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ANALYTICAL METHOD VALIDATION PROTOCOL: DNASE (EXONUCLEASE) ASSAY FOR 10N SODIUM HYDROXIDE

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1. PURPOSE:

1.1. To ensure the method of analysis for evaluating enzymatic activity of DNase (Exonuclease) by electrophoresis is adequately evaluated for suitability of use to detect exonuclease activity levels of 100 Unit/g in 10N Sodium Hydroxide.

2. SCOPE:

2.1. Applies to all 10N Sodium Hydroxide containing a specification for DNase (Exonuclease). This protocol may be executed multiple times to accommodate new or changing specifications. The limit of analysis in this protocol is 100 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 100 unit/g. System Suitability, Specificity and Detection Limit will be assessed to validate the procedure for routine use.

3. RESPONSIBILITIES:

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

3.2. Qualified Chemists or technicians 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 Chemical Preparation
4.4. BSI-SOP-0259, Fisher Scientific Isotemp Water Bath Operation and Calibration SOP
4.5. *USP Current*

5. EQUIPMENT:

5.1. 2.0 mL microcentrifuge tubes
5.2. Analytical Balance
5.3. E-Gel Power Snap Electrophoresis Device or equivalent
5.4. E-Gel Power Snap Camera
5.5. Eppendorf centrifuge
5.6. Laser Printer or equivalent
5.7. Micropipettes
5.8. Water Bath capable of maintaining 37° ± 2°C

6. REAGENTS:

6.1. **1% Agarose E-Gel Cassette:** Purchased Commercially.
6.2. **Bal-31 Enzyme:** Purchased Commercially.
6.3. **Bromophenol Blue:** Purchased Commercially.
6.4. **Calcium Chloride Dihydrate:** Purchased Commercially.
6.5. **Disodium EDTA Dihydrate:** Purchased Commercially.
6.6. **DNase 5x Reaction Buffer (Exonuclease):** Dissolve 1.21g of Tris Base in approximately 85mL of Sterile Water and adjust the pH to approximately 8.5 with concentrated Hydrochloric Acid. Add 1.2g of Magnesium Chloride Hexahydrate, 0.89g of Calcium Chloride Dihydrate, and 17.5g of Sodium Chloride. Adjust the pH to 8.1 with Hydrochloric Acid and dilute to 100mL with Sterile Water. Stable for 2 – 3 months refrigerated in a closed container.

6.7. **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.

6.8. **DNase Nuclease Buffer (Exonuclease):** Dissolve 0.242g of Tris Base in 40mL of Sterile Water and adjust to approximately pH 8.5 with concentrated Hydrochloric Acid. Then add 0.102g of Magnesium Chloride Hexahydrate, 0.075g of Calcium Chloride Dihydrate, 0.584g of Sodium Chloride, and 0.037g of Disodium EDTA Dihydrate. Adjust to pH 8.1 with Hydrochloric Acid, add 50mL of Glycerol, and dilute to 100mL with Sterile Water. Stable for 1 – 2 months refrigerated in a closed container.

6.9. **E-Gel 1Kb Plus DNA Ladder:** Purchased Commercially.

6.10. **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.

6.11. **Glacial Acetic Acid:** Purchased Commercially.

6.12. **Glycerol:** Purchased Commercially.

6.13. **HEPES:** Purchased Commercially or In-House.

6.13.1. **Note:** Must contain and meet specifications for DNase (Exonuclease) on Certificate of Analysis.

6.14. **Hydrochloric Acid, concentrated:** Purchased Commercially.

6.15. **Magnesium Chloride Hexahydrate:** Purchased Commercially.

6.16. **Sodium Acetate Trihydrate:** Purchased Commercially.

6.17. **Sodium Chloride:** Purchased Commercially.

6.18. **Sodium Hydroxide:** Purchased Commercially or Prepared In-House.

6.19. **Sterile Water:** Purchased Commercially.

6.20. **Tris Base:** Purchased Commercially.

7. VALIDATION PROCEDURE:

7.1. Assay

7.1.1. Prepare each sample utilizing the table below, in triplicate:

TABLE 1: VALIDATION SAMPLE / SPIKE SOLUTION PREPARATION

Sample ID	Sample Volume (µL)	HEPES Weight (g)	Nuclease Buffer Volume (µL)[1]	Volume of Bal-31 Enzyme (µL)
0% Spike	15	0.07	985	0
100% Limit Spike	15	0.07	983	2

[1]Volume of Nuclease buffer for Bal-31 enzyme at 1000 Units/ mL

7.1.2. Prepare standards utilizing the table below:

TABLE 2: DNASE: EXONUCLEASE STANDARDS PREPARATION

Purpose	Final Concentration (Unit/µL)	Volume of Bal-31 Enzyme (µL)	Volume of Nuclease Buffer (µL)[1]
100% Limit Std.	**$0.2x10^{-2}$**	2	998

[1]Volume of Nuclease buffer for Bal-31 enzyme at 1000 Units/ mL

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

TABLE 3: EXONUCLEASE REACTION MIX

Amount	Solution
(Y+1) x 3µL	E-Gel 1Kb Plus DNA Ladder
(Y+1) x 1µL	DNase 5x Reaction Buffer (Exonuclease)
(Y+1) x 1µL	Sterile Water

7.1.4. 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
Nuclease Buffer (µL)	5	-	-	-	5
Test Solution (µL)	-	5	5	-	-
Control Enzyme[1] (µL)	-	-	-	5[1]	-

[1]Appropriately diluted Bal-31

7.1.5. Mix thoroughly and immediately place the Control onto ice or into a temperature monitored refrigerator.
7.1.6. Incubate all others at 37°C for 4 hours.
7.1.7. Cool tubes on ice or in a temperature monitored refrigerator for approximately 5 minutes.
7.1.8. Centrifuge all tubes for 1 minute.
7.1.9. To each tube, add 4µL of Gel Loading Buffer.
7.1.10. Vortex thoroughly.
7.1.11. Centrifuge for 1 minute.

7.2. Electrophoresis
7.2.1. Utilize 1% Agarose E-GEL cassettes.
7.2.2. Remove the E-GEL cassette it's from packaging, carefully remove the well comb, and inspect under sufficient lighting.
7.2.2.1. Inspect for any damage to the wells and any defects. If any damage is found, discard properly.
7.2.2.2. Note: Load the gel within 15 minutes of opening the package and run gel within one minute of loading the samples
7.2.3. Place the E-GEL cassette into the E-Gel Power Snap Electrophoresis Device starting from the right edge.
7.2.4. Load the entire sample into to the well.
7.2.5. Load all empty wells with 14µL with Sterile Water.
7.2.6. Set up the run by selecting the E-Gel Protocol 1-2% on the E-Gel Power Snap Electrophoresis Device.
7.2.7. Ensure the run time is 15 min.
7.2.8. Run the gel by pressing "Start Run"

7.3. Photograph
7.3.1. Label the Cassette appropriately.
7.3.1.1. Exo, Initials / Date
7.3.2. Connect the E-Gel Power Snap Camera to the Electrophoreses unit.

7.3.3. Press Capture in the home screen view.
7.3.3.1. Note: Allow the gel to cool for 5-10 minutes before image capture to enhance sensitivity.
7.3.4. Export image to a USB thumb drive.
7.3.5. Print image with the wells labeled

8. VALIDATION PARAMETERS:

8.1. System Suitability:

8.1.1. All of the following system suitability acceptance criteria must be met for the assay results to be suitable for use:
8.1.1.1. The Blank lane demonstrates substrate degradation equal to or more pronounced than the control lane.
8.1.1.2. The 100% Limit Standard lane shows substrate degradation more pronounced than the Blank lane.
8.1.1.3. 100% Limit Standard lane substrate is visible and degraded.

8.2. Specificity:

8.2.1. Specificity will be demonstrated by comparing the control lane, 0% spiked sample solution lane, and 100% level spiked solution lane.
8.2.2. Acceptance Criteria:
8.2.2.1. The 100% level spike should demonstrate more pronounced degradation than the 0% spiked sample solution.
8.2.2.2. The 0% spike sample solution should demonstrate degradation equal to or more pronounced than the control.

8.3. Detection Limit:

8.3.1. Report the level of detectability in DNase units per gram as less than the standard.

$$\text{Units/g} = (1\text{x}10^{-2}\text{Unit})/(0.005\text{mL}*0.02\text{g/mL})$$

8.3.2. Acceptance Criteria:
8.3.2.1. No less than 100 Unit/g is acceptable.