Australian Government
Department of Industry, Science and Resources

National Measurement Institute

NATA
WORLD RECOGNISED ACCREDITATION

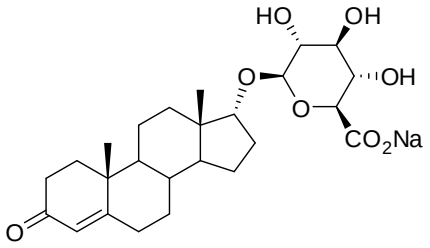
CERTIFIED REFERENCE MATERIAL CERTIFICATE OF ANALYSIS

NMIA S035: Epitestosterone glucuronide sodium salt

Report ID: S035.2025.01 (Ampouled 200520)

Chemical Formula: $C_{25}H_{35}O_8Na$

Molecular Weight: 486.5 g/mol



Certified value

Batch No.	CAS No.	Mass per ampoule
15-S10	16996-33-1 (free acid)	889 ± 28 μg

The uncertainty has been calculated according to ISO Guide 35 and is stated at the 95% confidence limit (k = 2).

IUPAC name: Sodium (17β)-3-oxoandrost-4-en-17-yl (5ξ)-β-L-xylo-hexopyranosiduronate.

Expiration of certification: The property values are valid till 22 July 2030, five years from the date of re-certification provided the **unopened** material is handled and stored in accordance with the recommendations below. The material as issued in the unopened container and stored as recommended below should be suitable for use beyond this date, subject to confirmation of batch stability from the issuing body. The expiry date/shelf life does not apply to ampoules that have been opened. In such cases it is recommended that the end-user conduct their own in-house stability trials.

Description: The compound is supplied as a dried aliquot in a sealed ampoule under an atmosphere of argon. The CRM is intended for a single use to prepare a standard solution containing S035. This material was sourced from an external supplier and certified for identity and purity by NMI Australia.

Intended use: This certified reference material is suitable for use as a primary calibrator.

Instructions for use: Open the ampoule and carefully rinse the interior at least three times with a suitable organic solvent (e.g. methanol). This will transfer 889 ± 28 μg of epitestosterone glucuronide sodium salt. The mass of analyte in each ampoule is calculated from the assigned purity of the bulk and the concentration of bulk material in a stock solution used to prepare the ampoules.

Recommended storage: When not in use, this material should be stored at or below 4 ºC in a closed container in a dry, dark area.

Metrological traceability: The certified purity value is traceable to the SI unit for mass (kg) through Australian national standards via balance calibration. In the mass balance all impurities are quantified as a mass fraction and subtracted from 100%. Quantitative NMR provides an independent direct measure of the mass fraction of the analyte of interest, calibrated with an internal standard certified for purity (mass fraction).

Stability: In the absence of long term stability data the measurement uncertainty at the 95% coverage interval has been expanded to accommodate any potential change in the property value. The stability component has been estimated from stability trials conducted on similar materials by NMI Australia over the last ten years. The long-term stability of the compound in solution has not been examined.

Homogeneity assessment: The homogeneity of the material was assessed using purity assay by HPLC with UV detection on seven randomly selected ampoules of the material. The material was judged to be sufficiently homogeneous at this level of sampling as the variation in analysis results between samples was not significantly different at a 95% confidence level from that observed on repeat analysis of the same sample.

Safety: Treat as hazardous substance. Use appropriate work practices when handling to avoid skin or eye contact, ingestion or inhalation of dust. Refer to the provided safety data sheet.

S. R. Davies

Dr Stephen R. Davies,
Team Leader,
Chemical Reference Materials, NMI.
24 July 2025

This report supersedes any issued prior to 24 July 2025.

NATA Accreditation No. 198 / Corporate Site No. 14214.

Characterisation Report:

HPLC:	Instrument:	Shimadzu Binary pump LC-20AB, SIL-20 A HT autosampler or Waters Separations Module 2695 or Thermo Scientific Ultimate 3000 RS pump, RS auto sampler
	Column:	Alltima C18 or ACE Super C18, 5 μm (4.6 mm × 150 mm)
	Column oven:	40 °C
	Mobile Phase:	MilliQ water with 0.1% formic acid/methanol (40:60)
	Flow rate:	1.0 mL/min
	Detector:	Shimadzu SPD-M20A PDA or Waters 2998 PDA or RS Diode Array Detector operating at 247 nm
	Relative mass fraction of the main component:	
	Initial analysis:	Mean = 98.7%, s = 0.01% (7 ampoules in duplicate, May 2020)
	Re-analysis:	Mean = 98.8%, s = 0.03% (7 ampoules in duplicate, June 2021)
	Re-analysis:	Mean = 98.5%, s = 0.01% (5 ampoules in duplicate, May 2022)
	Re-analysis:	Mean = 98.8%, s = 0.02% (5 ampoules in duplicate, April 2023)
	Re-analysis:	Mean = 98.8%, s = 0.02% (5 ampoules in duplicate, July 2025)

The following analytical data was obtained on the bulk material subsequently used in the preparation of the ampoules.

The identity was confirmed by a range of spectroscopic techniques, NMR, IR and MS. The certified purity value by qNMR was obtained using the one-proton doublet at 5.88 ppm measured against a certified internal standard of potassium hydrogen maleate.

Supporting evidence is provided by HPLC with UV detection at 247 nm, thermogravimetric analysis, Karl Fischer analysis, ^{1}H NMR spectroscopy, headspace GC-MS analysis of occluded solvents and elemental microanalysis.

HPLC:	Instrument:	Waters Model 1525 Binary pump, 717 plus auto sampler or Waters Separations Module 2695
	Column:	Alltima C18, 5 μm (4.6 mm × 150 mm)
	Column oven:	40 °C
	Mobile Phase:	MilliQ water with 0.1% formic acid/methanol (45:55)
	Flow rate:	1.0 mL/min
	Detector:	Waters 2998 PDA operating at 245 nm
	Relative mass fraction of the main component:	
	Initial analysis:	Mean = 98.9%, s = 0.05% (10 sub samples in duplicate, September 2015)
	Re-analysis:	Mean = 98.9%, s = 0.02% (5 sub samples in duplicate, November 2016)
	Re-analysis:	Mean = 99.0%, s = 0.04% (5 sub samples in duplicate, April 2018)
	Re-analysis:	Mean = 98.9%, s = 0.02% (5 sub samples in duplicate, April 2019)
	Re-analysis:	Mean = 99.1%, s = 0.05% (5 sub samples in duplicate, May 2020)
Karl Fischer analysis:		Moisture content 6.8% mass fraction (October 2016) Moisture content 8.7% mass fraction (April 2018) Moisture content 10.8% mass fraction (April 2019) Moisture content 7.8% mass fraction (May 2020) Moisture content 9.0% mass fraction (April 2023)
Thermogravimetric analysis:		Volatile content 2.9% mass fraction Due to the material being the sodium salt, the non-volatile content could not be determined by thermogravimetric analysis.
QNMR:	Instrument:	Bruker Avance-III-500
	Field strength:	500 MHz
	Solvent:	AcOH-d_4 (2.07 ppm)
	Internal standard:	Potassium hydrogen maleate (99.6% mass fraction)
	Initial analysis:	Mean (5.88 ppm) = 89.8%, s = 0.1% (5 sub samples, November 2016)

Spectroscopic and other characterisation data

GC-MS:

Bis-TMS derivative:
The free steroid was liberated upon treatment with β-glucuronidase enzyme (E. Coli K12) and derivatised with MSTFA.

Instrument:	Agilent 6890/5973	
Column:	TG-1MS, 30 m x 0.25 mm I.D. x 0.25 μm	
Program:	180 °C (1 min), 30 °C /min to 250 °C (10 min), 30 °C /min 300 °C (3 min)	
Injector:	250 °C	Transfer line temp: 280 °C
Carrier:	Helium, 1.0 mL/min	Split ratio: 20/1

The retention time of the *bis*-TMS derivative of epitestosterone is reported along with the major peaks in the mass spectra. The latter are reported as mass/charge ratios and (in brackets) as a percentage relative to the base peak.

Bis-TMS (10.1 min): 432 (100), 417 (16), 327 (12), 209 (17), 73 (62) *m/z*

ESI-MS:

Instrument:	Waters Acquity TQ API mass spectrometer
Operation:	Negative ion mode, infusion at 5 μL/min
Ionisation:	ESI spray voltage at 3.5 kV negative ion
EM voltage:	650 V
Cone voltage:	20 V
Peak:	463.4 ($M-H^+$) *m/z*

HS-GC-MS:

Instrument:	Agilent 6890/5973/G1888	
Column:	DB-624, 30 m x 0.25 mm I.D. x 1.4 μm	
Program:	50 °C (5 min), 7 °C/min to 120 °C, 15 °C/min to 220 °C (8.3 min)	
Injector:	150 °C	Transfer line temp: 280 °C
Carrier:	Helium, 1.2 mL/min	Split ratio: 50/1

Solvents detected: Propan-2-ol and diethyl ether

TLC:

Conditions: Kieselgel 60F_{254}. Chloroform/Methanol/Water (70/30/2)
Single spot observed, R_f = 0.2. Visualisation with UV at 254 nm

IR:

Instrument:	Bruker Alpha Platinum ATR
Range:	4000-400 cm^{-1}, neat
Peaks:	3394, 2932, 2870, 2847, 1663, 1611, 1408, 1231, 1159, 1113, 1066, 1043, 1020 cm^{-1}

^{1}H NMR:

Instrument:	Bruker Avance III-500
Field strength:	500 MHz
Solvent:	MeOH-d_4 (3.31 ppm)
Spectral data:	δ 0.78 (3H, s), 0.99 (1H, m), 1.11 (1H, m), 1.24 (3H, s), 1.27 (1H, m), 1.44-1.87 (9H, m), 1.93 (1H, m), 2.00 (1H, m), 2.11 (1H, m), 2.25-2.34 (2H, m), 2.43-2.54 (2H, m), 3.18 (1H, dd, *J* = 7.9, 8.9 Hz), 3.38 (1H, t, *J* = 8.8 Hz), 3.44 (1H, t, *J* = 9.6 Hz), 3.50 (1H, d, *J* = 9.7 Hz), 3.98 (1H, d, *J* = 5.7 Hz), 4.24 (1H, d, *J* = 7.7 Hz), 5.71 (1H, s) ppm

Propan-2-ol (0.8%), methanol (0.05%) and diethyl ether (0.1%) were quantified by ^{1}H NMR

^{13}C NMR:

Instrument:	Bruker Avance III-500
Field strength:	126 MHz
Solvent:	MeOH-d_4 (49.0 ppm)
Spectral data:	δ 17.3, 17.8, 21.7, 25.7, 29.8, 32.5, 33.7, 34.1, 34.7, 36.9, 37.2, 40.1, 45.9, 50.2, 55.3, 73.7, 74.9, 76.4, 78.0, 85.7, 101.6, 124.0, 175.6, 177.1, 202.5 ppm

Melting point: > 250 °C (decomposition.)

Microanalysis:

Found: C = 59.6%; H = 7.4% (September 2015)
Calculated: C = 55.9%; H = 7.5% (Calculated for $C_{25}H_{35}NaO_8$ + 3.7% water + 1.2% propan-2-ol + 0.2% diethyl ether)