Pregnanediol-3-Glucuronide (PDG) ELISA Kit

Item No. 501300

www.caymanchem.com
Customer Service 800.364.9897
Technical Support 888.526.5351
1180 E. Ellsworth Rd · Ann Arbor, MI · USA

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GENERAL INFORMATION

Materials Supplied

Item Number	Item	96 wells Quantity/Size
400162	Pregnanediol-3-Glucuronide ELISA Antiserum	1 vial/100 dtn
400160	Pregnanediol-3-Glucuronide AP Tracer	1 vial/100 dtn
400174	Pregnanediol-3-Glucuronide ELISA Standard	1 vial/2.5 µg
400080	Tris Buffer Concentrate (10X)	1 vial/10 ml
411007	AP Wash Buffer Concentrate (150X)	1 vial/5 ml
400004/400006	Mouse Anti-Rabbit IgG Coated Plate	1 plate
400089	*p*NPP Substrate Solution	2 vials/12 ml
400040	ELISA Tracer Dye	1 ea
400042	ELISA Antiserum Dye	1 ea
400012	96-Well Cover Sheet	1 cover

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

Fax: 734-971-3640

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 pure water; glass distilled water or deionized water is acceptable. *NOTE: Ultra-Pure water is available for purchase from Cayman (Item No. 400000).*
4. Materials used for **Sample Preparation** (see page 13).

INTRODUCTION

Background

Pregnanediol-3-glucuronide (5β-pregnane-3α,20α-diol-3α-glucuronide, PDG or P3G) is the major metabolite of progesterone, an endogenous steroid and the most important member of the class of steroid hormones known as progestogens. Progesterone is important in a variety of biological functions such as preparing the endometrium for implantation, maintaining pregnancy, differentiating breast tissue, and promoting normal development of neurons in the brain as a neuro steroid. Low progesterone levels are linked to chronic anovulation which is reported to be associated with female infertility, breast cancer and endometrial cancer.[1,2]

Levels of PDG in the urine correlate highly to levels of progesterone measured in the serum.[3,4] PDG analysis may be used as a non-invasive method to indirectly measure progesterone and therefore might be used as a biomarker for luteal activity, ovarian cancer, and a variety of other biological disorders.[5-7]

About This Assay

Cayman's Pregnanediol-3-Glucuronide (PDG) ELISA Kit is a competitive assay that can be used for quantification of pregnanediol metabolites in urine. The assay has a range from 0.4-50 ng/ml and a sensitivity (80% B/B_0) of approximately 0.94 ng/ml.

Principle of the Assay

This assay is based on the competition between pregnanediol-3-glucuronide and a pregnanediol-3-glucuronide-alkaline phosphatase (AP) conjugate (Pregnanediol-3-Glucuronide AP Tracer) for a limited amount of Pregnanediol-3-Glucuronide ELISA Antiserum. Because the concentration of the Pregnanediol-3-Glucuronide AP Tracer is held constant while the concentration of pregnanediol-3-glucuronide varies, the amount of Pregnanediol-3-Glucuronide AP Tracer that is able to bind to the Pregnanediol-3-Glucuronide ELISA Antiserum will be inversely proportional to the concentration of pregnanediol-3-glucuronide in the well. This antibody-pregnanediol-3-glucuronide complex binds to a mouse anti-rabbit IgG that has been previously attached to the well. The plate is washed to remove any unbound reagents and then ρNPP Substrate Solution 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 Pregnanediol-3-Glucuronide AP Tracer bound to the well, which is inversely proportional to the amount of free pregnanediol-3-glucuronide present in the well during the incubation; or

$$\text{Absorbance} \propto [\text{Bound Pregnanediol-3-Glucuronide AP Tracer}] \propto 1/[\text{Pregnanediol-3-Glucuronide}]$$

A schematic of this process is shown in Figure 1, on page 9

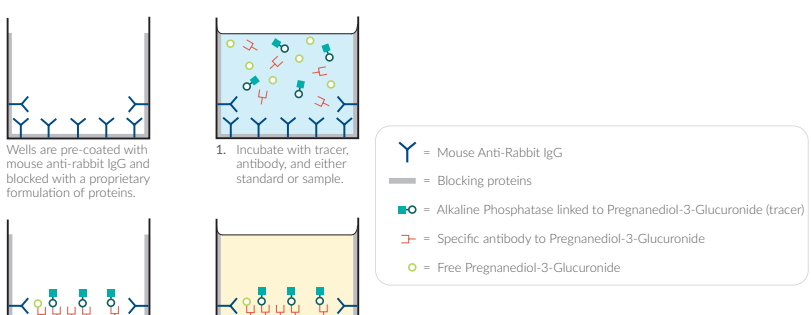


Figure 1. Schematic of the ELISA

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.

Total Activity (TA): total enzymatic activity of the alkaline phosphatase-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. Do not forget to subtract the Blank absorbance values.

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 will be stable for about two months.

1. **Tris Buffer 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 water.*

2. **AP Wash Buffer Preparation**

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

Sample Preparation

This assay has been demonstrated to work with human urine without extraction. Proper sample storage and preparation are essential for consistent and accurate results. Please read this section thoroughly before beginning the assay.

General Precautions

- All samples must be free of organic solvents prior to 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 which interfere with the assay by binding to the mouse anti-rabbit plate. We recommend that all rabbit samples be purified prior to use in this assay.

Testing for Interference

This assay has been tested using human urine. Other sample types will need to be assessed for interference by the end user. To test for interference, dilute one or two test samples to obtain at least two different dilutions of each sample between approximately 25 and 0.8 ng/ml (*i.e.*, between 20-80% B/B_0). If the two different dilutions of the sample show good correlation (differ by 20% or less) in the final calculated pregnanediol-3-glucuronide concentration, purification is not required. If you do not see good correlation of the different dilutions, purification is advised. Purification methods will need to be determined by the end user and tested for compatibility in the assay.

Sample Matrix Properties

Linearity

Human urine samples were serially diluted and evaluated for linearity using the Pregnanediol-3-Glucuronide (PDG) ELISA Kit. The results are shown in the table below.

Human Urine		
Dilution	**Concentration (ng/ml)**	**Dilutional Linearity (%)**
1:16	583.1	101.0
1:32	576.2	108.0
1:64	531.6	95.4
1:128	557.1	93.9
1:256	593.1	100.0

Table 1. Dilutional Linearity of human urine samples

Spike and Recovery

Human urine was spiked with pregnanediol-3-glucuronide, diluted as described in the **Sample Preparation** section and analyzed using the Pregnanediol-3-Glucuronide (PDG) ELISA Kit. The results are shown below. The y-intercept corresponds to the amount of endogenous pregnanediol-3-glucuronide in the sample.

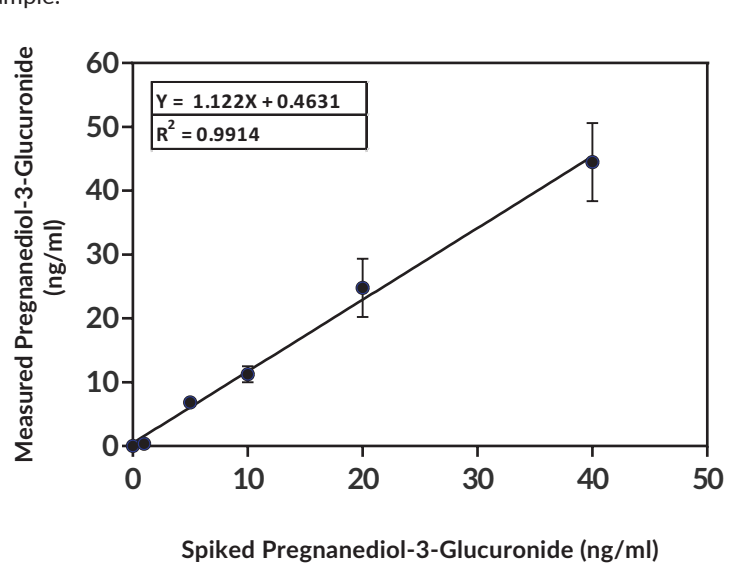


Figure 2. Spike and recovery in human urine

Sample	Spike Concentration (ng/ml)	% Recovery
Human Urine	40	110
	20	106
	10	107
	5	143

Table 2. Spike and recovery of human urine

Parallelism

To assess parallelism, a human urine sample was serially diluted and evaluated using the Pregnanediol-3-Glucuronide (PDG) 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.

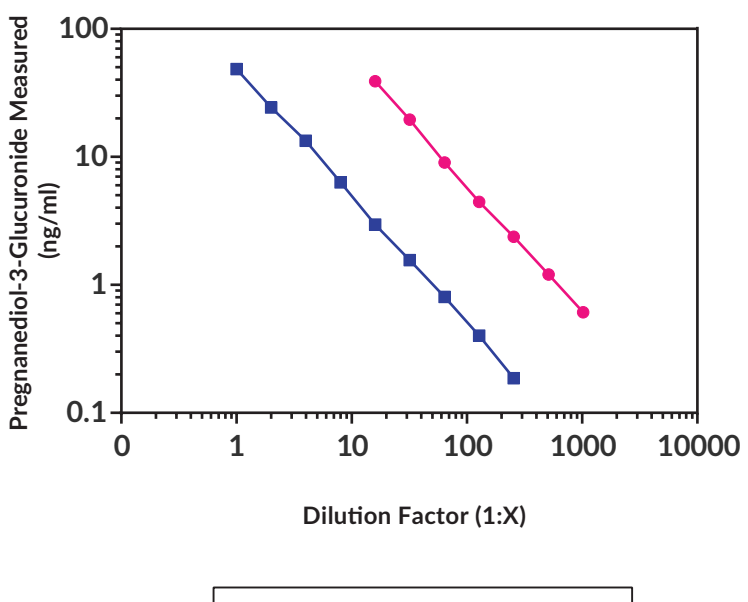


Figure 3. Parallelism of sample matrices in the Pregnanediol-3-Glucuronide ELISA Kit

ASSAY PROTOCOL

Preparation of Assay-Specific Reagents

Pregnanediol-3-Glucuronide ELISA Standard

Equilibrate a pipette tip by repeatedly filling and expelling the tip with Pregnanediol-3-Glucuronide ELISA Standard (Item No. 400174) several times. Using the equilibrated pipette tip, transfer 100 µl of the Pregnanediol-3-Glucuronide ELISA Standard (Item No. 400174) into a clean test tube containing 900 µl Pure Water and mix thoroughly. The concentration of this solution (the bulk standard) will be 500 ng/ml.

To prepare the standard for use in ELISA: Obtain eight clean test tubes and number them #1 through #8. Aliquot 900 µl Tris Buffer to tube #1 and 500 µl Tris Buffer to tubes #2-8. Transfer 100 µl of the bulk standard (500 ng/ml) to tube #1 and mix thoroughly. Serially dilute the standard by removing 500 µl from tube #1 and placing in tube #2; mix thoroughly. Next, remove 500 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 two hours.

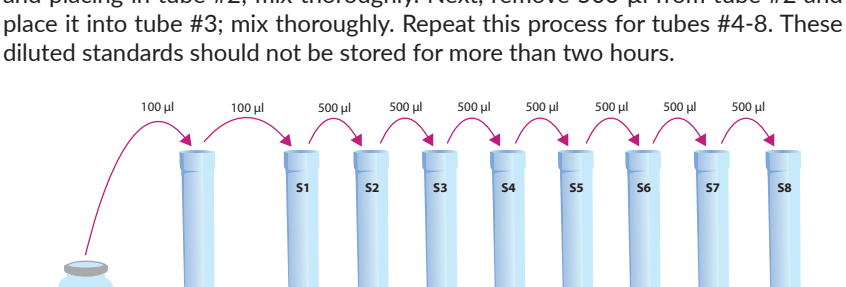


Figure 4. Preparation of the Pregnanediol-3-Glucuronide standards

Pregnanediol-3-Glucuronide AP Tracer

Reconstitute the Pregnanediol-3-Glucuronide AP Tracer (Item No. 400160) with 6 ml of Tris Buffer. A 20% surplus of tracer has been included to account for any incidental losses. Store the reconstituted Pregnanediol-3-Glucuronide ELISA AP Tracer at 4°C. It will be stable for at least three weeks.

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).

Pregnanediol-3-Glucuronide ELISA Antiserum

Reconstitute the Pregnanediol-3-Glucuronide ELISA Antiserum (Item No. 400162) with 6 ml of Tris Buffer. A 20% surplus of antiserum has been included to account for any incidental losses. Store the reconstituted Pregnanediol-3-Glucuronide ELISA Antiserum at 4°C. It will be stable for at least three weeks.

Antiserum Dye Instructions (optional)

This dye may be added to the antiserum, if desired, to aid in visualization of antiserum-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(s) 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 the strips at once, place 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 blanks (Blk), two non-specific binding wells (NSB), two maximum binding wells (B_0), and an eight-point standard curve run in duplicate. *NOTE: Each assay must contain this minimum configuration to ensure accurate and reproducible results. Each sample should be assayed at two dilutions and each dilution should be assayed in duplicate. For statistical purposes, we recommend assaying samples in triplicate.*

A suggested plate format is shown in Figure 5, below. The user may vary the location and type of wells present as necessary for each particular experiment. The plate format provided below has been designed to allow for easy data analysis using a convenient spreadsheet offered by Cayman (see page 21, for more details). We suggest you record the contents of each well on the template sheet provided (see page 31).

	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 5. 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**

   Add 100 µl Tris Buffer to Non-Specific Binding (NSB) wells. Add 50 µl Tris Buffer to Maximum Binding (B_0) wells.

2. **Pregnanediol-3-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). Continue with this procedure until all the standards are aliquoted. 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. **Pregnanediol-3-Glucuronide AP Tracer**

   Add 50 µl to each well *except* the Total Activity (TA) and the Blank (Blk) wells.

5. **Pregnanediol-3-Glucuronide ELISA Antiserum**

   Add 50 µl to each well except the Total Activity (TA), the Non-Specific Binding (NSB), and the Blank (Blk) wells.

Incubation of the Plate

Cover each plate with plastic film (Item No. 400012) and incubate for 2 hours at room temperature with shaking.

Development of the Plate

1. Empty the wells and rinse five times with Wash Buffer.
2. Add 200 µl of *p*NPP Substrate Solution (Item No. 400089) to each well.
3. Dilute 10 µl of Tracer into 90 µl Tris Buffer. Add 5 µl of the diluted tracer to the Total Activity wells.
4. Cover the plate with the supplied 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. Develop assay for 60 min.

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 plots 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 Chemical 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* pregnanediol-3-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 by identifying the %B/B_0 on the standard curve and reading the corresponding values on the x-axis. *NOTE: Remember to account for any 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.

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 value of your samples.

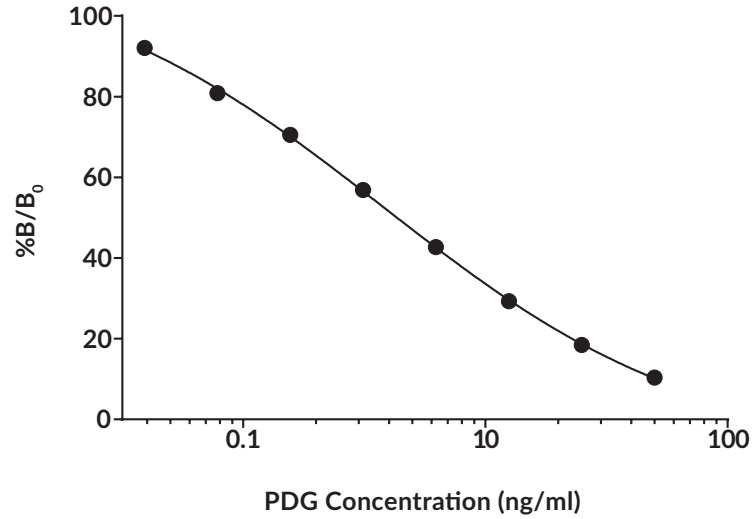
Absorbance at 405 nm at 60 minutes

Representative Data					
Pregnanediol-3-Glucuronide Standards (ng/ml)	Blank Corrected Absorbance	NSB Corrected Absorbance	%B/B_0	%CV* Intra-Assay Variance	%CV* Inter-Assay Variance
NSB	0.004	--	--	--	--
B_0	1.018	1.014	--	--	--
Total Activity	1.386	--	--	--	--
50	0.110	0.106	10.3	8.8	5.5
25	0.192	0.188	18.5	6.2	2.9
12.5	0.303	0.298	29.3	7.3	6.2
6.3	0.438	0.434	42.7	7.6	6.2
3.15	0.582	0.578	56.9	6.8	6.9
1.6	0.721	0.717	70.5	10.6	9.2
0.8	0.826	0.821	80.8	10.1	11.0
0.4	0.939	0.934	92.1	16.8	10.7

Table 3. Typical results
50% B/B_0–4.304 ng/ml

Sensitivity (80% B/B_0)–0.941 ng/ml

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



Assay Range = 0.4-50 ng/ml
Sensitivity (defined as 80% B/B_0) = 0.94 ng/ml
Mid-point (defined as 50% B/B_0) = 4.30 ng/ml
Lower Limit of Detection (LLOD) = 0.23 ng/ml

The sensitivity and mid-point were derived from the standard curve shown above. The standard was diluted with Tris Buffer.

Figure 6. Typical standard curve

Precision:

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

Intra-assay Precision	
Matrix Control (ng/ml)	%CV
14.7	11.1
3.04	11.0
1.07	15.7

Table 4. Intra-Assay Variation

The inter-assay precision was determined by analyzing four replicate of three matrix controls (pooled human urine) in separate assays spanning across several days.

Inter-assay Precision	
Matrix Control (ng/ml)	%CV
16.8	9.6
3.53	13.0
1.07	10.2

Table 5. Inter-Assay Variation

Cross Reactivity:

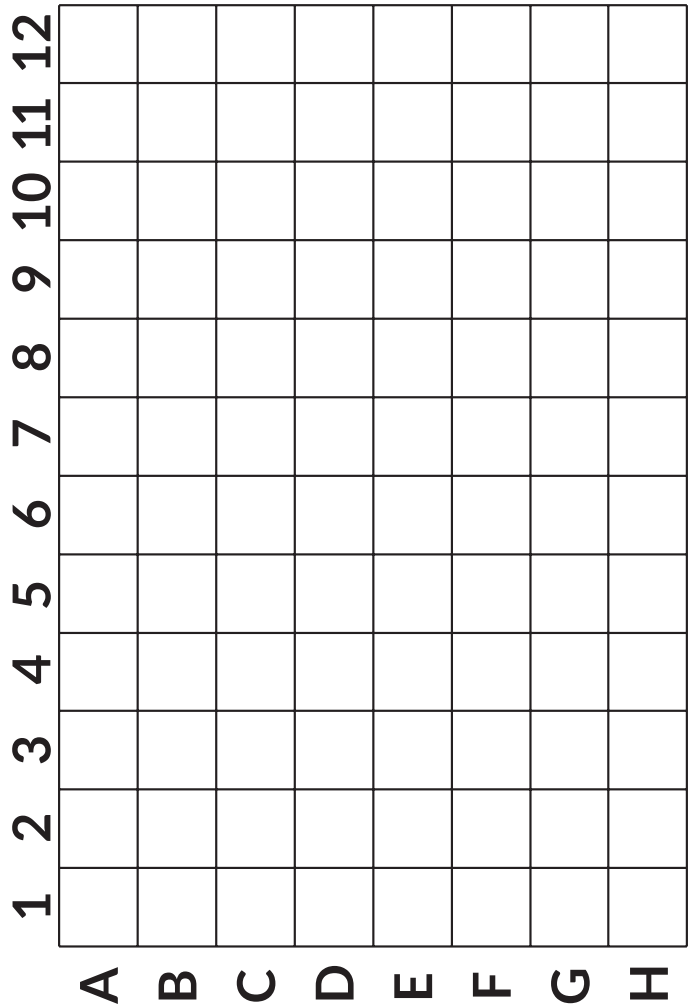
Compound	Cross Reactivity
Pregnanediol-3-Glucuronide	100%
Progesterone	25.8%
Corticosterone	0.03%
DHEA Sulfate	0.02%
Estriol	0.01%
Estradiol Glucuronide	0.002%
Aldosterone	0.006%
Cortisol	0.009%
Testosterone	0.35%
Testosterone Glucuronide	0.006%

Table 6. Specificity of the Pregnanediol-3-Glucuronide ELISA Antiserum

Assay Summary:

Procedure	Blk	TA	NSB	B_0	Standards/ Samples
Reconstitute and Mix	Mix all reagents gently				
Tris Buffer (1X)	--	--	100 µl	50 µl	--
Pregnanediol-3-Glucuronide Standards/Samples	--	--	--	--	50 µl
Pregnanediol-3-Glucuronide AP Tracer	--	--	50 µl	50 µl	50 µl
Pregnanediol-3-Glucuronide Antiserum	--	--	--	50 µl	50 µl
Seal	Seal the plate				
Incubate	Incubate for 2 hours at room temperature on orbital shaker				
Aspirate	Aspirate wells and wash 5 x ~300 µl				
Apply ρNPP Substrate	200 µl	200 µl	200 µl	200 µl	200 µl
Total Activity (TA) - Apply Tracer	--	5 µl	--	--	--
Seal	Seal plate and incubate for 60 min at room temperature on orbital shaker, protect from light				
Read	Remove seal and read absorbance at 405 nm				

Table 8. Pregnanediol-3-Glucuronide Assay Summary



RESOURCES

Troubleshooting

Problem	Possible Causes
Erratic values; dispersion of duplicates	A. Trace organic contaminants in the water source B. Poor pipetting/technique
High NSB (> 0.075)	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. Plate requires additional development time C. Dilution error in preparing reagents
Low sensitivity (shift in dose response curve)	Standard is degraded
Analysis of two dilutions of a biological sample do not agree (*i.e.*, more than 20% difference)	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.