Version 14-04/24

User's Manual

ACTH (Adrenocorticotropic Hormone) ELISA Kit

REF **DEIA1838**

Σ **96T**

RUO

This product is for research use only and is not intended for diagnostic use.

For illustrative purposes only. To perform the assay the instructions for use provided with the kit have to be used.

Creative Diagnostics

Address: 45-1 Ramsey Road, Shirley, NY 11967, USA

Tel: 1-631-624-4882 (USA) 44-161-818-6441 (Europe) **Fax: 1-631-938-8221**

Email: info@creative-diagnostics.com **Web: www.creative-diagnostics.com**

PRODUCT INFORMATION

Intended Use

The ACTH ELISA is intended for the quantitative determination of ACTH (Adreno-corticotropic Hormone) in human plasma. This assay is intended for research use only.

Principles of Testing

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 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. 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 patient samples are determined directly from this curve.

Reagents And Materials Provided

Kit Components	Description	Quantity
RGT 1 = Reagent 1	Biotinylated ACTH Antibody [affinity purified goat anti human ACTH]	1 x 2.7mL
RGT 2 = Reagent 2	Peroxidase (Enzyme) labeled ACTH Antibody [mouse monoclonal anti human ACTH]	1 x 2.7 mL
RGT A = *ELISA* Reagent A	ELISA Wash Concentrate [Saline with surfactant]	1 x 30 mL
RGT B = *ELISA* Reagent B	TMB Substrate [tetramethylbenzidine]	1 x 15.5 mL
SOLN = *Stopping solution*	ELISA Stop Solution [1 N sulfuric acid]	1 x 20 mL
PLA = Microplate	One holder with Streptavidin Coated Strips.	12 x 8-well strips
CAL = Calibrators A: 0 pg/mL B: C: *Refer to vial labels* D: *for exact* E: *concentrations* F:	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
CTRL = Controls 1 & 2 *Refer to vial labels for exact ranges*	Lyophilized. 2 Levels. Synthetic h-ACTH (1-39) in BSA/equine serum solution.	1 x 2 mL per level

Materials Required But Not Supplied

1. Microplate reader.

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

Storage

Store all kit components at 2-8°C except Wash Concentrate and Stop Solution.

Specimen Collection And Preparation

1. The determination of ACTH should be performed on EDTA plasma.
2. 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.
3. 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

1. All reagents except the non-zero calibrators, kit controls and the Wash Concentrate are ready-to-use. Store all reagents at 2-8°C, except the Wash Concentrate, which should be kept at room temperature (22-28°C) 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 2 mL 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. **ELISA Reagent A:** Wash Concentrate: Mix contents of wash concentrate thoroughly. If precipitate is present in the Wash 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 wash 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. 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.
2. Pipet **200 µL** of sample into the designated or mapped well. **Freeze (-20°C) the remaining calibrators and controls as soon as possible after use.**
3. Add or dispense **25 µL** of Reagent 1 (Biotinylated Antibody) into each of the wells which already contain the sample.
4. Add or dispense **25 µL** of Reagent 2 (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 + 10 rpm for 4 hours ± 30 minutes at room temperature (22-28°C).

5. First aspirate the fluid completely and then wash/aspirate each well five (5) times with the Working Wash Solution (prepared from **ELISA Reagent A**), using an automatic microplate washer. Blot dry by inverting the plate on an absorbent material. The wash solution volume should be set to dispense 0.35 mL into each well.
6. Add or dispense **150 µL** of the **ELISA Reagent B** (TMB Substrate) into each of the wells.
7. With appropriate cover to avoid light exposure, place the microplate(s) on an **orbital shaker or rotator** set at 170 + 10 rpm for 30 ±5 minutes at room temperature (22-28°C).
8. Add or dispense **100 µL** of the **ELISA Reagent C** (Stop Solution) into each of the wells. Mix gently.
9. 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, patient 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, patient and control samples should be read using the 450 nm for ACTH concentrations up to 150 pg/mL. ACTH concentrations above 150 pg/mL should be interpolated using the 405 nm reading.

10. By using the final absorbance values obtained in the previous step, construct a calibration curve via cubic spline, 4 parameter logistics, or point-to-point interpolation to quantify the concentration of the ACTH.

PROCEDURAL NOTES

1. 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.
2. It is recommended that all calibrators, controls, and patient 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.
3. 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 manufacturers of Pipettors is recommended.
4. The 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 reassayed. Multiply the result by the dilution factor.
5. Reagents from different lot numbers must not be interchanged.
6. If preferred, mix in equal volumes, in sufficient quantities for the assay, Reagent 1 (Biotinylated Antibody) and Reagent 2 (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.
7. When mixing avoid splashing of reagents from wells. This will affect assay precision and accuracy.

Quality Control

Control samples or serum pools should be analyzed with each run of calibrators and patient 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 patient sample may not be valid.

Calculation

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. Patient and control samples should be read using the 450 nm 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	*
Patient Sample 1	0.072	0.078	0.075	4.9	4.9
Patient Sample 2	0.185	0.177	0.181	14.0	14.0
Patient Sample 3	0.495	.491	.493	40.8	40.8
Patient 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
Patient Sample 1	0.030	0.032	0.031	< 150	¶
Patient Sample 2	0.068	0.062	0.065	< 150	¶
Patient Sample 3	0.165	0.159	0.162	< 150	¶
Patient 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.

Performance Characteristics

Accuracy

Three hundred (300) samples, with ACTH values ranging from 1.0 to 640 pg/mL were assayed by the previous ACTH kit and the updated ACTH kit ELISA. Linear regression analysis gives the following statistics:

ACTH ELISA = 1.02 - 1.58 pg/mL r = 0.995 N = 300

Precision

The precision (intra-assay variation) of the ACTH ELISA Test was calculated from 25 replicate determinations on each of the two samples.

Intra-Assay Variation

Sample	Mean Value (pg/mL)	N	Coefficient of variation %
A	42.2	25	6.71
B	269.9	25	2.27

The total precision (inter-assay variation) of the ACTH ELISA Test was calculated from data on two samples obtained in 21 different assays, by three technicians on three different lots of reagents, over a four-week period.

Inter-Assay Variation

Sample	Mean Value (pg/mL)	N	Coefficient of variation %
A	42.3	21	7.1
B	287.8	21	6.9

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 0.22 pg/mL.

Specificity

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	62.9 62.9 62.9 62.9	0.8 5.05 28.6 43.4	-62.1 -57.85 -34.3 -19.5	-0.06 % -0.58 % -3.43 % -9.75 %
ACTH (18-39)	5000 pg/ml 2000 pg/ml 500 pg/ml	61.2 61.2 61.2	2 13.6 24.3	-59.2 -47.6 -36.9	-1.2 % -2.4 % -7.4 %
a-MSH	100 000 pg/ml 10 000 pg/ml 1000 pg/ml 200 pg/ml	88.1 88.1 88.1 88.1	65.7 69.1 70.7 74.8	-22.4 -19 -17.4 -13.3	-0.02 % -0.19 % -1.7 % -6.7 %
b-ENDORPHIN	100 000 pg/ml 50 000 pg/ml	73.8 73.8	60.5 56.9	-13.3 -16.9	-0.01 % -0.03 %

Linearity

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	-	288	-
	1:2	144	150	104%
	1:4	72	70.9	98%
	1:8	36	35.7	99%
B	Undiluted	-	468	-
	1:2	234	278	119%
	1:4	117	135	115%
	1:8	58.5	65.5	112%
C	Undiluted	-	270	-
	1:2	135	146	108%
	1:4	67.5	68	101%
	1:8	33.75	33.5	99%
D	Undiluted	-	336	-
	1:2	168	149	89%
	1:4	84	83	99%
	1:8	42	47	112%
E	Undiluted	-	452	-
	1:2	226	268	119%
	1:4	113	126	112%
	1:8	56.5	68.9	122%

Recovery

Various amounts of ACTH were added to four different plasma 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	13.3	50.0 100.0	63.3 113.5	62.4 116	99 % 102 %
B	17.7	50.0 100.0	67.7 117.7	62.1 121.7	92 % 103 %
C	14.8	50.0 100.0	64.8 114.8	64.2 114.2	99 % 99 %
D	27.1	50.0 100.0	77.1 127.1	67.4 119	87 % 94 %

Precautions

1. Although the reagents provided in this kit has been specifically designed to contain no human blood components, the human patient 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 sample.
2. ELISA Reagent C, 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.

Limitations

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 reassayed for correct values.