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ANALYTICAL METHOD VALIDATION PROTOCOL: DNASE (ENDONUCLEASE) ASSAY

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1. PURPOSE:

1.1. To ensure the method of analysis for evaluating enzymatic activity of DNase (for nicking capability) 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 DNase (Endonuclease). 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. The sample is incubated for a period of time with the substrate (plasmid pBR322). The integrity of the substrate and the presence of any degradation products are examined using agarose gel electrophoresis containing ethidium bromide stain. DNase (Endonuclease or 'nicking activity') will cause breakdown of the relatively fast migrating, supercoiled, circular pBR322 plasmid DNA to the relatively slow migrating nicked ("relaxed", but still circular) form and finally to the intermediate, migrating linear form.

2.3. 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 implementation, control and maintenance of this protocol.

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

4. REFERENCES:

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
4.5. 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 included 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. EQUIPMENT:

6.1. 2.0 mL microcentrifuge tubes
6.2. 15mL to 50mL Centrifuge Tubes or equivalent

6.3. Analytical Balance
6.4. Calibrated Timer
6.5. E-Gel Power Snap Electrophoresis Device or equivalent
6.6. E-Gel Power Snap Camera
6.7. Laser Printer or equivalent
6.8. Microcentrifuge
6.9. Microcentrifuge Tubes Rack
6.10. Micropipettes
6.11. USB Drive or equivalent
6.12. Water Bath capable of maintaining 37° ± 2°C

7. REAGENTS:

7.1. **1% Agarose E-Gel Cassette:** Purchased Commercially.
7.2. **Bromophenol Blue:** Purchased Commercially.
7.3. **Calcium Chloride Dihydrate:** Purchased Commercially.
7.4. **Deoxyribonuclease (DNase) 1 Enzyme:** Purchased Commercially.
7.5. **Disodium EDTA Dihydrate:** Purchased Commercially.
7.6. **DNase 1 Buffer (Endonuclease):** Dissolve 0.272g of Sodium Acetate Trihydrate and 0.074g of Calcium Chloride Dihydrate in 45mL of Sterile Water and adjust the pH to 6.5 with Glacial Acetic Acid or Sodium Hydroxide. Add 50mL (61.5g) of Glycerol then dilute to a final volume of 100mL. Stable for 2 – 4 months refrigerated.
7.7. **DNase 10x Reaction Buffer (Endonuclease):** Dissolve 1.3g of Sodium Acetate Trihydrate and 0.1g of Magnesium Chloride Hexahydrate in 95mL of Sterile Water and adjust the pH to 5.0 with Glacial Acetic Acid. Dilute with Sterile Water to a final volume of 100mL. Stable for 2 – 3 months refrigerated in a closed container.
7.8. **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.
7.9. **DNase TE Buffer (Endonuclease):** Dissolve 0.121g of Tris Base and 0.037g of Disodium EDTA Dihydrate in approximately 85mL of Sterile Water. Adjust the pH to 7.5 with concentrated Hydrochloric Acid and dilute to 100mL with Sterile Water. Keep refrigerated in a closed container, stable for 3 – 4 months.
7.10. **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.
7.11. **Glacial Acetic Acid:** Purchased Commercially.
7.12. **Glycerol:** Purchased Commercially.
7.13. **HEPES:** Purchased Commercially or In-House.
7.13.1. **Note:** Must contain and meet specifications for DNase (Endonuclease) on the Certificate of Analysis.
7.14. **Hydrochloric Acid, concentrated:** Purchased Commercially.
7.15. **Magnesium Chloride Hexahydrate:** Purchased Commercially.
7.16. **Plasmid pBR322 DNA Substrate:** Purchased Commercially.
7.17. **Sodium Acetate Trihydrate:** Purchased Commercially.
7.18. **Sodium Hydroxide:** Purchased Commercially or Prepared In-House.
7.19. **Sterile Water:** Purchased Commercially.
7.20. **Tris Base:** Purchased Commercially.

8. VALIDATION PROCEDURE:

8.1. Assay

8.1.1. Prepare each sample (typically 2% or 0.2 g/mL) utilizing the table below, in triplicate:

TABLE 1: VALIDATION SAMPLE / SPIKE SOLUTION PREPARATION

Sample ID	Sample Volume (µL)	HEPES Weight (g)	DNase 1 Buffer Volume (µL)	Volume of Stock Solution B (µL)
0% Spike	15	0.07	985	0
100% Limit Spike	15	0.07	975	10

8.1.2. Prepare standards utilizing the table below:

TABLE 2: DNASE (ENDONUCLEASE) STANDARDS PREPARATION

Purpose	Final Concentration (Unit/µL)	Volume of DNase I Enzyme (µL)	Volume of DNase I Buffer (µL)
Stock Solution A	**0.2**	2 of DNase I	2120[1]
Stock Solution B	**$0.2x10^{-2}$**	10 of 0.2	990
100% Limit Std.	**$0.2x10^{-4}$**	10 of $0.2x10^{-2}$	990

[1]Volume of DNase I buffer for DNase I enzyme at 20,000 Units

8.1.3. Dilute Substrate prior to preparing reaction mix, as follows:

TABLE 3: DNASE (ENDONUCLEASE) SUBSTRATE PREPARATION

Final Concentration (µg/µL)	Volume of pBR 322 DNA Substrate (µL)	Volume of TE Buffer (µL)
0.1	8	12

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

TABLE 4: ENDONUCLEASE REACTION MIX

Amount	Solution
(Y+1) x 1µL	Diluted pBR322 DNA Substrate
(Y+1) x 1µL	DNase 10x Reaction Buffer (Endonuclease)
(Y+1) x 3µL	Sterile Water

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

TABLE 5: 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
DNase 1 Buffer (µL)	5	-	-	-	5
Test Solution (µL)	-	5	5	-	-
Control Enzyme[1] (µL)	-	-	-	5[1]	-

[1]Appropriately diluted DNase I. (Note, for instance, that 5 microliters of 0.2×10^{-4} Units DNase/µL represents 1×10^{-4} Units DNase.)

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

8.2. Electrophoresis
8.2.1. Utilize 1% 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. Place the E-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 set to 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. Endo, 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.
9.1.1.3. The 100% Limit Standard lane substrate is visible and degraded.

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 spike 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 DNase 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.