Testosterone Glucuronide ELISA Kit

Item No. 501740

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GENERAL INFORMATION

Materials Supplied

Item Number	Item	96 wells Quantity/Size
401742	Testosterone Glucuronide ELISA Antiserum	1 vial/100 dtn
401740	Testosterone Glucuronide AP Tracer	1 vial/100 dtn
401744	Testosterone Glucuronide ELISA Standard	1 vial
400080	Tris Buffer Concentrate (10X)	2 vials/10 ml
411007	AP Wash Buffer Concentrate (150X)	1 vial/5 ml
400004/400006	Mouse Anti-Rabbit IgG-Coated Plate	1 plate
400012	96-Well Cover Sheet	1 cover
400089	*p*NPP Substrate Solution	2 vials/12 ml
400040	ELISA Tracer Dye	1 vial
400042	ELISA Antiserum Dye	1 vial

If any of the items listed above are damaged or missing, please contact our Customer Service department at (800) 364-9897 or (734) 971-3335. We cannot accept any returns without prior authorization.

WARNING: THIS PRODUCT IS FOR RESEARCH ONLY - NOT FOR HUMAN OR VETERINARY DIAGNOSTIC OR THERAPEUTIC USE.

Safety Data

This material should be considered hazardous until further information becomes available. Do not ingest, inhale, get in eyes, on skin, or on clothing. Wash thoroughly after handling. Before use, the user must review the complete Safety Data Sheet, which has been sent *via* email to your institution.

Precautions

Please read these instructions carefully before beginning this assay.

This kit may not perform as described if any reagent or procedure is replaced or modified.

When compared to quantification by LC/MS or GC/MS, it is not uncommon for immunoassays to report higher analyte concentrations. While LC/MS or GC/MS analyses typically measure only a single compound, antibodies used in immunoassays sometimes recognize not only the target molecule, but also structurally related molecules, including biologically relevant metabolites. In many cases, measurement of both the parent molecule and metabolites is more representative of the overall biological response than is the measurement of a short-lived parent molecule. It is the responsibility of the researcher to understand the limits of both assay systems and to interpret their data accordingly.

If You Have Problems

Technical Service Contact Information

Phone: 888-526-5351 (USA and Canada only) or 734-975-3888

Email: techserv@caymanchem.com

In order for our staff to assist you quickly and efficiently, please be ready to supply the lot number of the kit (found on the outside of the box).

Storage and Stability

This kit will perform as specified if stored as directed at -20°C and used before the expiration date indicated on the outside of the box.

Materials Needed But Not Supplied

1. A plate reader capable of measuring absorbance at 405 nm.
2. Adjustable pipettes and a repeating pipettor.
3. A source of ultrapure water, with a resistivity of 18.2 MΩ·cm and total organic carbon (TOC) levels of <10 ppb, is recommended. Pure water - glass-distilled or deionized - may not be acceptable. *NOTE: UltraPure Water is available for purchase from Cayman (Item No. 400000).*
4. Materials used for Sample Preparation (see page 12).

INTRODUCTION

Background

Testosterone glucuronide is a urinary metabolite of the endogenous steroid hormone testosterone.[1] It is formed by glucuronide conjugation of testosterone primarily by the UDP-glucuronosyltransferase (UGT) isoform UGT2B17, which is found in the small intestine, colon, and liver.[1-3]

Testosterone glucuronide levels are lower in individuals with mutations in the UGT2B17 gene.[4] Glucuronidation of testosterone is decreased by the NSAIDs diclofenac and ibuprofen *in vitro* but not *in vivo*.[5,6]

About This Assay

Cayman's Testosterone Glucuronide ELISA Kit is a competitive assay that can be used for quantification of testosterone glucuronide in urine. The assay has a range of 4.9-3,000 pg/ml and a sensitivity (80% B/B_0) of approximately 25 pg/ml.

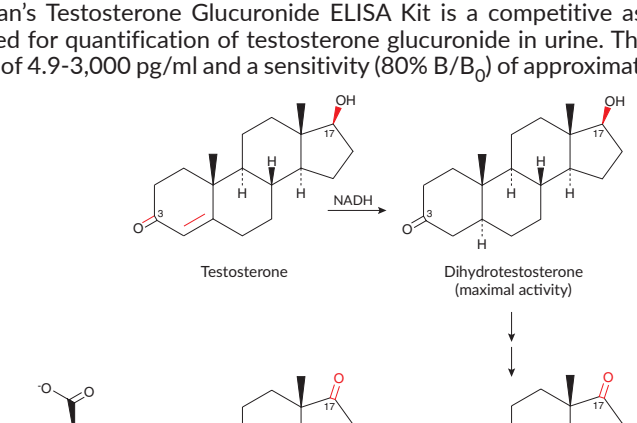


Figure 1. Metabolism of testosterone

Principle of the Assay

This assay is based on the competition between native free testosterone glucuronide and a testosterone glucuronide conjugate (Testosterone Glucuronide Alkaline Phosphatase (AP) Tracer) for a limited amount of Testosterone Glucuronide Antiserum. Because the concentration of the Testosterone Glucuronide AP Tracer is held constant while the concentration of native testosterone glucuronide varies, the amount of Testosterone Glucuronide AP Tracer that is able to bind to the Testosterone Glucuronide Antiserum will be inversely proportional to the concentration of native testosterone glucuronide in the well. This antibody-testosterone glucuronide complex binds to a mouse monoclonal anti-rabbit IgG that has been previously attached to the well. The plate is washed to remove any unbound reagents and *p*NPP Substrate Solution (which contains the substrate to AP) is added to the well. The product of this enzymatic reaction has a distinct yellow color and absorbs strongly at 405 nm. The intensity of this color, determined spectrophotometrically, is proportional to the amount of Testosterone Glucuronide Tracer bound to the well, which is inversely proportional to the amount of native testosterone glucuronide present in the well during the incubation, as described in the equation:

Absorbance $\propto$ [Bound Testosterone Glucuronide AP Tracer] $\propto$ 1/[testosterone glucuronide]

A schematic of this process is shown in Figure 2, on page 8.

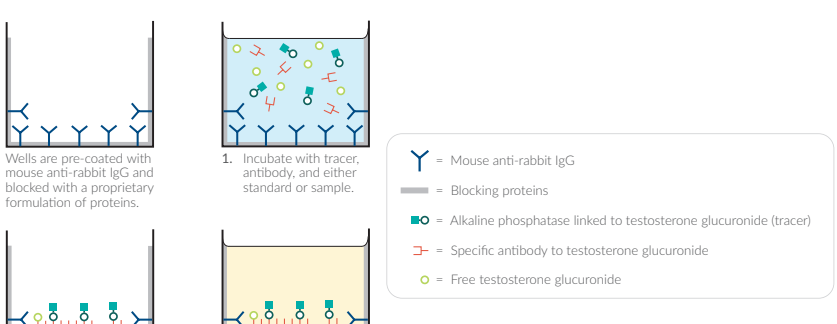


Figure 2. Schematic of the Testosterone Glucuronide ELISA

Definition of Key Terms

Blank: background absorbance caused by *p*NPP Substrate Solution. The blank absorbance should be subtracted from the absorbance readings of all the other wells, including non-specific binding (NSB) wells.

Total Activity (TA): total enzymatic activity of the AP-linked tracer.

NSB (Non-Specific Binding): non-immunological binding of the tracer to the well. Even in the absence of specific antibody a very small amount of tracer still binds to the well; the NSB is a measure of this low binding.

B_0 (Maximum Binding): maximum amount of the tracer that the antibody can bind in the absence of free analyte.

%B/B_0 (%Bound/Maximum Bound): ratio of the absorbance of a particular sample or standard well to that of the maximum binding (B_0) well.

Standard Curve: a plot of the %B/B_0 values *versus* concentration of a series of wells containing various known amounts of analyte.

Dtn: determination, where one dtn is the amount of reagent used per well.

Cross Reactivity: numerical representation of the relative reactivity of this assay towards structurally related molecules as compared to the primary analyte of interest. Biomolecules that possess similar epitopes to the analyte can compete with the assay tracer for binding to the primary antibody. Substances that are superior to the analyte in displacing the tracer result in a cross reactivity that is greater than 100%. Substances that are inferior to the primary analyte in displacing the tracer result in a cross reactivity that is less than 100%. Cross reactivity is calculated by comparing the mid-point (50% B/B_0) value of the tested molecule to the mid-point (50% B/B_0) value of the primary analyte when each is measured in assay buffer using the following formula.

$$\text{\% Cross Reactivity} = \left[\frac{\text{50\% } B/B_0 \text{ value for the primary analyte}}{\text{50\% } B/B_0 \text{ value for the potential cross reactant}} \right] \text{x } 100\%$$

Lower Limit of Detection (LLOD): the smallest measure that can be detected with reasonable certainty for a given analytical procedure. The LLOD is defined as a point two standard deviations away from the mean zero value.

PRE-ASSAY PREPARATION

Buffer Preparation

Store all diluted buffers at 4°C; they should be stable for approximately two months.

1. **Tris Buffer (1X) Preparation**

   Dilute the contents of one vial of Tris Buffer Concentrate (10X) (Item No. 400080) with 90 ml of pure water. Be certain to rinse the vial to remove any salts that may have precipitated. *NOTE: It is normal for the concentrated buffer to contain crystalline salts after thawing. These will completely dissolve upon dilution with pure water.*

2. **AP Wash Buffer (1X) Preparation**

   Dilute the contents of one vial of AP Wash Buffer Concentrate (150X) (Item No. 411007) with pure water to a total volume of 750 ml. Smaller volumes of AP Wash Buffer (1X) can be prepared by diluting the AP Wash Buffer Concentrate (150X) 1:150 with pure water.

Sample Preparation

Testing for Interference

This assay has been tested using human urine. Other sample types should be checked for interference to evaluate the need for sample purification before embarking on a large number of sample measurements. To test for interference, dilute one or two test samples to obtain at least two different dilutions of each sample between approximately 800 pg/ml and 25 pg/ml (*i.e.*, between 20-80% B/B_0, which is the linear portion of the standard curve). The two different dilutions of the sample should show good correlation (differ by 20% or less) in the final calculated testosterone glucuronide concentration.

Urine

It is recommended that urine samples be diluted at least 1:20 into Tris Buffer (1X) prior to testing in the assay.

General Precautions

All samples must be free of organic solvents prior to testing in the assay.

Samples should be assayed immediately after collection; samples that cannot be assayed immediately should be stored at -80°C.

Samples of rabbit origin may contain antibodies that interfere with the assay by binding to the mouse anti-rabbit plate. We recommend that all rabbit samples be purified prior to use in the assay.

Sample Matrix Properties

Linearity

A human urine sample with a biologically relevant level of testosterone glucuronide was serially diluted and evaluated for linearity using the Testosterone Glucuronide ELISA Kit. The results are shown in the table below.

Dilution Factor	Concentration (ng/ml)	Dilutional Linearity (%)
1:160	137.7	100
1:320	117.6	85
1:640	116.5	85
1:1,280	112.6	82
1:2,560	130.9	95

Table 1. Dilutional linearity of human urine samples

Spike and Recovery

Human urine samples were spiked with testosterone glucuronide, diluted as described in the Sample Preparation section (see page 12), and analyzed using the Testosterone Glucuronide ELISA Kit. The results are shown below. The y-intercept corresponds to the amount of endogenous testosterone glucuronide in the sample. Error bars represent standard deviations obtained from multiple dilutions of each sample.

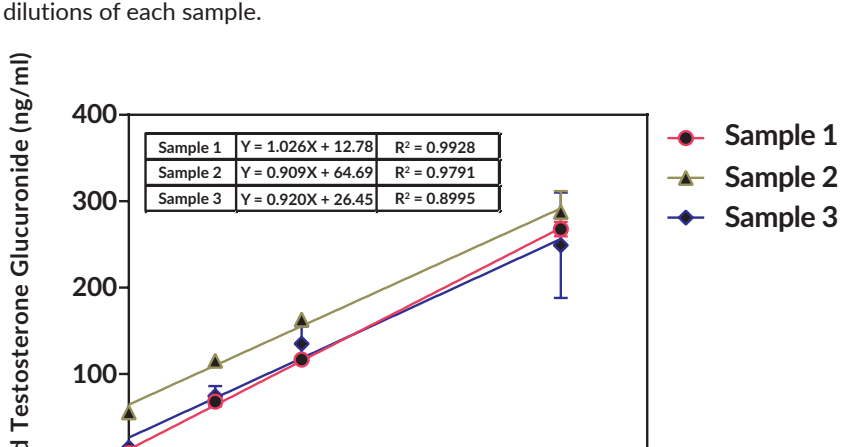


Figure 3. Spike and recovery in human urine

Parallelism

To assess parallelism, human urine was serially diluted and evaluated using the Testosterone Glucuronide ELISA Kit. Concentrations were plotted as a function of the sample dilution. The results are shown below. Parallelism of the curves demonstrates that the antigen binding characteristics are similar enough to allow the accurate determination of native analyte levels in diluted human urine samples.

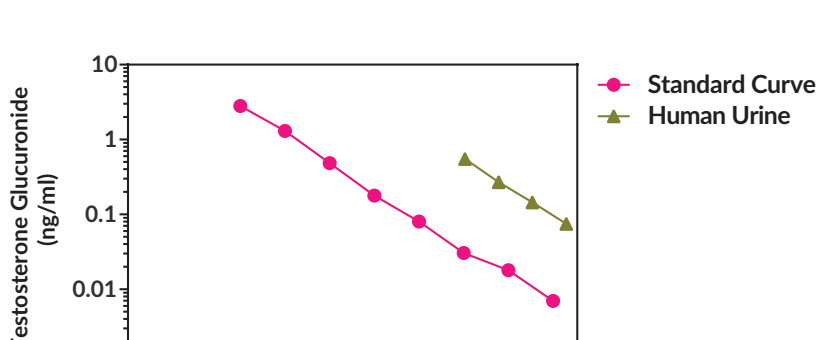


Figure 4. Parallelism of urine sample matrix in the Testosterone Glucuronide ELISA Kit

Preparation of Assay-Specific Reagents

Testosterone Glucuronide ELISA Standard

Equilibrate a pipette tip by repeatedly filling and expelling the tip with the Testosterone Glucuronide ELISA Standard (Item No. 401744) several times. Using the equilibrated pipette tip, transfer 100 µl of the standard into a clean test tube, then dilute with 900 µl pure water. The concentration of this solution (the bulk standard) will be 30 ng/ml. Do not store the bulk standard for more than 24 hours.

To prepare the standard for use in the ELISA: Obtain eight clean test tubes and label them #1-8. Aliquot 900 µl Tris Buffer (1X) to tube #1 and 600 µl Tris Buffer (1X) to tubes #2-8. Transfer 100 µl of the bulk standard (30 ng/ml) to tube #1 and mix thoroughly. The concentration of this standard, the first point on the standard curve, will be 3 ng/ml (3,000 pg/ml). Serially dilute the standard by removing 400 µl from tube #1 and placing in tube #2; mix thoroughly. Next, remove 400 µl from tube #2 and place it into tube #3; mix thoroughly. Repeat this process for tubes #4-8. These diluted standards should not be stored for more than 24 hours.

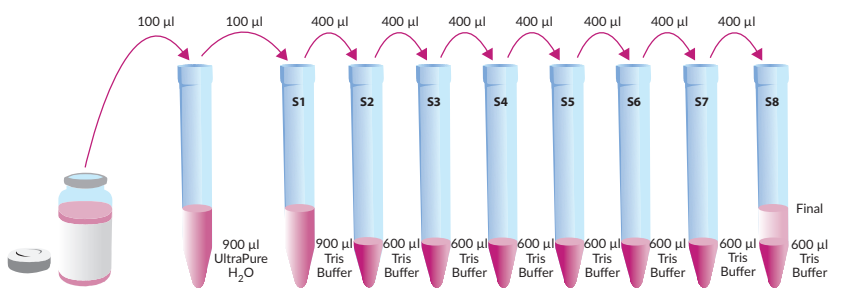


Figure 5. Preparation of the testosterone glucuronide standards

Testosterone Glucuronide AP Tracer

Reconstitute the Testosterone Glucuronide AP Tracer (Item No. 401740) with 6 ml of Tris Buffer (1X). Store the reconstituted Testosterone Glucuronide AP Tracer at 4°C (do not freeze!) and use within two weeks. A 20% surplus of tracer has been included to account for any incidental losses.

Tracer Dye Instructions (optional)

This dye may be added to the tracer, if desired, to aid in visualization of tracer-containing wells. Add the dye to the reconstituted tracer at a final dilution of 1:100 (add 60 µl of dye to 6 ml tracer).

Testosterone Glucuronide ELISA Antiserum

Reconstitute the Testosterone Glucuronide ELISA Antiserum (Item No. 401742) with 6 ml of Tris Buffer (1X). Store the reconstituted Testosterone Glucuronide ELISA Antiserum at 4°C (do not freeze!). It will be stable for at least two weeks. A 20% surplus of antiserum has been included to account for any incidental losses.

Antiserum Dye Instructions (optional)

This dye may be added to the antiserum, if desired, to aid in visualization of antibody-containing wells. Add the dye to the reconstituted antiserum at a final dilution of 1:100 (add 60 µl of dye to 6 ml antiserum).

Plate Set Up

The 96-well plate included with this kit is supplied ready to use. It is not necessary to rinse the plate(s) prior to adding the reagents. *NOTE: If you do not need to use all of the strips at once, plate the unused strips back in the plate packet and store at 4°C. Be sure the packet is sealed with the desiccant inside.*

Each plate or set of strips must contain a minimum of two Blk, two NSB, and two B_0 wells, and an eight-point standard curve run in duplicate. *NOTE: Each assay must contain this minimum configuration in order to ensure accurate and reproducible results.* Each sample should be assayed at a minimum of two dilutions and each dilution should be assayed in duplicate. For statistical purposes, we recommend assaying samples in triplicate. An optional TA well can be added for troubleshooting purposes, if desired.

A suggested plate format is shown in Figure 6, below. The user may vary the location and type of wells present as necessary for each particular experiment. We suggest you record the contents of each well on the template sheet provided (see page 29).

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blk	S1	S1	1	1	1	9	9	9	17	17	17
B	Blk	S2	S2	2	2	2	10	10	10	18	18	18
C	NSB	S3	S3	3	3	3	11	11	11	19	19	19
D	NSB	S4	S4	4	4	4	12	12	12	20	20	20
E	B_0	S5	S5	5	5	5	13	13	13	21	21	21
F	B_0	S6	S6	6	6	6	14	14	14	22	22	22
G	B_0	S7	S7	7	7	7	15	15	15	23	23	23
H	TA	S8	S8	8	8	8	16	16	16	24	24	24

Blk - Blank
TA - Total Activity
NSB - Non-Specific Binding
B_0 - Maximum Binding
S1-S8 - Standards 1-8
1-24 - Samples

Figure 6. Sample plate format

Performing the Assay

Pipetting Hints

- Use different tips to pipette each reagent.
- Before pipetting each reagent, equilibrate the pipette tip in that reagent (*i.e.*, slowly fill the tip and gently expel the contents, repeat several times).
- Do not expose the pipette tip to the reagent(s) already in the well.

Addition of the Reagents

1. **Tris Buffer (1X)**

   Add 100 µl of Tris Buffer (1X) to NSB wells. Add 50 µl Tris Buffer (1X) to B_0 wells.

2. **Testosterone Glucuronide ELISA Standard**

   Add 50 µl from tube #8 to both of the lowest standard wells (S8). Add 50 µl from tube #7 to each of the next two standard wells (S7). Repeat this process for the remaining standards (S6-S1). *The same pipette tip should be used to aliquot all the standards. Before pipetting each standard, be sure to equilibrate the pipette tip in that standard.*

3. **Samples**

   Add 50 µl of sample per well. Each sample should be assayed at a minimum of two dilutions. Each dilution should be assayed in duplicate (triplicate recommended).

4. **Testosterone Glucuronide AP Tracer**

   Add 50 µl to each well *except* the Blk and TA wells.

5. **Testosterone Glucuronide ELISA Antiserum**

   Add 50 µl to each well *except* the Blk, TA, and NSB wells.

Incubation of the Plate

Cover each plate with a 96-Well Cover Sheet (Item No. 400012) and incubate 18 hours at 4°C.

Development of the Plate

1. Equilibrate *p*NPP Substrate Solution to room temperature prior to developing the plate.
2. Empty the wells and rinse five times with ~300 µl AP Wash Buffer (1X).
3. Add 200 µl of *p*NPP Substrate Solution to each well.
4. Add 5 µl of the reconstituted tracer to the TA wells.
5. Cover the plate with 96-Well Cover Sheet. Optimum development is obtained by using an orbital shaker equipped with a large, flat cover to allow the plate(s) to develop in the dark for 90 minutes.

Reading the Plate

1. Wipe the bottom of the plate with a clean tissue to remove fingerprints, dirt, *etc*.
2. Remove the plate cover, being careful to keep *p*NPP Substrate Solution from splashing on the cover. *NOTE: Any loss of pNPP Substrate Solution will affect the absorbance readings.*
3. Read the plate at a wavelength of 405 nm.

ANALYSIS

Many plate readers come with data reduction software that plot data automatically. Alternatively, a spreadsheet program can be used. The data should be plotted as either %B/B_0 *versus* log concentration using a four-parameter logistic fit or as logit B/B_0 *versus* log concentration using a linear fit. *NOTE: Cayman has a computer spreadsheet available for data analysis. Please contact Technical Service or visit our website (www.caymanchem.com/analysis/elisa) to obtain a free copy of this convenient data analysis tool.*

Calculations

Preparation of the Data

The following procedure is recommended for preparation of the data prior to graphical analysis.

NOTE: If the plate reader has not subtracted the absorbance readings of the blank wells from the absorbance readings of the rest of the plate, be sure to do that now.

1. Average the absorbance readings from the NSB wells.
2. Average the absorbance readings from the B_0 wells.
3. Subtract the NSB average from the B_0 average. This is the corrected B_0 or corrected maximum binding.
4. Calculate the B/B_0 (Sample or Standard Bound/Maximum Bound) for the remaining wells. To do this, subtract the average NSB absorbance from the S1 absorbance and divide by the corrected B_0 (from Step 3). Repeat for S2-S8 and all sample wells. (To obtain %B/B_0 for a logistic four-parameter fit, multiply these values by 100.)

Plot the Standard Curve

Plot %B/B_0 for standards S1-S8 *versus* testosterone glucuronide concentration using linear (y) and log (x) axes and perform a 4-parameter logistic fit.

Alternative Plot: The data can also be linearized using a logit transformation. The equation for this conversion is shown below. *NOTE: Do not use %B/B_0 in this calculation.*

$$\text{logit } (B/B_0) = \ln [B/B_0/(1 - B/B_0)]$$

Plot the data as logit (B/B_0) *versus* log concentrations and perform a linear regression fit.

Determine the Sample Concentration

Calculate the B/B_0 (or %B/B_0) value for each sample. Determine the concentration of each sample using the equation obtained from the standard curve plot. *NOTE: Remember to account for any concentration or dilution of the sample prior to the addition to the well.* Samples with %B/B_0 values greater than 80% or less than 20% should be re-assayed as they generally fall out of the linear range of the standard curve. A 20% or greater disparity between the apparent concentration of two different dilutions of the same sample indicates interference, which could be eliminated by purification.

NOTE: If there is an error in the B_0 wells it is possible to calculate sample concentrations by plotting the absorbance values and back calculating sample absorbance off the standard curve.

Performance Characteristics

Representative Data

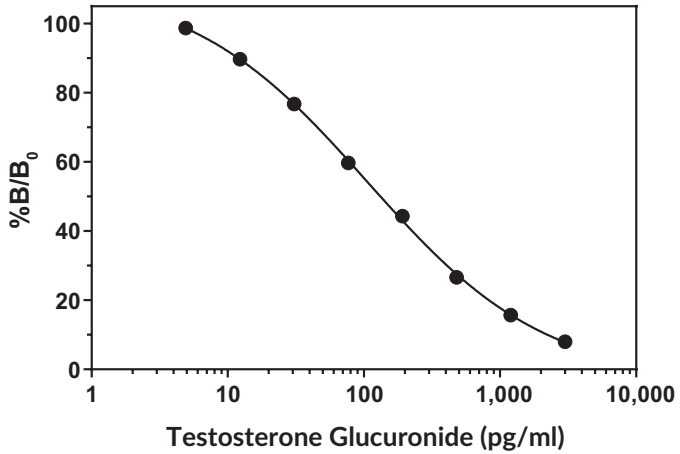
The standard curve presented here is an example of the data typically produced with this kit; however, your results will not be identical to these. You must run a new standard curve. Do not use the data below to determine the values of your samples. *Absorbance 405 nm at 90 minutes*

Testosterone Glucuronide Standards (pg/ml)	Blank-subtracted Absorbance	NSB-Corrected Absorbance	%B/B_0	%CV* Intra-assay precision	%CV* Inter-assay precision
NSB	0.010		--	--	--
B_0	1.220	1.210		--	--
3,000.0	0.108	0.098	8.0	10.3	13.6
1,200.0	0.200	0.190	15.7	8.3	5.0
480.0	0.332	0.322	26.6	9.7	6.0
192.0	0.547	0.537	44.3	10.2	6.2
76.8	0.732	0.722	59.7	12.1	5.6
30.7	0.939	0.929	76.7	18.9	3.3
12.3	1.095	1.085	89.7	30.1†	4.1
4.9	1.204	1.194	98.7	39.1†	6.0
TA	2.871				

Table 2. Typical Results

*%CV represents the variation in concentration (not absorbance) as determined using a reference standard curve.

† evaluate data in this range with caution



Assay Range = 4.9-3,000 pg/ml
Sensitivity (defined as 80% B/B_0) = 25 pg/ml
Mid-point (defined as 50% B/B_0) = 133 pg/ml
Lower Limit of Detection (LLOD) = 8.9 pg/ml

The sensitivity and mid-point were derived from the standard curve shown above. The standard was diluted in Tris Buffer (1X).

Figure 7. Typical standard curve for testosterone glucuronide

Precision:

Intra-assay precision was determined by analyzing 24 replicates of three matrix controls (human urine) in a single assay.

Matrix Control (pg/ml)	%CV
658.1	10.7
95.2	11.1
40.1	22.4

Table 3. Intra-assay variation

Inter-assay precision was determined by analyzing replicates of three matrix controls (human urine) in separate assays on different days.

Matrix Control (pg/ml)	%CV
638.4	8.7
99.4	10.3
38.8	8.4

Table 4. Inter-assay variation

Cross Reactivity:

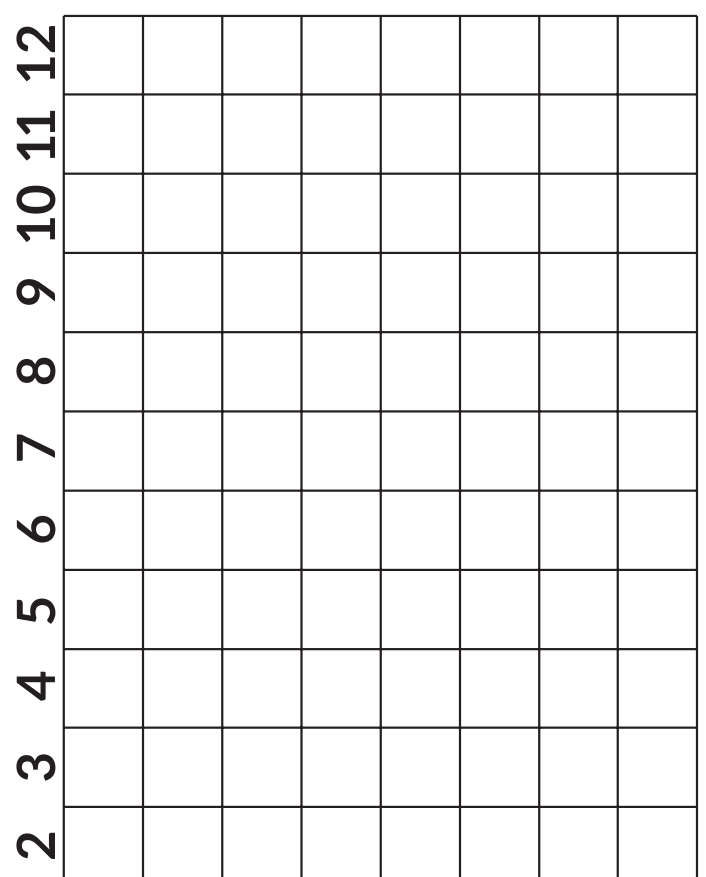
Compound	Cross Reactivity
Testosterone	30.8%
Progesterone	9.9%
Androstenedione	9.5%
epi-Testosterone	2.2%
Nandrolone	1.9%
Corticosterone	1.2%
11-keto Testosterone	0.8%
Aldosterone	0.5%
Cortisol	0.1%
epi-Testosterone Glucuronide	0.07%
Androsterone	0.02%
Pregnanediol-1-Glucuronide	0.01%
DHEA Sulfate	<0.01%
Estradiol 17-(β-Glucuronide)	<0.01%
Estradiol 17-Sulfate	<0.01%

Table 5. Cross reactivity of the Testosterone Glucuronide ELISA Kit

RESOURCES

Testosterone Glucuronide Assay Summary					
Procedure	**Blk**	**TA**	**NSB**	**B_0**	**Standards/ Samples**
Reconstitute and Mix	Mix all reagents gently				
Tris Buffer (1X)	-	-	100 µl	50 µl	-
Standards/Samples	-	-	-	-	50 µl
Testosterone Glucuronide AP Tracer	-	-	50 µl	50 µl	50 µl
Testosterone Glucuronide ELISA Antiserum	-	-	-	50 µl	50 µl
Seal	Seal the plate				
Incubate	Incubate 18 hours at 4°C				
Aspirate	Aspirate wells and wash 5 x ~300 µl				
*p*NPP Substrate Solution	200 µl	200 µl	200 µl	200 µl	200 µl
Testosterone Glucuronide AP Tracer	-	5 µl	-	-	-
Seal	Seal plate and incubate for 90 minutes at room temperature on orbital shaker, protect from light				
Read	Remove plastic seal and read absorbance at 405 nm				

Table 6. Assay Summary



Troubleshooting

Problem	Possible Causes
Erratic values; dispersion of duplicates	A. Trace organic contaminants in the water source B. Poor pipetting/technique
High NSB (>0.100 O.D.)	A. Poor washing; ensure proper washing is used B. Exposure of NSB wells to specific antibody
Very low B_0	A. Trace organic contaminants in the water source B. Dilution error in preparing reagents
Low sensitivity (shift in dose response curve)	Standard is degraded or contaminated
Analysis of two dilutions of a biological sample do not agree (*i.e.*, more than 20% differences)	Interfering substances are present; consider sample purification prior to analysis

References

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NOTES

Warranty and Limitation of Remedy

Buyer agrees to purchase the material subject to Cayman's Terms and Conditions. Complete Terms and Conditions including Warranty and Limitation of Liability information can be found on our website.

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