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ANALYTICAL METHOD VERIFICATION REPORT: PROTEASE ASSAY FOR 10N SODIUM HYDROXIDE

TABLE OF CONTENTS

1. PURPOSE:

1.1. To ensure that the Protease Assay procedure is adequately evaluated and reported for 10N Sodium Hydroxide.
1.2. To verify that the Protease Assay procedure meets requirements for System Suitability, Accuracy, Limit of Quantitation, Linearity, Precision, Specificity, and Range.

2. SCOPE:

2.1. This analytical method verification report applies to the Protease Assay using the UV/Vis Spectrophotometer for 10N Sodium Hydroxide solution.
2.2. The Protease Assay was verified as a Category II Quantitative Method.
2.3. Protease Specification: NMT 0.1 Unit/gram Protease.
2.4. This method covers protease activity for a 2% (0.02g/mL) sample solution from a range of 0.005 Unit/gram to 0.10 Unit/gram.
2.5. **Reaction Chemistry:** The sample is incubated for a period of time with the substrate (Azocasein). The presence of protease enzymes hydrolyzes the substrate into small fragments of casein peptides and the azo dye. The casein peptides and undigested substrate are precipitated with trichloroacetic acid (TCA) and separated from the supernatant liquid via centrifugation. The absorbance at 440nm of the supernatant liquid containing the azo dye is directly related to the concentration of protease present.

3. RESPONSIBILITIES:

3.1. The Senior Product Life Cycle Manager is responsible for the implementation, control, and maintenance of this report.

4. REFERENCES:

4.1. BSI-PRL-0857, Analytical Method Verification Protocol: Protease Assay for 10N Sodium Hydroxide
4.2. BSI-SOP-0090, Lambda 25 UV/Vis Operation and Calibration
4.3. BSI-SOP-0098, Balance SOP
4.4. BSI-SOP-0126, Laboratory Notebooks
4.5. BSI-SOP-0134, Pipette SOP
4.6. BSI-SOP-0135, Laboratory Chemicals
4.7. BSI-SOP-0139, Protease Assay
4.8. BSI-SOP-0259, Fisher Scientific Isotemp Water Bath Operation and Calibration
4.9. BSI-SOP-0436, Analytical Methods Validation Master Plan
4.10. BMV01 pp52-58

5. MATERIALS AND EQUIPMENT:

5.1. Equipment

Table 1: Equipment

Equipment	Model / Part Number	Manufacturer	Serial Number	Calibration Due Date	Date of Last Calibration
Analytical Balance	MSE224S	Sartorius	24801744	4/30/25	10/4/24
Calibrated Pipette	1000µL - 10000µL Research Plus	Eppendorf	I44338L	2/28/25	8/6/24
Calibrated Pipette	100µL - 1000µL Research Plus	Eppendorf	O39512B	7/31/25	1/10/25
Calibrated Pipette	20µL - 200µL Research Plus	Eppendorf	N41555G	3/31/25	9/9/24
Calibrated Pipette	2µL - 20µL Research Plus	Eppendorf	G24188D	7/31/25	1/10/25
Calibrated Pipette	500µL - 5000µL Research Plus	Eppendorf	K53394I	7/31/25	1/10/25
Calibrated Pipette	0.5µL - 10µL Research Plus	Eppendorf	N27646F	7/31/25	1/10/25
Calibrated Timer	14-649-17	Fisherbrand	230665671	9/29/25	9/29/23
Water Bath	Isotemp	Fisher Scientific	300004011	7/25	7/22/24
Microcentrifuge	accuSpin Micro 17	Fisher Scientific	41650138	Not Applicable	Not Applicable
UV/Vis Spectrophotometer	Lambda 25	Perkin Elmer	501S13110518	2/25	1/16/25

5.2. Supplies

Table 2: Supplies

Supply	Manufacturer	Part Number
Weigh Boat, Small, White	Cole-Parmer	01017-05
Transfer Pipettes	FisherBrand	13-711-9AM
epT.I.P.S. Racks 0.1 - 20µL	Eppendorf	0030 071 557
epT.I.P.S. Racks 50 - 1000µL	Eppendorf	0030 071 581
epT.I.P.S. Racks 0.1 – 5mL	Eppendorf	0030 075 293
epT.I.P.S. Racks 0.5 – 10mL	Eppendorf	0030 071 654
epT.I.P.S. Racks 2 - 200µL	Eppendorf	0030 071 565
Semi-Micro, 1.5mL, 10mm Pathlength, Disposable Cuvettes	Fisher Scientific	14-955-127

6. REAGENTS:

Table 3: Reagents and Standards

Reagent / Standard	Lot ID	Manufacturer	Part Number	Expiration Date	Date of Opening
10N NaOH	NAHY-M03-0125-0003	BioSpectra Inc.	Not Applicable	Not Applicable	1/12/25
Purified Water (Type 1 D.I.)	F9SA14284H	Millipore Sigma	Milli-Q IQ 7005	5/17/25	5/17/24
Proteinase K Solution	BSP44P79	In-House	Not Applicable	3/21/25	12/9/24
Protease Substrate Solution	BSP45P04	In-House	Not Applicable	3/21/25	1/21/25
Protease 10x Reaction Buffer	BSP44P23	In-House	Not Applicable	3/21/25	9/21/24
10% Trichloroacetic Acid	2406E94	Ricca	8685-32	6/25	8/31/24
0.5N Sodium Hydroxide	233401	Fisher Chemical	SS270-1	10/25	4/23/24
HEPES	SLCM4091	Sigma Aldrich	PHG0001-100G	1/31/26	7/8/24

7. PROCEDURE:

7.1. Assay:

7.1.1. Prepare test sample solution at a concentration of 2% (0.02 g/mL) utilizing the table below (Solution may be scaled as needed):

Table 4: Sample Test Solution Preparation			
Sample ID	**Sample Volume (µL)**	**HEPES Weight (g)**	**Sterile[1] Water Volume (µL)**
10N NaOH	750	3.5324	49250

[1]Type 1 DI Ultra Pure Water

7.1.2. Prepare standards solutions utilizing the table below:

Table 5: Protease Standards Preparation				
Standard Solution ID	**Final Concentration of Proteinase K (Unit/mL)**	**Proteinase K in Sample (Unit/g)**	***PKWSS* Solution Volume (µL)**	**Purified Water Volume (µL)**
Proteinase K Working Standard Solution (PKWSS)	0.200	Not Applicable	200 of *20 Unit/mL Proteinase K Solution*	19800
Calibration Standard 1	0.004	0.004	20µL of *PKWSS*	980
Calibration Standard 2	0.010	0.010	50µL of *PKWSS*	950
Calibration Standard 3	0.020	0.020	100µL of *PKWSS*	900
Calibration Standard 4	0.050	0.050	250µL of *PKWSS*	750
Calibration Standard 5	0.100	0.100	500µL of *PKWSS*	500

7.1.3. Prepare each Spike Solution utilizing the table below:

Table 6: Protease Spike Solution Preparation			
Spike Solution ID	**Number of Replicates**	***PKWSS* Volume (µL)**	**Purified Water Volume (µL)**
0% Spike	3	0	1000
10% Spike (0.005 Unit/g Proteinase K)	1	25	975
50% Spike (0.025 Unit/g Proteinase K)	1	125	875
80% Spike (0.040 Unit/g Proteinase K)	3	200	800
100% Spike (0.050 Unit/g Proteinase K)	6	250	750
200% Spike (0.100 Unit/g Proteinase K)	3	500	500

7.1.4. Make a Reaction Mix, where Y represents the total number of tubes to be prepared, as follows:

Table 7: Protease Reaction Mix	
Amount	**Solution**
(Y + 1) x 0.2mL: 6mL	Protease Substrate Solution
(Y + 1) x 0.05mL: 1.5mL	Protease 10x Reaction Buffer

7.1.5. Label an appropriate number of microcentrifuge tubes and add previously prepared solutions to each of the tubes as follows:

Table 8: Incubation Scheme				
	Blank	**0% Spike Solution**	**Calibration Standard**	**Validation Spike Solution**
Tube Numbers	1 Replicate	3 Replicates	1 Replicate	Refer to Table 2
Purified Water (µL)	250	-	250	-
Sample Test Solution (µL)	-	250	-	250
Control Enzyme[1] (µL)	-	-	5[1]	-
Control Enzyme[2] (µL)	-	-	-	5[2]
Reaction Mix (µL)	250	250	250	250

[1]Appropriately diluted Proteinase K from Table 2.
[2]Appropriately diluted Proteinase K from Table 3.

7.1.6. Mix thoroughly.
7.1.7. Ensured the water bath is at least ¾ full. Incubate at 37°C for 16-18 hours.
7.1.8. Cooled tubes in a temperature monitored refrigerator or on ice for approximately 5 minutes. Centrifuge for 5-10 seconds. Add to each tube 0.67 mL of 10% Trichloroacetic Acid and mix thoroughly.
7.1.9. Cool in a temperature monitored refrigerator or on ice for 30-60 minutes. Centrifuge for 5-10 seconds. Rotate tubes 180° in the centrifuge. Centrifuge for 1 minute.
7.1.10. Carefully remove 0.65 mL of the supernatant and add it to 0.65 mL of 0.5N Sodium Hydroxide and mix thoroughly.

7.2. Absorbance Measurements

7.2.1. Within 1 hour, zero the UV/Vis spectrophotometer at 440 nm with water in matched 1 cm pathlength cuvettes.
7.2.2. Calibrate the UV/Vis Spectrophotometer by ensuring that the Blank is assigned as "Blank", the Calibrations Standards 1 through 5 are assigned as "Standard", and all Samples/Spike solutions are assigned as "Sample" in the "Type" column on the "Sample Info" window of the Perkin Elmer UV Win Lab software.
7.2.3. Input the Calibration Standards Proteinase K concentration in Units/g into the "Concentration" column on the "Sample Info" window of the Perkin Elmer UV WinLab software.
7.2.4. Measure the absorbance of the blank at 440nm against purified water and record the average value.

7.2.4.1. The average value should not exceed 0.10 absorbance units (a.u.), if it does exceed 0.10 a.u. make a new Protease Substrate Solution with fresh Protease Buffer for Substrate and repeat the assay.

7.2.5. Measure the absorbance of the standards and samples at 440nm against purified water and record the results.

7.3. Quantitative Reporting:

7.3.1. Using linear regression of the standard solutions calculate the protease content of each spiked sample solution. Correct for endogenous protease activity if detected.

8. VERIFICATION PARAMETERS:

8.1. **System Suitability:**

8.1.1. The average absorbance value of the blank measurement does not exceed 0.10 absorbance units (a.u.).

8.1.2. The Calibration Coefficient (r^2) value must be greater than or equal to 0.90.

8.2. **Accuracy:**

8.2.1. Accuracy will be assessed using twelve (12) determinations over three (3) Analysis Levels with six (6) determinations at the 100% Level Analysis.

8.2.2. Accuracy is assessed as Percent Recovery. All samples must have a percent recovery of 50% to 150%.

8.3. **Limit of Quantitation (LoQ):**

8.3.1. Report the lowest level of protease detected that meets requirements for accuracy and precision. LoQ must be NMT 0.1U/g.

8.4. **Linearity:**

8.4.1. Linearity will be assessed across five (5) analysis levels.

8.4.2. Plot and report the Coefficient of Determination (r2), slope, y-intercept, and residual sum of squares of the average absorbance reading vs. the Proteinase K Content spiked into each sample in Units/gram.

8.5. **Precision:**

8.5.1. Precision will be assessed using twelve (12) determinations over three (3) Analysis Levels with six (6) determinations at the 100% Level Analysis.

8.5.2. Precision is assessed by reporting Standard Deviation (s), Relative Standard Deviation (%RSD), and the 95% Confidence Interval for each analysis level. Each analysis level must have a Relative Standard Deviation (%RSD) of NMT 25%.

8.6. **Specificity:**

8.6.1. Specificity will be assessed by meeting requirements for accuracy and precision.

8.7. **Range:**

8.7.1. Range will be established by confirming an acceptable degree of Accuracy, Precision, and Linearity.

9. CALCULATIONS:

9.1. Percent Recovery

$$Percent\ Recovery\ (\%) = \frac{Calculated\ Protease\ Content\ \left(\frac{Units}{gram}\right)}{Protease\ Spike\ \left(\frac{Units}{gram}\right)} \times 100$$

9.2. Standard Deviation (s)

$$Standard\ Deviation\ (s) = \sqrt{\frac{\sum(X_i - \bar{X})^2}{n-1}}$$

Where:

X_i = Each Individual Protease Content Value (Units/gram)
$\bar{X}$ = Average Protease Content Value (Units/gram)
n = Number of Protease Content determinations

9.3. Relative Standard Deviation (%RSD)

$$Relative\ Standard\ Deviation\ (\%RSD) = \frac{Standard\ Deviation\ \left(\frac{Units}{gram}\right)}{Average\ Protease\ Content\ Value\ \left(\frac{Units}{gram}\right)} \times 100$$

9.4. 95% Confidence Interval

$$95\%\ Confidence\ Interval = \bar{X} \pm z\left(\frac{Standard\ Deviation\ \left(\frac{Units}{gram}\right)}{\sqrt{n}}\right)$$

Where:

$\bar{X}$ = Average Protease Content Value (Units/gram)
z = 1.960 = Z Value at the 95% Confidence Interval
n = Number of Protease Content determinations

9.5. Residual Sum of Squares (RSS)

$$RSS = \sum(Actual\ Absorbance\ Value - Theoretical\ Absorbance\ Value)^2$$

9.5.1. Actual absorbance value is calculated using the average protease spike (Units/gram) and the theoretical absorbance value of the protease spiked value (Units/gram) is calculated from the linear regression line.

10. VERIFICATION SUMMARY:

Table 9: Verification Summary		
Performance Parameters	**Acceptance Criteria**	**Results**
System Suitability	• The average absorbance value of the blank measurement does not exceed 0.10 a.u. • The Calibration Coefficient (r^2) value must be greater than or equal to 0.90.	• Blank Absorbance = 0.0127 a.u. • r^2 = 0.9979
Accuracy	• All samples must have a percent recovery of 50% to 150%.	80% Level • Replicate 1 = 114% • Replicate 2 = 86% • Replicate 3 = 81% 100% Level • Replicate 1 = 99% • Replicate 2 = 93% • Replicate 3 = 96% • Replicate 4 = 99% • Replicate 5 = 96% • Replicate 6 = 98% 200% Spike • Replicate 1 = 93% • Replicate 2 = 98% • Replicate 3 = 94%
Limit of Quantitation	• Report the lowest level of protease detected that meets requirements for accuracy and precision. • Limit of Quantitation (LoQ) must be NMT 0.1 Unit/gram.	• LoQ = 0.040 Units/gram
Linearity	• Report the Coefficient of Determination (r^2), Slope, Y-Intercept, and Residual Sum of Squares. • The Coefficient of Determination (r^2) is NLT 0.90	• Coefficient of Determination (r^2) = 0.9994 • Slope = 1.6099 • Y-Intercept = 0.0158 • Residual Sum of Squares = 0.0158

Performance Parameters	Acceptance Criteria	Results
Precision	• Each analysis level must have a %RSD of NMT 25%. • Standard Deviation: Report • 95% Confidence Interval: Report	80% Level • Standard Deviation = 0.0071 Unit/g • %RSD = 19% • 95% Confidence Interval = 0.0080 Unit/g 100% Level • Standard Deviation = 0.0012 Unit/g • %RSD = 2% • 95% Confidence Interval = 0.0009 Unit/g 200% Level • Standard Deviation = 0.0024 Unit/g • %RSD = 3% • 95% Confidence Interval = 0.0027 Unit/g
Range	• The range will be established by showing an acceptable degree of accuracy, precision, and linearity	• 0.040 Units/gram Protease to 0.100 Units/gram Protease
Specificity	• Requirements for accuracy and precision are met.	• Requirements for accuracy and precision were met.

11. VALIDATION RESULTS:

11.1. System Suitability

11.1.1. System Suitability was assessed by calibrating the UV/Vis Spectrophotometer with the calibration standards. All acceptance criteria were met and are summarized in Table 7.

11.1.2. Acceptance Criteria:

11.1.2.1. The absorbance value of the blank measurement does not exceed 0.10 absorbance units (a.u.).

11.1.2.2. The Calibration Coefficient (r^2) value must be greater than or equal to 0.90.

Table 10: System Suitability Results

Standard Solution ID	Final Concentration of Proteinase K (Unit/mL)	Proteinase K in Sample (Unit/gram)	Absorbance @ 440nm (a.u.)
Blank	0.00	0.00	0.0127
Calibration Standard 1	0.004	0.004	0.0161
Calibration Standard 2	0.010	0.010	0.0247
Calibration Standard 3	0.020	0.020	0.0501
Calibration Standard 4	0.050	0.050	0.0890
Calibration Standard 5	0.100	0.100	0.1800
		Calibration Coefficient (r^2):	0.9979

11.2. **Accuracy**

11.2.1. Accuracy was assessed using twelve (12) determinations over three (3) analysis levels with six (6) determinations at the 100% level. The calculated protease content (Units/gram), corrected for the average intrinsic 0% Spike protease content, was compared to the protease spike (Unit/gram) by calculating the percent recovery of each determination. All acceptance criteria were met and are summarized in Table 8.

11.2.2. Acceptance Criteria:

11.2.2.1. Percent Recovery: All replicates are between 50% and 150%.

Table 11: Accuracy Results

Analysis Level (%)	Protease Spike (Unit/gram)	Replicate	Corrected Protease Concentration Result (Unit/gram)	Percent Recovery (%)
0%	0.000	1	0.0027	Not Applicable
		2	0.0048	Not Applicable
		3	0.0032	Not Applicable
80%	0.040	1	0.0925	114
		2	0.0742	86
		3	0.0704	81
100%	0.050	1	0.0991	99
		2	0.0942	93
		3	0.0969	96
		4	0.0933	99
		5	0.0965	96
		6	0.0988	98
200%	0.100	1	0.1735	93
		2	0.1808	98
		3	0.1742	94

11.3. **Limit of Quantitation (LoQ)**

11.3.1. Limit of Quantitation (LoQ) was assessed by reporting the lowest level of protease detected that meets requirements for accuracy (section 11.2.) and precision (section 11.5.). All acceptance criteria were met.

11.3.2. Acceptance Criteria:

11.3.2.1. LoQ must be NMT 0.1 Unit/gram.

Limit of Quantitation (LoQ): 0.040 Units/gram

11.4. **Linearity**

11.4.1. The average absorbance values at each protease spike level were analyzed to determine linearity across five (5) analysis levels.

11.4.2. Plotted the average absorbance (ordinate) value (a.u.) versus the Protease Spike (Unit/gram) and reported the Coefficient of Determination (r^2), Slope, Y-Intercept, and Residual Sum of Squares.

11.4.3. An acceptable range of linearity was demonstrated across the range of 0 Unit/gram (0% Level) to 0.100 Unit/gram (200% Level). All acceptance criteria were met for this range and are summarized in Figure 1 and Table 9.

11.4.4. Acceptance Criteria:

11.4.4.1. Coefficient of Determination (r^2): Report

11.4.4.2. Slope: Report

11.4.4.3. Y-Intercept: Report

11.4.4.4. Residual Sum of Squares: Report

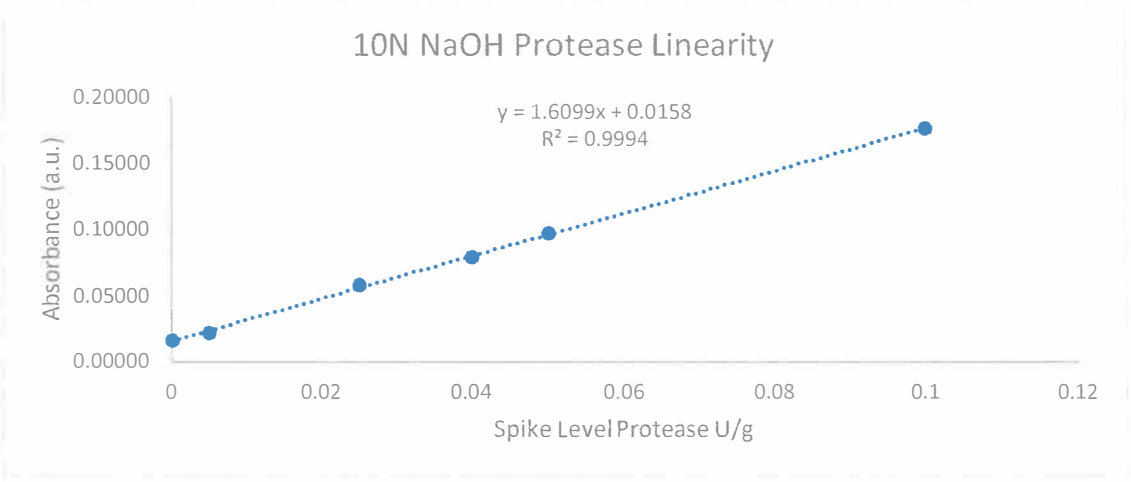

Figure 1: 10N NaOH Protease Linearity – Range: 0 Unit/gram (0% Level) to 0.100 Unit/gram (200% Level)

Table 12: Linearity Results

Analysis Level (%)	Protease Spike (Unit/gram)	Average Absorbance (a.u.)	Coefficient of Determination (r^2)	Slope	Y-Intercept	Residual Sum of Squares
0	0.000	0.01593	0.9994	1.6099	0.0158	0.0158
10	0.005	0.02210				
50	0.025	0.05800				
80	0.040	0.07903				
100	0.050	0.09747				
200	0.100	0.17617				

11.5. **Precision**

11.5.1. Precision was assessed using twelve (12) determinations over three (3) analysis levels with six (6) determinations at the 100% level. The Standard Deviation (s), Relative Standard deviation (%RSD), and 95% Confidence Interval were calculated at each analysis level. All acceptance criteria were met and are summarized in Table 10.

11.5.2. Acceptance Criteria:

11.5.2.1. Standard Deviation: Report

11.5.2.2. Relative Standard Deviation: NMT 25%

11.5.2.3. 95% Confidence Interval: Report

Table 13: Precision Results

Analysis Level (%)	Protease Spike (Unit/gram)	Replicate	Corrected Protease Concentration Result (Unit/gram)	Standard Deviation (Unit/gram)	%RSD	95% Confidence Interval (Unit/gram)
0	0	1	0.0027	0.0011	31	0.0012
		2	0.0048			
		3	0.0032			
80	0.040	1	0.0454	0.0071	19	0.0080
		2	0.0345			
		3	0.0322			
100	0.050	1	0.0493	0.0012	2	0.0009
		2	0.0464			
		3	0.0480			
		4	0.0494			
		5	0.0478			
		6	0.0491			
200	0.100	1	0.0934	0.0024	3	0.0027
		2	0.0978			
		3	0.0939			

11.6. **Range**

11.6.1. The range of the method is derived from linearity and is established by confirming an acceptable degree of linearity, accuracy, and precision. The range of the Protease Assay for 10N Sodium Hydroxide is:

Range of Analysis: 0.040 Units/gram Protease – 0.100 Units/gram Protease

11.7. **Specificity**

11.7.1. Specificity was determined by meeting requirements for accuracy (section 11.2.) and precision (section 11.5.). All acceptance criteria were met and are summarized in Table 11.

Table 14: Specificity Results

Acceptance Criteria	Result
All requirements were met for Accuracy (Refer to Section 11.2)	Pass
All requirements were met for Precision (Refer to Section 11.5)	Pass

12. CONCLUSION:

12.1. Performance Summary

Table 15: Performance Summary	
Method Performance Indicator	**Result**
System Suitability	Pass
Accuracy	Pass
Limit of Quantitation (LoQ)	0.040 Unit/gram
Linearity	Pass
Precision	Pass
Range	0.040 Unit/gram – 0.100 Unit/gram
Specificity	Pass

12.2. **Statement of Verification:** The Protease Assay for 10N Sodium Hydroxide is considered a verified method of analysis at all BioSpectra facilities and is approved for use.