
Control-I	74.35	2.40	3.22%	6.35	8.54%	6.79	9.13%
Control-II	203.82	5.68	2.79%	7.12	3.49%	9.11	4.47%

Linearity:

Equine, Bovine Follicular fluids, Canine testis extract and Calibrator F containing various Inhibin A levels were diluted with Calibrator A/Sample diluent. The % recovery on individual samples is represented in the following table.

Sample	Dilution Factor	Expected Conc. (pg/mL)	Observed Conc. (pg/mL)	% Recovery
Calibrator F Dilution	Neat	667.8	NA	NA
	1:02	333.9	355.4	106%
	1:04	167.0	185.9	111%
	1:08	83.5	94.3	113%
	1:16	41.7	47.5	114%
Equine FF	1:100	450.5	NA	NA
	1:200	225.3	234.4	104%
	1:400	112.6	117.2	104%
	1:800	56.3	59.2	105%
	1:1600	28.2	27.9	99%
Bovine FF	1:3200	14.1	13.2	94%
	1:100	278.4	NA	NA
	1:200	139.2	135.4	97%
	1:400	69.6	62.2	89%
	1:800	34.8	34.7	100%
Canine Testis Extract	1:1600	17.4	17.9	103%
	1:3200	8.7	8.0	92%
	1:10	34.3	NA	NA
	1:20	17.1	16.3	95%
	1:40	8.6	9.0	105%
	1:80	4.3	4.2	99%
	1:160	2.1	2.4	112%

Recovery

Three independent serum samples were spiked with three different levels of Inhibin A and assayed. The spike recovery is shown below.

Sample ID	Endogenous Conc. (pg/mL)	Expected Conc. (pg/mL)	Observed Conc. (pg/mL)	%Recovery
1	0	75.25	82.78	110%
		112.08	128.28	114%
		148.40	171.44	116%
2	2.38	77.36	75.94	98%
		114.06	116.19	102%
		150.25	155.17	103%
3	8.701	82.97	83.71	101%
		119.32	128.05	107%
		155.17	170.18	110%

Analytical Specificity:

The antibody pair used in the assay is specific to Inhibin A. Other related molecules at the concentration in the table below did not show any significant cross-reaction.

Cross-Reactant	Concentration	% Cross-reactivity
Inhibin A	1ng/mL	100
Activin A	1ng/mL	ND
Activin B	1ng/mL	ND
Activin AB	1ng/mL	ND
Follistatin-288	1ng/mL	ND
Follistatin-315	1ng/mL	ND
Inhibin B	1ng/mL	ND
Myostatin	1ng/mL	ND

Interference:

When potential interferents (hemoglobin, triglycerides, bilirubin and biotin) were added at their physiological concentration to known sample, Inhibin A concentration were within $\pm 15\%$ of the control as represented in the following table.

Interferent	Interferent Dose	Analyte Conc. (pg/mL)	Spiked Sample Value (pg/mL)	% Difference
Hemoglobin	1 mg/mL	95.8	89.4	-6.7
	0.5 mg/mL	100.6	95.0	-5.6
	0.1 mg/mL	101.6	101.7	0.1
Hemoglobin	1 mg/mL	9.5	8.6	-9.2
	0.5 mg/mL	10.1	9.2	-8.5
	0.1 mg/mL	10.3	9.9	-4.5
Biotin	1200 ng/mL	94.7	94.2	-0.5
	600 ng/mL	102.7	103.7	1.0
	1200 ng/mL	9.7	10.2	4.8
Biotin	600 ng/mL	10.6	9.9	-5.9
	20 mg/mL	91.4	96.7	5.8
	10 mg/mL	100.3	102.2	1.9
Intralipids	5 mg/mL	104.2	103.6	-0.7
	20 mg/mL	9.7	9.5	-1.6
	10 mg/mL	9.8	9.9	1.6
Bilirubin	0.66 mg/mL	69.5	65.3	-6.0
	0.2 mg/mL	96.7	94.2	-2.6
	0.66 mg/mL	8.0	7.5	-7.0
Bilirubin	0.2 mg/mL	10.9	10.0	-8.5

Expected Values:

Random serum samples from various species were analyzed using Inhibin A ELISA. The levels of Inhibin A detected are represented in the table below. Serum samples from other species i.e. caprine, ovine, rabbit, canine, bovine and porcine not shown in the table are detected by this assay.

Samples	Number of specimens	Median (pg/mL)	Range (pg/mL)
Mouse Female Intact	10	1013.6	385.6-3060.1
Mouse Female Ovariectomized	5	15.5	10.7-24.9
Equine (Mare)	432	38.7	1.9-305.7
Equine (Mare GCT)	43	67.1	4.9-760.8

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