Monobind Inc.
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AccuLite
CLIA Microwells

Adrenocorticotropic Hormone (ACTH) Test System
Product Code: 10675-300

1.0 INTRODUCTION

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

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):
ACTH immunoassay is an adapted two-site sandwich CLIA. In this assay, standards and patient samples are simultaneously incubated with the tracer labeled detection antibody and a biotin coupled capture antibody on a coated microplate well. At the end of the assay incubation, the microwell is washed to remove unbound components and the tracer bound to the solid phase is incubated with the with the signal reagent containing luminol. A dose response curve of RLU unit vs. concentration is generated using results obtained from the calibrators. Concentrations of ACTH present in the controls and patient samples are determined directly from a curve with the help of a Microplate Luminometer to obtain semi-quantitative results.
The interaction is illustrated by the following equation:

$$Enz_{Ab(p)} + Ag_{ACTH} + Ab(m) \underset{k\text{-}a}{\overset{ka}{\rightleftharpoons}} Enz_{Ab(p)}\text{-}Ag_{ACTH}\text{-}Ab_{(m)}$$

$Ab_{(m)}$ = Anti-ACTH (MoAb)(On the Microwells in Excess Quantity)
AgCT = Native Antigen (Variable Quantity)
$EnzAb_{(ACTH)}$=Enzyme labeled Mouse α ACTH(P) (Excess Quantity)
$EnzAb_{(ACTH)}$-Ag_{ACTH}-Ab(m)=Ag Antibodies Sandwich complex
ka = 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.

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

D. ACTH Light Reaction Wells – 96 wells – Icon
One 96-well white microplate coated with anti-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. Signal Reagent A – 7 ml/vial – Icon C^A
One (1) vial containing luminol in buffer. Store at 2-8°C.

G. Signal Reagent B – 7ml/vial - Icon C^B
One (1) vial containing hydrogen peroxide (H_2O_2) in buffer. 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.

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 Luminometer
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 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. Allow the blood to clot for samples. Centrifuge the specimen to separate the serum 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.
2. **Working Signal Reagent Solution** - Store at 2 - 30°C.
   Determine the amount of reagent needed and prepare by mixing equal portions of Signal Reagent A and Signal Reagent B in a clean container. For example, add 1 ml of A and 1ml of B per two (2) eight well strips (A slight excess of solution is made). **Discard the unused portion if not used within 36 hours after mixing.** If complete utilization of the reagents is anticipated, within the above time constraint, pour the contents of Signal Reagent B into Signal Reagent A and label accordingly.

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, 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 tracer 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.
5. Incubate 45 minutes 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 four (4) additional times for a total of five (5) 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 four (4) additional times.**
8. Add 0.100 ml (100 µl) of working signal reagent to all wells. (See Reagent Preparation Section) **Always add reagents in the same order to minimize reaction time differences between wells.**
   DO NOT SHAKE PLATE AFTER SIGNAL ADDITION
9. Read the relative light units (RLUs) in each well for 0.2 – 1.0 seconds. **The results should be read within thirty (30) minutes of adding the signal reagent solution.**

Note 1: Do not use the working signal reagent solution if older than 36 hours.
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.
Note 5: For reassaying specimens with concentrations greater than 2000 pg/ml, dilution should be performed.

10.0 CALCULATION OF RESULTS

A dose response curve is used to ascertain the concentration of ACTH in unknown specimens.
1. Record the RLU obtained from the printout of the microplate reader as outlined in Example 1.
2. Plot the RLU 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).

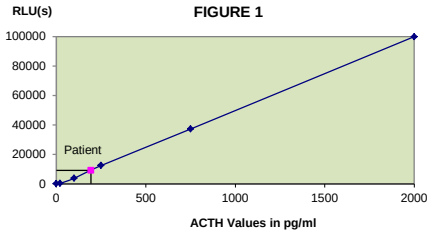
Note: Computer data reduction software designed for CLIA assay may also be used for the data reduction. If such software is utilized, the validation of the software should be ascertained.

EXAMPLE 1

Sample I.D.	Conc. (pg/ml)	RLU
Cal A	0	29
Cal B	20	276
Cal C	100	3806
Cal D	250	12410
Cal E	750	37214
Cal F	2000	100000
Patient	107	**4206**

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

FIGURE 1



* 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. In addition, the RLUs of the calibrators have been normalized to 100,000 RLUs for the F calibrator (greatest light output). This conversion minimizes differences caused by efficiency of the various instruments that can be used to measure light output.
*If the RLU readout is off-scale or higher than the average RLU 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 Dose Response Curve should be within established parameters.
2. Four out of six quality control pools should be within the established ranges.

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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. Failure to remove adhering solution adequately in the aspiration or decantation wash step(s) may result in poor replication and spurious results.
6. Use components from the same lot. No intermixing of reagents from different batches.
7. 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.
8. 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.
9. 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.
10. 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. For valid test results, adequate controls and other parameters must be within the listed ranges and assay requirements.
4. 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.
5. 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.
6. The CALCITONIN ELISA kit has exhibited no high dose hook effect with samples spiked with 2,000,000 pg/ml of Intact CALCITONIN. Samples with Intact CALCITONIN levels greater than the highest calibrator, however, should be diluted and assayed 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 AccuLite® CLIA test system were determined by analysis of three different levels of pool control and patient sera run in duplicate twice a day for twenty days (n=80). The data is presented in Table 2.

TABLE 2

	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	6.00
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 AccuLite® CLIA test system has LoB = 1.14 pg/ml and LoD = 5.52 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 an R^2 correlation factor of 0.994.

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

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Revision: 0 **Date: 2019-MAY-21** **DCO: N/A**
Product Code: 10675-300

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 (7ml)	2 (7ml)
	G)	1 (7ml)	2 (7ml)

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