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# ANALYTICAL METHOD VERIFICATION PROTOCOL: PROTEASE ASSAY FOR 10N SODIUM HYDROXIDE

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## 1. PURPOSE:

1.1. To ensure that the Protease Assay procedure is adequately evaluated and verified 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.
1.3. To ensure the proper reagents and testing materials are used and the correct documentation is provided for the evaluation.

## 2. SCOPE:

2.1. This analytical method verification protocol applies to the Protease Assay using the UV/Vis Spectrophotometer.
2.2. Applies to all 10N Sodium Hydroxide containing a specification for Protease. This protocol may be executed multiple times to accommodate new or changing specifications. The upper limit of analysis in this protocol is 0.1 Unit/gram and will represent the top end of the calibration curve.
2.3. 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 protocol.
3.2. Qualified laboratory personnel are responsible for performing the testing as stated in this protocol.
3.3. The qualified laboratory personnel performing this procedure, with help and training from the Senior Product Life Cycle Manager are responsible for documenting the results obtained from testing.
3.4. Safety: Standard laboratory safety regulations apply. Before working with any chemical, read and understand the Safety Data Sheet (SDS).

## 4. REFERENCES:

4.1. BSI-SOP-0090, Lambda 25 UV/Vis Operation and Calibration
4.2. BSI-SOP-0098, Balance SOP
4.3. BSI-SOP-0126, Laboratory Notebooks
4.4. BSI-SOP-0134, Pipette SOP
4.5. BSI-SOP-0135, Laboratory Chemicals
4.6. BSI-SOP-0139, Protease Assay
4.7. BSI-SOP-0259, Fisher Scientific Isotemp Water Bath Operation and Calibration
4.8. BSI-SOP-0436, Analytical Methods Validation Master Plan

## 5. PRE-VERIFICATION REQUIREMENTS:

5.1. Equipment
5.1.1. All equipment to be used in this verification is in proper working order and with current calibrations.
5.2. Personnel
5.2.1. All personnel who perform this verification will be properly trained in accordance with the Analytical Methods Validation Master Plan.

5.3. Supplies
5.3.1. All supplies used in the verification will be clean and appropriate for their intended use.
5.4. Reagents
5.4.1. All reagents will be current, meet required specifications, and be suitable for their intended use.
5.5. Reference Standards
5.5.1. All standards that will be used in this verification are listed in the materials and equipment section.

## 6. MATERIALS AND EQUIPMENT:

6.1. 2.0 mL Microcentrifuge tubes
6.2. Analytical Balance
6.3. Calibrated Timer
6.4. Lambda 25 UV/Vis Spectrophotometer
6.5. Laser Printer or equivalent
6.6. Microcentrifuge
6.7. Micropipettes
6.8. Temperature Monitored Refrigerator or equivalent
6.9. UV/Vis Spectrophotometer Cuvettes
6.10. Vortex
6.11. Water Bath capable of maintaining 37° ± 2°C

## 7. REAGENTS:

7.1. **Azocasein:** Purchased Commercially.
7.2. **Calcium Chloride Dihydrate:** Purchased Commercially.
7.3. **HEPES:** Purchased Commercially or In-House, Protease-Free
7.4. **Hydrochloric Acid, concentrated:** Purchased Commercially.
7.5. **Protease 10x Reaction Buffer:** Dissolve 1.21g of Tris Base in 90mL of Purified Water and adjust the pH to 7.8 with concentrated hydrochloric acid. Dilute to 100mL with Purified Water. Stable for 5-6 months if refrigerated in a closed container.
7.6. **Protease Buffer for Substrate:** Dissolve 1.21g of Tris Base in 80mL of Purified Water and adjust to approximately a pH of 7.5 with concentrated hydrochloric acid. Add 0.029g of Calcium Chloride Dihydrate and adjust the pH to 7.2 with hydrochloric acid. Add Purified Water to a final volume of 100mL. Stable for 5-6 months if refrigerated in a closed container.
7.7. **Protease Substrate Solution:** Dissolve 200mg of Azocasein in 40mL of Protease Buffer for Substrate to give 5.0mg/mL. Stable frozen for 2-3 months.
7.8. **Proteinase K:** 800U/mL (20mg/mL), Purchased Commercially.
7.9. **Proteinase K Solution (20U/mL):** Pipette 37.5µL of 800U/mL (20mg/mL) Proteinase K into 1.46mL of Proteinase Buffer for Substrate. Mix thoroughly. Transfer 0.3mL of solution to 5 separate vials. Stable in freezer for 3 – 4 months in a closed container.
7.10. **Proteinase K Working Standard Solution (PKWSS):** Dilute 200µL of Proteinase K Solution to 19.8mL with Purified Water, mix thoroughly for a concentration of 0.200Unit/mL Proteinase K. Prepare this solution at time of use. Solution can be scaled as necessary.
7.11. **Purified Water:** Generated In-House or Purchased Commercially.
7.12. **Sodium Hydroxide, 0.5N:** Purchased Commercially.
7.13. **Sterile Water:** Purchased Commercially.
7.14. **Trichloroacetic Acid (10%):** Purchased Commercially.
7.15. **Tris Base:** Purchased Commercially.

## 8. PROCEDURE:

8.1. Assay:

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

| Table 1: Sample Test Solution Preparation | | | |
|---|---|---|---|
| **Sample ID** | **Sample Volume (µL)** | **HEPES Weight (g)** | **Sterile Water Volume (µL)** |
| 10N NaOH | 750 | 3.5 | 49250 |

8.1.2. Prepare standards solutions utilizing the table below:

| Table 2: 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)** |
| 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 |

8.1.3. Prepare each Spike Solution utilizing the table below:

| Table 3: Protease Spike Solution Preparation | | | |
|---|---|---|---|
| **Spike Solution ID** | **Number of Replicates** | ***PKWSS* Volume (µL)** | **Purified Water Volume (µL)** |
| 0% Spike | 3 | 0.00 | 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 |

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

| Table 4: Protease Reaction Mix | |
|---|---|
| **Amount** | **Solution** |
| (Y + 1) x 0.2mL | Protease Substrate Solution |
| (Y + 1) x 0.05mL | Protease 10x Reaction Buffer |

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

| Table 5: 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.

8.1.6. Mix thoroughly.
8.1.7. Ensure the water bath is at least ¾ full. Incubate at 37°C for 16-18 hours.
8.1.8. Cool 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.
8.1.9. 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.
8.1.10. Carefully remove 0.65 mL of the supernatant and add it to 0.65 mL of 0.5N Sodium Hydroxide and mix thoroughly.

8.2. Absorbance Measurements
8.2.1. Within 1 hour, zero the UV/Vis spectrophotometer at 440 nm with water in matched 1 cm pathlength cuvettes.
8.2.2. 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.
8.2.3. 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.
8.2.4. Measure the absorbance of the blank at 440nm against purified water and record the average value.
8.2.4.1. 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.
8.2.5. Measure the absorbance of the standards and samples at 440nm against purified water and record the results.

8.3. Quantitative Reporting:
8.3.1. Using linear regression of the standard solutions calculate the protease content of each spiked sample solution. Correct for endogenous protease activity if detected.

## 9. VERIFICATION PARAMETERS:

9.1. **System Suitability:**

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

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

9.2. **Accuracy:**

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

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

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

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

9.4. **Linearity:**

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

9.4.2. 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.

9.5. **Precision:**

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

9.5.2. 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%.

9.6. **Specificity:**

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

9.7. **Range:**

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

## 10. CALCULATIONS:

10.1. Percent Recovery

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

10.2. 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

10.3. Relative Standard Deviation (%RSD)

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

10.4. 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

10.5. Residual Sum of Squares (RSS)

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

10.5.1. 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.

## 11. DOCUMENTATION PROCEDURES:

11.1. All data sheets, including notebooks, are to be signed and dated by the employee executing the protocol. Pages will be copied and uploaded as supporting material into MasterControl.
11.2. All testing equipment must be calibrated. Ensure that there is a certificate on file or appropriate standards are used if calibration is required.
11.3. Any critical changes that must be made to the analytical procedure should be noted in the Validation Report with supporting evidence for the change.