rCorticosterone [^{125}I] RIA KIT

(REF: RK-548)

For Research Use Only. Not for use in diagnostic procedures.

The [^{125}I]rCorticosterone RIA system provides direct quantitative *in vitro* determination of rat Corticosterone in plasma, tissue and cell culture. Rat Corticosterone can be measured in the range 0.039-10 ng/tube. Each kit contains materials sufficient for 120 determinations permitting the construction of one standard curve and the assay of 48 unknowns in duplicate.

Introduction

The adrenal cortex produces 3 types of steroid hormones: glucocorticoids, mineralocorticoids and androgens or oestrogens. The glucocorticoids, which include corticosterone and cortisol, have important effects on carbohydrate and proteinmetabolism in mammals. Glucocorticoids are mainly produced in the inner layers of the adrenal cortex, the zona fasciculata and zona reticularis, as they contain the appropriate steroidogenic enzymes.

Synthesis of all adrenal steroids starts with the conversion of the common precursor cholesterol into pregnenolone and this is the main rate-limiting step. Corticosterone (MW346.5) is the main secreted glucocorticoid in the rat and rabbit while in man, monkey and sheep cortisol is the main secreted product. Cow,ferret, dog and cat secrete equal mixtures of both. Interestingly, cortisol and corticosterone are found in different biosynthetic pathways in the adrenal cortex. A characteristic 17.-hydroxylation of pregnenolone eventually leads to cortisol while corticosterone is one product in the pathway leading to the mineralocorticoid aldosterone. However, corticosterone possesses clear glucocorticoid activity and this is the main function in the rat.

Corticosterone is transported in the blood bound to a specific corticosteroid-binding globulin (CBG) (transcortin) which serves as a carrier and is in equilibrium with free steroid which is the active molecule. The mode of action of corticosterone is, as usual for steroids, that after interacting with cytosolic receptors the steroidreceptor complex binds to nuclear DNA to initiate transcription of specific genes.

Corticosterone stimulates hepatic gluconeogenesis, increases hepatic glycogen content and blood glucose levels, promotes lipolysis and has a catabolic effect leading to a negative nitrogen balance. It is one of the mediators of the stress response to which these actions, which tend to oppose those of insulin, are an adaptation.

Glucocorticoid secretion by the adrenal is stimulated by adrenocorticotrophin (ACTH) which acts to increase the rate of conversion of cholesterol to pregnenolone. Corticosterone acts in a negative feedback loop to inhibit the secretion of ACTH and corticotrophin releasing hormone (CRH) by the pituitary and hypothalamus respectively. This regulatory system is also influenced by other factors such as diurnal rhythm and stress. In the rat corticosterone levels are at a peak in late afternoon and evening. Corticosterone concentrations can also be influenced by handling stress, anaesthesia, sex, and age in the rat.

Principle of method

The assay is based on the competition between unlabelled corticosterone and a fixed quantity of 125I-labelled corticosterone for a limited number of binding sites on a corticosterone specific antibody. With fixed amounts of antibody and radioactive ligand, the amount of radioactive ligand bound by the antibody will be inversely proportional to the concentration of added non-radioactive ligand.

The antibody bound corticosterone is then reacted with the separating reagent. Separation of the antibody bound fraction is effected by either magnetic separation or centrifugation of the separating reagent suspension and decantation of the supernatant.

Measurement of the radioactivity in the pellet enables the amount of labelled corticosterone in the bound fraction to be calculated. The concentration of unlabelled corticosterone in the sample is then determined by interpolation from a standard curve. The standard curve and samples should be prepared simultaneously.

Contents of the kit

1. 1 vial TRACER, lyophilized, reconstitution with 12.5 ml assay buffer, containing ~ 96 kBq, 2.6 µCi [^{125}I]corticosterone. The final solution contains [^{125}I]corticosterone in 0.02M borate buffer pH7.4 containing 0.1%(w/v) sodium azide. Store at 2-8 ^{o}C.

2. 1 vial STANDARD, lyophilized, reconstitution with 2.0 ml assay buffer. The final solution contains corticosterone at a concentration of 400 ng/ml. Store at 2-8 ^{o}C.

3. 1 vial ANTISERUM (12.5 ml), ready for use containing rabbit anti- corticosterone serum in buffer pH7.4 with 0.1% (w/v) sodium azide. Store at 2-8 ^{o}C.

4. 1 vial ASSAY BUFFER concentrate (10.0 ml), dilution to 100 ml. On dilution this will give 0.02 M borate buffer, pH7.4, containing 0.1% (w/v) sodium azide. Store at 2 -8 ^{o}C.

5. 1 vial MAGNETIC IMMUNOSORBENT (55 ml), ready for use, containing paramagnetic particles in buffer with 0.1% (w/v) sodium azide. Store at 2-8 ^{o}C.

Pack leaflet

Materials, tools and equipment required

Pipettes or pipetting equipment with disposable tips (100 µl, 200 µl, 400 µl, 500 µl, 2.0 ml and 12.5 ml); disposable polypropylene or polystyrene tubes (12 x 75 mm); refrigerator; glass measuring cylinder (100 ml); test tube rack; vortex mixer; plastic foil; separators, comprising magnetic base and assay rack - these are for use in the magnetic separation protocol; adsorbent tissue; gamma counter.

Note: For the centrifugal protocol, the following additional equipment will be required:

Decantation racks; refrigerated centrifuge capable of >1500 xg.

Specimen collection

This section is provided for guidance only. It remains the investigator's responsibility to validate the chosen sample collection technique.

Blood samples

It is advised that if measurements are to be made on plasma samples, blood should be collected into tubes containing heparin or EDTA. Blood should be centrifuged immediately to remove cells and the plasma stored below -15°C prior to analysis. Serum samples can also be assayed with this kit. Prior to assay, Corticosterone needs to be displaced from cortisol binding globulin (CBG) in plasma/serum. We recommend heating diluted samples to 60°C for 30 minutes., for example in a water bath, then cool to ambient temperature and assay. Samples may need to be diluted due to the high rat corticosterone plasma/serum concentration. We recommend dilutions of 1:10 or greater depending on expected concentration, for example dilute 50 µl sample with 450 µl buffer. Dilution of samples can be done before heating to inactivate CBG.

Tissue samples

Adrenal samples should be frozen immediately after removal and stored at -80°C until required. Prior to analysis, glands should be homogenized or sonicated in appropriate buffer. Buffers described in the literature include Krebs-Ringer bicarbonate buffer (KRB) containing glucose. Samples may need to be diluted depending on the expected concentration. Assay buffer remaining after reconstitution of assay components may be used for this.

Cell culture

Cell culture media should be collected and stored below -15°C prior to analysis. Samples may need to be diluted depending on the expected concentration. Assay buffer remaining after reconstitution of assay components may be used for this.

Preparation of reagents, storage

Storage: *see Contents of the kit.* At these temperatures each reagent is stable until expiry date. The actual expiry date is given on the package label and on the quality certificate.

Preparation: Equilibrate all reagents and samples to room temperature prior to use.

Assay buffer: Transfer the contents of the bottle to a 100 ml measuring cylinder and dilute to 100 ml with distilled or deionized water. Mix well. Assay buffer is used to redissolve the standard and the tracer.

Tracer and standard: Carefully add the required volume of assay buffer and replace stopper. Mix the contents of the bottles by inversion and swirling, taking care to avoid foaming.

Preparation of working standards

1. Label 8 polystyrene or polypropylene tubes 0.039, 0.078, 0.156, 0.312, 0.625, 1.25, 2.5 and 5.

2. Pipette 500 µl of assay buffer into all tubes.

3. Into the 5 tube pipette 500 µl of stock standard (400 ng/ml) and vortex thoroughly.

4. Transfer 500 µl from the 5 tube to the 2.5 tube and vortex thoroughly.

5. Repeat this doubling dilution successively with the remaining tubes.

6. 25 µl aliquots from each serial dilution give rise to 8 standard levels of corticosterone ranging from 0.039 to 5 ng/tube.

Note: Twenty five microlitres (25 µl) of the reconstituted stock standard provided serves as the top standard (10 ng/tube). Working standards should be freshly prepared before each assay, and not re-used.

Assay procedure

(For a quick guide, refer to Table 1.)

1. Equilibrate all reagents to room temperature.
2. Prepare reagents and assay standards as described in the previous section.
3. Label 12x75 mm disposable tubes in duplicate for total count (TC), non-specific binding (NSB), zero standard (Bo), standards and samples.
4. Pipette **125 µl** assay buffer into NSB tubes and **25 µl** assay buffer into Bo tubes.
5. Starting with the most dilute, pipette **25 µl** of each standard (S1-9) into the appropriately labelled tubes.
6. Pipette **25 µl** unknown sample (M_x) directly into appropriately labelled tubes.
7. Pipette **100 µl** of tracer into all tubes. The TC tubes should be stoppered and put aside for counting.
8. Pipette **100 µl** antiserum into all tubes except NSB and TC.
9. Vortex mix all tubes thoroughly. Cover the tubes and incubate for 2 hours at room temperature (15-30 °C).
10. Gently shake and swirl the bottle containing magnetic immunosorbent to ensure a homogeneous suspension. Add **400 µl** into each tube except the TC. Vortex mix all tubes thoroughly and incubate at room temperature for 15 minutes.
11. Separate the antibody bound fraction using either magnetic separation or centrifugation as described below.

Magnetic separation

Attach the rack on the magnetic separator base and ensure that all tubes are in contact with the base plate. Leave for 5 minutes. After separation, do not remove the rack from the separator base. Pour off and discard the supernatant. Keeping the separator inverted, place the tubes on a pad of absorbent tissues and allow to drain for 5 minutes.

Centrifugation

Centrifuge all tubes at 4°C for 10 minutes at 1500 xg or greater. After centrifugation, place the tubes carefully into suitable decantation racks, then pour off and discard the supernatant. Keeping the tubes inverted, place the tubes on a pad of absorbent tissues and allow to drain for 5 minutes.

12. On completion of either magnetic or centrifugal separation, firmly blot the rims of the inverted tubes on the tissue pad to remove any adhering liquid. Do not re-invert the tubes once they have been turned upright.
13. Determine the radioactivity present in each tube by counting for at least 60 seconds in a gamma scintillation counter.

Table 1. Assay Protocol, Pipetting Guide (all volumes are in microlitres)

Tubes	TC	NSB	Bo	Stan - dard	Sam-ple
Buffer	-	**125**	**25**	-	-
Stan-dard	-	-	-	**25**	-
Sample	-	-	-	-	**25**
Tracer	**100**	**100**	**100**	**100**	**100**
Anti-serum	**-**	**-**	**100**	**100**	**100**
Vortex mix and incubate for 2 hours at room temperature (15-30 °C).					
Magnetic immuno-sorbent	-	**400**	**400**	**400**	**400**
Vortex mix. Incubate for 15 minutes at room temperature.					
Separate either using magnetic separator for 5 minutes or by centrifugation for 10 minutes at >1500 xg .					
Decant tubes and blot on filter paper.					
Count radioactivity (60 sec/tube).					
Calculate the results.					

Calculation of results

Calculate the average count per minute (CPM) for each pair of assay tubes.
Calculate the percent NSB/TC using the following equation:

$$NSB/TC(\%) = \frac{NSB\ (cpm)}{TC\ (cpm)} \times 100$$

If the counter background is high, it should be subtracted from all counts.

Users may wish to subtract the average NSB cpm from all tubes except TC. If so the appropriate correction should be made. Calculate the percent Bo/TC using the following equation:

$$Bo/TC(\%) = \frac{Bo\ (cpm) - NSB\ (cpm)}{TC\ (cpm)} \times 100$$

Calculate the percent bound for each standard and sample using the following equation:

$$B/Bo(\%) = \frac{S1\text{-}9/M_x\ (cpm) - NSB\ (cpm)}{Bo\ (cpm) - NSB\ (cpm)} \times 100$$

A standard curve can be generated by plotting the percent B/Bo as a function of the log corticosterone concentration.

Plot B/Bo(%) (y-axis) against concentration ng standard per tube (x-axis). The concentration (ng per tube) value of the samples can be read directly from the graph (see Figure 1).

Table 2. Typical assay data

Tube	Conc. ng/tube	Mean counts (cpm)	B/TC (%)	B/Bo (%)
TC	-	28706	-	-
NSB	-	567	1.9	-
Bo	-	12742	42.4	-
S1	0.039	11824	-	92.4
S2	0.078	11181	-	87.2
S3	0.156	10217	-	79.3
S4	0.312	8865	-	68.2
S5	0.625	6903	-	52.1
S6	1.25	5363	-	39.4
S7	2.5	3551	-	24.5
S8	5	2613	-	16.8
S9	10	1782	-	10.0

Note: The counts were obtained using new tracer. The counts will decline in line with the age of the tracer.

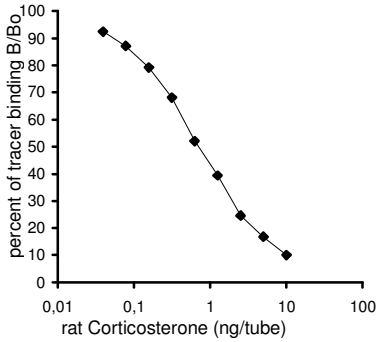


Figure 1: A typical standard curve
(Do not use to calculate unknown samples!)

Characterization of assay

Stability

The components of this assay system will have a shelf-life of at least 4 weeks from the date of despatch.
Upon arrival, all components should be stored at 2-8 C where they are stable until the expiry date printed on the end pack label. Once reconstituted, all reagents should de stored at 2-8 C where they are stable for at least 28 days.

Non-specific binding

The non-specific binding (NSB) defined as the proportion of tracer bound in the absence of antibody was determined to be 1.9%.
The NSB was independent of tracer batch and did not change over a 14-week storage period.

Sensitivity

The sensitivity, defined as the amount of corticosterone needed to reduce zero dose binding by two standard deviations was 0.01 ng/tube.

Specificity

The cross-reactivity, as determined by the concentration giving 50% B/B0 with a number of related compounds is shown below:

Compound	Cross-reactivity, %
Rat corticosterone	100
11-Deoxycortisol	<0.039
21-Deoxycortisol	0.078
Cortisol	0.016
11-Deoxycorticosterone	1.645
Progesterone	0.055
17-Hydroxyprogesterone	0.13

Precision

The within-assay precision for duplicate determinations was calculated by repeatedly measuring an unknown in the assay.The result obtained was 3.35±0.09 ng, CV=1.7%. The between assay precision was assessed by repeated measurement of the same samples in successive assays using different batches of reagents and different operators. These results are shown below:

Sample	(n)	Mean ± SD	CV %
1	10	0.643±0.098	4.1
2	10	1.693±0.254	5.6
3	10	4.965±0.077	3.5

Assay Parallelism

The following correlation was obtained between sample dilution and measured concentration in unextracted plasma and serum.

Sample dilution	plasma	
	Measured conc. (ng/tube)	Calculated conc. (ng/ml)
1:5	2.22	444
1:10	1.19	476
1:20	0.65	520
Sample dilution	serum	
	Measured conc. (ng/tube)	Calculated conc. (ng/ml)
1:5	2.81	562
1:10	1.41	564
1:20	0.59	472

Recovery

The following recovery was obtained when corticosterone was added to rat plasma and serum. Corticosterone was added at ~2 ng/tube at each dilution and endogenous levels accounted for in calculation of recovery.

Sample dilution	plasma		
	Expected conc. (ng/tube)	Measured conc. (ng/tube)	Recovery (%)
1:5	3.64	4.12	113.2
1:10	2.82	3.26	115.6
1:20	2.41	2.74	113.7
Sample dilution	serum		
	Expected conc. (ng/tube)	Measured conc. (ng/tube)	Recovery (%)
1:5	3.69	4.00	108.4
1:10	2.85	3.64	127.7
1:20	2.42	2.72	112.4

Additional information

Components from various lots or from kits of different manufacturers should not be mixed or interchanged.

Precautions

Radioactivity

This product contains radioactive material. It is the responsibility of the user to ensure that local regulations or code of practice related to the handling of radioactive materials are satisfied.

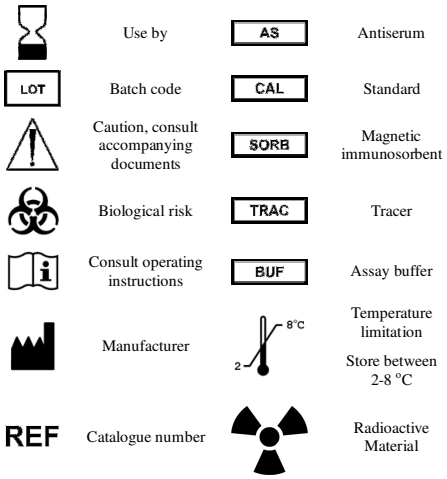
Chemical hazard

Components contain sodium azide as an antimicrobial agent. Dispose of waste by flushing with copious amount of water to avoid build-up of explosive metallic azides in copper and lead plumbing. The total azide present in each pack is 158 mg.

All chemicals should be considered as potentially hazardous. We therefore recommend that this product is handled only by those persons who have been trained in laboratory techniques and that it is used in accordance with the principles of good laboratory practice. Wear suitable protective clothing such as laboratory overalls, safety glasses and gloves. Care should be taken to avoid contact with skin or eyes. In the case of contact with skin or eyes wash immediately with water (see safety data sheet for specific advice).

Safety data sheet

Product name:
Sodium azide
CAS No. 26628-22-8
R: 22-32 Toxic if swallowed. Contact with acids liberates very toxic gas.
S: (1/2)-28-45 (Keep locked up and out of the reach of children). After contact with skin, wash immediately with plenty of water. In case of accident or if you feel unwell, seek medical advice immediately (show label where possible).
Composition:
Sodium azide solution.
Hazards identification:
Toxic if swallowed, inhaled, or absorbed through skin. May cause eye and skin irritation.
First aid measures:
In case of contact, immediately flush eyes or skin with copious amounts of water. If inhaled remove to fresh air. In severe cases seek medical attention.
Fire fighting measures:
Dry chemical powder. Do not use water.
Accidental release:
Wear suitable protective clothing including laboratory overalls, safety glasses and gloves. Mop up spill area, place waste in a bag and hold for waste disposal. Wash spill site area after material pick-up is complete.
Handling and storage:
Wear suitable protective clothing including overalls, safety glasses and gloves. Do not get in eyes, on skin, or on clothing. Wash thoroughly after handling.
Personal protection:
See above instructions for handling and storage.
Physical and chemical properties:
Formula weight: 65.01. Density: 1.850.
Stability and reactivity:
Avoid contact with metals and acid chlorides. This yields a very toxic gas.
Toxicological information:
LD50: 27 mg/kg oral, rat
LD50: 20 mg/kg skin, rabbit
Ecological information:
Not applicable
Disposal consideration:
Up to 5 vials worth of material may be disposed of directly down the sink with water. If 6 or more vials are to be disposed of they should pass through a chemical waste route.
Note: Inorganic azides will react with lead and copper plumbing fixtures to give explosive residues. Disposal of significant quantities of azides via such plumbing is not recommended.
Transport information:
No special considerations applicable.
Regulatory information:
The information contained in this safety data sheet is based on published sources and is believed to be correct. It should be used as a guide only. It is the responsibility of the user of this product to carry out an assessment of workplace risks, as may be required under national legislation.



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