Monobind Inc.
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AccuBind
ELISA Microwells

Adrenocorticotropic Hormone (ACTH) Test System

Product Code: 10625-300

1.0 INTRODUCTION

Intended Use: The Quantitative Determination of Adrenocorticotropic Hormone Concentration in Human Serum or Plasma by a Microplate Enzyme Immunoassay, Colorimetric

2.0 SUMMARY AND EXPLANATION OF THE TEST

Adrenocorticotropic hormone (ACTH) is a hormone produced in the anterior, or front, pituitary gland in the brain. The function of ACTH is to regulate the levels of steroid hormones that are released from the adrenal glands including cortisol, aldosterone, and androgen precursors.[1] ACTH is secreted in response to a variety of severe stressors such as pain or emotional stress and ultimately results in analgesic, anti-inflammatory, and tissue regeneration effects.[2]

An overactive pituitary gland can result in increased ACTH levels leading to excess cortisol production (hypercortisolism), also known as Cushing's syndrome.[3] Conversely, hypopituitarism characterized by reduced ACTH levels can lead to adrenocortical insufficiency.[4] Addison's disease, or primary adrenal insufficiency, can be diagnosed when ACTH levels are high, but there is insufficient cortisol produced by the adrenal gland.[4]

Monitoring ACTH levels is a key aspect of mediating symptoms in patients with adrenal abnormalities.

3.0 PRINCIPLE

Sandwich Equilibrium Method (Type 2):
The ACTH immunoassay is an adapted two-site sandwich ELISA. In this assay, standards and patient samples are simultaneously incubated with the enzyme labeled detection antibody and a capture antibody coated on a microplate well. At the end of the assay incubation, the microplate well is washed to remove unbound components and the enzyme bound to the solid phase is incubated with the substrate, tetramethylbenzidine (TMB). An acidic stopping 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. Standards are used to generate a dose response curve of absorbance unit vs. concentration. Concentrations of ACTH present in the controls and patient samples are determined directly from this curve.

The essential reagents required for a sandwich equilibrium assay include high affinity and specificity antibodies (signal and capture), with different and distinct epitope recognition, in excess, and native antigen. In this procedure, the calibrator, control or patient sample is added to the wells coated with anti-ACTH antibody. ACTH from the sample binds to the anti-ACTH (PoAb) on the wells. Subsequently an enzyme labeled anti-ACTH is added to the wells. ACTH from the sample forms a sandwich between the two antibodies. Excess enzyme and sample is removed via a wash step. The interaction is illustrated by the following equation:

$$^{Enz}Ab_{(p)} + Ag_{ACTH} + Ab_{(p)} \underset{k_{-a}}{\overset{k_a}{\rightleftharpoons}} {}^{Enz}Ab_{(p)} - Ag_{ACTH} - Ab_{(p)}$$

$Ab_{(p)}$ = Anti-ACTH (PoAb) (On the Microwells in Excess Quantity)
Ag_{ACTH} = Native Antigen (Variable Quantity)
$^{Enz}Ab_{(p)}$ = Enzyme labeled Mouse α ACTH(P) (Excess Quantity)
$^{Enz}Ab_{(p)} - Ag_{ACTH} - Ab_{(p)}$ = Antigen-Antibody Sandwich complex
k_a = Rate Constant of Association
k_{-a} = Rate Constant of Dissociation

The enzyme activity in the antibody bound fraction is directly proportional to the native antigen concentration. By utilizing several different serum references of known antigen values, a dose response curve can be generated from which the antigen concentration of an unknown can be ascertained.

A suitable substrate is added to the wells to generate color in varying intensity depending upon the concentration of ACTH in the wells. The intensity of the color in the sample can be visually compared to the known calibrators to obtain qualitative results or the color development can be read with the help of a microplate spectrophotometer to obtain semi -quantitative results.

4.0 REAGENTS

Materials Provided:

A. ACTH Calibrators (Dried) – 1.0 ml/vial – Icon A-F
Six (6) vials of references for ACTH at approximate concentration range of 0, 20, 100, 250, 750 and 2000 pg/ml. Store at 2-8°C. ***Reconstitute each vial with 1ml of distilled or deionized water***.. The reconstituted calibrators are stable for 1 hour at 2-8°C. A preservative has been added. For longer periods after reconstitution, aliquot into smaller portions and freeze (<-20°C) for up to 3 months. Freeze and thawed cycles should be minimized to one time only.

B. ACTH Control M (Dried) – 1.0 ml/vial – Icon M
One (1) vial of ACTH control containing lyophilized serum. Store at 2-8°C. ***Reconstitute with 1ml of distilled or deionized water***. The reconstituted control should be assayed immediately after reconstitution. A preservative has been added. For longer periods after reconstitution, aliquot into smaller portions and freeze (<-20°C) for up to 3 months. Freeze and thawed cycles should be minimized to one time only.

C. ACTH Enzyme Reagent – 6 ml/vial – Icon
One (1) vial contains ACTH-HRP (horseradish peroxidase) conjugated antibody in a protein-based buffer and a non-mercury preservative. Store at 2-8°C.

D. ACTH Antibody Coated Plate – 96 wells – Icon
One 96-well microplate coated with ACTH antibody and packaged in an aluminum bag with a drying agent. Store at 2-8°C.

E. Wash Solution Concentrate – 20 ml/vial – Icon
One (1) vial containing a surfactant in buffered saline. A preservative has been added. Store at 2-8°C

F. Substrate Reagent – 12 ml/vial – Icon S^N
One (1) vial containing tetramethylbenzidine (TMB) and hydrogen peroxide (H_2O_2) in buffer. Store at 2-8°C.

G. Stop Solution – 8 ml/vial – Icon
One (1) vial containing a strong acid (0.5M H_2SO_4). Store at 2-8°C

H. Product Instructions

Note 1: Do not use reagents beyond the kit expiration date.
Note 2: Do not expose reagents to heat, sun, or strong light. **Opened reagents are stable for sixty (60) days when stored at 2-8 °C, unless otherwise specified. Kit and component stability are identified on label.**
Note 3: The above components are for a single 96-well microplate. For other kit configurations, please refer to table at the end of the instructions.

4.1 Required But Not Provided:
1. Pipette capable of delivering 0.050ml (50µl) volumes with a precision of better than 1.5%.
2. Dispenser(s) for repetitive deliveries of 0.050ml (50µl), 0.100ml (100µl), and 0.350ml (350µl) volumes with a precision of better than 1.5%.
3. Microplate washers or a squeeze bottle (optional).
4. Microplate Reader with 450nm and 620nm wavelength absorbance capability.
5. Absorbent paper for blotting the microplate wells.
6. Plastic wrap or microplate covers for incubation steps.
7. Vacuum aspirator (optional) for wash steps.
8. Timer.
9. Quality control materials.

5.0 PRECAUTIONS

For In Vitro Diagnostic Use
Not for Internal or External Use in Humans or Animals

All products that contain human serum have been found to be non-reactive for Hepatitis B Surface Antigen, HIV 1&2 and HCV Antibodies by FDA licensed reagents. Since no known test can offer complete assurance that infectious agents are absent, all human serum products should be handled as potentially hazardous and capable of transmitting disease. Good laboratory procedures for handling blood products can be found in the Center for Disease Control / National Institute of Health, "Biosafety in Microbiological and Biomedical Laboratories," 2nd Edition, 1988, HHS Publication No. (CDC) 88-8395.

Safe Disposal of kit components must be according to local regulatory and statutory requirement.

6.0 SPECIMEN COLLECTION AND PREPARATION

The specimens shall be blood serum in type or heparanised plasma in type, and the usual precautions in the collection of venipuncture samples should be observed. For accurate comparison to established normal values, a fasting morning serum sample should be obtained. The blood should be collected in a plain redtop venipuncture tube without additives or anti-coagulants for serum; use evacuated tube(s) containing heparin for plasma. Allow the blood to clot for samples. Centrifuge the specimen to separate the serum or plasma from the cells.

Samples may be refrigerated at 2-8 °C for a maximum period of five (5) days. If the specimen(s) cannot be assayed within this time, the sample(s) may be stored at temperatures of -20 °C for up to 30 days. Avoid use of contaminated devices. Avoid repetitive freezing and thawing. When assayed in duplicate, 0.10 ml (100 µl) of the specimen is required.

7.0 QUALITY CONTROL

Each laboratory should assay controls at levels in the low, medium and high ranges of the dose response curve for monitoring assay performance. These controls should be treated as unknowns and values determined in every test procedure performed. Quality control charts should be maintained to follow the performance of the supplied reagents. Pertinent statistical methods should be employed to ascertain trends. Significant deviation from established performance can indicate unnoticed change in experimental conditions or degradation of kit reagents. Fresh reagents should be used to determine the reason for the variations.

8.0 REAGENT PREPARATION

1. **Wash Buffer**
Dilute contents of wash solution concentrate to 1000ml with distilled or deionized water in a suitable storage container. Diluted buffer can be stored at 2-30°C for up to 60 days.

9.0 TEST PROCEDURE

Before proceeding with the assay, bring all reagents, serum reference calibrators and controls to room temperature (20-27°C).
*****Test Procedure should be performed by a skilled individual or trained professional*****

1. Format the microplates' wells for each serum reference calibrator, control and patient specimen to be assayed in duplicate. **Replace any unused microwell strips back into the aluminum bag, seal and store at 2-8°C.**
2. Pipette 0.050 ml (50 µl) of the appropriate serum reference calibrator, control or specimen into the assigned well.
3. Pipette 0.050 ml (50 µl) of the ACTH Enzyme to each well. **It is very important to dispense all reagents close to the bottom of the coated well.**
4. Swirl the microplate gently for 20-30 seconds to mix (500-600 rpm) and gently cover (See Note 3).
5. Incubate 60 minutes (1 hour) at room temperature.
6. Discard the contents of the microplate by decantation or aspiration. If decanting, tap and blot the plate dry with absorbent paper.
7. Add 0.350 ml (350 µl) of wash buffer (see Reagent Preparation Section), decant (tap and blot) or aspirate. Repeat two (2) additional times for a total of three (3) washes. **An automatic or manual plate washer can be used. Follow the manufacturer's instruction for proper usage. If a squeeze bottle is employed, fill each well by depressing the container (avoiding air bubbles) to dispense the wash. Decant the wash and repeat two (2) additional times.**
8. Add 0.100 ml (100 µl) of Substrate Reagent to all wells. **Always add reagents in the same order to minimize reaction time differences between wells.**
DO NOT SHAKE PLATE AFTER SUBSTRATE ADDITION
9. Incubate at room temperature for twenty (20) minutes.
10. Add 0.050 ml (50 µl) of stop solution to each well and mix gently for 15-20 seconds. **Always add reagents in the same order to minimize reaction time differences between wells.**
11. Read the absorbance in each well at 450nm (using a reference wavelength of 630nm to minimize well imperfections) in a microplate reader. **The results should be read within fifteen (15) minutes of adding the stop solution.**

Note 1: For reassaying specimens with concentrations greater than 2000 pg/ml, dilution should be performed.
Note 2: Do not use reagents that are contaminated or have bacterial growth.
Note 3: Cycle (start and stop) mixing (4 cycles) for 5-8 seconds/cycle is more efficient than one continuous (20-30 seconds) cycle to achieve homogeneity. A plate mixer can be used to perform the mixing cycles.
Note 4: It is extremely important to accurately dispense the correct volume with a calibrated pipette and by adding near the bottom of the microwells at an angle while touching the side of the well.

10.0 CALCULATION OF RESULTS

A dose response curve is used to ascertain the concentration of ACTH in unknown specimens.
1. Record the absorbance obtained from the printout of the microplate reader as outlined in Example 1.
2. Plot the absorbance for each duplicate serum reference versus the corresponding ACTH Calibrators concentration in pg/ml on linear graph paper (do not average the duplicates of the serum references before plotting).
3. Draw the best-fit curve through the plotted points.
4. To determine the concentration of ACTH for an unknown, locate the average absorbance of the duplicates for each unknown on the vertical axis of the graph, find the intersecting point on the curve, and read the concentration (in pg/ml) from the horizontal axis of the graph (the duplicates of the unknown may be averaged as indicated).

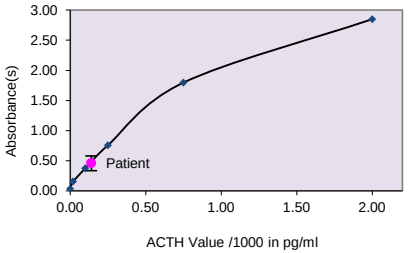
Note: Computer data reduction software designed for ELISA assays may also be used for the data reduction. If such software is utilized, the validation of the software should be ascertained.

*The data presented in Example 1 and Figure 1 is for illustration only and **should not** be used in lieu of a dose response curve prepared with each assay.

EXAMPLE 1

Sample I.D.	Well Number	Abs (A)	Mean Abs (B)	Value (pg/ml)
Cal A	A1	0.038	0.033	0
	B1	0.028		
Cal B	C1	0.155	0.154	20
	D1	0.152		
Cal C	E1	0.370	0.374	100
	F1	0.378		
Cal D	G1	0.761	0.758	250
	H1	0.756		
Cal E	A2	1.796	1.797	750
	B2	1.797		
Cal F	C2	2.879	2.850	2000
	D2	2.821		
	G2	0.459	0.458	**140.0**
Pat# 1	H2	0.457		

Figure 1



*If the absorbance readout is off-scale or higher than the average absorbance of the highest calibrator, sample should be repeated with dilution.

11.0 Q.C. PARAMETERS

In order for the assay results to be considered valid the following criteria should be met:

1. The absorbance (OD) of Calibrator 'F' (2000 pg/ml) should be ≥1.3
2. Four out of six quality control pools should be within the established ranges.

12.0 RISK ANALYSIS

The MSDS and Risk Analysis Form for this product are available on request from Monobind Inc.

12.1 Assay Performance

1. It is important that the time of reaction in each well is held constant to achieve reproducible results.
2. Pipetting of samples should not extend beyond ten (10) minutes to avoid assay drift.
3. Highly lipemic, hemolyzed or grossly contaminated specimen(s) should not be used.
4. If more than one (1) plate is used, it is recommended to repeat the dose response curve.
5. The addition of substrate solution initiates a kinetic reaction, which is terminated by the addition of the stop solution. Therefore, the substrate and stop solution should be added in the same sequence to eliminate any time-deviation during reaction.
6. Plate readers measure vertically. Do not touch the bottom of the wells.
7. Failure to remove adhering solution adequately in the aspiration or decantation wash step(s) may result in poor replication and spurious results.
8. Use components from the same lot. No intermixing of reagents from different batches.
9. Accurate and precise pipetting, as well as following the exact time and temperature requirements prescribed are essential. Any deviation from Monobind's IFU may yield inaccurate results.
10. All applicable national standards, regulations and laws, including, but not limited to, good laboratory procedures, must be strictly followed to ensure compliance and proper device usage.
11. It is important to calibrate all the equipment e.g. Pipettes, Readers, Washers and/or the automated instruments used with this device, and to perform routine preventative maintenance.
12. Risk Analysis- as required by CE Mark IVD Directive 98/79/EC - for this and other devices, made by Monobind, can be requested via email from Monobind@monobind.com.

12.2 Interpretation

1. ***Measurements and interpretation of results must be performed by a skilled individual or trained professional.***
2. Laboratory results alone are only one aspect for determining patient care and should not be the sole basis for therapy, particularly if the results conflict with other determinants.
3. The reagents for the procedure have been formulated to eliminate maximal interference; however, potential interaction between rare serum specimens and test reagents can cause erroneous results. Heterophilic antibodies often cause these interactions and have been known to be problems for all kinds of immunoassays. *(Boscato LM Stuart MC.'Heterophilic antibodies: a problem for all immunoassays' Clin. Chem 1988:3427-33).* For diagnostic purposes the results from this assay should be used in combination with clinical examination, patient's history, and, all other clinical findings.
4. For valid test results, adequate controls and other parameters must be within the listed ranges and assay requirements.
5. If test kits are altered, such as by mixing parts of different kits, which could produce false test results, or if results are incorrectly interpreted, Monobind shall have no liability.
6. If computer controlled data reduction is used to interpret the results of the test, it is imperative that the predicted values for the calibrators fall within 10% of the assigned concentrations.
7. The ACTH AccuBind® ELISA kit has exhibited no high dose hook effect with samples spiked with 2,000,000 pg/ml of Intact ACTH. Samples with Intact ACTH levels greater than the highest calibrator, however, should be diluted and reassayed for correct values.

13.0 EXPECTED RANGES OF VALUES

ACTH levels were measured in three hundred and fifty-four (354) apparently normal individuals. The values obtained ranged from 7.2 – 63.3 pg/ml.

It is important to keep in mind that establishment of a range of values, which can be expected to be found by a given method for a population of "normal" persons, is dependent upon a multiplicity of factors: the specificity of the method, the population tested and the precision of the method in the hands of the analyst. For these reasons, each laboratory should depend upon the range of expected values established by the Manufacturer only until an in-house range can be determined by the analysts using the method with a population indigenous to the area in which the laboratory is located.

14.0 PERFORMANCE CHARACTERISTICS

14.1 Precision

The within and between assay precisions of the ACTH AccuBind® ELISA test system were determined by analysis on three different levels of pool control sera and three levels of patient sera. The number, mean values, standard deviation and coefficient of variation for each of these control sera are presented in Table 1.

TABLE 1

	Mean Value (pg/ml)	Within-Run Precision		Total Precision (n=80)	
		SD	CV%	SD	CV%
Control 1	36.06	3.23	8.95	2.81	7.8
Control 2	191.28	3.08	1.61	14.56	7.61
Control 3	90.23	2.25	2.5	5.41	6
Patient 1	1181.18	33.31	2.82	109.64	9.28
Patient 2	22.59	1.25	5.51	1.21	5.37
Patient 3	292.36	5.95	2.04	26.28	8.99

14.2 Sensitivity

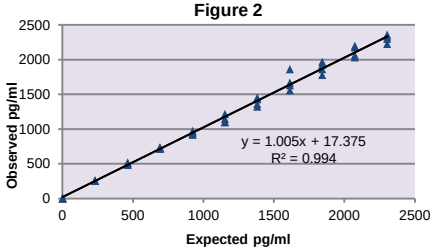
The ACTH AccuBind® ELISA test system has a LoB of 2.81 pg/ml, and a LoD=LoQ of 3.13 pg/ml.

14.3 Accuracy

14.3.1 Linearity

The linearity of the ACTH AccuBind® Microplate ELISA Test System was tested by diluting a human serum samples containing a high level of ACTH (~2300 pg/ml) with the "0 pg/ml" serum reference. The system was determined to have excellent linearity up to 2300pg/ml with a slope of 1.005 and a correlation factor of 0.994. The expected values were compared to the observed concentrations of the samples and graphed in Figure 2.

Figure 2



14.3.2 Recovery

The recovery of the ACTH AccuBind® Microplate ELISA Test System was calculated for five patient samples spiked with 25, 100, 400, 800, and 1600 pg/ml ACTH. Recoveries were determined to be within 15% of the expected values for all samples.

14.4 Specificity

The % cross reactivity of the ACTH antibody to selected substances was evaluated by adding the interfering substance to a serum matrix at various concentrations. No cross reactivity was detected for the following analytes up to 100,000 pg/ml concentrations.

Substance	%Cross Reactivity
ACTH (Fragment 18-39)	<0.001
ACTH (Fragment 1-10)	<0.001
ACTH (Fragment 1-24)	<0.001
α-MSH	<0.001
β-MSH	<0.001
β-Endorphin	<0.001

15.0 REFERENCES

1. Gallo-Payet, N. (2016). "60 YEARS OF POMC: Adrenal and extra-adrenal functions of ACTH", *Journal of Molecular Endocrinology*, *56*(4), T135-T156.
2. Tennant, F. (2017). "Adrenocorticotropin (ACTH) in chronic pain". *Journal of Applied Biobehavioral Research*, *22*(3) e12063
3. Findling, J.W. et al. (1991). "Routine Inferior Petrosal Sinus Sampling in the Differential Diagnosis of Adrenocorticotropin (ACTH)-Dependent Cushing's Syndrome: Early Recognition of the Occult Ectopic ACTH Syndrome", *The Journal of Clinical Endocrinology & Metabolism*, *73*(2), 408–413 https://doi.org/10.1210/jcem-73-2-408
4. Oelkers, W et al. (1992). "Diagnosis and therapy surveillance in Addison's disease: rapid adrenocorticotropin (ACTH) test and measurement of plasma ACTH, renin activity, and aldosterone", *The Journal of Clinical Endocrinology & Metabolism*, *75*(1), 259–264, https://doi.org/10.1210/jcem.75.1.1320051
5. HHS Publication, 5th ed., 2007. "Biosafety in Microbiological and Biomedical Laboratories". Available http://www.cdc.gov/biosafety/publications/bmbl5/BMBL5
6. DHHS (NIOSH) Publication No. 78–127, August 1976. "Current Intelligence Bulletin 13 - Explosive Azide Hazard". Available http:// www.cdc.gov/niosh.
7. Approved Guideline – Procedures for the Handling and Processing of Blood Specimens, H18-A3. 2004. Clinical and Laboratory Standards Institute.
8. Kricka L. "Interferences in immunoassays – still a threat". Clin Chem 2000; 46: 1037–1038.

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MP10625 **Product Code: 10625-300**

Size		96(A)	192(B)
Reagent (fill)	A)	1ml (Dried) set	1ml (Dried) set
	B)	1 (1ml/ Dried)	1 (1m/ Dried)
	C)	1 (6ml)	2 (6ml)
	D)	1 plate	2 plates
	E)	1 (20ml)	1 (20ml)
	F)	1 (12ml)	2 (12ml)
	G)	1 (8ml)	2 (8ml)

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IVD 2° 8° CE

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Please visit our website to learn more about our products and services.

Glossary of Symbols
(EN 980/ISO 15223)

IVD — In Vitro - Diagnostic Medical Device

2° 8° — Temperature Limitation Storage Condition (2-8° C)

Consult Instructions for Use

REF — Catalogue Number

Σ — Contains Sufficient Test for Σ

LOT — Batch Code

Used By (Expiration Day)

Date of Manufacturer

Manufacturer

EC REP — Authorized Rep in European Country

CE — European Conformity