



Manufactured for:  
**Immuno-Biological Laboratories, Inc.**  
8201 Central Ave. NE, Suite P  
Minneapolis, MN 55432, USA  
Phone: (888) 523-1246  
Email: info@ibl-america.com  
Web: www.ibl-america.com

*Direct ELISA Kit*

**5 $\alpha$ -ANDROSTANE-3 $\alpha$ , 17 $\beta$ -DIOL  
GLUCURONIDE (3 $\alpha$  DIOL G)**

Cat. No.: IB59102

Version: 5.0

Effective: October 25, 2010

**INTENDED USE**

For the direct determination of 3 $\alpha$  Diol G by enzyme immunoassay in human serum. For research use only, not for use in diagnostic procedures.

**PRINCIPLE OF THE TEST**

The principle of the following enzyme immunoassay test follows the typical competitive binding scenario. Competition occurs between an unlabeled antigen (present in calibrators, controls and unknowns being tested) and an enzyme-labelled 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 enzyme substrate is added. The enzymatic reaction is terminated by addition of the stopping solution. The absorbance is measured on a microtiter plate reader. The intensity of the colour formed is inversely proportional to the concentration of 3 $\alpha$  Diol G in the unknown. A set of calibrators is used to plot a calibration curve from which the amount of 3 $\alpha$  Diol G in the unknowns 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 unknowns.
5. All kit reagents and unknowns should be brought to room temperature and mixed gently but thoroughly before use. Avoid repeated freezing and thawing of reagents and unknowns.
6. A calibrator curve must be established for every run.
7. The controls 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 controls do not reflect established ranges.
9. When reading the microplate, the presence of bubbles in the microwells will affect the optical densities (ODs). Carefully remove any bubbles before performing the reading step.
10. The substrate solution (TMB) is sensitive to light and should remain colourless if properly stored. Instability or

- contamination may be indicated by the development of a blue colour, in which case it should not be used.
11. 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 and stopping solution, do not use pipettes in which these 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, unknown, calibrator 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 3 $\alpha$  Diol G in human serum. The kit is not calibrated for the determination of 3 $\alpha$  Diol G in saliva, plasma or other types of human or animal biologicals.
2. Do not use grossly hemolyzed, grossly lipemic, icteric or improperly stored serum.
3. Any unknowns or control sera containing azide or thimerosal are not compatible with this kit, as they may lead to inaccurate results.
4. Only calibrator A may be used to dilute any high serum unknowns. The use of any other reagent may lead to inaccurate results.
5. The occurrence of heterophilic antibodies in subjects regularly exposed to animals or animal products has the potential of causing interferences in immunological tests. The subject's background, including the frequency of exposure to animals/products, should be considered if false results are suspected.

**SAFETY CAUTIONS AND WARNINGS  
POTENTIAL BIOHAZARDOUS MATERIAL**

Human serum that may be used in the preparation of the calibrators and controls 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 biohazardous material.

**CHEMICAL HAZARDS**

Avoid contact with reagents containing TMB, hydrogen peroxide and sulfuric acid. If contacted with any of these reagents, wash with plenty of water. TMB is a suspected carcinogen.

**COLLECTION AND HANDLING OF UNKNOWN**

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

**PRETREATMENT OF UNKNOWN**

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

**REAGENTS AND EQUIPMENT NEEDED BUT NOT PROVIDED**

1. Precision pipettes to dispense 50, 100, 150 and 300  $\mu$ l
2. Disposable pipette tips
3. Distilled or deionized water
4. Plate shaker
5. Microwell plate reader with a filter set at 450nm and an upper OD limit of 3.0 or greater\* (see assay procedure step 10).

**REAGENTS PROVIDED**

**1. Rabbit Anti-3 $\alpha$  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°C  
Stability: 12 months or as indicated on label.

**2. 3 $\alpha$  Diol G-Horseradish Peroxidase (HRP) Conjugate Concentrate - Requires Preparation.**

Contents: 3 $\alpha$  Diol G-HRP conjugate in a protein-based buffer with a non-mercury preservative.  
Volume: 300  $\mu$ l/vial  
Storage: Refrigerate at 2-8°C  
Stability: 12 months or as indicated on label.  
Preparation: Dilute 1:50 in assay buffer before use (eg. 40  $\mu$ l of HRP in 2 ml of assay buffer). If the whole plate is to be used dilute 240  $\mu$ l of HRP in 12ml of assay buffer. Discard any that is left over.

**3. 3 $\alpha$  Diol G Calibrators - Ready To Use.**

Contents: Six vials containing 3 $\alpha$  Diol G in a protein-based buffer with a non-mercury preservative. Prepared by spiking buffer with a defined quantity of 3 $\alpha$  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  
Stability: 12 months in unopened vials or as indicated on label. Once opened, the calibrators should be used within 14 days or aliquoted and stored frozen. Avoid multiple freezing and thawing cycles.

**4. Controls - Ready To Use.**

Contents: Two vials containing 3 $\alpha$  Diol G in a protein-based buffer with a non-mercury preservative. Prepared by spiking buffer with defined quantities of 3 $\alpha$  Diol G. Refer to vial labels for 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 controls 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.  
\*Warm to completely dissolve before use.

**7. TMB Substrate - Ready To Use.**

Contents: One bottle containing tetramethylbenzidine and hydrogen peroxide in a non-DMF or DMSO containing buffer.  
Volume: 16 ml/bottle  
Storage: Refrigerate at 2-8°C  
Stability: 12 months or as indicated on label.

**8. Stopping Solution - Ready To Use.**

Contents: One vial containing 1M sulfuric acid.  
Volume: 6 ml/vial  
Storage: Refrigerate at 2-8°C  
Stability: 12 months or as indicated on label.

**ASSAY PROCEDURE**

Pretreatment of Unknowns:

**None.**

All reagents must reach room temperature before use. Calibrators, controls and unknowns should be assayed in duplicate. Once the procedure has been started, all steps should be completed without interruption.

1. Prepare working solutions of the 3 $\alpha$  Diol G-HRP conjugate and wash buffer.
2. Remove the required number of microwell strips. Reseal the bag and return any unused strips to the refrigerator.
3. Pipette 50  $\mu$ l of each calibrator, control and unknown into correspondingly labelled wells in duplicate.
4. Pipette 100  $\mu$ l 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 3 times with 300  $\mu$ l of diluted 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 150  $\mu$ l of TMB substrate into each well at timed intervals.
8. Incubate on a plate shaker for 10-15 minutes at room temperature (or until calibrator A attains dark blue colour for desired OD).
9. Pipette 50  $\mu$ l of stopping solution into each well at the same timed intervals as in step 7.
10. Read the plate on a microwell plate reader at 450nm within 20 minutes after addition of the stopping solution.

\* If the OD exceeds the upper limit of detection or if a 450nm filter is unavailable, a 405 or 415nm filter may be substituted. The optical densities will be lower, however, this will not affect the results of the controls or unknowns being tested.

**RESULTS**

1. Calculate the mean optical density of each calibrator duplicate.
2. Draw a calibrator curve on semi-log paper with the mean optical densities on the Y-axis and the calibrator concentrations on the X-axis. If immunoassay software is being used, a 4-parameter or 5-parameter curve is recommended.
3. Calculate the mean optical density of each unknown duplicate.
4. Read the values of the unknowns directly off the calibrator curve.
5. If an unknown 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.

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