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

1.0 PRODUCT DETAILS

1.1 **Enzyme Name**: Hyaluronidase

1.2 **Systematic Name**: Hyaluronate 4-glycanohydrolase

1.3 **E.C. Number**: 3.2.1.35

1.4 **Source**: Ovine testes and bovine testes

2.0 UNIT DEFINITION

That amount of enzyme causing the same turbidity reduction as the 'International Unit' (I.U.) as compared with the International Standard. For comparison purposes 1 I.U. = 3.3 V.R.U. (viscosity reducing units).

3.0 EQUIPMENT REQUIRED

Double beam UV/vis spectrophotometer with chart recorder
Water bath set to achieve a reaction temperature of 37°C (± 0.2°C)
Thermometer
Silica and disposable cuvettes
Test tubes and test tubes with stoppers
Pipettes and tips
Calibrated timer

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.
2M Sodium hydroxide	Fisher Scientific	71474-1L	N/A
6M Hydrochloric acid	Fisher Scientific	72033-1L	N/A
Sodium dihydrogen phosphate monohydrate	VWR	102454R	137.99
Sodium hyaluronate	F.I.P.	22	N/A
Sodium chloride	VWR	27810.262	58.44
Acetic acid, glacial	VWR	20104.298	60.05
Sodium acetate	VWR	27650.292	82.03
Bovine serum albumin (BSA)	Sigma-Aldrich	10735108001	N/A
Hyaluronidase (International standard)	F.I.P.	14	N/A

5.0 PREPARATION OF REAGENTS

5.1 2M Sodium hydroxide

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

5.2 0.3M Sodium phosphate pH 5.30 – 5.35

Dissolve 10.3g of Sodium dihydrogen phosphate monohydrate in approximately 200ml of water. Adjust to between pH 5.30 – 5.35 with 2M Sodium hydroxide and adjust to a final volume of 250ml with water.

Stable for 2 weeks at 2°C to 8°C.

5.3 Hyaluronic acid solution

Accurately weigh approximately 50mg of Sodium hyaluronate and dissolve to a concentration of 0.5mg/ml in 0.3M Sodium phosphate pH 5.30 – 5.35. Cover and allow to stand at 2°C to 8°C overnight. The following day stir for at least 1 hour on a magnetic stirrer until fully dissolved.

Stable for 5 days at 2°C to 8°C.

5.4 Enzyme diluent (0.02M sodium phosphate pH 6.9, containing 0.45% sodium chloride and 0.01% BSA)

Dissolve 2.76g of Sodium dihydrogen phosphate monohydrate in approximately 900ml of water. Adjust to pH 6.9 with 2M Sodium hydroxide. Add 4.5g of Sodium chloride and 100mg of bovine serum albumin (BSA) and adjust to a final volume of 1L with water.

Stable for 5 days at 2°C to 8°C.

5.5 6M Hydrochloric acid

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

5.6 Acid albumin solution

Carefully dilute 4.56ml of glacial acetic acid to 1000ml with water, then add 3.26g of Sodium acetate and stir until dissolved. Carefully add 1g of bovine serum albumin, allow to soak into the buffer, then stir gently until completely dissolved. Adjust to pH 3.75 with 6M Hydrochloric acid.

Stable for 5 days at 2°C to 8°C.

5.7 Standard hyaluronidase solution (10 I.U./ml)

Accurately weigh at least 10mg of hyaluronidase standard and dissolve up to a concentration of 1000 U/ml in enzyme diluent. Stir until completely dissolved. Dilute 0.5ml in enzyme diluent to a total volume of 50ml in a volumetric flask.
Store in a dark bottle and prepare fresh daily.

5.8 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 enzyme diluent. Immediately prior to incubation, dilute further in enzyme diluent to approximately 5 I.U./ml. Carry out two different dilutions for each weighing.

Process samples:

Immediately prior to incubation, dilute further in enzyme diluent to approximately 5 I.U./ml.

Note: 1st stage process supernatant is dialysed: 4ml vs 10litres of 0.02M potassium phosphate pH 7.0. This is to remove ammonium sulphate which interferes with the assay.

6.0 TEST PROCEDURE

Temperature = 37°C Wavelength = 600nm Light path = 10mm

Note: During 6.4, the absorbance of each solution is measured exactly 5 minutes after the addition of the acid albumin reagent. Allowing 30 seconds for each absorbance reading to be taken, only 10 tubes may be incubated at one time. To minimise the time taken to complete the assay, a second run may be started while the first run is still incubating.

Standard Curve

6.1 Into disposable test tubes pipette the following:

Standard hyaluronidase solution (ml)	**0.05**	**0.10**	**0.15**	**0.20**	**0.25**	**0.30**	**0.35**	**0.40**	**0.45**	**0.50**
Enzyme diluent (ml)	**0.45**	**0.40**	**0.35**	**0.30**	**0.25**	**0.20**	**0.15**	**0.10**	**0.05**	**0.00**
Hyaluronidase concentration (I.U./ml)	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**

6.2 Mix and allow solutions to equilibrate to 37°C for at least 5 minutes.

6.3 At zero time, leaving a 30 second time interval between subsequent additions, add 0.5ml of Hyaluronic acid solution to each of the tubes. Immediately mix and stopper each tube and incubate at 37°C for exactly 45 minutes.

6.4 After 45 minutes, add 5ml of Acid albumin solution to each tube and mix rapidly by inversion. Allow the solutions to stand for exactly 5 minutes at room temperature, then read the absorbances of each solution at 600nm versus water.

6.5 Construct a standard curve, plotting the measured absorbances given by the standard hyaluronidase solutions against the I.U./ml using a spectrophotometer. If R^2 value is <0.995 repeat standard curve.

Sample Measurement

6.6 Pipette 0.5 ml of each diluted sample into disposable tubes, then repeat steps 6.2 to 6.4.

6.7 Obtain the hyaluronidase concentration in I.U./ml for each sample solution from the standard curve using the absorbances at 600nm of each of the sample reaction mixes and use these values in the calculations below.

The concentration in I.U./ml must fall within the range 3 I.U./ml to 10 I.U./ml for all active samples. The sample must be repeated if outside this range.

7.0 CALCULATION

$$\text{Weight activity (U/mg material)} = \frac{\text{I.U.U/ml x dilution factor}}{\text{mg/ml}}$$

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

8.0 PROTEIN DETERMINATION

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

9.0 $A_{280}^{1\%}$ DETERMINATION

This is determined in accordance with Analytical Procedure AP63.

10.0 ASSOCIATED DOCUMENTS

AP62	Lowry Protein Determination
AP63	Spectrophotometric Measurements

11.0 REVISION HISTORY

Document version number	Section number	Summary of Changes
06	Header/ footer	Changed to reflect current format.
	Global	Formatting improved and key stages now in red for emphasis. Changes for previous versions removed for clarity.
	Approval	Approval and issue dates removed and replaced with Refer to Q-Pulse as per current format.
	3.0	'Stopwatch' replaced with 'Calibrated timer'. 'Manual' removed from pipettes.
	4.0	Statement of chemical use reworded. Table updated to reflect current suppliers.