Originating Department	QC
Approval Departments	QA, QC & Validation
Effective Date	Refer to Q-pulse

1.0 ASSAY PRINCIPLE

FAD, Flavine adenine dinucleotide, is separated from the D-Amino acid oxidase on a G25M Sephadex column. The FAD peak is collected as one fraction and the absorbance of this read at 449nm. The FAD content is then estimated using a pre-determined $A^{0.1\%}_{449}$ for the FAD used.

2.0 ASSOCIATED DOCUMENTS

EOP6756 AKTA Chromatography System
FM269 FAD determination on DOX

3.0 EQUIPMENT REQUIRED

Double beam UV/vis spectrophotometer
Pipettes and tips
Plastic cuvettes
AKTA Chromatography system
GE Healthcare XK16 column
UV monitor
0.2µm filter and 2ml disposable syringe
0.2µm filtration unit

4.0 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.
Flavine adenine dinucleotide disodium (only required if standard curve is to be prepared)	Ubichem	GM F005-H	N/A
G25M Sephadex resin	Fisher Scientific	17-00-33-01	N/A
Orthophosphoric acid (85%)	Merck	1.00573.1000	98.00
Sodium pyrophosphate decahydrate	Fisher Scientific	10144423	446.05

5.0 PREPARATION OF REAGENTS

5.1 8% Orthophosphoric acid

Add 9.4ml of 85% Orthophosphoric acid to 90.6ml of water.

Stable at ambient temperature for 1 month.

5.2 5mM Sodium pyrophosphate pH 8.0

Dissolve 2.23g of Sodium pyrophosphate decahydrate in approximately 950ml of water and adjust to pH 8.0 with 8% Orthophosphoric acid. Adjust to a final volume of 1L with water. Filter the buffer through a 0.2μm filter capsule.

Stable for two weeks at 2°C to 8°C.

5.3 Enzyme solution

Into new glass vials accurately weigh at least 20mg of freeze-dried D-amino acid oxidase and dissolve each to a concentration of 7mg/ml in 5mM Sodium pyrophosphate pH 8.0. Allow to dissolve completely then filter via a 0.2μm filter.
A reference must be run with each set of samples being analysed. Pilot samples must be weighed in duplicate and final product samples must be weighed in triplicate.

5.4 Column

Suspend, wash and filter the G25M Sephadex resin in 5mM sodium pyrophosphate pH 8.0. Pack the resin suspension to a height of 13.5 to 14.5cm into an XK16 column giving a column volume of 27 to 29ml.

6.0 TEST PROCEDURE

6.1 **Determination of $A_{449}^{0.1\%}$ for FAD used**

NB: If the $A_{449}^{0.1\%}$ for the FAD used in the preparation of the DOX test sample has already been calculated, continue with step 6.2.

6.1.1 Accurately weigh 50mg of the FAD and make up to 500ml volumetrically in 5mM sodium pyrophosphate, pH 8.0.

6.1.2 Record the A_{449} of the solution.

6.1.3 Calculate the $A_{449}^{0.1\%}$ by multiplying the A_{449} by 10. This value is referred to as ‘F’ in the calculation.

6.1.4 Add the ‘F’ value to the list held at the front of the FAD determination file.

6.2 Sample FAD determination

6.2.1 Connect the column to the UV monitor.

6.2.2 Equilibrate the column with at least two column volumes of 5mM sodium pyrophosphate, pH 8.0, until a steady baseline is attained.

6.2.3 Charge 500µl of the filtered enzyme solution and elute from the column using the AKTA pre-programmed method - 'G25 Sephadex for FAD on DOX'. Allow the initial output from the column to run to waste via the UV monitor.

6.2.4 Carefully monitor the chromatogram (see typical trace attached in Section 8). Just as the second peak starts to rise, note the 'accumulated volume' on the display screen and simultaneously collect the output in a measuring cylinder as a single fraction until the programme finishes.

6.2.5 Repeat steps 6.2.3 to 6.2.4 for each test sample.

6.2.6 Calculate the volume (V) of the fraction by subtracting the volume at the point of peak collection obtained in step 6.2.4 from 47.16ml (47.16ml being the final accumulated volume).

6.2.7 Measure the absorbance of the fraction at 449nm versus 5mM sodium pyrophosphate pH 8.0 and use this value in the calculation below.

7.0 CALCULATION

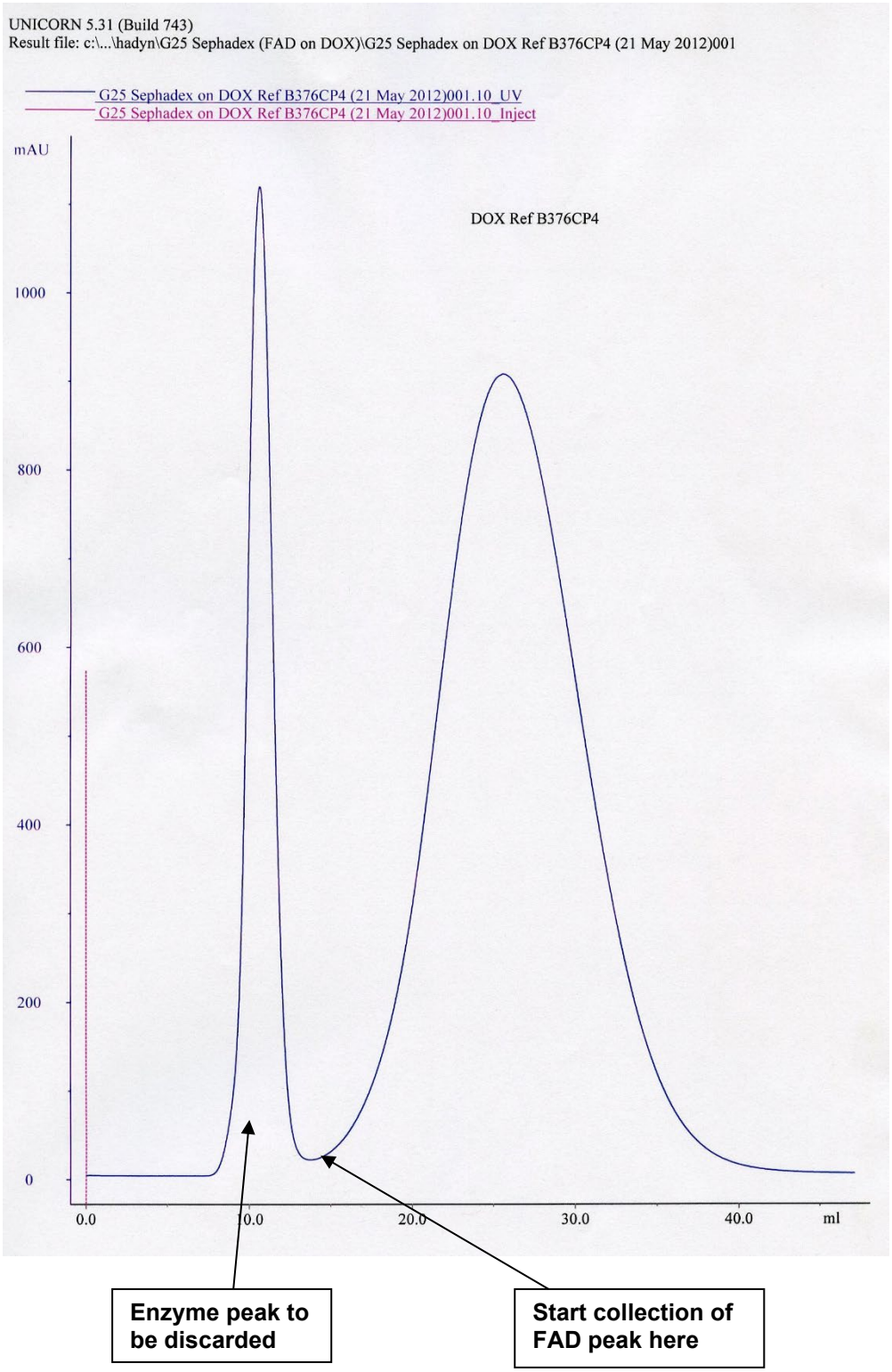
7.1 $$\text{mg FAD/1000U} = \frac{A \times V \times 1000}{F \times U \times 3.5}$$

Where:
A = Absorbance at 449nm of fraction
V = Volume of fraction in ml (see 6.2.6)
F = $A_{449}^{0.1\%}$ for FAD (see 6.1.3)
U = U/mg of DOX in oxygen electrode units (kinetic Units/1.95)
3.5 = mg of DOX in charge

7.2 Record the results on FM269.

7.3 Complete the 'FAD determination on DOX Summary' sheet at the front of the file.

8.0 TYPICAL CHROMATOGRAPH



9.0 REVISION HISTORY

Document version number	Section number	Summary of Changes
05	Global	Header changed; effective/approval date changed to refer to Q-pulse
	Header	Changed from AP111 issue 04 to AP111.5 to reflect current format.
	3.0	Manual pipettes changed to pipettes and tips.
	4.0	Statement for the handling of reagents reworded. Supplier and molecular weight changed.
	7.3	'in QA' removed as the file is held by QC
	Footer	"Part of BBI Group" Removed.