Originating Department	QC
Approval Departments	QA, QC & Validation
Approval Date	9th November 2017
Effective Date	18th December 2017

1.0 PRODUCT DETAILS

1.1 **Enzyme Name**: Ribonuclease (Kunitz)

1.2 **Systematic Name**: Ribonuclease 3'-pyrimidino-oligonucleotidohydrolase

1.3 **E.C. Number**: 3.1.27.5

1.4 **Source**: Bovine pancreas

2.0 ASSAY PRINCIPLE

The rate of decrease in absorbance at 300nm is a measure of the enzyme activity.

3.0 UNIT DEFINITION

That amount of enzyme which causes the hydrolysis of RNA at a rate such that 'k' (velocity constant) equals unity (Kunitz units) at 25°C and pH 5.0.

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 hazardous chemicals, handle in accordance with COSHH Regulations.

Reagent details

Chemical / Reagent	Supplier	Product No.	F.W.
Acetic acid, glacial	VWR	20104.298	60.05
Sodium acetate	VWR	27653.260	82.03
RNA (from Yeast)	Roche	10109223001	N/A

6.0 PREPARATION OF REAGENTS

6.1 0.05M Sodium acetate pH 5.0

Dissolve 2.05g of Sodium acetate in water and adjust to a final volume of 500ml.

Add 0.73ml of Glacial acetic acid to water, stirring for at least 5 minutes, and adjust to a final volume of 250ml.

Titrate the Sodium acetate with the acetic acid to obtain a pH of 5.0.
Stable for one month at 2 to 8°C.

6.2 RNA solution

The RNA substrate concentration needs to be optimized before use in the procedure. Each lot number of RNA is qualified to provide an acceptable separation of the A_f and A_0 values[1].
See the AP54 addendum for details for the current RNA concentration.
Dissolve the RNA to the required concentration in 0.05M Sodium acetate pH 5.0.
Prior to weighing the RNA should be inverted several times to ensure material uniformity.

6.3 Enzyme solution

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 2mg/ml in water. Immediately prior to assay, dilute to approximately 2.0 U/ml in water (typical dilutions are 1/91, 1/96, 1/100) for each vial.

7.0 TEST PROCEDURE

Temperature = 25°C. Wavelength = 300nm Light path = 10mm

Steady State (A_f)

The steady state absorbance (A_f) is measured by diluting the references and samples 1/76 in water and adding 0.1ml of the enzyme to 2.9ml of RNA substrate. These are capped and incubated at 25°C for 6.5 hours after which the A_f is read against 0.05M Sodium acetate, pH 5.0.
(NB: These need to be set up early in the day to allow time for final readings to be taken).

Into disposable test tubes pipette the following:

	Test	**Reference**
RNA solution	2.90ml	0.00ml
0.05M Sodium acetate, pH 5.0	0.00ml	3.00ml
Enzyme solution, diluted 1/76	0.10ml	0.00ml
Total volume (V_t):	3.00ml	3.00ml

[1] QCSR007

Assay (A_t)

Into disposable test tubes pipette the following:

	Test	**Reference**
RNA solution	2.90ml	0.00ml
0.05M Sodium acetate, pH 5.0	0.00ml	3.00ml

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

Enzyme solution, diluted to ~2.0U/ml at zero time	0.10ml	0.00ml
Total volume (V_t):	3.00ml	3.00ml

Mix, then transfer the solutions into glass cuvettes and record the decrease in absorbance at 300nm. Start to read the test versus reference solutions every 15 seconds from exactly 30 seconds to 4 minutes (15 intervals) (A_t).

8.0 CALCULATION

NB: An excel program has been set up to perform the calculations below

8.1 Plot log ($A_t - A_f$) versus time (t)

Where: $A_t = A_{300}$ at time t
$A_f = A_{300}$ at steady state

$$\text{Volume activity (U/ml)} = -2.3 \times S \times \frac{V_t}{V_s} \times \text{dilution factor}$$

Where: V_t = Final volume (ml) = 3.00
V_s = Sample volume (ml) = 0.10
S = Slope of graph
'-2.3' See ref. 1

8.2 Weight activity (U/mg material) = $\dfrac{\text{U/ml}}{\text{mg material/ml}}$

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

This is determined in accordance with Analytical Procedure AP63.

10.0 ASSOCIATED DOCUMENTS

AP63 Spectrophotometric Measurements

11.0 REFERENCES

1. Kunitz, M., (1946) *J. Biol. Chem*. **164**, 563 – 568

12.0 REVISION HISTORY

Document version number	Section number	Summary of Changes
05	Global	Reformat throughout
	1.5	Section Removed
	4.0	Equipment required amended to reflect current requirements
	5.0	Reagent details amended to reflect current suppliers, reagents now tabular
	6.0	Reference to numbered reagents removed
	6.2	Reworded to include reference to the addendum that will be used to record concentrations of the current RNA 'Prior to weighing the RNA should be inverted several times to ensure material uniformity.' Added in red to the point to allow more consistent weighing
	6.3	Approximately 20mgs amended to at least 10mgs Suitable vials changed to new glass vials Reference to analytical grade water removed Reference to storing on ice and using within one hour removed to reflect current practice. Typical dilutions included as they are currently used
	7.0	E_{300} amended to A_{300} to reflect current nomenclature. Light path changed from 1cm to 10mm Test tubes changed to disposable test tubes, reference to 'at 25^{o}C' removed 0.05M Acetate corrected to 0.05M Sodium acetate Allow solutions to equilibrate for approximately 5 minutes added Reagent 1 changed to reference solution, reference to numbered reagents removed E_t, E_f and extinction changed to A_t, A_f and absorbance to reflect current nomenclature. A_f is read against 0.05M Sodium acetate, pH 5.0. has been added to provide clarity for the steady state test The steady state section of the test has been set out like the assay section of the test to provide clarity and correct ordering The direction of the mixing and timing of the test samples has been further clarified with the number of intervals
	8.1	E_t and E_s changed to A_t and A_s to reflect current nomenclature
	8.2	Point deleted to bring into line with new AP format, now contains information on weight activity calculation
	8.3	Point deleted to bring into line with new AP format
	9.0	Details removed and reference made to the relevant Analytical Procedure AP63 $E_{280}^{1\%}$ amended to $A_{280}^{1\%}$ to reflect current nomenclature.
	10.0	New section for Associated Documents added
	11.0 (was 10.0)	References reformatted