Adrenocorticotropic Hormone (ACTH) ELISA Assay Kit

Catalog Number:
ACT31-K01 (1 x 96 wells)
For Research Use Only. Not for use in diagnostic procedures.
v. 2.0 (03/21/18)

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INTENDED USE

The Eagle Biosciences Human Adrenocorticotrophic Hormone (ACTH) ELISA Assay Kit is intended for use in the quantitative determination of human adrenocorticotropic hormone (ACTH) in EDTA-plasma. The test is useful for detecting elevated and deficient ACTH levels. The Eagle Biosciences Human Adrenocorticotrophic Hormone (ACTH) ELISA Assay Kit is for research use only and not to be used in diagnostic procedures.

Indications for use:

Samples may have a higher than normal levels of ACTH with

1. Addison's disease or primary adrenal insufficiency
2. Congenital adrenal hyperplasia
3. Cushing's syndrome
4. Cushing's disease
5. Multiple endocrine neoplasia (MEN), type I

Samples may have a lower than normal levels of ACTH with

6. Hypopituitarism and/or secondary adrenal insufficiency
7. Adrenal gland tumor
8. Other tumors that produce cortisol

INTRODUCTION

ACTH is a 39 amino acid polypeptide with a molecular weight of 4540 Dalton. ACTH is secreted from corticotropes in the anterior lobe (or adenohypophysis) of the pituitary gland in response to corticotropin-releasing hormone (CRH) released by the hypothalamus. ACTH is synthesized from pre-pro-opiomelanocortin (pre-POMC). The removal of the signal peptide during translation produces the 241-amino acid polypeptide POMC, which undergoes a series of post-translational modifications such as phosphorylation and glycosylation before it is proteolytically cleaved by endopeptidases to yield various polypeptide fragments with varying physiological activity.

ACTH is an important component of the hypothalamic-pituitary-adrenal axis and is often produced in response to biological stress. It stimulates secretion of glucocorticoid steroid hormones from adrenal cortex cells especially in the zona fasciculata of the adrenal. ACTH acts by binding to cell surface ACTH receptors, which are located primarily on adrenocortical cells of the adrenal cortex.

PRINCIPLE OF THE ASSAY

The Eagle Biosciences Human Adrenocorticotrophic Hormone (ACTH) ELISA Assay Kit is designed, developed and produced for the quantitative measurement of human ACTH in EDTA-plasma sample. The assay utilizes the two-site "sandwich" technique with selected antibodies that bind to N-terminal and C-terminal epitopes of ACTH.

Assay standards, controls and patient samples are added directly to wells of a microtiter plate that is coated with antibody to the C-terminal of human ACTH. Immediately, a horseradish

peroxidase (HRP) conjugated anti N-terminal of human ACTH antibody is added to each well. After the first incubation period, a "sandwich" of solid-phase polyclonal antibody - human ACTH – HRP conjugated monoclonal antibody" is formed. The unbound antibodies and buffer matrix are removed in the subsequent washing step. For the detection of this immunocomplex, the well is then incubated with a substrate solution in a timed reaction, which is terminated with an acidic reagent (i.e. ELISA stop solution). The absorbance is then measured in a spectrophotometric microplate reader. The enzymatic activity of the immunocomplex bound to the wall of each microtiter well is directly proportional to the amount of human ACTH in the test sample. A standard curve is generated by plotting the absorbance versus the respective human ACTH concentration for each standard on a point-to-point or 4-parameter curve fitting. The concentration of human ACTH in test

REAGENTS: Preparation and Storage
The ACTH ELISA Assay kit must be stored at 2 – 8°C upon receipt. For the expiration date of the kit refer to the label on the ACTH ELISA Assay kit box. All components are stable until the expiration date.

Allow all reagents to come to room temperature prior to use. Regents from different kit lot numbers should not be combined or interchanged.

1. Anti-ACTH Antibody Coated Microplate
One microplate with twelve by eight strips (96 wells total) coated with monoclonal antihuman ACTH antibody. The plate is framed and sealed in a foil zipper bag with a desiccant. This reagent should be stored at 2 – 8 °C and is stable until the expiration date on the ACTH ELISA Assay Kit box.

2. HRP Conjugated Anti-ACTH Antibody (Tracer Antibody)
One vial containing 0.25 mL HRP labeled anti-human ACTH antibody in a stabilized protein matrix. This reagent should be stored at 2 – 8°C and is stable until the expiration date on the ACTH ELISA Assay Kit box.

3. ELISA Wash Concentrate
One bottle contains 30 mL of 30-fold concentrate. Before use the contents must be diluted with **870 mL** of demineralized water and mixed well. Upon dilution, this yields a working wash solution containing a surfactant in phosphate buffered saline with a non-azide, non-mercury preservative. The diluted wash solution may be stored at room temperature and is stable until the expiration date on the ACTH ELISA Assay Kit box.

4. ELISA HRP Substrate
One bottle contains 25 mL of tetramethylbenzidine (TMB) with hydrogen peroxide. This reagent should be stored at 2 – 8°C and is stable until the expiration date on the kit box.

5. ELISA STOP SOLUTION
One bottle contains 12 mL of stop solution. This reagent may be stored at 2 – 8°C or room temperature and is stable until the expiration date on the ACTH ELISA Assay Kit box.

6. Human ACTH Standards
Six vials containing human ACTH in a lyophilized bovine serum based matrix with a non-azide, non-mercury preservative. **Refer to the vial for exact concentration of the**

standard. These standards should be stored at 2 – 8°C and is stable until the expiration date on the ACTH ELISA Assay Kit box. Refer to assay procedure section for dilution direction.

7. ACTH Controls

Two vials containing human ACTH in a lyophilized bovine serum based matrix with a non-azide preservative. **Refer to vials for exact concentration range for each control.** Both controls should be stored at 2 – 8 °C and are stable until the expiration date on the kit box. Refer to assay procedure section for dilution direction.

8. Tracer Antibody Diluent

One vial containing 5mL ready to use buffer. It should be used only for tracer antibody dilution according to the assay procedures. This reagent should be stored at 2 – 8°C and is stable until the expiration date on the ACTH ELISA Assay Kit box.

SAFETY PRECAUTIONS

The Human Adrenocorticotrophic Hormone (ACTH) Assay Kit reagents must be used in a professional laboratory environment and is for Research Use Only and is not to be used in diagnostic procedures. Only source material from which reagents of bovine serum was derived in the contiguous 48 United States. It was obtained only from donor health animals maintained under veterinary supervision and found free of contagious diseases. Wear gloves while performing this assay and handle these reagents as if they were potentially infectious. Avoid contact with reagents containing TMB, hydrogen peroxide, or sulfuric acid. TMB may cause irritation to skin and mucous membranes and cause an allergic skin reaction. TMB is a suspected carcinogen. Sulfuric acid may cause severe irritation on contact with skin. Do not get in eyes, on skin, or on clothing. Do not ingest or inhale fumes. On contact, flush with copious amounts of water for at least 15 minutes. Use Good Laboratory Practices**.**

MATERIALS REQUIRED BUT NOT PROVIDED

- Precision single channel pipettes capable of delivering 25 µL, 200 µL, etc.
- Disposable pipette tips suitable for above volume dispensing.
- Aluminum foil.
- Deionized or distilled water.
- Plastic microtiter well cover or polyethylene film.
- ELISA multichannel washes bottle or automatic (semi-automatic) washing system.
- Spectrophotometric microplate reader capable of reading absorbance at 450/650 nm.

SPECIMEN COLLECTION

Since the circulation ACTH shows a 24 hours circadian rhythms, it is recommend drawing blood sample early morning or before 8 a.m. Patient should stop taking steroid drugs before drawing blood sample according to physician's prescription.

EDTA-plasma is a suitable specimen for human ACTH measurement. Totally 0.4 mL EDTA-plasma is required for duplicate determination of human ACTH with this test kit. Whole blood should be collected with lavender-top Vaccutainer and separate the plasma from cells according to manufacturer's instruction. The EDTA-plasma should be separated from the cells right after collection or at least within one hour of blood collection. The plasma should be transferred to a clean test tube right after centrifugation. **Plasma samples should be stored at – 20ºC** if the

assay is not to be performed within 3 hours. Avoid more than three times freeze-thaw cycles of specimen.

Samples of serum, heparin plasma and citrate plasma should not be used for ACTH measurement.

Reagent Preparation

1. Prior to use allow all reagents to come to room temperature. Reagents from different kit lot numbers should not be combined or interchanged.
2. ELISA Wash Concentrate must be diluted to working solution prior use. Please see REAGENTS section for details.
3. Reconstitute assay standards and controls by adding **2.0 mL** of demineralized water to each standard and control bottle. Allow the standard and controls to sit undisturbed for 5 minutes, and then mix well by inversions or gentle vortexing. One must make sure that all solid is dissolved completely prior to use. These reconstituted standard and controls may be stored at 2- 8 °C for up to 24 hours or below –10 °C for long-term storage. Do not exceed 3 freeze-thaw cycles.
4. Prepare Tracer Antibody working solution by 1:21 fold dilution of the ACTH Tracer Antibody by adding the tracer antibody into the Tracer Antibody Diluent. Following is a table that outlines the relationship of strips used and antibody mixture prepared. NOTE: The tracer antibody should be prepared just prior to the beginning of the assay.

Dilution Scheme	Tracer Antibody Diluent	Tracer Antibody
1	0.4 mL	20 µl
2	0.8 mL	40 µl
3	1.2 mL	60 µl
4	1.6 mL	80 µl
5	2.0 mL	100 µl
6	2.4 mL	120 µl
7	2.8 mL	140 µl
8	3.2 mL	160 µl
9	3.6 mL	180 µl
10	4.0 mL	200 µl
11	4.4 mL	220 µl
12	4.8 mL	240 µl

5. Test Configuration

ROW	STRIP 1	STRIP 2	STRIP 3	STRIP 4
A	STD 1	STD 5	SAMPLE 1	SAMPLE 5
B	STD 1	STD 5	SAMPLE 1	SAMPLE 5
C	STD 2	STD 6	SAMPLE 2	SAMPLE 6
D	STD 2	STD 6	SAMPLE 2	SAMPLE 6
E	STD 3	C 1	SAMPLE 3	
F	STD 3	C 1	SAMPLE 3	
G	STD 4	C 2	SAMPLE 4	
H	STD 4	C 2	SAMPLE 4	

6. Place a sufficient number of streptavidin coated microwell strips in a holder to run human Adrenocorticotrophic Hormone (ACTH) standards, controls and unknown samples in duplicates.

Assay Procedure

1. Add **200 µl** of Standards, Controls and patient samples into the designated microwells.
2. Immediately add **25 µL** of HRP Conjugated Anti-ACTH Antibody to each well.
3. Seal the plate wells securely, cover with foil or other material to protect from light, and rotate on an ELISA plate shaker (small orbit radius) for 2 hr. ± 5 minutes at 400 to 450 rpm.
4. Wash each well 5 times by dispensing 350 µL of working wash solution into each well and then completely aspirating the contents. Alternatively, an automated microplate washer can be used.
5. Add **200 µL** of ELISA HRP Substrate into each of the wells.
6. Cover the plate with aluminum foil to or other material to avoid exposure to light. Incubate plate static, at room temperature for **20 minutes**.
7. Immediately add **50 µL** of ELISA Stop Solution into each of the wells. Mix gently.
8. Read the absorbance at 450 nm with reference filter at 620 nm or 650 nm.

PROCEDURAL NOTES

1. It is recommended that all standards, controls and unknown samples be assayed in duplicate. The average absorbance reading of each duplicate should be used for data reduction and the calculation of results.
2. Keep light sensitive reagents in the original bottles and avoid unnecessary exposure to the light.
3. Store any unused antibody coated strips in the foil Ziploc bag with desiccant to protect from moisture.
4. Careful technique and use of properly calibrated pipetting devices are necessary to ensure reproducibility of the ACTH ELISA Assay Test.
5. Incubation times or temperatures other than those stated in this insert may affect the results.
6. An orbital mixer with a larger orbit radius (e.g. > 1 cm) may be used at speeds of 150 to 200 rpm.
7. Avoid air bubbles in the microwell as this could result in lower binding efficiency and higher CV% of duplicate reading

8. All reagents of the ACTH ELISA Assay Kit should be mix gently and thoroughly prior use. Avoid foaming.
9. Adapting this assay to automated ELISA system such as DS-2 (Diamedix Corp., Miami), a procedural validation is necessary if there is any modification of the assay procedure.

INTERPRETATION OF RESULTS

It is recommended to use a point-to-point or 4-parameter standard curve fitting.

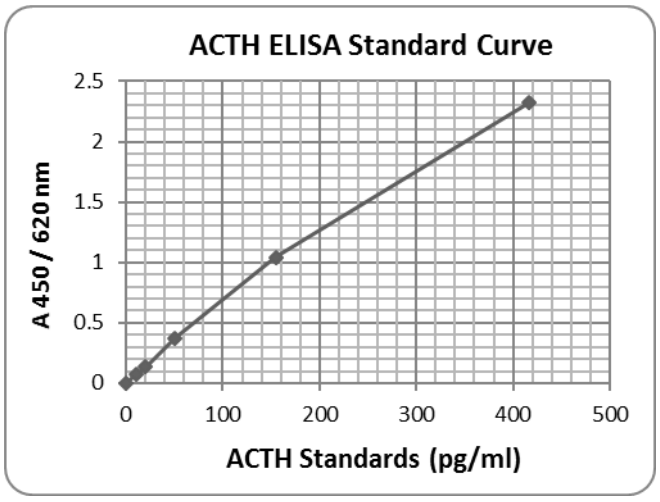
1. Calculate the average absorbance for each pair of duplicate test results.
2. Subtract the average absorbance of the level 1 standard (0 ng/mL) from the average absorbance of all other readings to obtain corrected absorbance.
3. The standard curve is generated by the corrected absorbance of all standard levels on the ordinate against the standard concentration on the abscissa using point-to-point or log-log paper. Appropriate computer assisted data reduction programs may also be used for the calculation of results.

The human ACTH concentrations for the controls and the samples are read directly from the standard curve using their respective corrected absorbance.

EXAMPLE DATA AND STANDARD CURVE

A typical absorbance data and the resulting standard curve from Human Adrenocorticotrophic Hormone (ACTH) ELISA Assay Kit are represented. This curve should not be used in lieu of standard curve run with each assay.

Well I.D.	OD 450/650 nm Absorbance			Results
	Readings	Average	Corrected	
Std-1: 0 pg/mL	0.025 0.029	0.027	0.000	
Std-2: 11 pg/mL	0.107 0.102	0.105	0.074	
Std-3: 20 pg/mL	0.160 0.160	0.160	0.133	
Std-4: 51 pg/mL	0.400 0.395	0.398	0.371	
Std-5: 155 pg/mL	1.057 1.081	1.069	1.042	
Std-6: 416 pg/mL	2.316 2.375	2.346	2.319	
Control 1	0.244 0.260	0.252	0.225	32.5 pg/mL
Control 2	0.676 0.714	0.695	0.668	97.1 pg/mL



EXPECTED VALUES

EDTA plasma samples from normal healthy adults ages 20 – 60 were collected and measured with this ELISA. The recommended **normal range** for ACTH concentration by using this ELISA is between 1 – 72 pg/mL. We strongly recommend for each clinical laboratory to establish its own normal range by measuring EDTA plasma samples with this ELISA. Please note that sample collection time of the day may have impact on the ACTH normal range.

LIMITATION OF THE PROCEDURE

1. This ACTH ELISA Assay kit requires EDTA-plasma sample for testing. Serum sample may show a lower ACTH level and must not be used, because ACTH is not stable in serum.
2. Because of 24 hours circadian rhythms of circulation ACTH level, the sample collection time of the day should be considered in interpreting test result. Therefore, a normal ACTH test result doesn't rule out related diseases.
3. For sample values reading greater than highest standard, it is recommended to re-assay samples with dilution (i.e. 1:10 or 1:100 with standard zero).
4. Water deionized with polyester resins may inactivate the horseradish peroxidase enzyme.

QUALITY CONTROL

To assure the validity of the results each assay should include adequate controls.

PERFORMANCE CHARACTERISTICS

Sensitivity

The analytical sensitivity (LLOD) of the ACTH ELISA Assay kit as determined by the 95% confidence limit on 8 replicate determination of zero standard is less than 1 pg/mL.

High Dose "hook" effect

This assay has showed that it did not have any high dose "hook" for ACTH level up to 10,000 pg/mL.

Precision
The intra-assay precision was validated by measuring three control samples with 16 replicate determinations.

Sample #	Mean ACTH Value (pg/mL)	CV (%)
1	36.1	7.6
2	66.5	8.6
3	276.9	10.3

The inter-assay precision was validated by measuring two control levels in duplicate in 16 individual assays.

Sample #	Mean ACTH Value (pg/mL)	CV (%)
1	32.1	7.1
2	261.0	5.3

Linearity
Two ACTH standard levels were diluted with assay buffer and tested. The results of ACTH percent recovery value in pg/mL are as follows:

DILUTION	OBSERVED Value (pg/mL)	RECOVERY %
Neat A	416	-
1:2	231.4	111
1:4	102.1	98
1:8	52.1	100
1:16	26.3	101
Neat B	155	-
1:2	80.3	104
1:4	38.6	100
1:8	20.5	106
1:16	9.3	96

Two EDTA plasma samples were collected and spiked with a high ACTH standard and tested. The results of ACTH percent recovery value in pg/mL are as follows:

DILUTION	OBSERVED VALUE (pg/mL)	RECOVERY %
Neat A	184.1	-
1:2	105.8	115
1:4	58.5	127
1:8	22.6	98
Neat B	33.3	-
1:2	17.1	103
1:4	9.4	113
1:8	5.1	121

Spike and Recovery

Two EDTA plasma samples and three assay standards (45, 135 and 405 pg/mL) were combined at equal volumes and tested. The results are as follows:

DILUTION	OBSERVED VALUE (pg/mL)	EXPECTED VALUE (pg/mL)	RECOVERY %
Neat A	12.5	-	-
Std-3	25.1	28.8	87
Std-4	73.5	73.8	100
Std-5	254.0	208.8	122
Neat B	21.5	-	-
Std-3	26.1	33.3	79
Std-4	66.8	78.3	85
Std-5	208.1	213.3	98

REFERENCES

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4. Kreek MJ, Wardlaw SL, Hartman N, Raghunath J, Friedman J, Schneider B, Frantz AG. Circadian rhythms and levels of beta-endorphin, ACTH, and cortisol during chronic methadone maintenance treatment in humans. Life Sci. 1983;33 Suppl 1:409-11

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For further information about this kit, its application or the procedures in this kit, please contact the Technical Service Team at Eagle Biosciences, Inc. at info@eaglebio.com or at 866-411-8023.