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Cortisol 20,000 tests

For in vitro research use only
Storage temperature : 2-8°C

www.htrf.com

HTRF® package insert

Document reference : 62CO2PEC rev01 (July 2008)

Packaging details :

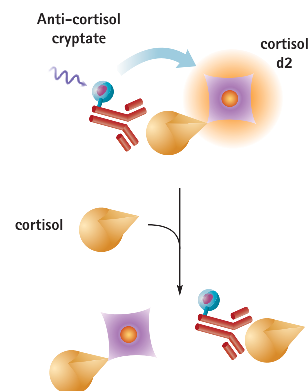
	384-well low volume plate (20 µL)
62CO2PEC	20,000 tests

1. Assay description and intended use

This kit is intended for quantitative determination of cortisol in buffered solution or in cell culture supernatants.

Its principle is based on HTRF® technology (Homogeneous Time-Resolved Fluorescence). The method is a competitive immunoassay in which native cortisol produced by cells and d2-labeled cortisol, compete for binding to a monoclonal anti-cortisol antibody labeled with europium-Cryptate.

The specific signal (i.e. energy transfer) is inversely proportional to the concentration of cortisol in the calibrator or in the sample.



2. Background

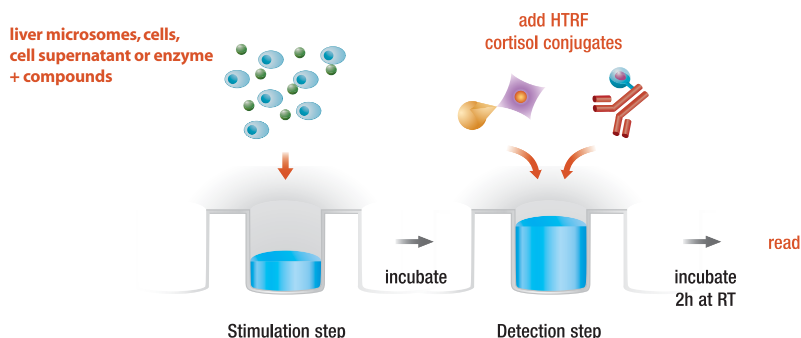
Cortisol (MW 362.5), also called hydrocortisone or compound F, is the major glucocorticoid produced by the adrenal cortex. As other adrenal steroids, cortisol synthesis starts from cholesterol which is modified through a series of enzymatic steps. The first enzymatic and rate-limiting step of steroidogenesis from cholesterol is modulated by the adrenocorticotropic hormone (ACTH) produced by the pituitary, which is in turn regulated by hypothalamic corticotropin releasing factor (CRF). As a negative feedback, cortisol controls steroidogenesis through the hypothalamic-pituitary-adrenal axis. As cortisol levels in blood rise, ACTH and CRF secretion is inhibited. In plasma, only a small amount of cortisol circulates freely as most of the molecules are bound to either corticosteroid-binding globulin (CBG) or albumin.

Cortisol acts through specific intracellular receptors and modulates numerous physiological processes including regulation of carbohydrate metabolism, electrolyte balance, vascular tone, bone metabolism. Cortisol plays also a role in inflammation, hypersensitivity, immunosupresion , and disease resistance. Cortisol production has an ACTH-dependent circadian rhythm with a peak level in the morning and a nadir at night. However cortisol levels can be influenced by stress.

Elevated levels of cortisol have been described in patients with cushing's disease (ACTH hypersecretion) or adrenal tumors. Low cortisol level stems primarily from adrenal insufficiency (Addison's disease).

3. Biochemical or cell based assay protocol

In case of supernatant assay protocol, the assay must be run in two distinct microplates :i) a culture plate for cell stimulation and ii:) an assay plate in which cortisol detection is carried out.



3.1. Supplied reagents

Allow the reagents to come to room temperature for at least 30 minutes before reconstitution.

Supplied reagents	Reagent reconstitution (stock solutions)		Working solutions
Anti-cortisol-Cryptate** 1 vial, lyophilized*	Reconstitute each vial with 5 mL of distilled water. Mix gently.	⇨	For each vial dilute 1 volume of reconstituted reagent in 19 volumes of reconstitution buffer (e.g. for 10,000 tests: 2.5 mL of reconstituted reagent + 47.5 mL of reconstitution buffer). Mix gently.
Cortisol-d2 1 vial, lyophilized			
Cortisol calibrator. Concentrated cortisol. 1 vial, lyophilized	See label indication for reconstitution volume and concentration. Mix gently after reconstitution	⇨	See calibration curve preparation for further dilution
Cortisol control. Concentrated cortisol. 1 vial, lyophilized		⇨	To be used directly after reconstitution

Reconstitution buffer (200 mL) 50 mM Phosphate buffer, pH 7.0, 0.8M KF, 0.2 % BSA
Diluent (20 mL) 50 mM Phosphate buffer, pH 7.0, 0.2 % BSA, 0.02 % NaN_3, preservatives

* All reagents were lyophilized in 50 mM phosphate buffer, pH 7.0, containing protease free BSA and stabilizers.
** The Cryptate conjugate concentration was optimized for a maximum assay sensitivity and to ensure an average counting of 40,000 cps at 620 nm (384-well low volume format), using the reference PHERAstar Plus reader (BMG LABTECH).

Precaution : HTRF® reagent concentrations have been set for optimal assay performances. Note that any dilution or improper use of the d2 and Cryptate-conjugates will impair the assay's quality.

3.2. Reagent storage and stability

All reagents should be stored at 2-8°C until reconstituted. Under proper storage conditions, they are stable until the expiry date indicated on the labels.

Reconstituted reagents and working solutions are stable for seven days at 4°C. They can be refrozen (at -80°C) and thawed twice.

3.3. Calibration curve preparation

Reconstitute the maximum calibrator according to the indications printed on the label and follow the dilution sequence shown in the following table to draw up the calibration curve. Dilution must be carried out with the diluent (or with freshly made PO_4 50 mM, BSA 0.2% pH 7.0).

Calibrator	Calibrator concentration in ng/mL	Preparation
Cal 6	100	100 µL reconstituted calibrator + 400 µL diluent
Cal 5	25	100 µL Cal 6 + 300 µL diluent
Cal 4	6.25	100 µL Cal 5 + 300 µL diluent
Cal 3	1.56	100 µL Cal 4 + 300 µL diluent
Cal 2	0.39	100 µL Cal 3 + 300 µL diluent
Cal 1	0.097	100 µL Cal 2 + 300 µL diluent
Cal 0	0	300 µL diluent

3.4. Sample preparation

Dilute all samples to be assayed with the diluent (or with freshly made PO4 50 mM, BSA 0.2% pH 7.0). Consecutive dilutions should be made within the 0-100 ng/mL range (working solution).

3.5. Assay protocol for 384-well low volume plate (20 µL)

Dispense the reagents in the following order :
· 10 µL standard or sample *
· 5 µL cortisol-d2
· 5 µL anti-cortisol Cryptate
** For negative control, replace the first reagent by 10 µL of diluent and cortisol-d2 by 5 µL of reconstitution buffer*
** For positive control, replace the standard by 10 µL of diluent*

· Cover the plate with a plate sealer and incubate for 2 hours at room temperature.

· Remove the plate sealer and read on a compatible HTRF® reader (more information about compatible reader at www.htrf.com/technology/htrfmeasurement/compatible_readers).

3.6. Data reduction and example of a calibration curve

This data should not be substituted for that obtained in the laboratory

	A (665nm)	B (620nm)	Ratio (1)	Mean Ratio (2)	CV % (3)	Delta F % (4)
Negative control	1,835 1,759	43,701 42,939	420 410	415	1.7	
[calibrator] ng/mL						
0 positive control	103,751 106,026	33,990 35,029	30,524 30,268	30,396	0.6	7,228
0.1	98,697 95,779	36,030 34,298	27,393 27,926	27,659	1.4	6,569
0.39	87,098 89,301	35,400 36,618	24,604 24,387	24,496	0.6	5,806
1.56	58,160 61,937	37,542 39,032	15,492 15,868	15,680	1.7	3,680
6.25	29,111 28,488	41,478 40,909	7,018 6,964	6,991	0.6	1,586
25	10,106 10,083	41,942 41,722	2,410 2,417	2,413	0.2	482
100	4,249 4,194	43,399 44,182	979 949	964	2.2	132
Cortisol control	72,649 72,570	38,193 38,426	19,022 18,886	18,954	0.5	4,470

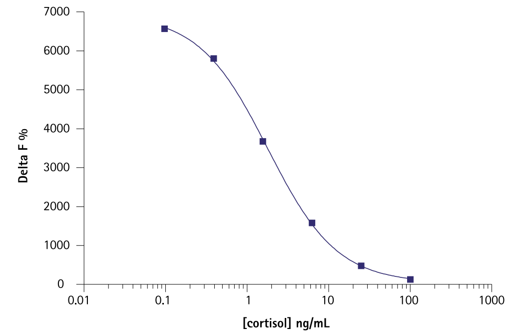
1. $\text{Ratio} = \frac{A_{665nm}}{B_{620nm}} \times 10^4$

2. $\text{Mean Ratio} = \frac{\Sigma \text{ratios}}{2}$

3. $CV = \frac{\text{Std deviation}}{\text{Mean ratio}} \times 100$

4. $\text{Delta F} = \frac{\text{Calibrator or sample Ratio} - \text{Ratio}_{neg}}{\text{Ratio}_{neg}} \times 100$

(Ratio_{neg} = negative control)



3.7. Detection limit and conversion to nmol/L

This data has been obtained using the reference RYBYstar reader (BMG Labtech), after one hour incubation RT.

Detection limit (dose of mean zero-2SD)	EC50 (cortisol concentration which allows the displacement of 50% of binding)	Signal to background (S/B)
0.08 ng/mL	≈ 2,3 ng/mL	≈ 30

(1 ng/mL = ≈ 2,758 mmol/L)

3.8. Cross-reactivity

	Cross-reactivity (%)
Cortisol	100
Prednisolone	5.7
Cortisone	0.9
11-deoxycortisol	27.6
Progesterone	2.7
Prednisone	< 0.3
Testosterone	< 0.002
21-deoxycortisol	13.1
DHEA	< 0.02
Corticosterone	7
Dexamethasone	< 0.0007

3.9 Assay flexibility and miniaturization

When used as suggested, the kit will provide sufficient reagents for 20,000 tests using a 384- well low volume plate in 20 µL final assay volume (HTRF® packaged basis).

To move to other plate formats (96 half-well or 1536-well) and final volumes (100 µL to less than 10 µL), the volume of each assay component is simply proportionally adjusted in order to maintain the reagent concentrations as for the 20 µL final assay volume. For instance, in the case of the 1536-well format in 10 µL final volume, half as much material per well is used, thereby allowing 40,000 tests to be run. The performances of the HTRF® assay remain the same whatever the level of miniaturization.

Assay components	Volume proportion	Assay format 1536-well (10 µL)	 384-well low volume (20 µL)	 96 half-well (100 µL)
Sample	2 volumes	5 µL	10 µL	50 µL
d2 conjugate	1 volume	2.5 µL	5 µL	25 µL
Cryptate conjugate	1 volume	2.5 µL	5 µL	25 µL
	Bulk size	40,000 tests	20,000 tests	4,000 tests

Plate references : 96 half-well plate (Costar # 3694 or equivalent), 384-well low volume plate (Greiner # 784076), 1536-well (Greiner # 782086)

4. Case study: quantification of 11 beta-hydroxysteroid-dehydrogenase type 1 in microsomes

4.1. Calibration curve preparation

Reconstitute the maximum calibrator with the buffer used for the dilution of the microsomes (e.g. Tris 20 mM EDTA 5 mM pH=6) . The volume of reconstitution is indicated on the product label. Follow the same dilution sequence as shown in the table in § 3.3., replacing the diluent by tris 20 mM EDTA 5mM pH=6.

4.2. Sample preparation

A 11b-HSD1 microsomal suspension (0.5 mg/mL) is prepared in tris 20 mM, EDTA 5 mM pH=6.

4.3. Biochemical assay protocol for 384-well low volume plate

Dispense the reagents in the following order:

- 6 µL enzymatic buffer (tris 20 mM EDTA 5mM pH=6 supplemented with NADPH 333 µM, Cortisone 266 nM)
- 2 µL compound in tris 20 mM EDTA 5 mM pH=6
- 2 µL microsomal preparation in tris 20 mM EDTA 5 mM pH=6

Incubate the plate for 2 hours at 37°C

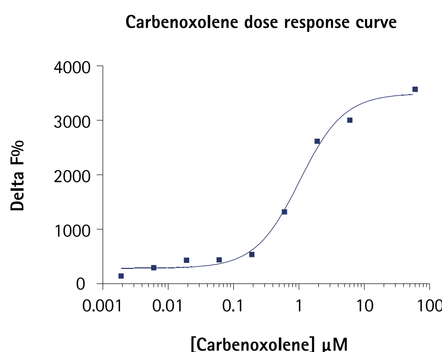
- 5 µL cortisol-d2
- 5 µL anti-cortisol-Cryptate

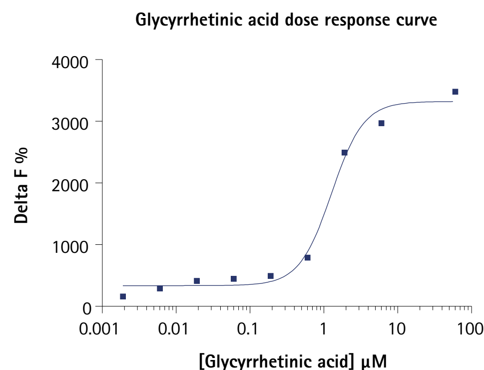
Incubate the plate for 2 hours at room temperature.

Read on a compatible HTRF® reader (more information about compatible reader at www.htrf.com/technology/htrfmeasurement/compatible_readers).

4.4. Dose response curves of 2 known inhibitors

Two inhibitors of the cortisol production (Carbenoxolene and Glycyrrhetinic acid) are tested at different concentrations in order to establish the dose response curves.





	IC_{50} µM
Carbenoxolene	1.03
Glycyrrhetinic acid	1.17

The data shows that this assay is particularly adapted to the 11b-HSD1 inhibitors sreening in microsomal suspension.

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