Inhibin B ELISA

RUO

AL-107

INTENDED USE

The Inhibin B enzyme linked immunuosorbent assay (ELISA) kit provides materials for the quantitative measurement of Inhibin B in human serum and other biological fluids. This kit is intended for laboratory research use only and is not for use in diagnostic or therapeutic procedures.

SUMMARY AND EXPLANATION

Inhibin B is a dimeric hormone that is composed of alpha (α) and beta B (β_B) subunits. The free alpha subunits usually do not have any physiological effect. Therefore, the bioactivity of the inhibins depends on the formation of a dimeric α-β structure, and only dimeric forms of inhibins are biologically active. Inhibins are protein hormones secreted by granulosa cells of the ovary in the female and sertoli cells of the testis in the male. They selectively suppress the secretion of pituitary follicle stimulating hormone (FSH) and also have local paracrine actions in the gonads. Inhibin B levels have been reported in sertoli cell function (potential marker for spermatogenesis and testicular function), ovarian reserve and granulosa cell tumors.

PRINCIPLE OF THE TEST

The Inhibin B ELISA is a quantitative three-step sandwich type immunoassay. In the first step Calibrators, Controls and unknown samples are added to Inhibin B antibody coated microtiter wells and incubated. After the first incubation and washing, the wells are incubated with biotinylated Inhibin B 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 B in the samples and calibrators.

MATERIALS SUPPLIED

CAL-107A - CAL-107F **Inhibin B Calibrators A thru F (Lyophilized)**
Six vials, labeled A-F, containing concentrations of approximately 10-1200 pg/mL Inhibin B in animal sera and a non-mercury preservative. Refer to **calibration card** for exact concentrations. Store unopened at 2 to 8°C until the expiration date. Reconstitute calibrators A-F with 1 mL deionized water. Solubilize, Mix well and use after reconstitution. Aliquot and freeze at -20°C or colder for up to one year. Avoid repeated freeze thaws. Discard after 5 days, if stored at 2 to 8°C.

CTR-107-I & CTR-107-II **Inhibin B Controls I & II (Lyophilized)**
Two vials, labeled Levels I and II containing low and high Inhibin B concentrations in animal sera and a non-mercury preservative. Refer to **calibration card** for exact control ranges. Store unopened at 2 to 8°C until the expiration date. Reconstitute control Levels I and II with 1 mL deionized water. Solubilize, mix well and use after reconstitution. Aliquot and freeze at -20°C or colder for up to one year. Avoid repeated freeze thaws. Discard after 5 days, if stored at 2 to 8°C.

PLT-107 **Inhibin B Coated Microtitration Strips**
One stripholder, containing 12 strips and 96 microtitration wells with Inhibin B 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-207A **Inhibin B Assay Buffer A**
One bottle, 8 mL, containing a protein-based (BSA)-buffer with a non-mercury preservative. Store at 2-8°C until expiration date.

ASB-207B **Inhibin B Assay Buffer B**
One bottle, 8 mL, containing a buffer solution with a non-mercury preservative. Store at 2-8°C until expiration date.

BCC-107 **Inhibin B 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 B Conjugate diluent. Store at 2-8°C until expiration date.

CND-207 **Inhibin B 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-107 **Inhibin B Streptavidin-Enzyme Conjugate—Ready-to-Use (RTU)**
One amber 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, 11 mL, containing a solution of tetramethylbenzidine (TMB) in buffer with hydrogen peroxide. Store at 2 to 8°C until expiration date.

STP-100 **Stopping Solution**
One bottle, 11 mL, containing 0.2 M sulfuric acid. Store at 2 to 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 to 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, 405nm 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 Research Use Only. Not for use in diagnostic procedures*.**

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.

WARNING: Potential Biohazardous Material

This reagent may contain some human source material (e.g. serum) or materials used in conjunction with human source materials. Handle all reagents and patient samples at a Biosafety Level 2, as recommended for any potentially infectious human material in the Centers for Disease Control/National Institutes of Health manual "Biosafety in Microbiological and Biomedical Laboratories," 5th Edition, 2007[1].

WARNING: Potential Chemical Hazard

Some reagents in this kit contain Pro-Clean 400 and Sodium azide[2] 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. Thaw samples no more than 3 times.

f) 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.

PROCEDURAL NOTES

1. A thorough understanding of this package insert is necessary for successful use of the Inhibin B 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 B Calibrators A-F and Inhibin B Controls I & II**: Tap and reconstitute Inhibin B Calibrator A-F and Inhibin B Controls I & II each with 1 mL deionized water. Solubilize, 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 ± 2°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 B Antibody-Biotin Conjugate Solution:** The Inhibin B Antibody-Biotin Conjugate Concentrate should be diluted at a ratio of 1 part conjugate to 50 parts of Inhibin B 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 ± 2°C) and mix thoroughly by gentle inversion before use. Calibrators, controls, and unknowns should be assayed in duplicate.

NOTE:

(i) Use alternative procedure for hemolyzed samples. Hemolyzed samples may cause excessive bubbling in the well.

(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. Reconstitute Inhibin B Calibrator A-F and Inhibin B Controls I & II each with **1 mL** deionized water. Solubilize for **10 minutes**, Mix well.
2. Label the microtitration strips to be used.
3. Pipette **50 µL** of the Calibrator, Controls and Unknowns to the appropriate wells.
4. Add **50 µL** of the Inhibin B Assay Buffer A to each well using a repeater pipette.
5. Add **50 µL** of the Inhibin B Assay Buffer B to each well using a repeater pipette.

   Note: *Samples that are hemolyzed or contain catalases are susceptible to foaming. Such foaming does not impact sample results. If such samples are present, incubate for 30 minutes without shaking at room temperature (23 ± 2°C) for foaming to subside.*
6. Incubate the plate, shaking at a fast speed (**600-800 rpm**) on an orbital microplate shaker, for **2 hour** at room temperature (23 ± 2°C).
7. During the last **20-30 minutes** of incubation, prepare the Inhibin B Antibody-Biotin Conjugate Solution by diluting the Inhibin B Biotin Conjugate Concentrate in Inhibin B Conjugate Diluent as described under the Preparation of the Reagents section of this package insert.

8. Aspirate and wash each strip **5 times** with Washing Solution **(350 µL/per well**) using an automatic microplate washer.
9. Add **100 µL** of the Antibody-Biotin Conjugate Solution to each well using a repeater pipette.
10. Incubate the plate, shaking at a fast speed (**600-800 rpm**) on an orbital microplate shaker, for **1 hour** at room temperature (23 ± 2°C).
11. Aspirate and wash each strip **5 times** with the Wash Solution **(350 µL/per well)** using an automatic microplate washer.
12. Add **100 µL** of the Streptavidin-Enzyme Conjugate-RTU to each well using a repeater pipette.
13. Incubate the plate, shaking at a fast speed (**600-800 rpm**) on an orbital microplate shaker, for **30 minutes** at room temperature (23 ± 2°C).
14. Aspirate and wash each strip **5 times** with the Wash Solution **(350 µL/per well**) using an automatic microplate washer.
15. Add **100 µL** of the TMB chromogen solution to each well using a precision pipette. Avoid exposure to direct sunlight.
16. Incubate the wells, shaking at **600–800 rpm** on an orbital microplate shaker, for **8-12 min** at room temperature (23 ± 2°C).
    ***NOTE**: Visually monitor the color development to optimize the incubation time.*
17. Add **100 µL** of the stopping solution to each well using a precision pipette. Read the absorbance of the solution in the wells within **20 minutes**, using a microplate reader set to **450 nm.**
    ***NOTE**: While reading the absorbance of the microtitration well, it is necessary to program the zero calibrator as a "Blank".*

ALTERNATIVE ASSAY PROCEDURE

1. Reconstitute Inhibin B Calibrator A-F and Inhibin B Controls I & II each with **1 mL** deionized water. Solubilize for **10 minutes**, Mix well.
2. For each unknown serum sample, calibrators and controls label one 12 X 75 culture tubes.
3. Pipette **75 µL** of the Calibrator, Controls and samples to the pre-labeled tube.
4. Add **75 µL** of the Inhibin B Assay Buffer A to each the pre-labeled tube using a repeater pipette.
5. Add **75 µL** of the Inhibin B Assay Buffer B to each pre-labeled tube using a repeater pipette and vortex well.
6. Place the tubes in a tight fitting tube rack and incubate the tubes, shaking at a slow speed **(100-200 rpm)** at room temperature (23 ± 2°C) for **30 minutes**.
7. The pre-mixed samples are now ready for analysis.
8. Label the microtitration strips to be used.
9. Pipette **150 µL** of the pre-mixed samples from **step 7** to the appropriate wells.
10. Incubate the plate, shaking at a fast speed (**600-800 rpm**) on an orbital microplate shaker, for **2 hour** at room temperature (23 ± 2°C).
11. During the last **20-30 minutes** of incubation, prepare the Inhibin B Antibody-Biotin Conjugate Solution by diluting the Inhibin B Biotin Conjugate Concentrate in Inhibin B Conjugate Diluent as described under the Preparation of the Reagents section of this package insert.
12. Aspirate and wash each strip **5 times** with Washing Solution **(350 µL/per well**) using an automatic microplate washer.
13. Add **100 µL** of the Antibody-Biotin Conjugate Solution to each well using a repeater pipette.
14. Incubate the plate, shaking at a fast speed (**600-800 rpm**) on an orbital microplate shaker, for **1 hour** at room temperature (23 ± 2°C).
15. Aspirate and wash each strip **5 times** with the Wash Solution **(350 µL/per well)** using an automatic microplate washer.
16. Add **100 µL** of the Streptavidin-Enzyme Conjugate-RTU to each well using a repeater pipette.
17. Incubate the plate, shaking at a fast speed (**600-800 rpm**) on an orbital microplate shaker, for **30 minutes** at room temperature (23 ± 2°C).
18. Aspirate and wash each strip **5 times** with the Wash Solution **(350 µL/per well**) using an automatic microplate washer.
19. Add **100 µL** of the TMB chromogen solution to each well using a precision pipette. Avoid exposure to direct sunlight.
20. Incubate the wells, shaking at **600–800 rpm** on an orbital microplate shaker, for **8-12 min** at room temperature (23 ± 2°C).
    ***NOTE**: Visually monitor the color development to optimize the incubation time.*
21. Add **100 µL** of the stopping solution to each well using a precision pipette. Read the absorbance of the solution in the wells within **20 minutes**, using a microplate reader set to **450 nm.**
    ***NOTE**: While reading the absorbance of the microtitration well, it is necessary to program the zero calibrator as a "Blank".*

RESULTS

***NOTE:** The results in this package insert were calculated by plotting the data on a log vs. log scale using a cubic regression curve-fit. Other data reduction methods may give slightly different results.*

1. Optimum results can be obtained at incubation temperature of (23 ± 2°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 B concentrations in pg/mL along the x-axis, using a cubic regression curve-fit.
4. Determine the Inhibin B concentrations of the Controls and unknowns from the calibration curve by matching their mean OD readings with the corresponding Inhibin B 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 B levels in human serum and lithium heparin plasma. 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[4].

QUALITY CONTROL

- Each laboratory should establish mean values and acceptable ranges to assure proper performance.
- Inhibin B ELISA controls or other commercial controls should fall within established confidence limits.
- The confidence limits for Inhibin B 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	Well Contents	Mean OD	Conc (pg/mL)
	Calibrators	(Blank)	
A1, A2	A	0.04	0
B1, B2	B	0.085	12.7
C1, C2	C	0.176	34
D1, D2	D	0.527	129
E1, E2	E	1.595	446
F1, F2	F	3.432	1390

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

ANALYTICAL CHARACTERISTICS

Limit of Detection (LoD):

The Limit of detection in the assay as calculated by the interpolation of mean plus two standard deviation of 24 replicates of calibrator A (0 pg/mL) and calibrator B (12.7 pg/mL) is 1.6 pg/mL.

Limit of Quantitation (LoQ):

The estimated minimum Inhibin B dose achieved at 20% total imprecision is 4.6 pg/mL. The value was determined by processing seven samples in the range of 2.95-364.12 pg/mL with seven runs in quadruplets (n=28).

Imprecision:

Reproducibility of the Inhibin B assay was determined in a study using two serum pools and two kit controls. The study included a total of 20 assays, four replicates of each per assay (n=78-80). Representative data were calculated based on NCCLS EP5-A guidelines and are presented in the following table.

Sample	Mean Conc.	Within Run		Between Run		Total	
	(pg/mL)	SD	%CV	SD	%CV	SD	%CV
Pool-1	68.898	2.680	3.89	4.353	6.32%	5.112	7.42%
Control I	99.388	4.352	4.38%	3.422	3.44%	5.536	5.57%
Pool-2	121.576	4.899	4.03%	5.143	4.23%	7.103	5.84%
Control II	308.103	12.322	4.00%	9.394	3.05%	15.495	5.03%

Recovery:

Known amounts of Inhibin B were added to four serum samples containing different levels of endogenous Inhibin B. The concentration of Inhibin B was determined before and after the addition of exogenous Inhibin B and the percent recovery was calculated.

Sample	Endogenous Conc. (pg/mL)	Expected Conc.(pg/mL)	Observed Conc. (pg/mL)	% Recovery
1	50.86	112.44	106.53	95
		168.42	151.23	90
		219.530	209.90	96
2	66.97	127.78	127.75	100
		183.06	180.78	99
		233.54	239.06	102
3	144.88	201.98	190.5	94
		253.89	235.8	93
		301.29	301.8	100
4	159.89	216.28	213.28	99
		267.54	252.14	94
		314.34	307.33	98

Linearity:

Based on NCCLS EP-6-P multiple dilutions of the three serum samples containing various Inhibin B levels were diluted with calibrator A. The % recovery on individual samples is represented in the following table.

Sample	Dilution Factor	Expected Conc. (pg/mL)	Observed Conc. (pg/mL)	% Recovery
1	Neat	1318.4	N/A	N/A
	1:2	659.2	674.6	102
	1:4	329.6	310.8	94
	1:8	164.8	173.3	105
	1:16	82.4	84.5	103
	1:32	41.2	47.1	114
2	Neat	319.8	N/A	N/A
	1:2	159.9	174.0	109
	1:4	79.9	91.9	115
	1:8	40.0	47.8	120
	1:16	20.0	19.3	97
	1:32	10.0	8.9	89
3	Neat	224.960	N/A	N/A
	1:2	112.480	122.580	109
	1:4	56.240	60.080	107
	1:8	28.120	30.970	110
	1:16	14.060	12.110	86

Analytical Specificity:

This monoclonal antibody pair used in the assay detects Inhibin B. Other related molecules at the concentration in the table below below did not show any significant cross-reaction. Specificity to other species has not been determined.

Sample	Cross-reactant	Concentration	% Cross-reactivity
1	Inhibin A	100 ng/mL	ND
2	Activin A	50 ng/mL	ND
3	Activin B	50 ng/mL	0.04%
4	Activin AB	50 ng/mL	ND
5	AMH	50 ng/mL	ND

Interference:

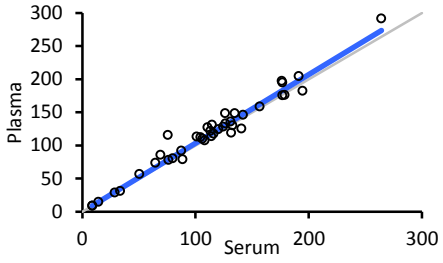
When hemoglobin and triglycerides were added at a greater than two folds of their physiological concentration to control sample, Inhibin B concentration were within ± 10% of the control as represented in the following table. This study was based on NCCLS EP7-P.

Interferents	Analyte Conc. (mg/mL)	Unspiked Sample Value (pg/mL)	Spiked Sample Value (pg/mL)	% Difference
Hemoglobin	1.35	133.99	124.83	-6.8
		31.83	30.01	-5.7
Triglygerides	5.0	133.99	141.57	5.7
		31.83	32.48	2.0

Sample Type:

Forty matched serum and Lithium heparin plasma specimens were compared in Ansh Inhibin B ELISA. Passing Bablok analysis of the results yielded the following Regression:

Plasma=1.04 (serum)-0.23
(r=0.98; P<0.0001)

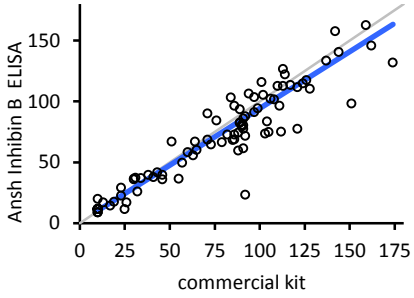


Method Comparison:

The Inhibin B ELISA has been compared to commercially available Inhibin B kit (Method A) using 97 random male and female serum samples in the range of 10-174 pg/mL.

Passing Bablok analysis of the results yielded the following Regression:

Inhibin B ELISA (AL-107) = 0.93 (Method A) + 1.08
(r=0.97; P<0.0001)



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Research Use Only

Not for use *in-vitro* diagnostic procedures.

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