5α-androstane-3α, 17β-diol glucuronide (3α diol G) LIA

For the direct quantitative determination of 3α diol G in human serum by chemiluminescence immunoassay (LIA)

For Research Use Only. Not For Use In Diagnostic Procedures.

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INTENDED USE

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INTRODUCTION

5α-Androstane-3α, 17β-diol glucuronide is a C19 steroid and is either abbreviated as 3α diol G, 5α diol G or simply, α diol G. It is produced mainly as a metabolite of testosterone and dihydrotestosterone (DHT). It is largely produced in target peripheral tissues such as the skin, especially around hair follicles. The stimulation by large amounts of 3α diol G leads to excessive hair formation, notably where hair is not normally present in women.

In recent years the interest in the measurement of this steroid has increased among clinical investigators studying women suffering from idiopathic hirsutism.

Among the steroids known to be precursors for 3α diol G are dehydroepiandrosterone (DHEA), dehydroepiandrosterone sulphate (DHEAS), dihydrotestosterone (DHT), androstenedione and testosterone. Only 3α diol G has been shown to increase with hirsutism and decrease with treatment. This correlation has also been demonstrated in patients with polycystic ovarian syndrome (PCO). 3α diol G determinations have therefore proved to be a useful indicator in a variety of ways including monitoring the progress of treatment of idiopathic hirsutism and women with PCO.

Furthermore, diabetic patients (both men and women) under cyclosporine A therapy have shown increased 3α diol G levels, a side effect resulting in the appearance of hair in previously hairless areas.

PRINCIPLE OF THE TEST

The principle of the following chemiluminescence immunoassay (LIA) test follows the typical competitive binding scenario. Competition occurs between an unlabeled antigen (present in standards, control and samples) and an enzyme-labeled antigen (conjugate) for a limited number of antibody binding sites on the microwell plate. The washing and decanting procedures remove unbound materials. After the washing step, the luminescence substrate solution is added. The relative luminescence units (RLUs) are measured on a microtiter plate luminometer. The RLU values are inversely proportional to the concentration of 3α diol G in the sample. A set of calibrators are used to plot a standard curve from which the amount of 3α diol G in samples and controls can be directly read.

PROCEDURAL CAUTIONS AND WARNINGS

1. Users should have a thorough understanding of this protocol for the successful use of this kit. Reliable performance will only be attained by strict and careful adherence to the instructions provided.
2. Control materials or serum pools should be included in every run at a high and low level for assessing the reliability of results.
3. When the use of water is specified for dilution or reconstitution, use deionized or distilled water.
4. In order to reduce exposure to potentially harmful substances, gloves should be worn when handling kit reagents and human specimens.
5. All kit reagents and specimens should be brought to room temperature and mixed gently but thoroughly before use. Avoid repeated freezing and thawing of reagents and specimens.
6. A calibrator curve must be established for every run.
7. The kit control should be included in every run and fall within established confidence limits.
8. Improper procedural techniques, imprecise pipetting, incomplete washing as well as improper reagent storage may be indicated when assay values for the control do not reflect established ranges.
9. The luminescence substrate solutions (A and B) are sensitive to light and should be stored in the original dark bottle away from direct sunlight.
10. The assay buffer is sensitive to light and should be stored in the original dark bottle away from direct sunlight.
12. When dispensing the substrate, do not use pipettes in which this liquids will come into contact with any metal parts.
13. To prevent contamination of reagents, use a new disposable pipette tip for dispensing each reagent, sample, standard and control.
14. Do not mix various lot numbers of kit components within a test and do not use any component beyond the expiration date printed on the label.
15. Kit reagents must be regarded as hazardous waste and disposed of according to national regulations.

LIMITATIONS

1. All the reagents within the kit are calibrated for the direct determination of 3α diol G in human serum. The kit is not calibrated for the determination of 3α diol G in saliva, plasma or other specimens of human or animal origin.
2. Do not use grossly hemolyzed, grossly lipemic, icteric or improperly stored serum.
3. Any samples or control sera containing azide or thimerosal are not compatible with this kit, as they may lead to false results.
4. Only calibrator A may be used to dilute any high serum samples. The use of any other reagent may lead to false results.
5. This kit is for research use only. The occurrence of heterophilic antibodies in individuals regularly exposed to animals or animal products has the potential of causing interferences in immunological tests.

SAFETY CAUTIONS AND WARNINGS

Human serum that may be used in the preparation of the standards and control has been tested and found to be non-reactive for Hepatitis B surface antigen and has also been tested for the presence of antibodies to HCV and Human Immunodeficiency Virus (HIV) and found to be negative. However no test method can offer complete assurance that HIV, HCV and Hepatitis B virus or any infectious agents are absent. The reagents should be considered a potential biohazard and handled with the same precautions as applied to any blood specimen.

CHEMICAL HAZARDS

Avoid direct contact with reagents. In case of contact, wash with plenty of water.

SPECIMEN COLLECTION AND STORAGE

Approximately 0.2 ml of serum is required per duplicate determination. Collect 4-5 ml of blood into an appropriately labeled tube and allow it to clot. Centrifuge and carefully remove the serum layer. Store at 4 ^{o}C for up to 24 hours or at -10^{o}C or lower if the analyses are to be done at a later date. Consider all human specimens as possible biohazardous materials and take appropriate precautions when handling.

SPECIMEN PRETREATMENT

This assay is a direct system; no specimen pretreatment is necessary.

REAGENTS AND EQUIPMENT NEEDED BUT NOT PROVIDED

1. Precision pipettes to dispense 50, 100, 150 and 300 µl
2. Disposable pipette tips
3. Distilled or deionized water
4. Plate shaker
5. Microwell plate luminometer

REAGENTS PROVIDED AND PREPARATION

1. Rabbit Anti-3α Diol G Antibody Coated Microwell Plate-Break Apart Wells - Ready To Use.
Contents: One 96 well (12x8) polyclonal antibody-coated microwell plate in a resealable pouch with desiccant.
Storage: Refrigerate at 2-8^{o}C
Stability: 12 months or as indicated on label.

2. 3α Diol G-Horseradish Peroxidase (HRP) Conjugate Concentrate - Requires Preparation.
Contents: 3α Diol G-HRP conjugate in a protein-based buffer with a non-mercury preservative.
Volume: 300 µl/vial
Storage: Refrigerate at 2-8^{o}C
Stability: 12 months or as indicated on label.
Preparation: Dilute 1:50 in assay buffer before use (eg. 40 µl of HRP in 2 ml of assay buffer). If the whole plate is to be used dilute 240 µl of HRP in 12ml of assay buffer. Discard any that is left over.

3. 3α Diol G Calibrators - Ready To Use.
Contents: Six vials containing 3α diol G in a protein-based buffer with a non-mercury preservative. Prepared by spiking buffer with a defined quantity of 3α diol G.
*Listed below are approximate concentrations, please refer to vial labels for exact concentrations.

Calibrator	Concentration	Volume/Vial
Calibrator A	0 ng/ml	2.0 ml
Calibrator B	0.25 ng/ml	0.6 ml
Calibrator C	1 ng/ml	0.6 ml
Calibrator D	3 ng/ml	0.6 ml
Calibrator E	10 ng/ml	0.6 ml
Calibrator F	50 ng/ml	0.6 ml

Storage: Refrigerate at 2-8°C
Std vials or as indicated on label. Once opened, the standards should be used within 14 days or aliquoted and stored frozen. Avoid multiple freezing and thawing cycles.

4. Control - Ready To Use.
Contents: One vial containing 3α diol G in a protein-based buffer with a non-mercury preservative. Prepared by spiking bufer with a defined quantity of 3α diol G. Refer to vial label for expected value and acceptable range.
Volume: 0.6 ml/vial
Storage: Refrigerate at 2-8 °C
Stability: 12 months in unopened vial or as indicated on label. Once opened, the control should be used within 14 days or aliquoted and stored frozen. Avoid multiple freezing and thawing cycles.

5. Wash Buffer Concentrate - Requires Preparation.
Contents: One bottle containing buffer with a non-ionic detergent and a non-mercury preservative.
Volume: 50 ml/bottle
Storage: Refrigerate at 2-8°C
Stability: 12 months or as indicated on label.
Preparation: Dilute 1:10 in distilled or deionized water before use. If the whole plate is to be used dilute 50 ml of the wash buffer concentrate in 450 ml of water.

6. Assay Buffer - Ready To Use
Contents: One bottle containing a protein-based buffer with a non-mercury preservative.
Volume: 15 ml/bottle
Storage: Refrigerate at 2-8°C
Stability: 12 months or as indicated on label.

7. LIA Substrate Reagent A - Requires Preparation.
Contents: One bottle containing luminol enhancer.
Volume: 1 ml/bottle
Storage: Refrigerate at 2-8°C
Stability: 12 months or as indicated on label.
Preparation: See preparation of LIA Working Substrate Solution

8. LIA Substrate Reagent B - Requires Preparation.
Contents: One vial containing peroxide solution.
Volume: 1 ml/vial
Storage: Refrigerate at 2-8°C
Stability: 12 months or as indicated on label.
Preparation: See preparation of LIA Working Substrate Solution

9. LIA Substrate Reagent C - Requires Preparation.
Contents: One vial containing buffer with a non-mercury preservative.
Volume: 15 ml/vial
Storage: Refrigerate at 2-8°C
Stability: 12 months or as indicated on label.
Preparation: See preparation of LIA Working Substrate Solution

Preparation of LIA Working Substrate Solution:
Mix 1 part of the chemiluminescence substrate reagent A with 1 part of reagent B and dilute this mixture 1:6 with reagent C. This gives the ready to use substrate solution. Prepare fresh for each use.

If the whole plate is to be used prepare working substrate solution as follows:

Combine 1 ml of reagent A with 1 ml of reagent B. To the 2 ml of this mixture add 12 ml of reagent C. Total volume = 14 ml of working substrate solution.

Stability: Working substrate solution is stable for 24 hours at room temperature.

ASSAY PROCEDURE

Important Notes:

1. **All reagents must reach room temperature before use.**
2. **Once the procedure has been started, all steps should be completed without interruption to ensure equal elapsed time for each pipetting step.**
3. **The washing procedure influences the precision markedly; it is essential to ensure the washing is effective and thorough.**

1. **Prepare working solutions of the conjugate, wash buffer, and LIA substrate (refer to reagents provided and preparation section.)**
2. **Remove the required number of microwell strips. Reseal the bag and return any unused strips to the refrigerator.**
3. **Pipette 50 µl of each calibrator, control, and specimen sample into correspondingly labeled wells in duplicate.**
4. **Pipette 100 µl of the conjugate working solution into each well (We recommend using a multichannel pipette).**
5. **Incubate on a plate shaker (approximately 200 rpm) for 30 minutes at room temperature.**
6. **Wash the wells 5 times with 300 µl ofdiluted wash buffer per well and tap the plate firmly against absorbent paper to ensure that it is dry (The use of a washer is recommended).**
7. **Pipette 100 µl of LIA working substrate solution into each well (We recommend using a multichannel pipette).**
8. **Shake for 5 seconds. Incubate for 15 minutes at room temperature without shaking.**
9. **Measure the RLUs in each well on a microplate luminometer within 20 minutes after addition of the substrate.**

CALCULATIONS

1. Calculate the mean RLU of each calibrator duplicate.

2. Draw a calibrator curve on semi-log paper with the mean RLUs on the Y-axis and the calibrator concentrations on the X-axis. If immunoassay software is being used, a 4-parameter curve is recommended.

3. Calculate the mean RLU of each unknown duplicate.

4. Read the values of the unknowns directly off the calibrator curve.

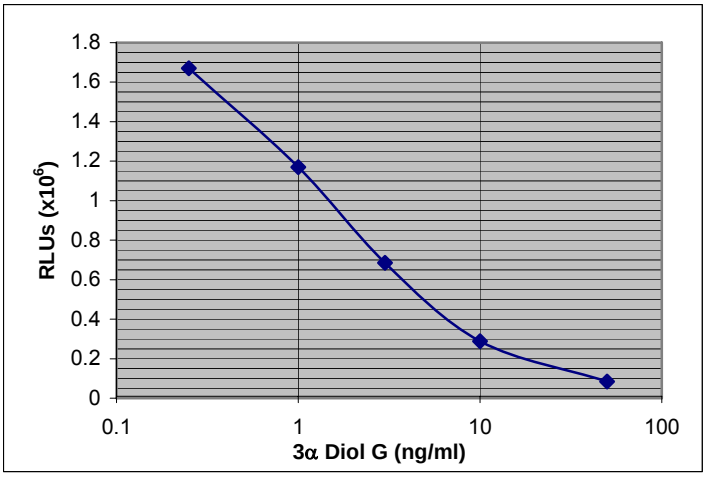
5. If a sample reads more than 50 ng/ml then dilute it with calibrator A at a dilution of no more than 1:8. The result obtained should be multiplied by the dilution factor.

TYPICAL TABULATED DATA**

Calibrator	RLU 1 x 10^3	RLU 2 x 10^3	Mean RLU x 10^3	RLU/RLU_{MAX} (%)
A, 0 ng/ml	1914	1917	1916	100
B, 0.25ng/ml	1669	1663	1671	87
C, 1 ng/ml	1181	1159	1170	61
D, 3 ng/ml	693.8	677.1	685.4	36
E, 10 ng/ml	280.1	296.0	288.0	15
F, 50 ng/ml	84.84	83.32	84.01	4.3

**- It is recommended to use the RLU/RLU_{MAX} values for comparative purposes since luminometers vary considerably between manufacturers. Results from different luminometers will show quite different RLU values, however, the RLU/RLU_{MAX} values remain consistent.

TYPICALCALIBRATORCURVE
Sample curve only. **Do not** use to calculate results.



PERFORMANCE CHARACTERISTICS
SENSITIVITY
The lower detection limit is calculated from the standard curve by determining the resulting concentration of the mean RLU of Calibrator A (based on 10 replicate analyses) minus 2 SD. Therefore, the sensitivity of this 3α diol G LIA kit is **0.1 ng/ml.**

SPECIFICITY (CROSS REACTIVITY)
The following compounds were tested for cross-reactivity with the 3α diol G LIA kit with 3α diol G cross-reacting at 100%.

Steroid	%Cross Reactivity
3α Diol G	100
Testosterone	0.2
Progesterone	0.16
Androstenedione	0.14
Cortisol	0.05

The following steroids were tested but cross-reacted at less than 0.01%: Corticosterone, Dehydroepiandrosterone, Dihydrotestosterone, Epiandrosterone, 17β-Estradiol and Estrone.

INTRA-ASSAY PRECISION

Three serum samples were assayed ten times each on the same calibrator curve. The results (in ng/ml) are tabulated below:

Sample	Mean	SD	CV%
1	1.21	0.10	7.96
2	8.86	0.32	3.58
3	32.23	1.95	6.06

INTER-ASSAY PRECISION

Three serum samples were assayed ten times over a period of four weeks. The results (in ng/ml) are tabulated below:

Sample	Mean	SD	CV%
1	1.57	0.10	6.4
2	10.49	0.87	8.34
3	43.19	3.81	8.82

RECOVERY

Spiked samples were prepared by adding defined amounts of 3α diol G to three serum samples. The results (in ng/ml) are tabulated below:

Sample	Obs.Result	Exp.Result	Recovery%
1 Unspiked	1.7	-	-
+0.25(1:1,v/v)	0.9	0.98	91.8
+3.0(1:1,v/v)	2.6	2.35	102.4
+15.0(1:1,v/v)	25.4	25.85	98.3
2 Unspiked	8.8	-	-
+1.0(1:1,v/v)	4.7	4.9	95.9
+3.0(1:1,v/v)	6.0	5.9	105.3
+10(1:1,v/v)	9.9	9.4	101.7
3 Unspiked	2.9	-	-
+0.25(1:1,v/v)	1.4	1.5	93.3
+3.0(1:1,v/v)	2.9	2.95	98.3
+50(1:1,v/v)	29.3	26.3	111.4

LINEARITY

Three serum samples were diluted with calibrator A. The results (in ng/ml) are tabulated below:

Sample	Obs.Result	Exp.Result	Recovery%
1	21.8	-	-
1:2	10.2	10.9	93.6
1:4	5.5	5.45	99.1
1:8	2.7	2.73	98.9
2	25.2	-	-
1:2	13.4	12.6	106.3
1:4	7.4	6.3	117.5
1:8	3.5	3.2	109.4
3	27.4	-	-
1:2	14.8	13.7	108.0
1:4	7.4	6.85	108.0
1:8	3.5	3.43	103.0

EXPECTED NORMAL VALUES

As for all clinical assays each laboratory should collect data and establish their own range of expected normal values.

Group	Range (ng/ml)
Males	1.53-14.82
Premenopausal	0.22-4.64
Postmenopausal	0.61-3.71
Puberty (Female)	0.51-4.03

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Notes