ELISA PRODUCT INFORMATION & MANUAL

ACTH

NBP2-61287

Enzyme-linked Immunosorbent Assay for quantitative detection of Human ACTH.
For research use only. Not for diagnostic or therapeutic procedures.

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INTENDED USE

The ACTH ELISA is intended for the quantitative determination of ACTH (Adrenocorticotropic Hormone) in human plasma.

SUMMARY AND EXPLANATION

ACTH (Adrenocorticotropic hormone) or corticotropin is a 39- amino acid peptide hormone (MW=4500) secreted by the pituitary to regulate the production of steroid hormones by the adrenal cortex. ACTH secretion from the anterior pituitary is controlled by both a classical negative feedback control mechanism and CNS- stress mediated control system. Various types of stress or pain perceived in higher levels of the brain modulate secretion of the hypothalamic neurosecretory hormone, corticotropin releasing hormone (CRH), a 41-amino acid peptide. CRH stimulates pituitary ACTH secretion. The second peptide that modulates ACTH secretion is vasopressin (AVP). AVP secretion is also stimulated by stress and acts synergistically with CRH to increase ACTH secretion in the pituitary portal circulation. ACTH increases the synthesis and release of all adrenal steroids, aldosterone, cortisol and adrenal androgens. It is the principal modulator of cortisol, the most important glucocorticoid in man. As the cortisol level in blood increases, release of ACTH is inhibited directly at the pituitary level. Through this same mechanism, decreasing cortisol levels lead to elevated ACTH levels.[2,3,4,5]

Biologically active ACTH results from enzymatic cleavage of a large precursor molecule, pro-opiomelanocortin (POMC). This molecule contains within its structure the amino acid sequences of ACTH, Pro-ACTH, ß-melanocyte stimulating hormone, lipotropin, as well as endorphin and the enkephalins. Because the reaction in immunoassays is determined by antigenic structure, not biological function, the usual ACTH RIA reacts with POMC, Pro-ACTH, ACTH and some fragments of the ACTH. Like other pituitary hormones, ACTH is secreted in a pulsatile manner. These small pulses are superimposed on a characteristic diurnal fluctuation of greater amplitude. In healthy individuals, ACTH reaches a peak in the early morning (6:00 - 8:00 hour) and levels become lowest late in the day and near the beginning of the sleep period. Because of this diurnal rhythm it is customary to draw plasma ACTH samples between 8:00 and 10:00 hour.

CLINICAL SIGNIFICANCE

Plasma ACTH assays are useful in the differential diagnosis of pituitary Cushing's disease, Addison's disease, autonomous ACTH producing pituitary tumors (e.g., Nelson's syndrome), hypopituitarism with ACTH deficiency and ectopic ACTH syndrome.[5,6,7,8,9,10]

PRINCIPLE OF THE TEST

The ACTH Immunoassay is a two-site ELISA [Enzyme-Linked ImmunoSorbent Assay] for the measurement of the biologically active 39 amino acid chain of ACTH. A goat polyclonal antibody to human ACTH, purified by affinity chromatography, and a mouse monoclonal antibody to human ACTH are specific for well-defined regions on the ACTH molecule. One antibody is prepared to bind only the C-terminal ACTH 34-39 and this antibody is biotinylated. The other antibody is prepared to bind only the mid-region and N- terminal ACTH 1-24 and this antibody is labeled with horseradish peroxidase [HRP] for detection.

Streptavidin Well - Biotinylated Anti-ACTH (34-39) -- ACTH -- HRP conjugated Anti-ACTH (1-24)

In this assay, calibrators, controls, or samples are simultaneously incubated with the enzyme labeled antibody and a biotin coupled antibody in a streptavidin-coated microplate well. At the end of the assay incubation, the microwell is washed to remove unbound components and the enzyme bound to the solid phase is incubated with the substrate, tetramethylbenzidine (TMB). An acidic stop solution is then added to stop the reaction and converts the color to yellow. The intensity of the yellow color is directly proportional to the concentration of ACTH in the sample. A dose response curve of absorbance unit vs. concentration is generated using results obtained from the calibrators. Concentrations of ACTH present in the controls and samples are determined directly from this curve.

KIT COMPONENTS

Description	Quantity
Biotinylated ACTH Antibody [affinity purified goat anti human ACTH]	1 x 2.7 mL
Peroxidase (Enzyme) labeled ACTH Antibody [mouse monoclonal anti human ACTH]	1 x 2.7 mL
Wash Buffer Concentrate [Saline with surfactant]	1 x 30 mL
TMB Substrate [tetramethylbenzidine]	1 x 15 mL
Stop Solution [1 N sulfuric acid]	1 x 20 mL
Microplate - One holder with Streptavidin Coated Strips.	12 x 8-well strips
Calibrators - Lyophilized [except zero calibrator] synthetic h-ACTH. Zero calibrator [BSA/equine serum solution] is in liquid form, ready to use. All other calibrators consist of synthetic h-ACTH (1-39) in BSA/equine serum solution 1 x 4 mL for the zero calibrator.	1 x 2 mL for all other calibrators
Controls 1 & 2 (refer to vial labels) - Lyophilized. 2 Levels. Synthetic h-ACTH (1-39) in BSA/equine serum solution.	1 x 2 mL per level

OTHER MATERIALS NEEDED

- Microplate reader.
- Microplate washer [if washer is unavailable, manual washing may be acceptable].
- Precision Pipettors to deliver 25, 100, 150 and 200 µL.
- *(Optional):* A multi-channel dispenser or a repeating dispenser for 25, 100 and 150 µL.

SAFETY WARNINGS & PRECAUTIONS

Although the reagents provided in this kit has been specifically designed to contain no human blood components, the human samples, which might be positive for HBsAg, HBcAg or HIV antibodies, must be treated as potentially infectious biohazard. Common precautions in handling should be exercised, as applied to any untested patient sample.

The Stop Solution consists of 1 N Sulfuric Acid. This is a strong acid. Although diluted, it still must be handled with care. It can cause burns and should be handled with gloves and eye protection, with appropriate protective clothing. Any spill should be wiped immediately with copious quantities of water. Do not breathe vapor and avoid inhalation.

SAMPLE COLLECTION AND STORAGE

The determination of ACTH should be performed on EDTA plasma. To assay the specimen in duplicate, 400 µL of EDTA plasma is required. Collect whole blood in a lavender [EDTA] tube. The plasma should be promptly separated, preferably in a refrigerated centrifuge, and stored at -20°C or lower. EDTA plasma samples may be stored up to 8 hours at 2-8°C. EDTA plasma samples frozen at -20°C are stable for up to 4 months.

REAGENT PREPARATION AND STORAGE

Store all kit components at 2-8°C except the Wash Buffer Concentrate.

1. All reagents except the non-zero calibrators, kit controls and the Wash Buffer Concentrate are ready-to-use. Store all reagents at 2-8°C, except the Wash Buffer Concentrate, which should be kept at room temperature until dilution to avoid precipitation.
2. For each of the non-zero calibrators (Calibrator B through F) and kit controls 1 and 2, reconstitute each vial with 2ml of distilled or deionized water and mix. Allow the vial to stand for 10 minutes and then mix thoroughly by gentle inversion to insure complete reconstitution. Use the calibrators and controls as soon as possible upon reconstitution. Freeze (-20°C) the remaining calibrators and controls as soon as possible after use. Standards and controls are stable at -20°C for 6 weeks after reconstitution with up to 3 freeze thaw cycles when handled as recommended in “Procedural Notes” section.
3. Wash Buffer Concentrate: Mix contents of the wash buffer concentrate thoroughly. If precipitate is present in the Wash Buffer Concentrate due to storage at lower temperature such as 4°C, dissolve by placing

the vial in a 37°C water bath or oven with swirling or stirring. Add the wash buffer concentrate (30 mL) to 570 mL of distilled or deionized water and mix. The diluted working wash solution is stable for 90 days when stored at room temperature.

ASSAY PROCEDURE

1. Please ensure all reagents are at room temperature before you start the assay.
2. Place sufficient **Streptavidin Coated Strips** in a holder to run all six (6) ACTH calibrators, A - F of the ACTH CALIBRATORS [Exact concentration is stated on the vial label], Quality Control Plasma and samples.
3. Pipet **200 µL** of sample, calibrators and controls into the designated or mapped wells. ***Freeze (-20°C) the remaining calibrators and controls as soon as possible after use.***
4. Add or dispense **25 µL** of the Biotinylated Antibody into each of the wells which already contain the sample.
5. Add or dispense **25 µL** of the Enzyme Labeled Antibody into each of the same wells. Cover the microplate(s) with aluminum foil or a tray to avoid exposure to light and place it on an **orbital shaker or rotator** set at 170 + 10rpm for **4 hours** + 30 minutes at room temperature (22-28°C).
6. First aspirate the fluid completely and then wash/aspirate each well five (5) times with the Working Wash Solution (prepared from the wash buffer concentrate), using an automatic microplate washer. The wash solution volume should be set to dispense 0.35 mL into each well.
7. Add or dispense **150 µL** of the TMB Substrate into each of the wells.
8. With appropriate cover to avoid light exposure, place the microplate(s) on an **orbital shaker or rotator** set at 170 + 10rpm for **30 +5 minutes** at room temperature (22-28°C).
9. Add or dispense **100 µL** of the Stop Solution into each of the wells. Mix gently.
10. Read the absorbance of the solution in the wells within 10 minutes, using a microplate reader set to **450 nm** against 250 µL of distilled or deionized water. **Read** the plate **again** with the reader set to **405 nm** against distilled or deionized water**.**

    Note*: The second reading is designed to extend the analytical validity of the calibration curve to the value represented by the highest calibrator, which is approximately 500 pg/mL. Hence, samples with ACTH >150 pg/mL can be quantified against a calibration curve consisting of the readings all the way up to the concentration equivalent to the highest calibrator using the 405 nm reading, away from the wavelength of maximum absorbance. In general, samples and control should be read using the 450nm for ACTH concentrations up to 150 pg/mL. ACTH concentrations above 150 pg/mL should be interpolated using the 405 nm reading.*

11. By using the final absorbance values obtained in the previous step,

construct a calibration curve via Assay Blaster!, 4 parameter logistics, or point-to-point interpolation to quantify the concentration of the ACTH.

PROCEDURAL NOTES

- ACTH 1-39 is a very labile molecule. Set up the assay immediately upon the reconstitution or the thawing of all calibrators, controls, and samples.
- It is recommended that all calibrators, controls, and samples are assayed in duplicate. The average absorbance units of duplicate sets should then be used for reduction of data and the calculation of results.
- The samples should be pipetted into the well with minimum amount of air bubble. To achieve this, “reverse pipet” described in the package insert of the Pipettor’s manufacturer is recommended.
- Samples with values greater than the highest calibrator (Calibrator F), which is approximately 500 pg/mL (see exact concentration on vial label), may be diluted with Calibrator A (Zero Calibrator) and re-assayed. Multiply the result by the dilution factor.
- Reagents from different lot numbers must not be interchanged.
- If preferred, mix in equal volumes, in sufficient quantities for the assay, the Biotinylated Antibody and the Enzyme Labeled Antibody in a clean amber bottle, then use 50 µL of the mixed antibody into each well. This alternative method should replace Step (3) and (4), to be followed with the incubation with orbital shaker.
- When mixing avoid splashing of reagents from wells. This will affect assay precision and accuracy.

CALCULATION OF RESULTS

Manual Method

1. For the 450 nm readings, construct a dose response curve (calibration curve) using the first five calibrators provided, i.e., Calibrators A, B, C, D and E. For the 405 nm readings, construct a second dose response curve using the three calibrators with the highest concentrations, i.e., Calibrators D, E and F.
2. Assign the concentration for each calibrator stated on the vial in pg/mL. Plot the data from the calibration curve on linear graph paper with the concentration on the X-axis and the corresponding A.U. on the Y-axis.
3. Draw a straight line between 2 adjacent points. This mathematical algorithm is commonly known as the "point-to- point" calculation. Obtain the concentration of the sample by locating the absorbance unit on the Y-axis and finding the corresponding concentration value on the X-axis. Samples and control should be read using the 450nm for ACTH concentrations up to 150 pg/mL. ACTH concentrations above 150 pg/mL should be interpolated using the 405 nm reading.

Automated Method:

Computer programs using cubic spline or 4 PL [4 Parameter Logistics] or Point-to-Point can generally give a good fit.

Sample Data *at 450 nm* [raw A.U. readout against distilled or deionized water]

Microplate Well	**1st Reading** Absorbance Unit	**2nd Reading** Absorbance Unit	**Average** Absorbance Unit	**ACTH pg/mL**	**ACTH pg/mL Result to report**
Calibrator A	0.020	0.018	0.019		0
Calibrator B	0.077	0.074	0.076		5
Calibrator C	0.221	0.229	0.225		18
Calibrator D	0.624	0.692	0.685		55
Calibrator E	1.802	1.934	1.868		165
Control 1	0.417	0.398	0.408	33.5	33.5
Control 2	2.868	2.774	2.821	>150	*
Sample 1	0.072	0.078	0.075	4.9	4.9
Sample 2	0.185	0.177	0.181	14.0	14.0
Sample 3	0.495	.491	.493	40.8	40.8
Sample 4	2.090	2.122	2.106	>150	*

* Because the concentration readout is >150 pg/mL, it is recommended to use the data obtained at 405 nm as shown in **Sample Data** *at 405 nm* in the table below.

Sample Data *at 405 nm* [raw A.U. readout against distilled or deionized water]

Microplate Well	**1st Reading** Absorbance Unit	**2nd Reading** Absorbance Unit	**Average** Absorbance Unit	**ACTH pg/mL**	**ACTH (pg/mL) Result to report**
Calibrator A	0.011	0.008	0.0095		0
Calibrator D	0.032	0.032	0.032		55
Calibrator E	0.074	0.081	0.078		165
Calibrator F	1.838	1.817	1.828		500
Control 1	0.138	0.132	0.135	<150	
Control 2	0.921	0.894	0.908	256	256
Sample 1	0.030	0.032	0.031	<150	
Sample 2	0.068	0.062	0.065	<150	
Sample 3	0.165	0.159	0.162	<150	
Sample 4	0.663	.677	0.670	188	188

For samples with readout <150 pg/mL, it is recommended to use the data obtained at 450 nm as shown in **Sample Data** *at 450 nm* in the table above. This practice should give the results with optimum sensitivity of the assay.

NOTE: The data presented are for illustration purposes only and must not be used in place of data generated at the time of the assay.

QUALITY CONTROL

Control plasma or plasma pools should be analyzed with each run of calibrators and samples. Results generated from the analysis of the control samples should be evaluated for acceptability using appropriate statistical methods. In assays in which one or more of the quality control sample values lie outside the acceptable limits, the results for the sample may not be valid.

LIMITATIONS OF THE PROCEDURE

The ACTH ELISA kit has exhibited no “high dose hook effect” with samples spiked with 20,000 pg/mL of ACTH. Samples with ACTH levels greater than the highest calibrator, however, should be diluted and re-assayed for correct values.

EXPECTED VALUES

ACTH levels were measured in eighty-three (83) apparently normal individuals in the U.S. with the ACTH ELISA. The values obtained ranged from 7.9 to 66.1 pg/mL. Based on statistical tests on skewness and kurtosis, the population, when transformed logarithmically, follows the normal or Gaussian distribution as shown in the histograms. The geometric mean + 2 standard deviations of the mean were calculated to be 8.3 to 57.8 pg/mL.

PERFORMANCE CHARACTERISTICS

Accuracy

One hundred seventeen (117) samples, with ACTH values ranging from 1.5 to 1045 pg/mL were assayed by this ELISA procedure and the IRMA (immunoradiometric assay) ACTH kit. Linear regression analysis gives the following statistics:

ELISA = 0.976 IRMA Kit + 4.2 pg/mL
r = 0.995 N = 117

Sensitivity

The sensitivity, or minimum detection limit, of this assay is defined as the smallest single value, which can be distinguished from zero at the 95% confidence limit. The ACTH ELISA has a calculated sensitivity of 0.46 pg/mL.

Precision and Reproducibility

The precision (intra-assay variation) of the ACTH ELISA Test was calculated from 21 replicate determinations on each of the two samples.

Intra-Assay Variation

Sample	Mean Value (pg/mL)	N	Coefficient of variation %
A	35.7	21	3.1
B	255.0	21	4.2

The total precision (inter-assay variation) of the ACTH ELISA Test was calculated from data on two samples obtained in 35 different assays, by three technicians on three different lots of reagents, over a nine-week period.

Inter-Assay Variation

Sample	Mean Value (pg/mL)	N	Coefficient of variation %
A	35.2	35	5.8
B	230.0	35	6.2

Specificity and Cross-Reactivity

Cross-reactivity in the ACTH was studied by the addition of various materials to an ACTH standard. The results are as follows:

Cross-reactant	Concentration of Cross-reactant	ACTH without Cross-reactant [pg/mL]	ACTH With Cross-reactant [pg/mL]	Change in ACTH [pg/mL]	% Cross-reactivity
ACTH (1-24)	**100 000 pg/mL** **10 000 pg/mL** **1000 pg/mL** **200 pg/mL**	74.5 74.5 74.5 74.5	3.1 17.1 60.9 68	-71.4 -57.4 -13.6 -6.5	-0.07 -0.57 -1.36 -3.25
ACTH (18-39)	**5000 pg/mL** **2000 pg/mL** **500 pg/mL**	67 67 67	19 26.8 43.3	-48 -40.2 -23.7	-0.96 -2.01 -4.74
a-MSH	**100 000 pg/mL** **10 000 pg/mL** **1000 pg/mL** **200 pg/mL**	72.3 72.3 72.3 72.3	1.3 9.8 44.5 61	-71 -62.5 -27.8 -11.3	-0.07 -0.63 -2.78 -5.65
b-ENDORPHIN	**100 000 pg/mL** **50 000 pg/mL**	76.3 76.3	69.3 73.5	-7 -2.8	-0.01 -0.01

Recovery

Various amounts of ACTH were added to four different plasma samples to determine the recovery. The results are described in the following table:

Plasma Sample	Endogenous ACTH (pg/mL)	ACTH added (pg/mL)	Expected Value (pg/mL)	Measured Value (pg/mL)	Recovery (%)
A	23.3 21.0 18.6	-- 50.0 100.0	-- 71.0 118.6	-- 75.0 126.0	-- 105.6 106.2
B	28.1 25.3 22.5	-- 50.0 100.0	-- 75.3 122.5	-- 80.7 142.0	-- 107.2 115.9
C	21.8 19.6 17.4	-- 50.0 100.0	-- 69.6 117.4	-- 67.6 125.0	-- 97.1 106.4
D	9.8 8.8 7.8	-- 50.0 100.0	-- 58.8 107.8	-- 51.6 96.4	-- 87.7 89.4

Kinetic Effect of the Assay

To determine whether there is any systematic kinetic effect between the beginning of the run and the end of the run, three spiked sample pools, selected to represent a good cross section of the ACTH concentration, were placed in sequence throughout the run of one microplate or 96 wells [with twelve 8-well strips].

Linearity of Sample Dilutions: Parallelism

Five plasma samples were diluted with Calibrator A (Zero Calibrator). Results in pg/mL are shown below:

Sample	Dilution	Expected pg/mL	Observed pg/mL	% Observed ÷ Expected
A	Undiluted 1:2 1:4 1:8	- 118.0 59.0 29.5	236.0 110.0 54.9 26.3	- 93% 93% 89%
B	Undiluted 1:2 1:4 1:8	- 96.5 48.3 24.1	193.0 101.0 44.9 230.	- 105% 93% 99%
C	Undiluted 1:2 1:4 1:8	- 132.0 66.0 33.0	264.0 128.0 53.5 25.2	- 97% 81% 76%
D	Undiluted 1:2 1:4 1:8 1 :16	153.0 76.5 38.3 >1000	306.0 162.0 77.6 41.7	- - 106% 101% 109%
D	Undiluted 1:2 1:4 1:8 1 :16	- - 212.0 106.0 52.9	>1000 423.0 217.0 109.0 49.2	- - 103% 103% 93%

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NOTES