Originating Department	QA
Approval Departments	QA, QC
Approval Date	31st August 2016
Effective Date	7th October 2016

1.0 PRODUCT DETAILS

1.1 **Enzyme Name**: **Cholesterol esterase**

1.2 **Systematic Name**: Steryl-ester acylhydrolase

1.3 **E.C. Number**: 3.1.1.13

1.4 **Source**: Porcine pancreas

1.5 **Suitable for BBI Solutions code**: CE2

2.0 ASSAY PRINCIPLE

A Peroxidase/Cholesterol oxidase coupled system measures the formation of cholesterol during the hydrolysis of Cholesteryl acetate:

$$\text{Cholesteryl Acetate} + H_2O \xrightarrow{\text{Cholesterol esterase}} \text{Cholesterol} + \text{Acetic acid}$$

$$\text{Cholesterol} + O_2 + H_2O \xrightarrow{\text{Cholesterol oxidase}} \text{Cholestenone} + H_2O_2$$

$$H_2O_2 + \text{4-Aminophenazone} + \text{Phenol} \xrightarrow{\text{Peroxidase}} \text{Quinoneimine dye} + H_2O$$

The rate of increase in absorbance at 505nm is followed spectrophotometrically.

3.0 UNIT DEFINITION

That amount of enzyme which causes the production of one micromole of cholesterol per minute at 37°C under conditions described in the assay procedure.

4.0 EQUIPMENT REQUIRED

Double beam UV/vis recording spectrophotometer, with temperature control set at 37°C (± 0.2°C).
Water bath at 37°C (± 0.2°C), thermometer.
Disposable cuvettes, silica cuvettes, disposable test tubes, glass test tubes and automatic pipettes.

5.0 REAGENTS REQUIRED

When using the following reagents please refer to the manufacturer's instructions for safe handling and disposal.

Reagent details

4-Aminoantipyrine
Supplier: Sigma
Product No.: A 4382
F.W.: 203.2

Cholesteryl acetate
Supplier: Sigma
Product No.: 151114
F.W.: 428.7

Cholesterol oxidase from Streptomyces sp.
Supplier: Sorachim
Product No.: COO-321

Peroxidase
Supplier: BBI Solutions, Blaenavon
Product No.: HRP2

Phenol AnalaR
Supplier: VWR International
Product No.: 10188
F.W.: 94.11

Nonaethylene glycol monododecyl ether
Supplier: Sigma
Product No.: P 9641
F.W.: 582.8

Sodium cholate hydrate
Supplier: Sigma
Product No.: C1254
F.W.: 430.55

Sodium hydroxide (2M NaOH)
Supplier: Sigma
Product No.: P6279

Sodium di-hydrogen orthophosphate (1 hydrate) AnalaR
Supplier: VWR International
Product No.: 10245
F.W.: 137.99

6.0 PREPARATION OF REAGENTS

6.1 2M Sodium hydroxide

Use as required and refer to the manufacturer's expiry date.

6.2 0.1M Sodium phosphate pH 7.0.

Dissolve 13.79g of Sodium di-hydrogen orthophosphate in 900ml of analytical grade water and adjust to pH 7.0 at 37°C with 2M Sodium hydroxide. Make up to 1 litre with analytical grade water. Stable at 2-8°C for 1 week.

6.3 4-Aminophenazone/phenol solution

Dissolve 18.5mg of 4-Aminophenazone (4-Aminoantipyrine) and 56.0mg of Phenol in 100ml of 0.1M Sodium phosphate pH 7.0. Prepare fresh daily.

6.4 Sodium cholate solution (0.6625M)

Dissolve 2.85g of Sodium cholate in 4mls of analytical grade water, heat with stirring to dissolve and make up to 10mls with water. Stable for 3 days at 2-8°C.

6.5 Cholesterol acetate solution

Heat 5g of Nonaethylene glycol monododecyl ether in a glass beaker at approximately 55°C and add 100mg of cholesterol acetate. When dissolved, add 40mls of 0.1M $NaPO_4$, pH 7.0 in 5ml aliquots, cool to 4°C and make up to 50ml with 0.1M 1M $NaPO_4$, pH 7.0. Stable for approximately 7 days at 2-8°C.

6.6 Peroxidase solution

Dissolve the freeze dried powder up to a concentration of 820 purpurogallin U/ml in 0.1M Sodium phosphate pH 7.0. Stable at 2-8°C for 1 week.

6.7 Enzyme solution

Into unused vials accurately weigh approximately 20mg of freeze-dried powder, each test sample to be weighed in triplicate. Dissolve at 5mg/ml in 0.1M Sodium phosphate pH 7.0, store on ice and use within 1 hour of dissolution. Immediately prior to assay, dilute to between 0.1 and 0.2U/ml with 0.1M Sodium phosphate pH 7.0.

6.8 Cholesterol oxidase solution

Dissolve the freeze-dried powder up to 5mg/ml in 0.1M Sodium phosphate pH 7.0. Stable at 2-8°C for 1 week.

7.0 TEST PROCEDURE

Temperature = 37°C Wavelength = 505nm Light path = 10mm

Pipette into 10m cuvettes at 37°C:

	TEST	REF
4-Aminophenazone/phenol solution	2.50ml	2.50ml
Sodium cholate solution	0.20ml	0.20ml
Cholesterol acetate solution	0.50ml	0.50ml
Peroxidase solution	0.10ml	0.10ml
0.1M Sodium phosphate pH 7.0.	-	0.10ml
Cholesterol oxidase	0.05ml	0.05ml
Equilibrate at 37°C for approximately 5 minutes, then add:		
Diluted enzyme	0.10ml	-
(Vt) =	3.45ml	3.45ml

Mix, then record the increase in absorbance at 505nm, reading the test solution versus the reference solution for approximately 5 minutes. Measure the change of absorbance per minute over the linear portion of the curve and use this value in the calculation.

8.0 CALCULATION

$$\text{Volume activity (U/ml)} = \frac{\Delta A_{505}/\text{min} \times Vt \times \text{dilution factor}}{Vs \times \varepsilon}$$

or $$\text{U/ml} = \Delta A_{505}/\text{min} \times 5.07 \times \text{dilution factor}$$

Where:

Vt = final volume of reaction mix (ml) = 3.45
Vs = sample volume (ml) = 0.10

ε = extinction coefficient (cm^2/micromole) = 6.8
Dilution factor = factor by which the enzyme solution is diluted

Weight activity (U/mg material) = $\frac{\text{U/ml}}{\text{mg solid/ml}}$

9.0 PROTEIN DETERMINATION

Protein is determined by the method Lowry *et.al*[1] (see Procedure No, AP62, Analytical Procedures Manual).

10.0 $A_{280}^{1\%}$ DETERMINATION

The $A_{280}^{1\%}$ is determined according to Proc. No. AP63 (Analytical Procedures Manual).

11.0 REFERENCES

1. Lowry, O.H., Rosebrough, N.J., Farr, A.L. & Randall, R.J. (1951) J. Biol. Chem. **193**, 256

12.0 REVISION HISTORY

Document Issue Number	Section Number	Summary of Changes
05	Global	Header and footer updated to current format and Approval Panel added.
	1.4	Remove *recombinant E coli*.
	5.0	Change of Sodium hydroxide and supplier. Removed F.W.: 40.0 Update Cholesterol oxidase supplier information.
	6.1	Paragraph amended for preparing Sodium Hydroxide to read 'Use as required and refer to the manufacturer's expiry date'.
	8.0 & 10.0	Amend 'E' to 'A' for absorbance
	12.0	New section added for 'Revision History'.
	Global	Font changed to Arial.