Originating Department	QC
Approval Departments	QA, QC & Validation
Approval Date	8th February 2017
Effective Date	16th March 2017

1.0 PRODUCT DETAILS

1.1 **Enzyme Name**: Glucose Oxidase

1.2 **Systematic Name**: β–D-Glucose : oxygen 1-oxidoreductase

1.3 **E.C. Number**: 1.1.3.4

1.4 **Source**: Aspergillus niger

1.5 **Suitable for BBI Solutions codes**: All Glucose Oxidase codes

2.0 ASSAY PRINCIPLE

The procedure for the analysis of glucose oxidase is based on the method of Bergmeyer.[1]

Glucose Oxidase (GO) catalyses the oxidation of glucose to produce D-glucono-1,5-lactone and hydrogen peroxide. This procedure utilises the hydrogen peroxide produced to convert a reduced dye (o-dianisidine) to an oxidised dye in the presence of peroxidase, the formation of which can be followed spectrophotometrically.

$$\beta\text{-D-glucose} + O_2 + H_2O \xrightarrow{\text{glucose oxidase}} \text{D-glucono-1,5-lactone} + H_2O_2$$

$$H_2O_2 + \text{reduced dye} \xrightarrow{\text{peroxidase}} 2\,H_2O + \text{oxidised dye}$$

3.0 UNIT DEFINITION

That amount of enzyme causing the oxidation of one micromole of glucose per minute at 25°C and pH 7.0

4.0 EQUIPMENT REQUIRED

Double beam UV/vis spectrophotometer with chart recorder.
Water bath set to achieve a reaction temperature of 25°C (± 0.1°C).
Thermometer
Silica cuvettes
Test tubes
Manual pipettes and tips

5.0 REAGENTS REQUIRED

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

Reagent details

Chemical / Reagent	Supplier	Product No.	F.W.
Di-potassium hydrogen phosphate	VWR	26931.263	174.18
Potassium dihydrogen phosphate	VWR	26936.293	136.09
D-Glucose	Sigma	G8270	180.16
O-Dianisidine dihydrochloride (3,3'-Dimethoxybenzidine dihydrochloride	Acros	184470050	317.2
Peroxidase	BBI Solutions	HRP2 or HRP3C	N/A
Oxygen	British Oxygen Company	N/A	N/A

6.0 PREPARATION OF REAGENTS

6.1 0.1M potassium phosphate pH7.0

Dissolve 8.71g of di-potassium hydrogen phosphate in water and adjust to a final volume of 500ml.

Dissolve 5.44g of potassium di-hydrogen phosphate in water and adjust to a final volume of 400ml.

Titrate the di-potassium hydrogen phosphate with the potassium di-hydrogen phosphate to obtain a pH of 7.0.
Stable for 2 weeks at 2 to 8°C.

6.2 0.0208M o-Dianisidine.dihydrochloride

Caution:
This material is a possible carcinogen, may cause inheritable genetic damage, and can cause irritation to the eyes, skin and respiratory system.
Do not breathe dust. Handle the powder in the fume hood.
Seek medical advice if you feel unwell after usage.

Accurately weigh approximately 70mg of o-dianisidine into a new glass vial and dissolve to a concentration of 6.6mg/ml in water. Store in a dark bottle.

Stable for 5 days at 2°C to 8°C.

6.3 Dye-Buffer solution

To approximately 95ml of 0.1M potassium phosphate, pH 7.0 in a 100ml volumetric flask, add 1ml of 0.0208M o-dianisidine. Mix, make up to 100ml with the 0.1M potassium phosphate pH 7.0 and store in a dark bottle.

Stable for 1 week at 2°C to 8°C.

6.4 10% β-D-glucose solution.

Rinse all equipment thoroughly with 3M sulphuric acid followed by water prior to making glucose solution in order to prevent contamination by glucose oxidase. Alternatively use a new container.

Pour ~90ml of water into a glass beaker and stir on a magnetic stirrer. Weigh 10.0g of β-D-glucose and add to the water whilst still stirring. Stir until completely dissolved. Make up to 100ml with water. Allow to stand at room temperature for at least one hour to mutarotate before using.

Stable for 1 month stored at 2°C to 8°C.

6.5 Peroxidase solution. (approximately 60 pyrogallol U/ml)

Weigh into a new glass vial either:

Code HRP2: Dissolve to a concentration of 1mg/ml in 0.1M potassium phosphate pH7.0

Code HRP3C: Dissolve to a concentration of 60 pyrogallol U/ml in 0.1M potassium phosphate pH7.0.

Stable at 2 to 8°C for 1 week.

6.6 Enzyme solution.

Freeze-dried powders:

Into new glass vials accurately weigh at least 10mg of freeze-dried powder, each test sample to be weighed in triplicate. Dissolve each to a concentration of 5mg/ml in 0.1M potassium phosphate pH 7.0. Immediately prior to assay, dilute to approximately 0.2 U/ml in 0.1M potassium phosphate pH 7.0.

Liquid preparations:

Immediately prior to assay, dilute to approximately 0.2 U/ml in 0.1M potassium phosphate pH 7.0.

Process samples:

Dilute to approximately 0.2U/ml, ensuring the concentration is within the range of 0.0497 U/ml to 0.349 U/ml (equivalent to reaction rates (ΔA_{436}/min) of 0.013 to 0.085)[1].

[1] Taken from Analytical Test Method Validation (ATMV 012).

7.0 TEST PROCEDURE

Temperature = 25°C. Wavelength = 436nm Light path = 10mm

Always sparge the Dye-Buffer solution with oxygen for at least 10 minutes before use.

Into disposable test tubes pipette the following:

	Test	**Reference**
Oxygenated Dye-Buffer:	2.40ml	2.40ml
0.1M potassium phosphate, pH 7.0:	0.00ml	0.10ml
10% β-D-glucose:	0.50ml	0.50ml
Peroxidase solution:	0.10ml	0.10ml

Allow the solutions to equilibrate to 25°C for approximately 5 minutes, then add:

Enzyme solution, diluted to ~0.2U/ml:	0.10ml	0.00ml
Total volume (V_t):	3.10ml	3.10ml

Transfer to a disposable cuvette as follows:

Gently pour the reaction mixture into a cuvette then back to the disposable test tube and back to the cuvette again.
This method of mixing is crucial to prevent de-oxygenation of the reaction mixture.

Place the cuvette in the spectrophotometer and record the increase in absorbance at 436nm, reading the test solution against the reference solution for approximately 3 minutes. Measure the change in absorbance per minute (ΔA_{436}/min) over the linear portion of the curve and use this value in the calculation.

8.0 CALCULATION

8.1 Volume activity (U/ml) = $\dfrac{\Delta A_{436}/\text{min} \times V_t \times \text{dilution factor}}{V_s \times \varepsilon}$

Where: V_t = final volume of the reaction mix (3.10ml)
V_s = sample volume (0.10ml)
ε = micromolar extinction coefficient for o-dianisidine (8.3cm^2/µmole)

Volume activity (U/ml) = ΔA_{436}/min x 3.72 x dilution factor

8.2. For freeze-dried samples: Weight activity (U/mg material) = $\dfrac{\text{U/ml}}{\text{mg/ml}}$

Specific activity (U/mg protein) = $\dfrac{\text{U/mg material}}{\text{mg protein/mg material}}$

8.3 For liquid samples: Specific activity (U/mg protein) = $\dfrac{\text{U/ml}}{\text{mg protein/ml}}$

9.0 PROTEIN DETERMINATION

Protein is determined by the method of Lowry et al in accordance with Analytical Procedure AP62 [2].

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

This is determined in accordance with Analytical Procedure AP63.

11.0 ASSOCIATED DOCUMENTS

ATMV012	Analytical Test Method Validation for Glucose Oxidase
AP62	Lowry Protein Determination
AP63	Spectrophotometric Measurements

12.0 REFERENCES

1. Bergymeyer, H.U., Gawehn, K., & Grassl, M., (1974) *Methods in Enzymatic Analysis*. 2nd edn (Bergmeyer, H.U., ed) Vol 1, p457, Academic Press, New York.

2. Lowry, O.H., Rosebrough, N.J., Farr, A.L., & Randall, R.J. (1951) *J. Biol. Chem*. **193**, 265

13.0 REVISION HISTORY

Document version number	Section number	Summary of Changes
04	Global	Reformat throughout
	4.0	Equipment required amended to reflect current requirements
	5.0	Reagent details amended to reflect current suppliers
	5.2.1 (now 6.1)	Calculations adjusted to account for the different formula weights of the chemicals currently used. Removed reference to USP Purified water. Removed reference to pH at 25°C since pH of the phosphate buffer is not sensitive to temperature. Volume of potassium di hydrogen phosphate prepared reduced to 400ml to avoid unnecessary waste.
	5.2.2 (now 6.2)	Removed reference to USP Purified water and rounded weight of O- dianisidine to 70mg. Added the statement to store in a dark bottle.
	5.2.4 (now 6.4)	Removed reference to USP Purified water Added statement with regard to rinsing equipment with 3M sulphuric acid followed by water or use of new container. Added to reduce chances of contamination. Instruction to mutarotate for 1 hour put in red type. Extended the expiry date to 1 month (based on Quality Control Study Report QCSR001/J).
	5.2.5 (now 6.5)	Extended expiry to 1 week and removed the need to store on ice to reflect current practice. Added statement to weigh into a new glass vial.
	5.2.6 (now 6.6)	Changed clean glass vial to new glass vial. Changed weigh 10-15mg to at least 10mg Removed statement to store sample on ice to reflect current practice. Added a section on dilution of process samples to include the range determined from Analytical Test Method Validation.
	6.0 (now 7.0)	Sparging with Dye buffer changed from approximately 10 minutes to at least 10 minutes. Removed statement to hold at 2 to 8°C and sparge every 30 minutes since current practice is to hold at ambient temperature and always sparge at least 10 minutes before use. More detail provided with regard to how to transfer the reaction mixture to the cuvette and statement of importance added in red type. $E_{436/min}$ amended to $A_{436/min}$ to reflect current nomenclature.
	7.0 (now 8.0)	$E_{436/min}$ amended to $A_{436/min}$ to reflect current nomenclature.
	9.0 (now 10.0)	Details removed and reference made to the relevant Analytical Procedure AP63 $E_{280}^{1\%}$ amended to $A_{280}^{1\%}$ to reflect current nomenclature.
	11.0	New section for Associated Documents added