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ANALYTICAL METHOD VALIDATION REPORT: 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 was responsible for the implementation, control and maintenance of this report.

4. REFERENCES:

4.1. BMV01 pp40-42, 47
4.2. BSI-PRL-0856, Analytical Method Validation Protocol: DNase (Exonuclease) Assay for 10N Sodium Hydroxide
4.3. BSI-SOP-0098, Balance SOP
4.4. BSI-SOP-0134, Pipette SOP
4.5. BSI-SOP-0135, Laboratory Chemical Preparation
4.6. BSI-SOP-0259, Fisher Scientific Isotemp Water Bath Operation Calibration SOP

5. MATERIALS AND EQUIPMENT:

TABLE 1: EQUIPMENT

Equipment	Serial Number	Calibration Due	Manufacturer	Date of Last Calibration
Analytical Balance	24801744	4/30/25	Sartorius	10/4/24
100µL - 1000µL Pipette	R14419C	2/28/25	Eppendorf Research Plus	8/6/24
0.5µL - 10µL Pipette	R32893H	4/30/25	Eppendorf Research Plus	10/15/24
20µL - 200µL Pipette	N41555G	3/31/25	Eppendorf Research Plus	9/9/24
2µL - 20µL Pipette	R12216C	6/30/25	Eppendorf Research Plus	12/10/24
Calibrated Timer	230665671	9/29/25	FisherBrand	9/29/23
Centrifuge	41650138	Not Applicable	Fisher Scientific accuSpin Micro 17	Not Applicable
Water Bath	300004011	7/25	Fisher Scientific Isotemp	7/22/24
E-Gel Power Snap Electrophoresis Device	2848022120117	Not Applicable	Invitrogen	Not Applicable
E-Gel Power Snap Camera	2848122060090	Not Applicable	Invitrogen	Not Applicable

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.

TABLE 2: REAGENTS					
Reagent	**Lot Number**	**Expiration Date**	**Manufacturer**	**Date of Opening**	**Part Number**
Bal-31 Enzyme (Concentration: 2 Units/µL)	AN91182A	4/25	Takara	5/17/24	2510A
HEPES	SLCM4091	1/31/26	Sigma Aldrich	7/8/24	PHG0001-100G
DNase Nuclease Buffer (Exonuclease)	BSP44P67	1/27/25	In-House Solution	11/27/24	Not Applicable
E-Gel 1 Kb Plus DNA Ladder	2930338	3/31/26	Invitrogen	8/15/24	10488090
DNase 5x Reaction Buffer (Exonuclease)	BSP45P02	4/15/25	In-House Solution	1/15/25	Not Applicable
Sterile Water	6402006	2/26	Ricca	1/21/25	R9145000-1G
Gel Loading Buffer	BSP44P68	1/27/25	In-House Solution	11/27/24	Not Applicable
1% Agarose E-Gel Cassette	T300724-01	7/30/25	Invitrogen	1/22/25	G401001
10N NaOH	NAHY-M03-0125-0003	Not Applicable	BioSpectra Inc.	1/12/25	Not Applicable

7. VALIDATION PROCEDURE:

7.1. Assay

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

TABLE 3: VALIDATION SAMPLE / SPIKE SOLUTION PREPARATION				
Sample ID	**Sample Volume (µL)[2] NAHY-M03-0125-0003**	**HEPES Weight (g)**	**Nuclease Buffer Volume (µL)[1]**	**Volume of Bal-31 Enzyme (µL)**
0% Spike 1	15	0.0716	985	0
0% Spike 2	15	0.0710	985	0
0% Spike 3	15	0.0701	985	0
100% Limit Spike 1	15	0.0723	984	1
100% Limit Spike 2	15	0.0728	984	1
100% Limit Spike 3	15	0.0746	984	1

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

[2]Equivalent to 0.02g based on density of 1.33g/mL for 10N NaOH

7.1.2. Prepare standards utilizing the table below:

TABLE 4: DNASE: EXONUCLEASE STANDARDS PREPARATION			
Purpose	**Final Concentration (Unit/µL)**	**Volume of Bal-31 Enzyme (µL)**	**Volume of Nuclease Buffer (µL)[1]**
100% Limit Std.	**$0.2x10^{-2}$**	1	999

[1]Volume of Nuclease buffer for Bal-31 enzyme at 2000 Units/ mL (2 Units/µL)

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

TABLE 5: 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. Labelled an appropriate number of microcentrifuge tubes and added previously prepared solutions to each of the tubes as follows:

TABLE 6: 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. Mixed thoroughly and immediately placed the Control onto ice or into a temperature monitored refrigerator.
7.1.6. Incubated all others at 37°C for 4 hours.
7.1.7. Cooled tubes on ice or in a temperature monitored refrigerator for approximately 5 minutes.
7.1.8. Centrifuged all tubes for 1 minute.
7.1.9. To each tube, added 4µL of Gel Loading Buffer.
7.1.10. Vortexed thoroughly.
7.1.11. Centrifuged for 1 minute.

7.2. Electrophoresis
7.2.1. Utilized 1% Agarose E-GEL cassettes.
7.2.2. Removed the E-GEL cassette it's from packaging, carefully remove the well comb, and inspect under sufficient lighting.
7.2.2.1. Inspected for any damage to the wells and any defects. If any damage is found, discard properly.
7.2.2.2. Note: Loaded the gel within 15 minutes of opening the package and ran gel within one minute of loading the samples
7.2.3. Placed the E-GEL cassette into the E-Gel Power Snap Electrophoresis Device starting from the right edge.
7.2.4. Loaded the entire sample into to the well.

7.2.5. Loaded 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. Ensured the run time was 15 min.
7.2.8. Ran the gel by pressing "Start Run"

7.3. Photograph
7.3.1. Labelled the Cassette appropriately.
7.3.1.1. Exo, Initials / Date
7.3.2. Connected the E-Gel Power Snap Camera to the Electrophoreses unit.
7.3.3. Pressed Capture in the home screen view.
7.3.3.1. Note: Allowed the gel to cool for 5-10 minutes before image capture to enhance sensitivity.
7.3.4. Exported image to a USB thumb drive.
7.3.5. Printed image with the wells labelled

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.

9. VALIDATION RESULTS:

TABLE 7: 10N SODIUM HYDROXIDE EXONUCLEASE VALIDATION RESULTS	
System Suitability	
Parameter	**Result**
0% (Blank) lane demonstrates substrate degradation equal to or more pronounced than the control lane.	Pass
The 100% level standard lane shows substrate degradation more pronounced than the 0% (Blank).	Pass
The 100% standard lane substrate is visible and degraded.	Pass
Specificity	
Parameter	**Result**
The 100% level spike should demonstrate more pronounced degradation than the 0% spiked sample solution.	Pass
The 0% spike sample solution should demonstrate degradation equal to or more pronounced than the control.	Pass
Limit of Detection	
Parameter	**Result**
Report the level of detectability in DNase units per gram as less than the standard. NMT 100 Units/g is acceptable. $\frac{Units}{g} = \frac{1 \times 10^{-2}\ Unit}{(0.005mL \times 0.02\frac{g}{mL})}$	100 Units/gram

10. CONCLUSION:

10.1. The method "DNase (Exonuclease) Assay" is considered validated and suitable for use for 10N NaOH at all BioSpectra, PA facilities. All acceptance criteria for System Suitability, Specificity, and Limit of Detection were met. The Limit of Detection was determined to be 100 Units/gram.