A GloMax® Multi Microplate Absorbance Method for

Monobind's Cortisol EIA Assay Kit

INTRODUCTION

Cortisol (hydrocortisone, compound F) is the most potent glucocorticoid produced by the human adrenal complex. As with most other adrenal steroids, cortisol is synthesized from cholesterol by the adrenal cortex (1,2) via a series of enzymatic-mediation steps. The first and rate-limiting step in adrenal steroidogenesis is conversion of cholesterol to pregnenolone. Step one is stimulated by pituitary adrenocorticotropic hormone (ACTH) which is, in turn, regulated by the hypothalamic corticotrophin-releasing factor (CRF). ACTH and CRF secretion are inhibited by high levels of cortisol. In plasma, most cortisol is bound with high affinity to corticosteroid-binding gobulin (CBG, transcortin), with much of the remainder loosely bound to albumin. Physiologically effective in anti-inflammatory activity and blood-pressure maintenance, cortisol is involved in gloconeogenesis. Cortisol acts through specific intracellular receptors and affects numerous other physiologic systems including: immune function, glucose-counter regulation, vascular tone, substrate utilization and bone metabolism[1-3]. Cortisol is excreted primarily in urine and in an unbound (free) form.

The GloMax® Multi Microplate Reader used in conjunction with the Monobind Cortisol EIA Assay Kit allows for rapid and accurate measurement of cortisol concentrations in a 96-well format. Detect as low as 0.25 µg/dL of cortisol by using of the GloMax® Multi Microplate Absorbance Reader.

MATERIALS REQUIRED

- GloMax® Multi Microplate Multimode Reader
- Absorbance Optical Module
- Cortisol EIA Assay Kit (Monobind Cat.# 3625-300), including:
  - **Human Serum references,1 mL/vial.** Six vials of serum reference for Cortisol at concentrations of 0 (A), 1.0 (B), 4.0 (C), 10.0 (D), 20.0 (E), and 50.0 (F) µg/dL. A preservative has been added. Store at 2 - 8°.
  - **Cortisol Enzyme Reagent, 1 mL/vial**. One vial of Cortisol (Analog)-horseradish peroxides (HRP) conjugate in a protein-stabilizing matrix with blue dye. Store at 2-8°C.
  - **Cortisol Conjugate Buffer, 7 mL/vial.** One vial of reagent containing buffer,red dye, preservative, and binding protein inhibitors. Store at 2-8°C.
  - **Cortisol Biotin Reagent, 7 mL bottle.** One bottle of reagent containing anti-cortisol biotinylated mIgG conjugate in buffer, green dye, and preservative. Store at 2 - 8° C.
  - **Streptavidin-Coated Plate. 96-well plate.** One 96-well microplate coated with 1.0 µg/mL streptavidin and packaged in an aluminum bag with drying agent. Store at 2 - 8° C.
  - **Wash Solution, 20 mL vial.** One vial containing a surfactant in buffered saline. A preservative has been added. Store at 2 - 8°C.
  - **Substrate A, 7 mL/vial.** One vial containing tetramethylbenzidine (TMB) in buffer. Store at 2 - 8° C.
  - **Substrate B, 7 mL/vial.** One vial containing hydrogen peroxide (H_2O_2) in buffer. Store at 2 - 8° C.
  - **Stop Solution, 8 mL/vial.** One vial containing a strong acid (11N HCL). Store at 2 - 30° C.

PRINCIPLE

Competitive Enzyme Immunoassay (Type 7)

The essential reagents required for an enzyme immunoassay include: antibody, enzyme-antigen conjugate and native antigen Mixing together biotinylated antibody, enzyme-antigen conjugate and a serum containing the native antigen, results in a competitive reaction between the native antigen and the enzyme-antigen conjugate;a limited number of antibody binding sites are affected. The interaction is illustrated by the following equation:

$$^{Enz}Ag + Ag + Ab_{Btn} \underset{k_{-a}}{\overset{k_a}{\rightleftharpoons}} AgAb_{Btn} + {}^{Enz}AgAb_{Btn}$$

$Ab_{C.W.}$= Monospecific Immolilized Antibody (Constant Quantity)
Ag = Native Antigen (Variable Quantity)
^{Enz}Ag = Enzyme-Antigen Conjugate (Constant Quantity)
$AgAb_{Btn}$ = Antigen-Antibody Complex
$^{Enz}AgAb_{Btn}$ = Enzyme-Antigen Conjugate-Antibody Complex
k_a = Rate of Constant Association
k_{-a} = Rate of Constant Disassociation
$K = k_a/k_{-a}$ = Equilibrium Constant

A simultaneous reaction occurs between the biotin attached to the antibody and the streptavidin immobilized on the microwell. This effects the separation of the antibody-bound fraction after decantation or aspiration.

$AgAb_{Btn} + {}^{Enz}AgAb_{Btn} + \underline{Streptavidin_{CW}} \rightarrow \underline{Immobilized\ complex}$
$\underline{Streptavidin_{CW}}$ = Streptavidin immobilized on well
Immobilized complex = sandwich complex bound to the solid surface

The enzyme activity in the antibody-bound fraction is inversely proportional to the native antigen concentration. Ascertain unknown antigen concentrations by utilizing various serum references of known antigen concentrations. Use this data to generate a dose-response curve.

EXPERIMENTAL PROTOCOL

Specimen Collection and Preparation

All specimens must be blood, either serum or plasmaand observe the usual precautions of venipuncture sample procurement.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) or in an evacuated tube containing EDTA or heparin. Allow the blood to clot for serum 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 days. If the specimen(s) cannot be assayed within this time, sample(s) may be stored at temperatures of -20°for up to 30 days. Avoid repetitive freezing and thawing. When assayed in duplicate, 50 µL of specimen is required.

Reagent Preparation

Working Enzyme Reagent

Add 0.7 ml of Cortisol Enzyme Reagent to the vial containing Cortisol Enzyme Buffer. Store at 2-8° C.

Wash Buffer

Dilute contents of wash solution to 1000 ml with distilled or deionized water in a suitable storage container. Store at room temperature (20 - 27° C) for up to 60 days.

Working Substrate Solution

Pour the contents of the amber vial labeled “Solution A” into the clear vial labeled “Solution B.” Place the yellow cap on the clear vial for easy identification. Mix and label accordingly. Store at 2-8° C.

Test Procedure

Before proceedingwith the assay, bring all reagents, serum references, and controls to room temperature (20-27°C).

1. Format microplate wells for each serum reference, control, and patient specimen to be assayed in duplicate.

   Note: Replace any unused microwell strips into the sealed aluminum bag and store at 2 - 8° C.

2. Pipette 25 µL of the appropriate serum reference, control, or specimen into the assigned well.

3. Add 50 µL of the working Cortisol Enzyme Reagent to all wells (see Reagent Preparation Section).

4. Swirl the microplate gently for 20 - 30 seconds to mix.

5. Add 50 µL of the Cortisol Biotin Reagent to all wells.

6. Swirl the microplate gently for 20 - 30 seconds to mix.

7. Cover and incubate for 60 minutes at room temperature.

8. Discard microplate contents by decantation or aspiration. If decanting, blot the plate dry with absorbent paper.

9. Add 300 µL of wash buffer (see Reagent Preparation Section), decant (tap and blot) or aspirate. Repeat two additional times for a total of three washes.

10. Add 100 µL of working substrate solution to all wells (see Reagent Preparation Section).

    Note: To minimize reaction time difference between wells, always add reagents in the same order.

    Note: Do not shake the plate after substrate addition.

11. Incubate at room temperature for 15 minutes.

12. Add 50 µL of stop solution to each well and gently mix for 15-20 seconds.

13. Using the GloMax® Multi Microplate Reader, determine the absorbance in each well at 450 nm. Use a reference wavelength of 650 nm to minimize well imperfections.

    Note: Results should be read within 30 minutes of adding the stop solution.

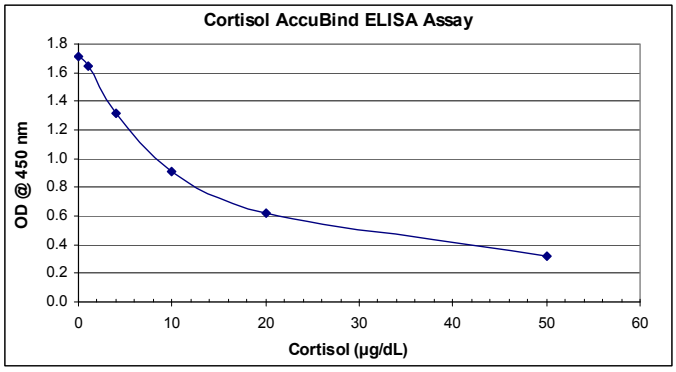

Figure 1. Dose-response curve of cortisol standards performed using the GloMax® Multi Microplate Absorbance Reader.

REFERENCES

1. Burtis, C.A., Ashweed, E.R. *Tietz Textbook of Clinical Chemistry*. Philadelphia: W.B. Saunders Company, 2nd edition, 1994: 1825 - 1827.

2. Foster, L., Dunn, R. “Single antibody technique for radioimmunoassay of cortisol in unextracted serum or plasma.” *Clin. Chem.*20 (1974): 365.

3. *Williams Textbook of Endocrinology*. Edited by Wilson, J.D., Foster, D.W. Philadelphia: WB Saunders, 7th edition, 1985.

4. Monobind Data Sheet 3625-300: Monobind Cortisol EIA Kit.

RESULTS

Sensitivity:< 0.25 µg/dL of cortisol (data not shown)

Instrument Dynamic Range: 0 – 4.0 OD at 450–700 nm

CONCLUSION

The GloMax® Multi Microplate offers both superior sensitivity and dynamic range. The GloMax® Multi Microplate Absorbance Reader provides measurements that are highly sensitive and cover a wide dynamic range. Superior performance is achieved by use of a large-area photodiode. The instrument can detect linear dynamic ranges from 0 – 4.0 OD, allowing

undiluted samples of both low and high OD to be read simultaneously in the same microplate. The modular approach of the GloMax® Multi Microplate Multimode reader allows for instrument capability expansion as needs in the lab change. Luminescence and/or Fluorescence Detection Modules as well as other accessories can be added after the initial purchase.

Superior performance, ease of use, and the utmost flexibility of the GloMax® Multi Microplate make it an ideal microplate reader for today's life science laboratory.

WARNINGS AND PRECAUTIONS

Note: For *in vitro* diagnostic use only. 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 and 2, and HCV Antibodies per tests required by the FDA. Since no known test can offer complete assurance that infectious agents are absent, all human serum products should be handled as if 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 publication entitled *Biosafety in Microbiological and Biomedical Laboratories* HHS Publication No. (CDC), 2nd Edition (1988): 88 - 8395.

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