SIGMA QUALITY CONTROL TEST PROCEDURE

Enzymatic Assay of CHOLESTEROL ESTERASE
(EC 3.1.1.13)

PRINCIPLE:

$$\text{Cholesterol Oleate} + H_2O \xrightarrow{\text{Cholesterol Esterase}} \text{Cholesterol} + \text{Oleic Acid}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{Cholesterol Oxidase}} H_2O_2 + \text{Cholestenone}$$

$$2H_2O_2 + \text{4-AAP} + \text{Phenol} \xrightarrow{\text{Peroxidase}} 4H_2O + \text{Quinoneimine Dye}$$

Abbreviation:
4-AAP = 4-Aminoantipyrine

CONDITIONS: T = 37°C, pH = 7.0, A_{500nm}, Light path = 1 cm

METHOD: Continuous Spectrophotometric Rate Determination

REAGENTS:

A. 400 mM Potassium Phosphate Buffer, pH 7.0 at 37°C
(Prepare 100 ml in deionized water using Potassium Phosphate, Monobasic, Anhydrous, Sigma Prod. No. P-5379. Adjust to pH 7.0 at 37°C with 1 M KOH.)

B. 0.9% (w/v) Sodium Chloride Solution
(Prepare 25 ml in deionized water using Sodium Chloride, Sigma Prod. No. S-9625.)

C. 8.6 mM Cholesteryl Oleate Solution (Chol-Oleate)
(Prepare 10 ml by first dissolving the Cholesteryl Oleate, Sigma Prod. No. C-9253, in 1 ml of Polyoxyethylene, 9 Lauryl Ether, Sigma Prod. No. P-9641. Stir gently, with heat, until the solution is clear and colorless. Then add 9 ml of hot Reagent B and continue for 5 minutes. Allow the solution to return to ambient temperature prior to use. The solution clears upon cooling.)

REAGENTS: (continued)

D. 15% (w/v) Taurocholic Acid Solution (Tauro)
(Prepare 10 ml in deionized water using Taurocholic Acid, Sodium Salt, Sigma Prod. No. T-4009.)

E. 15% (w/v) Cholic Acid Solution (Chol)
(Prepare 10 ml in deionized water using Cholic Acid, Sodium Salt, Sigma Prod. No. C-1254.)

F. 1.76% (w/v) 4-Aminoantipyrine Solution (4-AAP)
(Prepare 1 ml in deionized water using 4-Aminoantipyrine, Free Base, Sigma Prod. No. A-4382.)

G. 5% (w/v) Phenol Solution (Phenol)
(Prepare 10 ml in deionized water using Phenol, Sigma Prod. No. P-4161.)

H. Cholesterol Oxidase Enzyme Solution (Chol Oxid)
(Immediately before use, prepare a solution containing 20 - 30 units/ml of Cholesterol Oxidase, Sigma Prod. No. C-1512, in cold Reagent A.)

I. Peroxidase Enzyme Solution (POD)
(Immediately before use, prepare a solution containing 40 - 60 units/ml of Peroxidase Type II from Horseradish, Sigma Prod. No. P-8250 in cold deionized water.)

J. Cholesterol Esterase Enzyme Solution
(Immediately before use, prepare a solution containing 0.25 - 0.85 unit/ml of Cholesterol Esterase in cold Reagent A.)

PROCEDURE:

Pipette (in milliliters) the following reagents into suitable cuvettes[1]:

	Test	Blank
Reagent A (Buffer)	2.10	2.10
Reagent D (Tauro)	0.05	0.05
Reagent E (Chol)	0.05	0.05
Reagent I (POD)	0.10	0.10
Reagent C (Chol-Oleate)	0.50	0.50
Reagent G (Phenol)	0.05	0.05

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PROCEDURES: (continued)

Mix by inversion and equilibrate to 37°C. Then add:

Reagent F (4-AAP)	0.05	0.05
Reagent H (Chol Oxid)	0.05	0.05

Mix by inversion and obtain the baseline at 500 nm. After approximately 5 minutes add:

	Test	Blank
Reagent J (Cholesterol Esterase)	0.05	------
Reagent A (Buffer)	------	0.05

Immediately mix by inversion and record the increase in A_{500nm} for approximately 10 minutes.[2] Obtain the ΔA_{500nm}/minute using the maximum linear rate for both the Test and Blank.

CALCULATIONS:

$$\text{Units/ml enzyme} = \frac{(\Delta A_{500nm}/\text{min Test} - \Delta A_{500nm}/\text{min Blank})(3)(df)}{(0.5)(13.78)(0.05)} >$$

3 = Total volume (in milliliters) of assay
df = Dilution factor
0.5 = Conversion factor based on one mole of H_2O_2 produces half a mole of Quinoneimine Dye
0.05 = Volume (in milliliters) of enzyme used
13.78 = Millimolar extinction coefficient of Quinoneimine Dye at 500 nm under the assay conditions

$$\text{Units/mg solid} = \frac{\text{units/ml enzyme}}{\text{mg solid/ml enzyme}}$$

$$\text{Units/mg protein} = \frac{\text{units/ml enzyme}}{\text{mg protein/ml enzyme}}$$

UNIT DEFINITION:

One unit will hydrolyze 1.0 µmole of cholesteryl oleate to cholesterol and oleic acid per minute at pH 7.0 at 37°C in the presence of taurocholate.

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FINAL ASSAY CONCENTRATION:

In a 3.00 ml reaction mix, the final concentrations are 287 mM potassium phosphate, 0.25% (w/v) taurocholic acid, 0.25% (w/v) cholic acid, 4 - 6 units peroxidase, 1.4 mM cholesteryl oleate, 1.7% (v/v) polyoxyethylene 9 lauryl ether, 0.14% (w/v) sodium chloride, 0.083% (w/v) phenol, 0.03% (w/v) 4-aminoantipyrine, 1 - 1.5 units cholesterol oxidase and 0.013 - 0.043 unit cholesterol esterase.

REFERENCE:

Allain, C.C. et al., (1974) *Clinical Chemistry,* **20,** 470-475

NOTES:

1. Add the reagents in the order written.

2. The fastest rate is usually between 4-8 minutes after addition of the Cholesterol Esterase.

3. Cholesterol Oxidase Unit Definition: One unit will convert 1.0 µmole of cholesterol to 4-cholesten-3-one per minute at pH 7.5 at 25°C.

4. Peroxidase Unit Definition: One unit will form 1.0 mg purpurogallin from pyrogallol in 20 sec at pH 6.0 at 20°C.

5. This assay is based on the cited reference.

6. Where Sigma Product or Stock numbers are specified, equivalent reagents may be substituted.