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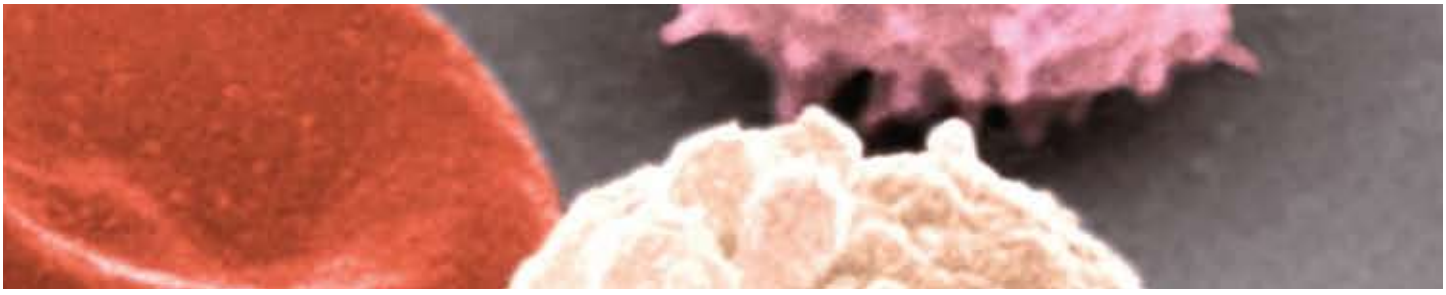
ACTH ELISA

Catalog Number M046006

For the quantitative determination of adrenocorticotropic hormone (ACTH) in human, mouse, and rat plasma samples.

For research use only.
This product insert must be read in its entirety before using this product.



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 [1]. 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 sterioids, aldosterone, cortisol and adrenal androgens. It is the principal modulator of cortisol, the most important glucocorticoid in human. 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, proopiomelanocortin (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 [5].

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 iscustomary to draw plasma ACTH samples between 8:00 and 10:00 hour. However, differentiation of patients with Cushing's disease from normal individuals may be best achieved on samples obtained in the evening (16:00 - 18:00 hour). In Cushing's disease and in ectopic ACTH syndromes, the diurnal pattern of ACTH secretion is generally absent. Stress may also override the diurnal variation.

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].

Cushing's syndrome is caused by the effects of excess glucocorticoid actions. All causes of Cushing's syndrome, with the exception of glucocorticoid medication, are associated with incresed 24 hour urinary cortisol. The most common cause of Cushing's syndrome is bilateral adrenal hyperplasia, due to pituitary ACTH hypersecretion (Cushing's disease) from a pituitary adenoma or corticotroph hyperplasia [5,6,7,8,9,10]. Laboratory diagnosis of Cushing's disease is supported by the following: (1) suppression of plasma ACTH and cortisol concentrations, by high-dose (2.0 mg q 6h x 8) dexamethasone administration, (2) absence of ACTH and cortisol suppression with low-dose (0.5 mg q 6h x 8 or 1 mg given at 23:30 hour) dexamethasone, (3) larger than normal response to metyrapone (Metopirone) stimulation and normal or elevated plasma ACTH levels [4].

When Cushing's syndrome is caused by primary adrenal abnormality (adenoma or carcinoma), the adrenal gland acts independently of ACTH and pituitary ACTH secretion is suppressed [5,6,7,8,9,10]. Hence, there is no response to dexamethasone suppresion or metyrapone stimulation. This type of Cushing's syndrome is characterized by very low or undetectable levels of ACTH.

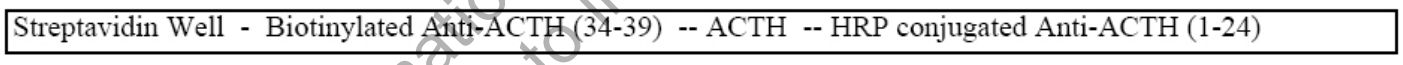
Therefore, measurement of plasma ACTH is helpful in differential diagnosis of pituitary Cushing's syndrome. In patients with adrenal tumors ACTH levels are low. High levels of ACTH are seen in patients with ectopic ACTH syndrome. Patients with bilateral adrenal hyperplasia will have ACTH levels inappropriately elevated for their degree of hypercortisolism, which should suppress ACTH. However, in most cases the ACTH concentration will be within the normal range.

Adrenocortical insufficiency or inadequate cortisol production can be due to destruction of the adrenal cortex or to abnormalities of the pituitary or hypothalamus which result in inadequate ACTH production of release [5,6,7,8,9,10]. Primary adrenocortical insufficiency, Addison's disease, is characterized by markedly elevated plasma ACTH levels and adrenal unresponsiveness to stimulation with exogenous ACTH. Hypopituitarism with ACTH deficiency, which is secondary adrenocortical insufficiency, is characterized by low plasma ACTH and cortisol concentrations, and a subnormal, but usually distinct adrenal response to stimulation with synthetic ACTH (Cortrosyn®) . If hypoglycemic stress or metyrapone stimulation is required for diagnosis, ACTH and cortisol responses are less than normal.

Aggressive and invasive ACTH producing pituitary tumors occurring before or following bilateral adrenalectomy for Cushing's disease (Nelson's syndrome) are characterized by the development of Addisonian pigmentation, often in an adrenalectomized patient who is taking adequate glucocorticoid replacement therapy. In these patients, plasma ACTH levels are markedly elevated and do not respond well to dexamethasone suppression.

PRINCIPLE OF THE ASSAY

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.



In this assay, calibrators, controls, or patient 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.

KIT COMPONENTS

Microtiter Plate - The plate contains 12 x 8-well strips coated with streptavidin. Ready for use.

Calibrator A - 1 vial (0 pg/mL) of solution containing BSA/equine serum. Ready to use.

Calibrators B through F - Synthetic human ACTH (1-39) in a BSA/equine serum solution. Lyophilized. See vial label for concentrations.

Controls - Synthetic human ACTH (1-39) in a BSA/equine serum solution. Lyophilized. See vial label for concentration ranges.

Biotinylated ACTH Antibody - 1 vial of Biotinylated ACTH Antibody containing blue dye. Ready to use.

Enzyme Conjugate - 1 vial of Peroxidase labeled ACTH Antibody containing red dye. Ready to use.

Wash Buffer Concentrate - 1 vial of 20-fold concentrated saline solution with surfactant.

Substrate - 1 vial of TMB Substrate. Ready to use.

Stop Solution - 1 vial of 1 N Sulfuric Acid. Ready to use.

STORAGE

<table>
<tr><td>Unopened Kit</td><td colspan="2">Store at 2 - 8° C. Do not use past the kit expiration date.</td></tr>
<tr><td rowspan="8">Opened Reagents</td><td>Calibrators</td><td rowspan="2">Store unused calibrators and controls at -20° C for up to 6 weeks.</td></tr>
<tr><td>Controls</td></tr>
<tr><td>Biotinylated ACTH Antibody</td><td rowspan="3">Store at 2 - 8° C.</td></tr>
<tr><td>Enzyme Conjugate</td></tr>
<tr><td>Substrate</td></tr>
<tr><td>Stop</td><td rowspan="2">Store at room temperature.</td></tr>
<tr><td>Wash Buffer</td></tr>
<tr><td>Microtiter wells</td><td>Return unused wells to the foil pouch containing the desiccant and seal. Store at 2 - 8° C.</td></tr>
</table>

SUPPLIES REQUIRED BUT NOT PROVIDED

- Microplate Reader
- Microplate Washer
- Pipettes or pipetting equipment with disposable polypropylene tips
- Multi-channel pipette
- Disposable polypropylene test tubes
- Glass measuring cylinders
- Distilled or deionised water

PRECAUTIONS

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 patient sample.

The Stop Solution provided with this kit 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 breath vapor and avoid inhalation.

CRITICAL PARAMETERS

- Allow samples and all reagents to equilibrate to room temperature (22–25 °C) prior to performing the assay. This is especially important for the TMB Substrate.
- Adhere to recommended incubation temperatures in the protocol as variations may cause inconsistent or poor assay results.
- It is essential that all wells are washed thoroughly and uniformly. When washing is done by hand, use a squeeze bottle and ensure that all wells are completely filled and emptied at each step.
- Use only reagents from the same lot for each assay. This is especially important when running more than one plate per sample group.
- A separate standard curve must be run on each plate.
- Reagents can easily be trapped in the caps of small microfuge tubes. It is recommended to briefly centrifuge these reagents before use.
- Mix all reagents thoroughly prior to use, but avoid foaming!
- Keep the wells sealed with the plate sealer except when adding reagents and during reading.
- Any variation in the protocol can cause variation in binding!
- The kit should not be used beyond the expiration date on the kit label.
- The values obtained by the samples should be within the standard range. If this is not the case, dilute the sample and repeat the assay.
- We take great care to ensure that this product is suitable for all validated sample types, as designated in this manual. Other sample types may be tested and validated by the user.

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

Note: All reagents should be stored at the recommended temperatures. Bring all reagents to room temperature (22 - 25°C) before use. Reagents can easily be trapped in the caps of small microfuge tubes. It is recommended to briefly centrifuge these reagents before use.

Wash Buffer Concentrate - Bring to room temperature before use. Wash Buffer may exhibit precipitation when stored at cold temperatures. Mix thoroughly before use. Add 30 mL of Wash Buffer Concentrate to 570 mL deionized or distilled water and mix.

Calibrators - For Calibrators B through F, 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 ensure complete reconstitution. **Use immediately following reconstitution.** Store unused calibrators at -20°C for up to 6 weeks.

Controls - 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 ensure complete reconstitution. **Use immediately following reconstitution.** Store unused controls at -20°C for up to 6 weeks.

ASSAY PROTOCOL

Read the entire protocol before beginning the assay. It is recommended that all standards and samples be assayed in duplicate. Reagents can easily be trapped in the caps of small microfuge tubes. It is recommended to briefly centrifuge these reagents before use. *Note: Reagents and samples may require specific handling temperatures and need preparation prior to the assay. See the Reagent and Sample Preparation sections before proceeding*.

1. Prepare all reagents and samples as described in the previous sections.
2. Remove any excess microtiter strips from the plate fram and return them to the foil pouch containing the desiccant pack.

Calibrator/Sample/Antibody Incubation

3. Pipet 200 µL of calibrator, control or sample into duplicate wells. Freeze (-20 °C) the remaining calibrators and controls as soon as possible after use.
4. Pipette 25 µL of Biotinylated Antibody into each of the wells which already contain the sample.
5. Pipette 25 µL of Enzyme Conjugate into each of the same wells.
6. Cover the microplate(s) with aluminum foil or a tray to avoid exposure to light. Incubate on an orbital shaker or rotator set at 170 ± 10 rpm for 4 hours ± 30 minutes at room temperature (22 - 25°C).

Wash

7. Aspirate and wash each well five (5) times with the Working Wash Solution, using an automatic microplate washer. Blot dry by inverting the plate on an absorbent material. The wash solution volume should be set to dispense 350 µL into each well.

Substrate Incubation

8. Add or dispense 150 µL of the Substrate Solution into each of the wells.
9. 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.

Stop Reaction

10. Add or dispense 100 µL of the Stop Solution into each of the wells. Mix gently.
11. 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

SUMMARY

Prepare reagents and samples as previously described.

Pipette 200 µL calibrator, control or sample in duplicate into the wells.

Pipette 25 µL Biotinylated Antibody into the wells.

Pipette 25 µL Enzyme Conjugate into the wells.

Cover and Incubate 4 hours at RT (22 - 25 °C) on a shaker.

Aspirate and wash 5 times.

Add 150 µL of Substrate to each well. Cover and Incubate 30 minutes at RT on a shaker.

Add 100 µL of Stop Solution to each well. Read at 405 nm

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 patient samples.
- 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.
- 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.
- Patient 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.
- Reagents from different lot numbers must not be interchanged.
- If preferred, mix in equal volumes, in sufficient quantities for the assay, Biotinylated Antibody and Enzyme Conjugate in a clean amber bottle, Then use 50 µL of the mixed antibody into each well. This alternative method should replace Step (4) and (5), to be followed with the incubation with orbital shaker.

CALCULATION OF RESULTS

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

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 Parameter Logistic (4 PL) can generally give a good curve fit.

SAMPLE DATA

Data obtained at 450 nm (raw A.U. readout against distilled or deionized water):

Well	1st Reading (A.U.)	2nd Reading (A.U.)	Average A.U.	ACTH pg/mL	ACTH result to report (pg/mL)
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	0.491	0.493	40.8	40.8
Sample 4	2.090	2.122	2.106	> 150	*

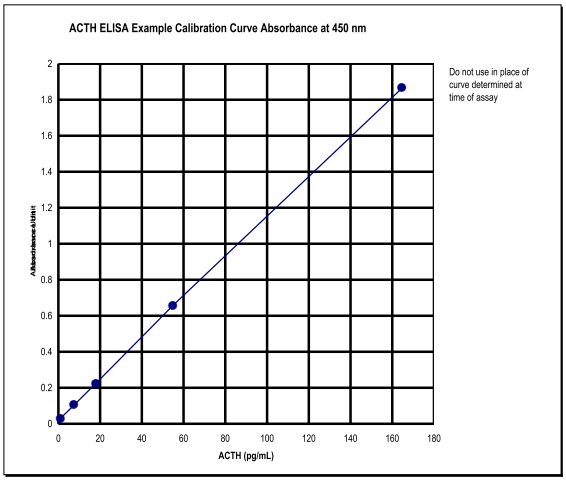
**Because the concentration is >150 pg/mL, it is recommended to use the data obtained at 405 nm.*

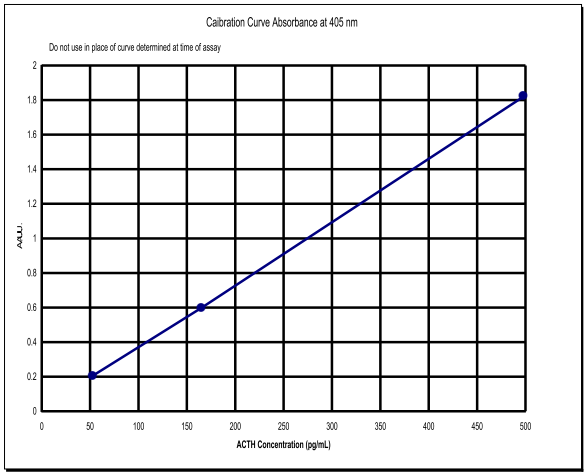
Data obtained at 405 nm (raw A.U. readout against distilled or deionized water):

Well	1st Reading (A.U.)	2nd Reading (A.U.)	Average A.U.	ACTH pg/mL	ACTH result to report (pg/mL)
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	0.677	0.670	188	188

For samples with a readout <150 pg/mL, it is recommended to use the data obtaind at 450 nm.This practice should give the results with optimum sensitivity of the assay.

NOTE: The data presented is 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 patient 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 reassayed for correct values.

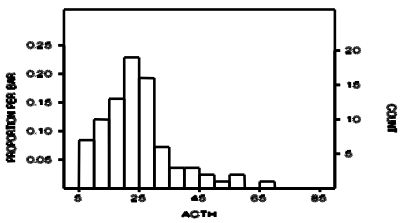
Like any analyte used as a diagnostic adjunct, ACTH results must be interpreted carefully with the overall clinical presentations and other supportive diagnostic tests.

EXPECTED VALUES

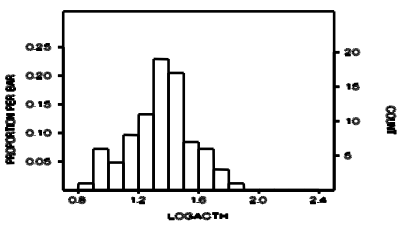
ACTH levels were measured in eighty-three (83) apparently normal individuals in the U.S. with the ACTH ELISA supplied by Morwell Diagnostics. The values obtained ranged from 7.9 to 66.1 pg/mL. Based on statistical tests on skewness and kurtosis, the population, when transformed logarithmically, follows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

ACTH Histograms

Raw ACTH Results



Logarithmically Transformed

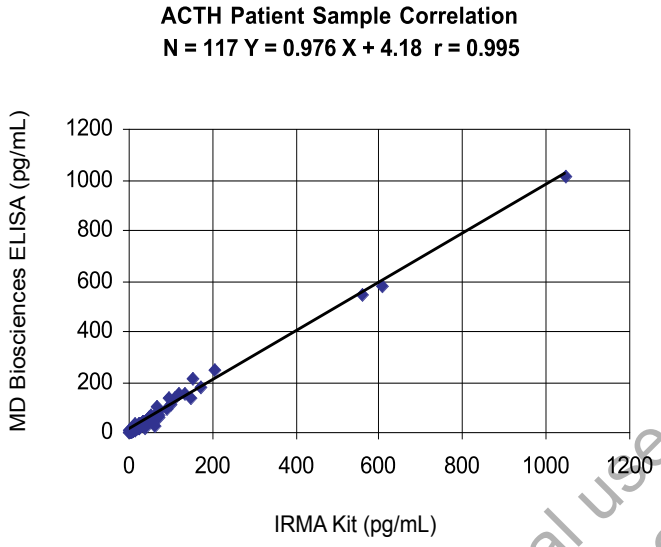


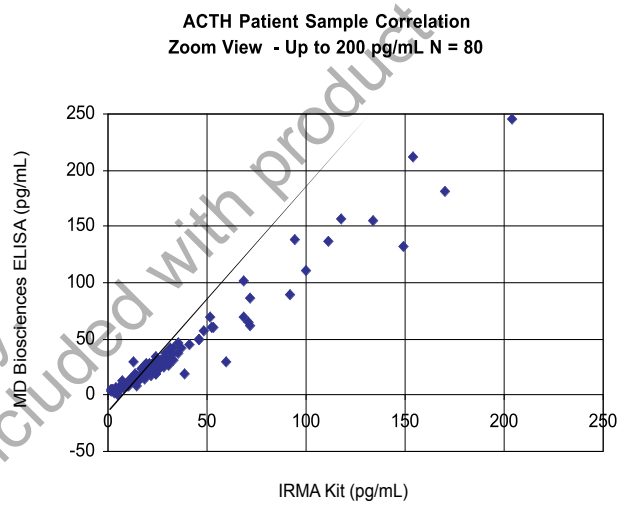
PERFORMANCE CHARACTERISTICS

Accuracy

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

ACTH ELISA = 0.976 IRMA kit + 4.2 pg/m
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.

Reproducibility

Intra-assay Precision (Precision within an assay) - The intra-assay precision was measured by assaying two control samples 21 times on one plate.
Inter-assay Precision (Precision between assays) - The inter-assay precison was assessed by repeated measurements of two control samples in 35 different assays by 3 technicians on 3 different lots of reagents, over a 9 week period.

	Intra-assay Precision		Inter-assay Precision	
Control	A	B	A	B
Mean (pg/mL)	35.7	255	35.2	230
CV (%)	3.1	4.2	5.8	6.2

SPECIFICITY AND CROSS-REACTIVITY

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

Cross-reactant	Concentration of Cross-reactant (pg/mL)	ACTH without cross-reactant (pg/mL)	ACTH with cross-reactant (pg/mL)	Change in ACTH (pg/mL)	% Cross-reactivity
ACTH (1-24)	100,000	74.5	3.1	-71.4	-0.07%
	10,000	74.5	17.1	-57.4	-0.57%
	1,000	74.5	60.9	-13.6	-1.36%
	200	74.5	68	-6.5	-3.25%
ACTH (18-39)	5,000	67	19	-48	-0.96%
	2,000	67	26.8	-40.2	-2.01%
	200	67	43.3	-23.7	-4.74%
a-MSH	100,000	72.3	1.3	-71	-0.07%
	10,000	72.3	9.8	-62.5	-0.63%
	1,000	72.3	44.5	-27.8	-2.78%
	200	72.3	61	-11.3	-5.65%
b-Endorphin	100,000	76.3	69.3	-7	-0.01%
	50,000	76.3	73.5	-2.8	-0.01%

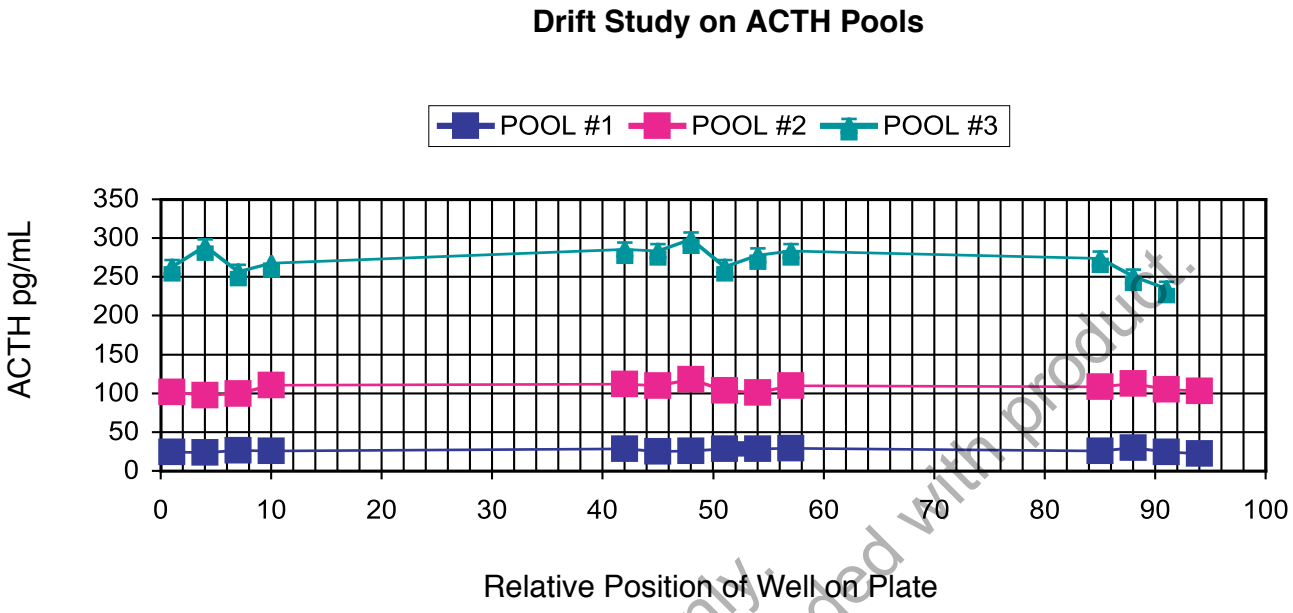
RECOVERY

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

Plasma Sample	Endogenous	ACTH added (pg/mL)	Expected Value (pg/mL)	Measured Value (pg/mL)	Recovery (%)
A	23.3	-	-	-	-
	21.0	50	71.0	75.0	105.6%
	18.6	100	118.6	126.0	106.2%
B	28.1	-	-	-	-
	25.3	50	75.3	80.7	107.2%
	22.5	100	122.5	142.0	115.9%
C	21.8	-	-	-	-
	19.6	50	69.6	67.6	97.1%
	17.4	100	117.4	125.0	106.4%
D	9.8	-	-	-	-
	8.8	50	58.8	51.6	87.7%
	7.8	100	107.8	96.4	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 patient 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]. The results, displayed in the following graphs, show no significant assay drift.



LINEARITY OF PATIENT SAMPLE DILUTIONS: PARALLELISM

Five patient 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	-	236	-
	1:2	118	110	93%
	1:4	59.0	54.9	93%
	1:8	29.5	26.3	89%
B	Undiluted	-	193	-
	1:2	96.5	101	105%
	1:4	48.3	44.9	93%
	1:8	24.1	23.9	99%
C	Undiluted	-	264	-
	1:2	132	128	97%
	1:4	66.0	53.5	81%
	1:8	33.0	25.2	76%
D	Undiluted	-	>1000	-
	1:2	-	306	-
	1:4	153	162	106%
	1:8	76.5	77.6	101%
	1:16	38.3	41.7	109%
E	Undiluted	-	>1000	-
	1:2		423	-
	1:4	212	217	103%
	1:8	106	109	103%
	1:16	52.9	49.2	93%

TROUBLESHOOTING

Problem	Recommendation
Low Absorbance	• Check reagents for proper storage • Control expiration date. • Check preparation of reagents. • Control incubation times and temperature. • Check reader wavelength.
High Absorbance/high zero standard value	• Check preparation of reagents. • Control incubation times and temperature. • Equilibrate ELISA reagents to room temperature (22 - 25 °C). • Ensure that every well of the ELISA plate is completely filled and emptied at every wash step. • Check that plates are blotted on tissue paper after washing.
Flat cure/poor reproduciblity	• Check reagents for proper storage. • Control expiration date. • Check preparation of working standards. • Check incubation times and temperatures. • Use separate reservoirs for pipetting different solutions with multichanned pipettes. Always use new pipette tips. • Check pipette calibration. • Ensure efficient washing procedure.

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