Parameter™

Corticosterone Assay

Catalog Number KGE009

For the quantitative determination of Corticosterone concentrations in cell culture supernates, serum, plasma, and urine.

This package insert must be read in its entirety before using this product.
For research use only. Not for use in diagnostic procedures.

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USA & Canada | R&D Systems, Inc.
614 McKinley Place NE, Minneapolis, MN 55413, USA
TEL: (800) 343-7475 (612) 379-2956 FAX: (612) 656-4400
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DISTRIBUTED BY:

UK & Europe | R&D Systems Europe, Ltd.
19 Barton Lane, Abingdon Science Park, Abingdon OX14 3NB, UK
TEL: +44 (0)1235 529449 FAX: +44 (0)1235 533420
E-MAIL: info@RnDSystems.co.uk

China | R&D Systems China Co., Ltd.
24A1 Hua Min Empire Plaza, 726 West Yan An Road, Shanghai PRC 200050
TEL: +86 (21) 52380373 FAX: +86 (21) 52371001
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INTRODUCTION

Corticosterone is the primary glucocorticoid steroid hormone in rodents, birds, amphibians, and reptiles, and is also present in significant amounts in sheep, pigs, and dogs (1). In humans, Corticosterone has weak glucocorticoid activity and instead acts as an intermediate in the steroidogenic pathway leading to Aldosterone synthesis. Glucocorticoids regulate diverse biological processes in nearly all cell types and are critical mediators of the physiological stress response (2).

Glucocorticoids are synthesized *de novo* from Cholesterol by steroidogenic enzymes. They are primarily synthesized in the adrenal cortex but can also be synthesized from steroid precursors by steroidogenic enzymes present in some peripheral tissues (3). Like all steroid hormones, glucocorticoids are released into the circulation. While in the circulation, the majority of glucocorticoid molecules, up to 95%, are bound by Corticosteroid Binding Protein (CBP), which limit the bioavailability of the steroid (4). Glucocorticoids not bound by CBP freely diffuse from the blood stream into peripheral tissues and across the cellular plasma membrane. The primary mechanism of glucocorticoid action is through the activation of the cytosolic Glucocorticoid Receptor (GR/NR3C1), a member of the superfamily of ligand-binding nuclear receptor transcription factors (5). Corticosterone also binds and activates a membrane-localized receptor to initiate rapid intracellular signaling (6-8).

Serum glucocorticoid concentrations follow circadian patterns. They can show a 3-5 fold change over a 24-hour period and are highest just prior to waking (1). Glucocorticoids are also released in response to emotional or physical stress and mediate both the acute and chronic stress responses. In humans, Cushing's Syndrome is associated with hypersecretion of glucocorticoids, while the autoimmune Addison's Disease is associated with insufficient glucocorticoid secretion (9, 10).

The Parameter Corticosterone assay is a 3.5 hour competitive enzyme immunoassay designed to measure Corticosterone in cell culture supernates, serum, plasma, and urine.

PRINCIPLE OF THE ASSAY

This assay is based on the competitive binding technique. A polyclonal antibody specific for Corticosterone becomes bound to the donkey anti-sheep antibody coated onto the microplate. Following a wash to remove excess polyclonal antibody, Corticosterone present in a sample competes with a fixed amount of horseradish peroxidase (HRP)-labeled Corticosterone for sites on the polyclonal antibody. This is followed by another wash to remove excess conjugate and unbound sample. A substrate solution is added to the wells to determine the bound enzyme activity. The color development is stopped and the absorbance is read at 450 nm. The intensity of the color is inversely proportional to the concentration of Corticosterone in the sample.

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- The kit should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.
- If samples generate values higher than the highest standard, further dilute the samples with Calibrator Diluent and repeat the assay.
- Any variation in standard diluent, operator, pipetting technique, incubation time or temperature, and kit age can cause assay variability.
- Variations in sample collection, processing, and storage may cause sample value differences.
- This assay is designed to eliminate interference by other factors present in biological samples. Until all factors have been tested in this assay, the possibility of interference cannot be excluded.
- **Samples containing sheep IgG may interfere with this assay.**

TECHNICAL HINTS

- When mixing or reconstituting protein solutions, always avoid foaming.
- To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
- Pre-rinse the pipette tip when pipetting.
- Pipette standards and samples to the bottom of the wells. Add all other reagents to the side of the wells to avoid contamination.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- When using an automated plate washer, adding a 30 second soak period following the addition of wash buffer, and/or rotating the plate 180 degrees between wash steps may improve assay precision.
- Substrate Solution should remain colorless until added to the plate. Keep Substrate Solution protected from light. Substrate Solution should change from colorless to gradations of blue.
- Stop Solution should be added to the plate in the same order as the Substrate Solution. The color developed in the wells will turn from blue to yellow upon addition of the Stop Solution.

MATERIALS PROVIDED & STORAGE CONDITIONS

Store unopened kit at 2-8 °C. Do not use past kit expiration date.

PART	PART #	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Donkey anti-sheep IgG Microplate	894823	96 well polystyrene microplate (12 strips of 8 wells) coated with a donkey anti-sheep IgG antibody.	Return unused wells to the foil pouch containing the desiccant pack. Reseal along entire edge of the zip-seal. May be stored for up to 1 month at 2-8 °C.*
Corticosterone Standard	893566	2 vials of Synthetic Corticosterone in a buffered protein base with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	Discard after use. Use a fresh standard for each assay.
Corticosterone Conjugate	893564	6 mL of Corticosterone conjugated to horseradish peroxidase with red dye and preservatives.	May be stored for up to 1 month at 2-8 °C.*
Corticosterone Primary Antibody Solution	893565	6 mL of sheep polyclonal antibody to Corticosterone in a buffered protein base with blue dye and preservatives.	
Calibrator Diluent RD5-43	895903	21 mL of a buffered protein base with preservatives.	
Pretreatment E	895996	11 mL of 0.6 N Trichloroacetic acid	
Pretreatment F	895496	6 mL of buffer with preservatives.	
Wash Buffer Concentrate	895003	21 mL of a 25-fold concentrated solution of buffered surfactant with preservative. *May turn yellow over time.*	
Color Reagent A	895000	12 mL of stabilized hydrogen peroxide.	
Color Reagent B	895001	12 mL of stabilized chromogen (tetramethylbenzidine).	
Stop Solution	895926	11 mL of 2 N sulfuric acid.	
Plate Sealers	N/A	4 adhesive strips.	

* Provided this is within the expiration date of the kit.

OTHER SUPPLIES REQUIRED

- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm.
- Pipettes and pipette tips.
- Deionized or distilled water.
- Squirt bottle, manifold dispenser, or automated microplate washer.
- 500 mL graduated cylinder.
- Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 500 ± 50 rpm.
- **Polypropylene** test tubes for dilution of standards and samples.
- Corticosterone Controls (optional; R&D Systems, Catalog # QC205).

SAMPLE COLLECTION & STORAGE

The sample collection and storage conditions listed below are intended as general guidelines. Sample stability has not been evaluated.

Cell Culture Supernates - Remove particulates by centrifugation. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Human Serum - Use a serum separator tube (SST) and allow samples to clot for 30 minutes at room temperature before centrifugation for 15 minutes at 1000 x g. Remove serum and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Mouse/Rat Serum - Allow blood samples to clot for 2 hours at room temperature before centrifuging for 20 minutes at 2000 x g. Remove serum and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Human Plasma - Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge for 15 minutes at 1000 x g within 30 minutes of collection. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Mouse/Rat Plasma - Collect plasma using EDTA as an anticoagulant. Centrifuge for 20 minutes at 2000 x g within 30 minutes of collection. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Note: *Mouse/rat heparin plasma has not been validated for use in this assay.*
Human/mouse/rat citrate plasma has not been validated for use in this assay.

Human Urine - Aseptically collect the first urine of the day (mid-stream), voided directly into a sterile container. Centrifuge to remove particulate matter, and assay immediately or aliquot and store at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Mouse/Rat Urine - Collect urine using a metabolic cage. Remove any particulates by centrifugation and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles. Centrifuge again before assaying to remove any additional precipitates that may appear after storage.

PRECAUTIONS

The Stop Solution and Pretreatment E provided in this kit are acid solutions.

Some components in this kit contain ProClin® which may cause an allergic skin reaction. Avoid breathing mist.

Color Reagent B may cause skin, eye, and respiratory irritation. Avoid breathing fumes.

Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Please refer to the MSDS on our website prior to use.

SAMPLE PREPARATION

Human urine samples require a 10-fold dilution. A suggested 10-fold dilution is 30 µL of sample + 270 µL of Calibrator Diluent RD5-43.

Mouse and rat urine samples require a 100-fold dilution. A suggested 100-fold dilution is 10 µL of sample + 990 µL of Calibrator Diluent RD5-43.

Note: *If assaying mouse or rat urine samples, more Calibrator Diluent may be needed to complete the dilution listed above. Please contact Technical Service to receive an additional vial of Calibrator Diluent RD5-43.*

SAMPLE PRETREATMENT

Note: *For serum and plasma samples only. Sample pretreatment removes potentially interfering proteins and protein-bound Corticosterone.* **Samples must be assayed within 8 hours after pretreatment.**

1. **For human samples**, add 300 µL of sample and 300 µL Pretreatment E to a microcentrifuge tube. **For mouse/rat samples**, add 150 µL of sample and 150 µL Pretreatment E to a microcentrifuge tube.The tubes will have a precipitate. Mix well.
2. Incubate for 15 minutes at room temperature.
3. Centrifuge at ≥ 12,000 x g for 4 minutes.
4. Carefully **decant** and retain the supernate. The supernate may be cloudy.
5. The concentration read off the standard curve must be multiplied by the dilution factor, 2.

Note: *Mouse and rat serum and plasma samples may require further dilution due to endogenous levels (see Sample Values section). If further dilution is required, use Calibrator Diluent RD5-43 to perform the dilution.*

REAGENT PREPARATION

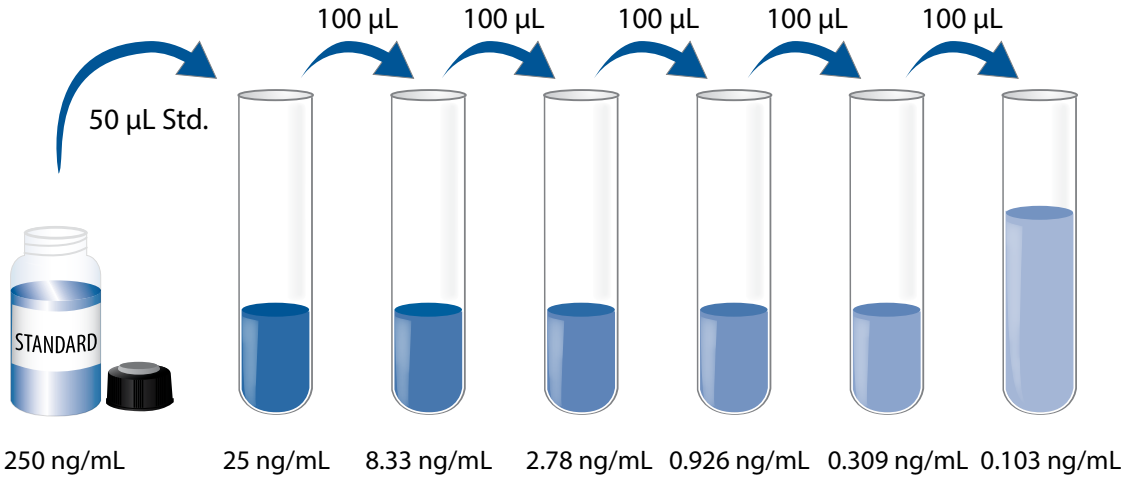
Bring all reagents to room temperature before use.

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to deionized or distilled water to prepare 500 mL of Wash Buffer.

Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200 µL of the resultant mixture is required per well.

Corticosterone Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Corticosterone Standard with deionized or distilled water. This reconstitution produces a stock solution of 250 ng/mL. Allow the standard to sit for a minimum of 15 minutes with gentle mixing prior to making dilutions.

Use polypropylene tubes. Pipette 450 µL of Calibrator Diluent RD5-43 into the 25 ng/mL tube. Pipette 200 µL into the remaining tubes. Use the stock solution to produce a 3-fold dilution series (below). Mix each tube gently but thoroughly before the next transfer. The 25 ng/mL standard serves as the high standard. Calibrator Diluent RD5-43 serves as the zero standard (0 ng/mL).



ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all samples, controls, and standards be assayed in duplicate.

Note: *Serum and plasma samples must be assayed within 8 hours after pretreatment.*

1. Prepare all reagents, working standards, and samples as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.
3. Add 50 µL of Corticosterone Primary Antibody Solution to each well **(excluding the NSB wells)**. Cover with the adhesive strip provided. Incubate for 1 hour at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 500 ± 50 rpm.
4. Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with Wash Buffer (400 µL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
5. Add 100 µL of Pretreatment F to each well.
6. Add 50 µL of Standard, control, or sample* to the appropriate wells.
7. Add 50 µL of Calibrator Diluent RD5-43 to the zero standard (B_0) wells and NSB wells.
8. Add 50 µL of the Corticosterone Conjugate to all wells. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature on the shaker.
9. Repeat the aspiration/wash as in step 4.
10. Add 200 µL of Substrate Solution to each well. Incubate for 30 minutes at room temperature **on the benchtop. Protect from light.**
11. Add 100 µL of Stop Solution to each well. The color in the wells should change from blue to yellow. If the color change does not appear uniform, gently tap the plate to ensure thorough mixing.
12. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

*Samples may require dilution or pretreatment. See Sample Preparation or Sample Pretreatment section.

CALCULATION OF RESULTS

Average the duplicate readings for each standard, control, and sample then subtract the average NSB optical density (O.D.).

Create a standard curve by reducing the data using computer software capable of generating a four paramater logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on a linear y-axis against the concentration on a logarithmic x-axis and draw the best fit curve through the points on the graph. Do not include the B_0 in the standard curve.

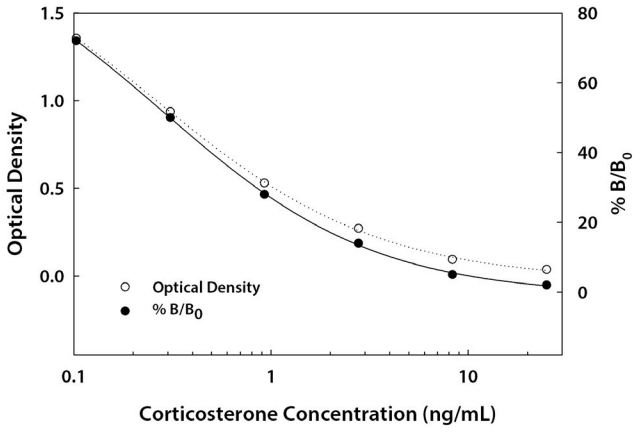
If desired, % B/B_0 can be calculated by dividing the corrected O.D. for each standard or sample by the corrected B_0 O.D. and multiplying by 100.

Calculate the concentration of Corticosterone corresponding to the mean absorbance from the standard curve.

If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

TYPICAL DATA

This standard curve is provided for demonstration only. A standard curve should be generated for each set of samples assayed.



(ng/mL)	O.D.	Average	Corrected	% B/B_0
NSB	0.012 0.018	0.015	——	——
0 (B_0)	1.878 1.923	1.901	1.886	——
0.103	1.347 1.395	1.371	1.356	72
0.309	0.942 0.961	0.952	0.937	50
0.926	0.531 0.559	0.545	0.530	28
2.78	0.271 0.300	0.286	0.271	14
8.33	0.100 0.118	0.109	0.094	5
25	0.049 0.055	0.052	0.037	2

PRECISION

Intra-assay Precision (Precision within an assay)
Three samples of known concentration were tested twenty times on one plate to assess intra-assay precision.

Inter-assay Precision (Precision between assays)
Three samples of known concentration were tested in twenty separate assays to assess inter-assay precision. Assays were performed by at least three technicians using two lots of kit components.

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	20	20	20
Mean (ng/mL)	1.24	3.98	8.05	1.39	4.08	8.21
Standard deviation	0.078	0.179	0.605	0.099	0.241	0.462
CV (%)	6.3	4.5	7.5	7.1	5.9	5.6

RECOVERY

The recovery of Corticosterone spiked to levels throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range
Cell culture media (n=4)	114	107-124%
Human serum* (n=4)	110	92-130%
Human EDTA plasma* (n=4)	108	87-127%
Human heparin plasma* (n=4)	107	97-122%
Human urine* (n=4)	105	92-121%
Mouse serum* (n=4)	96	79-113%
Mouse EDTA plasma* (n=4)	104	96-111%
Rat serum* (n=4)	99	87-108%
Rat EDTA plasma* (n=4)	98	77-114%

*Samples were diluted or pretreated prior to assay.

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of Corticosterone were diluted with Calibrator Diluent to produce samples with values within the dynamic range of the assay.

Human Samples		Cell culture media (n=4)	Serum* (n=4)	EDTA plasma* (n=4)	Heparin plasma* (n=4)	Urine* (n=4)
1:2	Average % of Expected	94	87	91	92	94
	Range (%)	89-99	82-92	85-96	88-97	91-98
1:4	Average % of Expected	86	82	86	86	96
	Range (%)	79-91	79-87	82-90	83-88	88-110
1:8	Average % of Expected	81	76	79	82	103
	Range (%)	76-84	72-79	77-81	79-83	92-114

Mouse Samples		Serum* (n=4)	EDTA plasma* (n=4)	Urine* (n=4)
1:2	Average % of Expected	101	98	101
	Range (%)	94-108	96-100	97-106
1:4	Average % of Expected	99	97	103
	Range (%)	85-105	92-102	99-108
1:8	Average % of Expected	99	98	114
	Range (%)	89-109	89-105	109-121

Rat Samples		Serum* (n=4)	EDTA plasma* (n=4)	Urine* (n=4)
1:2	Average % of Expected	98	101	98
	Range (%)	90-104	91-115	91-111
1:4	Average % of Expected	92	99	96
	Range (%)	81-97	96-113	86-102
1:8	Average % of Expected	95	109	102
	Range (%)	83-101	99-120	85-113

*Samples were diluted or pretreated prior to assay.

SENSITIVITY

Thirty-eight assays were evaluated and the minimum detectable dose (MDD) of Corticosterone ranged from 0.012-0.047 ng/mL. The mean MDD was 0.028 ng/mL.

The MDD was determined by subtracting two standard deviations from the mean optical density value of twenty zero standard replicates and calculating the corresponding concentration.

SAMPLE VALUES

Serum/Plasma/Urine - Samples from apparently healthy volunteers were evaluated for the presence of Corticosterone in this assay. No medical histories were available for the donors used in this study.

Human Samples	Mean (ng/mL)	Range (ng/mL)	Standard Deviation (ng/mL)
Serum (n=35)	1.43	0.428-2.74	0.646
EDTA plasma (n=35)	1.06	0.282-3.65	0.813
Heparin plasma (n=35)	1.19	0.328-2.92	0.618
Urine (n=11)	13.9	2.3-44.0	14.0

Mouse Samples	Mean (ng/mL)	Range (ng/mL)	Standard Deviation (ng/mL)
Serum (n=10)	106	59.2-154	33.5
EDTA plasma (n=10)	54.1	12.5-114	35.5
Urine (n=10)	127	82.0-169	28.0

Rat Samples	Mean (ng/mL)	Range (ng/mL)	Standard Deviation (ng/mL)
Serum (n=10)	69.5	13.7-127	38.7
EDTA plasma (n=10)	61.7	47.0-101	16.6
Urine (n=10)	207	79.0-513	120

Cell Culture Supernates - Human peripheral blood lymphocytes (1 x 10^6 cells) were cultured in DMEM supplemented with 5% fetal calf serum, 50 mM β-mercaptoethanol, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL of streptomycin sulfate. Cells were cultured unstimulated or stimulated with 10 µg/mL PHA for 1 and 5 days. Aliquots of the cell culture supernates were removed and assayed for levels of Corticosterone. No detectable levels were observed.

SPECIFICITY

The factors listed below were prepared at 250 ng/mL in Calibrator Diluent and assayed for cross-reactivity. Preparations of the following factors at 250 ng/mL in a mid-range Corticosterone control were assayed for interference. No significant cross-reactivity or interference was observed.

Substance:

Andosterone
Cortisone
Esterone
Estradiol
Estriol
Prednisone
Testosterone

The factors listed below showed cross-reactivity:

Substance	% Cross-Reactivity
Progesterone	0.10
Aldosterone	0.31
Hydrocortisone	0.09
Deoxycorticosterone	0.08
Prednisolone	0.11
Dexamethasone	0.04

The following factors showed interference:

Substance	Concentration
Aldosterone	> 62.5 ng/mL
Corticosteroid Binding Protein (untreated)	> 20,000 ng/mL
Progesterone	> 125 ng/mL

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PLATE LAYOUT

Use this plate layout to record standards and samples assayed.