

# Appendix 1 - 7- $\alpha$ -hydroxy-4-cholesten-3-one (C4)

## PRINCIPLE OF THE ASSAY

Manual sample preparation:

1. Pipette 250  $\mu$ l of patient sample into a 1.5ml tube.
2. Add 250  $\mu$ l of protein precipitation reagent.
3. Add 500  $\mu$ l of ice cold acetonitrile and vortex for 30 seconds.
4. Centrifuge for 10 minutes at 12000 RPM.
5. Transfer the supernatant to a glass HPLC injection vial and inject 100  $\mu$ l into the LC-MS/MS system.

Samples are resolved using a Supelco Analytical, Ascentis Express C18 fused core column (15 cm x 4.6 mm, 2.7  $\mu$ m, SigmaAldrich) on a ThermoScientific TLX-2 system using water and methanol mobile phases (each with 0.1% (v/v) formic acid). A ThermoScientific Hot Pocket is used to maintain the column at 40°C. The TLX-2 system is coupled to a ThermoScientific Vantage TSQ triple stage quadrupole mass spectrometer (ThermoScientific, UK) operated with atmospheric pressure chemical ionisation (APCI) source in positive ionisation mode.

## TECHNICAL DATA

### Sample requirements

Serum - Allow samples to clot for two hours at room temperature before centrifugation for 15 minutes at approximately 3000rpm. Assay immediately or store samples in aliquots at -20°C or -80°C for longer term storage. Avoid repeated freeze/thaw cycles.

To assay the sample a minimum of 250  $\mu$ L is required. However, the sample tube should contain at least a volume of 300  $\mu$ L to account for the dead volume.

### Intra-assay precision

	Level 1	Level 2	Level 3
n	10	10	10
Mean nmol/L	6.06	24.68	147.18
CV%	3.44	2.16	1.65

### Inter-assay Precision

	Level 1	Level 2	Level 3
n	10	10	10
Mean nmol/L	7.06	25.50	149.32
CV%	7.66	4.16	0.07

**Linearity**

Up to 1000 nmol/L, any results above this concentration are reported as >1000. The samples if necessary can be further diluted and repeated in the next batch.

**Sensitivity**

The minimal detectable concentration of C4 is 2.5 nmol/L.

**Reference range**

n	C4 (nmol/L)
100	2.9 – 134