Testosterone Immunoassay

Catalog Number DE2300

For the quantitative determination of testosterone concentrations in cell culture supernates, saliva, serum and plasma.

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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INTRODUCTION

Testosterone is one of the most important androgens secreted into the blood stream and is synthesized from pregnenolone, which is formed from cholesterol (1 - 3). In adult humans, approximately 5 mg of testosterone is synthesized per day and circulates in plasma predominantly bound to proteins, including specific sex hormone binding globulin (SHBG) and nonspecific proteins such as albumin. It is believed that the bioavailable testosterone includes the free steroid and the albumin bound steroid, equalling about 35% of the total testosterone (4). Both testosterone and SHBG exhibit rhythmic variations. In serum, testosterone concentrations peak between 4 am and 8 am, while SHBG concentration is affected by such factors as posture (5, 6). Testosterone is the main androgen secreted by the Leydig cells of the testes and effects both primary and secondary sexual development such as muscle mass and sex drive (7, 8).

R&D Systems' Testosterone Immunoassay is a 3 hour competitive enzyme immunoassay designed to measure testosterone in cell culture supernates, saliva, serum and plasma.

PRINCIPLE OF THE ASSAY

This assay is based on the competitive binding technique in which testosterone present in a sample competes with a fixed amount of alkaline phosphatase-labeled testosterone for sites on a mouse monoclonal antibody. During the incubations, the monoclonal antibody becomes bound to the goat anti-mouse antibody coated onto the microplate. Following a wash to remove excess conjugate and unbound sample, a substrate solution is added to the wells to determine the bound enzyme activity. Immediately following color development, the absorbance is read at 405 nm. The intensity of the color is inversely proportional to the concentration of testosterone in the sample.

TECHNICAL HINTS

- **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 reagents from different lot numbers.
- Allow reagents to warm to room temperature before use.
- The unopened bottle of pNPP Substrate should be colorless to pale yellow.
- Stop Solution should be added to the plate in the same order as the pNPP Substrate. The color developed in the wells will remain yellow upon the addition of Stop Solution.
- When mixing or reconstituting protein solutions, always avoid foaming.
- Pre-rinse the pipette tips when pipetting standards.
- 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.
- Pipette standards and samples to the bottom of the wells.
- Add all other reagents to the side of the wells to avoid contamination.
- If samples generate values higher than the highest standard, further dilute the samples with Assay Buffer ED3 and repeat the assay.
- Any variation in operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- Alkaline Phosphatase is a temperature sensitive enzyme. Optical Density (OD) units may vary with temperature changes.

REAGENTS

Microplate (Part R80-0050) - 96 well microplate coated with a goat antibody specific to the Fc portion of mouse IgG.

Testosterone Conjugate (Part R80-0431) - 6 mL of testosterone conjugated to alkaline phosphatase, with blue dye and preservatives.

Testosterone Standard (Part R80-0430) - 0.5 mL of testosterone (50,000 pg/mL) in buffer.

Testosterone Antibody Solution (Part R80-0429) - 6 mL of mouse monoclonal antibody to testosterone, with yellow dye and preservatives.

Assay Buffer ED3 (Part R80-0145) - 30 mL of a buffered protein base with preservatives.

Steroid Displacement Reagent (R80-0120) - 1 mL of a specially formulated displacer to inhibit testosterone binding to proteins.

Wash Buffer Concentrate (Part R80-1286) - 30 mL of a 20-fold concentrated solution of a buffered surfactant with preservatives.

pNPP Substrate (Part R80-0075) - 20 mL of p-nitrophenyl phosphate in a buffered solution.

Stop Solution (Part R80-0247) - 5 mL of a trisodium phosphate (TSP) solution. **Keep tightly capped. Caution: caustic.**

Plate Cover - 1 adhesive strip.

STORAGE

<table>
<tr><td>Unopened Kit</td><td colspan="2">Store at 2 - 8° C. Do not use past kit expiration date.</td></tr>
<tr><td rowspan="8">Opened Reagents</td><td>Diluted Wash Buffer</td><td>May be stored at room temperature for up to 3 months.*</td></tr>
<tr><td>Stop Solution</td><td rowspan="6">May be stored at 2 - 8° C until the expiration date of the kit.</td></tr>
<tr><td>Assay Buffer ED3</td></tr>
<tr><td>Steroid Displacement Reagent</td></tr>
<tr><td>pNPP Substrate</td></tr>
<tr><td>Conjugate</td></tr>
<tr><td>Antibody Solution</td></tr>
<tr><td>Standard</td></tr>
<tr><td>Microplate Wells</td><td>Return unused wells to the pouch containing the desiccant pack, reseal along the entire edge of the zip-seal. May be stored at 2 - 8° C until the expiration date of the kit.</td></tr>
</table>

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

OTHER SUPPLIES REQUIRED

- Microplate reader capable of measuring absorbance at 405 nm with wavelength correction set between 570 nm and 590 nm.
- Pipettes and pipette tips.
- 1000 mL graduated cylinder for preparation of Wash Buffer.
- Deionized or distilled water.
- 37° C incubator.
- Squirt bottle, manifold dispenser, or autowasher.
- Horizontal orbital microplate shaker (0.12” orbit) capable of maintaining a speed of 500 $\pm$ 50 rpm.

Reagents required for sample extraction:

- Diethyl ether (ACS Grade)

PRECAUTIONS

Some components of this kit contain sodium azide which may react with lead and copper plumbing to form explosive metallic azides. Flush with large volumes of water during disposal.

The Stop Solution provided with the kit is a caustic material. Wear eye, hand, face, and clothing protection when using this material.

The Testosterone Standard provided with this kit is supplied in ethanolic buffer at a pH optimized to maintain testosterone integrity. Care should be taken when handling this material because of the known and unknown effects of steroids.

The activity of the Testosterone Conjugate is dependent on the presence of Mg^{2+} and Zn^{2+} ions and is affected by high concentrations of chelators, such as EDTA and EGTA. Samples that contain < 10 mM EDTA or EGTA can be assayed without interference. Samples containing higher concentrations of chelators must be diluted prior to assay.

Inorganic phosphate is a strong competitive inhibitor of alkaline phosphatase; avoid the use of PBS based wash buffers and other sources of inorganic phosphate contamination.

SAMPLE COLLECTION AND STORAGE

Samples containing mouse IgG may interfere with this assay.

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

Saliva - Collect 2 - 3 mL of saliva from each donor in polypropylene tubes. Vortex briefly and centrifuge for 6 minutes at 14,000 rpm in microcentrifuge tubes. Collect the aqueous layer only (no pellet) and assay immediately or aliquot and store samples at ≤ -20° C. Avoid repeated freeze-thaw cycles.

Serum - Use a serum separator tube (SST). Allow samples to clot for 30 minutes before centrifugation at 1600 x g for 15 minutes. Assay immediately or aliquot and store samples at ≤ -20° C. Avoid repeated freeze-thaw cycles.

Plasma - Collect plasma using EDTA as an anticoagulant. Centrifuge at 1600 x g for 15 minutes. Assay immediately or aliquot and store samples at ≤ -20° C. Avoid repeated freeze-thaw cycles.

SAMPLE PREPARATION

Samples containing steroid binding proteins (such as serum and plasma) need to be treated with Steroid Displacement Reagent. Add 5 µL of Displacement Reagent to 495 µL of sample.

Saliva, serum, and plasma samples require at least a 20-fold dilution. A suggested 20-fold dilution is 15 µL sample + 285 µL Assay Buffer ED3.

Cell culture supernate samples require at least a 2-fold dilution. A suggested 2-fold dilution is 125 µL sample + 125 µL cell culture media.

SAMPLE EXTRACTION

If diluted samples measure below the lowest standard, then it is recommended that the samples be extracted for accurate measurement of testosterone. The following protocol is a general extraction method for small peptides.

1. Add sufficient Testosterone to a typical sample for determination of extraction efficiency.
2. In a fume hood, add 1 mL of diethyl ether for every 1 mL of sample. Stopper and shake sample. **Caution: Diethyl ether is extremely flammable.**
3. Allow layers to separate. Carefully pipette off the top layer and place in a clean test tube.
4. Repeat steps 1 - 3 two more times, combining the ether layers.
5. Evaporate the ether to dryness under nitrogen.
6. Dissolve the extracted testosterone with at least 250 µL of Assay Buffer ED3 by vortexing well. Allow to sit for 5 minutes at room temperature. Repeat twice more.
7. Run the reconstituted samples in the assay immediately or keep the dried samples desiccated at ≤ -20° C.

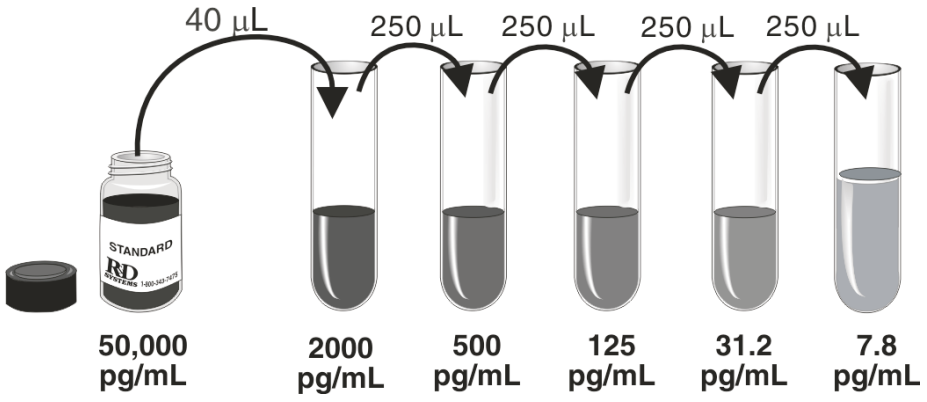
REAGENT PREPARATION

Bring all reagents to room temperature before use.

Wash Buffer - Dilute 30 mL of Wash Buffer Concentrate into deionized or distilled water to prepare 600 mL of Wash Buffer (1X).

Testosterone Standard - Pipette 960 µL of Assay Buffer ED3 into the 2000 pg/mL tube. Pipette 750 µL of Assay Buffer ED3 into the remaining tubes. *When pipetting standards, it is important to pre-rinse the pipette tips.* Use the 50,000 pg/mL standard stock to produce a dilution series (below). Mix each tube thoroughly and change pipette tips between each transfer. The 2000 pg/mL standard serves as the high standard and the Assay Buffer ED3 serves as the zero standard (B_0) (0 pg/mL). **Diluted standards should be used within 60 minutes of preparation.**

When running cell culture supernate samples, it is recommended to use cell culture media (CCM) instead of Assay Buffer ED3 to produce the standard dilution series and as the zero standard. Changes in binding have been associated with running the standards and samples in media.



ASSAY PROCEDURE

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

Total Activity (TA), Non-Specific Binding (NSB), Maximum Binding (B_0), and Substrate Blank wells should be included in the assay as a means of quality control for each assay.

1. Prepare all reagents, working standards, and samples as directed in the previous sections.
2. Remove excess microplate wells from the plate frame, return them to the pouch containing the desiccant pack, reseal.
3. Reserve wells for TA and Substrate Blank.
4. Add 150 µL of Assay Buffer ED3 (or CCM) to the NSB wells.
5. Add 100 µL of Assay Buffer ED3 (or CCM) to the zero standard (B_0) wells.
6. Add 100 µL of Standard or sample* to the remaining wells.
7. Add 50 µL of Testosterone Antibody Solution to each well (excluding the NSB, TA, and Substrate Blank wells). Incubate for 1 hour at room temperature on a horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 500 ± 50 rpm.
8. Add 50 µL of Testosterone Conjugate to each well (excluding the TA and Substrate Blank wells). Incubate for 1 hour at room temperature on the shaker.

   Note: *The TA and Substrate Blank wells are empty at this point. The NSB wells should be blue and all other wells should be green.*
9. Aspirate or decant each well and wash, repeating the process twice for a total of three 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 toweling.
10. Add 5 µL of Testosterone Conjugate to the TA wells.
11. Add 200 µL of pNPP Substrate to **all wells**. Cover with an adhesive strip and incubate for 1 hour at 37° C. **Do not shake.**
12. Add 50 µL of Stop Solution to each well.
13. Determine the optical density of each well **immediately** using a microplate reader set to 405 nm with wavelength correction set between 570 nm and 590 nm.

*Samples require dilution as directed in the Sample Preparation Section.

CALCULATION OF RESULTS

Average the duplicate readings for each standard and sample and subtract the average NSB optical density.

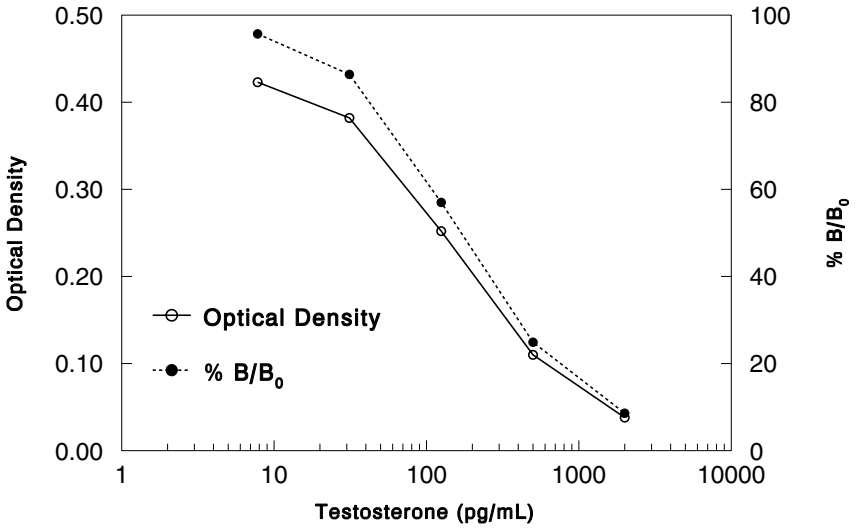
Create a standard curve by reducing the data using computer software capable of generating a four paramater logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on a linear y-axis against the concentration on a logarithmic x-axis and draw the best fit curve through the points on the graph.

% B/B_0 can be calculated by dividing the corrected OD for each standard or sample by the corrected B_0 OD and multiplying by 100.

Calculate the concentration of testosterone corresponding to the mean absorbance or % B/B_0 from the standard curve. Since 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.



pg/mL	OD	Average	Corrected	% B/B_0
Blank	0.176 0.178	(0.177)	—	—
NSB	0.181 0.183	0.005	—	—
0 (B_0)	0.619 0.629	0.447	0.442	100
7.8	0.602 0.607	0.428	0.423	95.7
31.2	0.557 0.570	0.387	0.382	86.4
125	0.432 0.435	0.257	0.252	57.0
500	0.291 0.292	0.115	0.110	24.9
2000	0.216 0.223	0.043	0.038	8.6

QUALITY CONTROL

Each laboratory should establish a quality control program to monitor the performance of the Testosterone Immunoassay. As a part of this program, TA, NSB, B_0, and Substrate Blank wells should be run in each assay.

Typical Quality Control Parameters

Substrate Blank (O.D.)	=	0.177
TA (TA x 10)	=	1.73
%NSB (NSB/TA x 100)	=	0.289%
% B_0 (B_0/TA x 100)	=	25.5%
Quality of Fit	=	0.999

PRECISION

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

Inter-assay Precision (Precision between assays)
Three samples of known concentration were tested in eight separate assays to assess inter-assay precision.

	Intra-assay Precision			Inter-assay Precision		
Sample	1	2	3	1	2	3
n	8	8	8	8	8	8
Mean (pg/mL)	38.3	91.0	270	32.0	81.2	259
Standard deviation	4.14	9.10	21.1	4.67	9.18	24.1
CV (%)	10.8	10.0	7.8	14.6	11.3	9.3

RECOVERY

The recovery of testosterone spiked into samples in various matrices was evaluated.

Sample Type	Average % Recovery	Range
Cell Culture Media*	104	99 - 110%
Porcine Serum*	103	102 - 104%
Porcine EDTA Plasma*	101	90 - 111%
Human Saliva*	101	93 - 105%

*Samples were diluted prior to assay as directed in Sample Preparation

LINEARITY

To assess the linearity of the assay, Assay Buffer ED3 spiked with testosterone was assayed using serial 2-fold dilutions.

Dilution	Observed (pg/mL)	Expected (pg/mL)	% $\frac{\text{Observed}}{\text{Expected}}$
Neat	236	—	—
1:2	115.7	118.0	98.1
1:4	51.9	59.0	88.0
1:8	27.5	29.5	93.2
1:16	15.6	14.7	105.8

SENSITIVITY

The sensitivity of the Testosterone assay is typically less than 5.67 pg/mL.

Sensitivity was determined by subtracting two standard deviations from the mean optical density value of twenty zero standard (B_0) replicates and calculating the corresponding concentration.

CROSS-REACTIVITY

Cross-reactivity for the following compounds was determined by adding the cross-reactant to Assay Buffer ED3 at concentrations ranging from 10 pg/mL to 50,000 pg/mL. The cross-reactivity was calculated at 50% B/B_0.

Compound	% Cross-reactivity
19-hydroxytestosterone	14.6
Androstendione	7.2
Dehydroepiandrosterone	0.72
Estradiol	0.4
Dihydrotestosterone	< 0.001
Estriol	< 0.001
Aldosterone	< 0.001
Corticosterone	< 0.001
Cortisol	< 0.001
Cortisone	< 0.001
Estrone	< 0.001
Progesterone	< 0.001
Pregnenolone	< 0.001

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