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ANALYTICAL METHOD VALIDATION PROTOCOL: RNASE (RIBONUCLEASE) ASSAY FOR 10N SODIUM HYDROXIDE

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1. PURPOSE:

1.1. To ensure the method of analysis for evaluating enzymatic activity of RNase by electrophoresis is adequately evaluated for suitability of use to detect levels of 1 Unit/g in 10N Sodium Hydroxide.

2. SCOPE:

2.1. Applies to all 10N Sodium Hydroxide containing a specification for Rnase. This protocol may be executed multiple times to accommodate new or changing specifications. The limit of analysis in this protocol is 1 Unit/g.

2.2. For a Category 2 limit test, it is only necessary to show that a compound of interest is either present or not above and below the limit of 1 unit/g. Specificity and Detection Limits will be assessed to validate the procedure for routine use.

3. RESPONSIBILITIES:

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

3.2. Qualified laboratory personnel are responsible for compliance with the terms of this protocol.

4. REFERENCE:

4.1. BSI-SOP-0098, Balance SOP
4.2. BSI-SOP-0134, Pipette SOP
4.3. BSI-SOP-0135, Laboratory Chemicals
4.4. BSI-SOP-0259, Fisher Scientific Isotemp Water Bath Operation and Calibration SOP
4.5. BSI-SOP-0436, Analytical Methods Validation Master Plan
4.6. *USP Current*

5. PRE-VALIDATION REQUIREMENTS:

5.1. Equipment

5.1.1. All equipment to be used in this Validation must be in proper working order and with current calibrations. This will be documented in the Materials and Equipment portion of the Analytical Method Validation Report.

5.2. Personnel

5.2.1. All personnel performing this Validation will be properly trained in accordance with the Analytical Methods Validation Master Plan.

5.3. Supplies

5.3.1. Any supplies to be used in the Validation must be clean and appropriate for the intended use. A list of supplies used will be in the Materials and Equipment section of the Analytical Method Validation Report.

5.4. Reagents

5.4.1. All reagents must be current, meet required specifications, and be suitable for the intended use. A list of reagents used will be included in the Analytical Method Validation Report. This should include: Reagent Name, lot number, and expiration.

6. MATERIALS AND EQUIPMENT:

6.1. All materials and equipment utilized in this Validation will be outlined in this section. This is a list of the anticipated materials and equipment required. As part of the Analytical Method Validation Report, all materials and equipment used should be documented in a list that will serve as an attachment to the report. Any other pertinent information regarding the materials and equipment should be documented in this attachment.

6.2. Equipment

6.2.1. 2.0 mL microcentrifuge tubes

6.2.2. 15mL to 50mL Centrifuge Tubes or equivalent
6.2.3. Analytical Balance
6.2.4. Calibrated Timer
6.2.5. E-Gel Power Snap Electrophoresis Device or equivalent
6.2.6. E-Gel Power Snap Camera
6.2.7. Laser Printer or equivalent
6.2.8. Microcentrifuge
6.2.9. Micropipettes
6.2.10. Water Bath capable of maintaining 37° ± 2°C

7. REAGENTS:

7.1. **2% Agarose E-Gel Cassette:** Purchased Commercially.
7.2. **Bromophenol Blue:** Purchased Commercially.
7.3. **Disodium EDTA Dihydrate:** Purchased Commercially.
7.4. **DNase and RNase 50x Gel Buffer (Endonuclease and Exonuclease):** Dissolve 12.1g of Tris Base, 0.68g of Sodium Acetate Trihydrate, and 1.86g of Disodium EDTA Dihydrate in approximately 35mL of Sterile Water. Adjust to pH 8.1 with Glacial Acetic Acid or Sodium Hydroxide. Dilute to a final volume of 50mL with Sterile Water. Stable for 1 – 2 months refrigerated in a closed container.
7.5. **Gel Loading Buffer:** Mix 3mL of Glycerol, 10mg of Bromophenol Blue, 0.2mL of DNase and RNase 50x Gel Buffer (Endonuclease and Exonuclease), and dilute to 10mL with Sterile Water. Stable for 1 – 2 months refrigerated in a closed container.
7.6. **Glacial Acetic Acid:** Purchased Commercially.
7.7. **Glycerol:** Purchased Commercially.
7.8. **HEPES:** Purchased Commercially, protease free.
7.9. **Hydrochloric Acid, concentrated:** Purchased Commercially.
7.10. **RNA Millennium Marker:** Purchased Commercially.
7.11. **RNase (Ribonuclease A):** Purchased Commercially.
7.12. **RNase 10x Reaction Buffer:** Dissolve 6.8g of Sodium Acetate Trihydrate and 0.074g of Disodium EDTA Dihydrate in approximately 80mL of Sterile Water and adjust the pH to 4.5 with Glacial Acetic Acid or Sodium Hydroxide. Dilute to 100mL with Sterile Water. Stable for 2 – 3 months refrigerated in a closed container.
7.13. **RNase Buffer:** Dissolve 0.121g of Tris Base and 0.088g of Sodium Chloride in 90mL of Sterile Water and adjust to pH 7.5 with concentrated Hydrochloric Acid. Add Sterile Water to a final volume of 100mL. Stable for 6 – 8 months refrigerated in a closed container.
7.14. **RNase Solution:** Dissolve 2.0mg of RNase (Ribonuclease A) in 0.2mL of RNase Buffer. Stable for 6 – 8 months in a freezer in a closed container.
7.15. **Sodium Acetate Trihydrate:** Purchased Commercially.
7.16. **Sodium Chloride:** Purchased Commercially.
7.17. **Sodium Hydroxide, 10N:** Purchased Commercially or Prepared In-House.
7.18. **Sterile Water:** Purchased Commercially.
7.19. **Tris Base:** Purchased Commercially.

8. VALIDATION PROCEDURE:

8.1. Assay

8.1.1. Prepare each sample (typically 2% or 0.02 g/mL) utilizing the table below, in triplicate:

TABLE 1: VALIDATION SAMPLE / SPIKE SOLUTION PREPARATION				
Sample ID	**Sample Volume (µL)**	**HEPES Weight (g)**	**RNase Buffer Volume (µL)**	**Volume of Stock Solution B (µL)**
0% Spike	15	0.07	985	0
100% Limit Spike	15	0.07	885	100

8.1.2. Prepare standards utilizing the table below:

TABLE 2: RNASE (RIBONUCLEASE) STANDARDS PREPARATION			
Purpose	**Final Concentration (Unit/µL)**	**Volume of RNase Solution[1](µL)**	**Volume of RNase Buffer (µL)**
Stock Solution A	**$1x10^{-3}$**	2.5 of RNase Solution[2]	1997.5[2]
Stock Solution B	**$0.2x10^{-3}$**	200 of 0.2	800
100% Limit Std.	**$0.2x10^{-4}$**	100 of $0.2x10^{-2}$	900

[1]10 mg/mL

[2]Volumes will vary based on the concentration reported on the RNase vendor's C of A.

8.1.2.1. **Note:** RNase (Ribonuclease) Standard preparation is dependent on RNase (Ribonuclease A) enzyme activity (found on the Certificate of Analysis or on the reagent container). The volume of RNase Solution used will be determined using the following equation (may be scaled as needed):

$$Volume\ of\ RNase\ Solution\ (\mu L) = \frac{\left(1 \times 10^{-3}\frac{Units}{\mu L}\right) \times \left(Final\ Volume\ (\mu L)\right)}{\left(RNase\ Solution\ Concentration\ \left(\frac{mg}{\mu L}\right)\right) \times \left(RNase\ Enzyme\ Activity\ \left(\frac{Units}{mg}\right)\right)}$$

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

TABLE 3: RIBONUCLEASE REACTION MIX	
Amount	**Solution**
(Y+1) x 1µL	Millennium RNA Marker
(Y+1) x 1µL	RNase 10x Reaction Buffer
(Y+1) x 3µL	Sterile Water

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

TABLE 4: REACTION SCHEME

	Blank	Test Solution	100% Spike Sample	100% Limit Standard	Control
Tube #	**1**	**2**	**3**	**4**	**5**
Reaction Mix (µL)	5	5	5	5	5
RNase Buffer (µL)	5	-	-	-	5
Test Solution (µL)	-	5	5	-	-
Control Enzyme[1] (µL)	-	-	-	5[1]	-

[1]Appropriately diluted RNase. (Note, for instance, that 5 microliters of 0.2×10^{-4} Units RNase per microliter represents 1×10^{-4} Units RNase.)

8.1.5. Mix thoroughly and immediately place the Control onto ice or into a temperaturemonitored refrigerator.
8.1.6. Incubate all others at 37°C for 4 hours.
8.1.7. Cool tubes on ice or in a temperature monitored refrigerator for approximately 5 minutes.
8.1.8. Centrifuge all tubes for 1 minute.
8.1.9. To each tube, add 4µL of Gel Loading Buffer.
8.1.10. Vortex thoroughly.
8.1.11. Centrifuge for 1 minute.

8.2. Electrophoresis
8.2.1. Utilize 2% Agarose E-GEL cassettes.
8.2.2. Remove the E-GEL cassette from its packaging, carefully remove the well comb, and inspect under sufficient lighting.
8.2.2.1. Inspect for any damage to the wells and any defects.
8.2.2.2. **Note:** Load the gel within 15 minutes of opening the package and run gel within one min of loading the samples
8.2.3. Insert the gel cassette into the E-Gel Power Snap Electrophoresis Device, starting from the right edge.
8.2.4. Load the entire sample into to the well.
8.2.5. Load all empty wells with 14µL of Sterile Water.
8.2.6. Set up the run by selecting the E-Gel Protocol 1 – 2% on the E-Gel Power Snap Electrophoresis Device.
8.2.7. Ensure the run time is 10 minutes.
8.2.8. Run the gel by pressing "Start Run".

8.3. Photograph
8.3.1. Label the cassette appropriately.
8.3.1.1. RNase, Initials / Date
8.3.2. Connect the E-Gel Power Snap Camera to the Electrophoreses unit.
8.3.3. Press Capture in the home screen view.
8.3.3.1. Note: Allow the gel to cool for 5-10 minutes before image capture to enhance sensitivity.
8.3.4. Export image to a USB thumb drive.
8.3.5. Print image with the wells labeled.

9. VALIDATION PARAMETERS:

9.1. System Suitability:

9.1.1. All of the following system suitability acceptance criteria must be met for the assay results to be suitable for use:

9.1.1.1. The Blank lane demonstrates substrate degradation equal to or more pronounced than the Control lane.

9.1.1.2. The 100% Limit Standard lane shows substrate degradation more pronounced than the Blank lane.

9.2. Specificity:

9.2.1. Specificity will be demonstrated by comparing the control lane, 0% spiked sample solution lane, and 100% level spiked solution lane.

9.2.2. Acceptance Criteria:

9.2.2.1. The 100% level spiked sample should demonstrate more pronounced degradation than the 0% spiked sample solution.

9.2.2.2. The 0% spike sample solution should demonstrate degradation equal to or more pronounced than the control.

9.3. Detection Limit:

9.3.1. Report the level of detectability in RNase units per gram.

$$\text{Units/g} = (1\text{x}10^{-4}\text{Unit})/(0.005\text{mL}*0.02\text{g/mL})$$

9.3.2. Acceptance Criteria:

9.3.2.1. No less than 1 Unit/g is acceptable.