# CERTIFICATE OF ANALYSIS

## ERM®- DA346a

| Frozen Human Serum – Testosterone, Low Level | | |
|---|---|---|
| Constituent | Certified value (µg/kg) | Uncertainty (µg/kg) |
| Testosterone (17ß-Hydroxyandrost-4-en-3-one) | 0.25 | 0.04 |

1) The certified value and its uncertainty were determined using isotope dilution mass spectrometry (IDMS), and are traceable to the SI through the use of a traceable, high accuracy testosterone standard for instrument calibration.

2) The quoted uncertainty is the half-width of the expanded uncertainty calculated using a coverage factor, *k*, of 2.1, which gives a level of confidence of approximately 95 %.

This certificate is valid for 12 months from the date of shipment provided the sample is stored under the recommended conditions.

The minimum amount of sample to be used is 0.8 g.

European Reference Material ERM®-DA346a was produced and certified under the responsibility of LGC according to the principles laid down in the Technical Guidelines of the European Reference Materials® co-operation agreement between BAM-LGC-IRMM. Information on these guidelines is available on the Internet (http://www.erm-crm.org).

Accepted as an ERM®, Teddington, August 2007.
Certificate revised June 2008

**Signed:** _____

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| Calculated Value | | |
|---|---|---|
| Constituent | Concentration [1] (nmol/L) | Uncertainty [2] (nmol/L) |
| Testosterone | 0.89 | 0.12 |

1) The concentration and uncertainty have been calculated using the density of serum: 1.0207 kg/L, expanded uncertainty ($k$ = 2): 0.0018 kg/L measured at LGC and a molecular weight of 288.41 g (Merck Index, 12th Edition, 1996).

2) The quoted uncertainty is the half-width of the expanded uncertainty calculated using a coverage factor, $k$, of 2.1, which gives a level of confidence of approximately 95 %.

## DESCRIPTION OF THE SAMPLE

Time expired human blood serum from donors to the National Blood Transfusion Service, Bristol was prepared at the University Hospital in Wales using their standard method for the General Chemistry EQA (External Quality Assessment) samples for the WEQAS scheme (http://www.weqas.co.uk/dsp_frame.cfm?link=schemes). Female blood serum was used with a concentration within the normal range for female human serum. The material was screened to ensure it was negative for HIV and Hepatitis B and C, then mixed and sterile filtered to 0.2 µm. Gentamicin was added as a preservative.

The serum was thoroughly mixed and portions of 0.8 mL (minimum) were sub-sampled into 3 mL screw-cap plastic vials, which were stored at (-70 ± 10)°C.

## INTENDED USE

The material is intended for use in the validation and ongoing monitoring of methods of analysis for the determination of testosterone in human blood samples.

## ANALYTICAL METHOD USED FOR CHARACTERISATION AT LGC

Testosterone CRM No M914 (99.4 ± 0.4) % was obtained from NMIA, New South Wales, Australia. Deuterated isotopically-labelled d2-testosterone DLM 683-0 (≥ 98 %) was obtained from Cambridge Isotope Laboratories, Massachusetts, USA. Stock standards were prepared in ethanol. Dilutions were prepared in methanol: water (70:30). The high purity water (>18 MΩ cm) used for this and all other stages of the method was taken from an ELGA water purification system.

22 samples were removed from the freezer and allowed to equilibrate to room temperature, and then inverted several times to ensure thorough mixing of the contents. A pooled sample was prepared for homogeneity determination by taking 12 vials, adding their contents to a 15 mL centrifugation tube, and mixing using an end-over-end mixer for 3 hours. One aliquot of each remaining vial, and 10 aliquots of the pooled sample (900 mg), were placed in separate silanised amber glass vials (4 mL). Each aliquot was spiked with 700 mg of 3 µg/kg isotopically-labelled d2-testosterone, which was estimated to be equimolar in concentration to that of the natural testosterone in the serum sample. The spiked samples were then mixed using a vortex mixer for 10 seconds and left to equilibrate in a refrigerator overnight. The extracts were transferred to a 15 mL centrifugation tube, 2 mL saturated sodium acetate buffer (0.5 M, pH 5.5) was added to the vial and then added to the extracts. 5 mL diethyl ether was then added to the extract and vortexed for 10 seconds, followed by mixing using an end-over-end type mixer for 10 minutes. The extract was centrifuged at 4 °C for 30 minutes at 3900 G. The tubes were stored overnight in a polystyrene box containing dry ice, freezing the lower aqueous layer. The supernatant (organic) layer was transferred to a



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15 mL centrifuge tube, and evaporated to dryness at 40 °C under nitrogen before being reconstituted in 2 mL de-ionised water.

The reconstituted extract was subject to clean-up using an SPE manifold with a Strata X cartridge. The cartridge was conditioned firstly with 6 mL methanol and then with 6 mL water. 2 mL of extract was loaded, and the cartridge washed firstly with 6 mL water and then with 6 mL 2 % by volume aqueous methanol. The cartridge was dried under 20 kPa pressure and eluted with 6 mL methanol into a 15 mL centrifuge tube. The methanol extract was evaporated to dryness under nitrogen at 40 °C and reconstituted in 300 µL 50 mM ammonium acetate: methanol solution (40:60), vortex mixed and then centrifuged at 4000 rpm for 5 minutes. The extracts were transferred to a total recovery autosampler vial for LC-MS/MS analysis.

LC-MS was performed using a Waters, 2690 Separations Module (Watford, UK) connected to a Micromass Ultima tandem mass spectrometer (Manchester, UK). The chromatography was performed on a Polaris C18-A 150 x 2.0 mm, 3 µm analytical chromatography column (Varian, Walton-on-Thames, UK) and the mobile phase consisted of water:methanol (40:60) maintained at a flow rate of 0.2 mL/min. The column temperature was 30 °C and the sample rack temperature 20 °C. The injection volume was 15 µL.

The LC was coupled to a Micromass Quattro Ultima tandem mass spectrometer (Manchester, UK) via an electrospray probe, operated at 3.0 kV with the combined hexapole and cone voltage 40 V. The source block and desolvation temperatures were 130 °C and 400 °C respectively. Analyte ionisation was by protonation resulting in $[M+H]^+$ ions at 289 and 291 m/z for the natural and deuterated compounds respectively.

The material was first issued for sale in December 2007. In June 2008 an error was identified in the calculated value for the concentration of testosterone and its associated uncertainty (page 2, and in Table 1 on page 4), so the certificate was corrected accordingly and reissued.

## CONFIRMATORY DATA

The University Hospital of Wales (UHW), UK, organise a proficiency testing scheme, the Wales External Quality Assessment Scheme (WEQAS) in which over 300 laboratories participate. Serum ERM-DA346a was used for 1 round of the WEQAS scheme for the determination of testosterone, in which 22 laboratories participated. Further confirmatory data was also obtained from University Hospital Wales, Department of Medical Biochemistry, determined using a Bayer Advia Centaur automated immunoassay system.

Confirmatory data was also obtained from the National Institute of Standards and Technology (NIST), Gaithersburg, Maryland, USA, using liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS).

The results are shown in Table 1; although there is a statistically significant difference to the value obtained by LGC, this is due to the relative lack of specificity and accuracy of the routine procedures used at UHW and by scheme participants compared with the traceable primary methods used by LGC and NIST.





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*Table 1 LGC, WEQAS, UHW and NIST results for testosterone*

| | Concentration | | Standard deviation* | |
|---|---|---|---|---|
| | nmol/L | µg/kg | nmol/L | µg/kg |
| LGC | 0.89 | 0.25 | 0.04 | 0.01 |
| WEQAS | 1.37 | 0.39 | 0.29 | 0.08 |
| UHW | 1.6 | 0.45 | 0.15 | 0.04 |
| NIST | 0.88 | 0.25 | 0.02 | 0.01 |
| * expanded uncertainty value (k=2), excluding contributions for potential inhomogeneity or long-term instability quoted for LGC and NIST data. | | | | |

## HOMOGENEITY

The material was tested for homogeneity by analysing 0.9 g aliquots from 10 individual samples plus 10 aliquots from a pooled sample of 12 randomly selected individual samples for testosterone, using the method detailed above. The material was judged to be homogeneous as the variation between the samples tested was not significantly greater than the method variation.

## STABILITY

The nature of the material is such that deterioration is not anticipated over the life of the material. ERM-DA346a was analysed three years after the first certification values and it was concluded that there had been no significant changes. An uncertainty value has been calculated to represent possible long-term instability and added to the total uncertainty budget.

## ANALYTICAL METHOD USED FOR THE DETERMINATION OF DENSITY

The density of the serum was determined in order to be able to calculate the concentration on a unit and volume basis. Water and 10 serum vials were placed in a water bath at 20 °C for 2 hours. An Eppendorff Multipipette was calibrated, using high purity water and calibrated balance, to dispense 0.5 mL. The weight of 0.5 mL of serum was determined by dispensing 0.5 mL portions into a pre-weighed empty container. This process was repeated 10 times for water and 10 times for the serum (each from a different serum vial).

## PARTICIPANTS

The University Hospital of Wales (UHW) collaborated with LGC in the production and characterisation of this material, through their proficiency testing scheme, the Wales External Quality Assessment Scheme (WEQAS). This collaboration included the preparation of materials and the provision of confirmatory data. The National Institute of Standards and Technology (NIST) collaborated through the provision of confirmatory data.

## SAFETY INFORMATION

Refer to material safety data sheet.





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## INSTRUCTIONS FOR USE

Prior to use, the material should be thoroughly thawed by equilibration at room temperature for at least 2 hours, and mixed by inverting the vial several times.

## STORAGE

The material should be stored at (-20 ± 5) °C in the original closed vial until it is first used.





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| Unit Number: | Shipment Date: |
|---|---|

## LEGAL NOTICE

(revised April 2008)

The values quoted in this certificate are the best estimate of the true values within the stated uncertainties and based on the techniques described herein. No warranty or representation, express or implied, is made that the use of the product or any information, material, apparatus, method or process which is the subject of or referred to in this certificate does not infringe any third party rights. Further, save to the extent: (a) prohibited by law; or (b) caused by a party's negligence; no party shall be liable for the use made of the product, any information, material, apparatus, method or process which is the subject of or referred to in this certificate. In no event shall the liability of any party exceed whichever is the lower of: (i) the value of the product; or (ii) £500,000; and any liability for loss of profit, loss of business or revenue, loss of anticipated savings, depletion of goodwill, any third-party claims or any indirect or consequential loss or damage in connection herewith is expressly excluded.

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