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ANALYTICAL METHOD VALIDATION REPORT: DNASE (NICKASE) ASSAY MES HYDRATE

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1. PURPOSE:

1.1. The purpose of this method validation report is to:

1.1.1. 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 as a category 2 limit test.

1.1.2. To summarize the findings for all applicable products from the DNase (NICKase) Assay validation including:

1.1.2.1. System Suitability

1.1.2.2. Specificity

1.1.2.3. Limit of Detection

2. SCOPE:

2.1. This analytical method validation report applies to MES Hydrate.

2.2. The DNase (NICKase) Assay was validated as a category II Limit Test.

2.3. The Analytical Method Validation Master Plan dictates that this report will include an assessment and conclusive statements of validation on the following: System Suitability, Specificity, and Limit of Detection.

2.4. The sample was incubated for a period of time with the substrate (plasmid pBR322). The integrity of the substrate and the presence of any degradation products were examined using agarose gel electrophoresis containing Sybr gold 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 in excessive amounts.

3. RESPONSIBILITIES:

3.1. The Associate Director Product Life Cycle and/or qualified designee(s) were responsible for completing the method validation report.

3.2. The Chemist Product Life Cycle and/or the qualified designee(s) were responsible for performing the testing as stated in the Analytical Method Validation Protocol.

4. REFERENCES:

4.1. BSI-PRL-0770, DNase NICKase Assay Method Validation Protocol MES Hydrate

4.2. BSI-SOP-0098, Balance SOP

4.3. BSI-SOP-0134, Pipette SOP

4.4. BSI-SOP-0135, Laboratory Chemicals

4.5. BSI-SOP-0259, Fisher Scientific Isotemp Water Bath Operation and Calibration SOP

4.6. BSI-SOP-0436, Analytical Methods Validation Master Plan

5. MATERIALS AND EQUIPMENT:

5.1. All materials and equipment utilized in this Validation are outlined in this section:

TABLE 1: REAGENTS

Reagent	Lot Number	Expiration Date	Manufacturer	Date of Opening	Part Number
DNase 1 Enzyme (Activity: 338.5 Units/µL)	2407903	3/8/24	Invitrogen	12/13/23	18047-019
DNase 1 Buffer	BSP40P23	3/28/24	In-House Solution	Not Applicable	Not Applicable
pBR 322 DNA Substrate	2441895	5/11/24	Invitrogen	12/13/23	15367-014
TE Buffer	BSP39P92	1/31/24	In-House Solution	Not Applicable	Not Applicable
Endonuclease Free Tris Base	190374	1/31/24	Fisher Scientific	10/23/19	BP152-500
Sterile Water	6210004	10/24	Ricca	12/13/23	R9145000-1G
DNase 10x Reaction Buffer (Endonuclease)	BSP40P18	2/22/24	In-House Solution	Not Applicable	Not Applicable
Gel Loading Buffer	BSP39P75	12/17/23	In-House Solution	Not Applicable	Not Applicable
1% Agarose E-Gel Cassette	T-280623-01	6/28/24	Invitrogen	12/13/23	G401001

TABLE 2: EQUIPMENT				
Equipment	**Serial Number**	**Calibration Due**	**Manufacturer**	**Date of Last Calibration**
Analytical Balance	24801744	4/30/24	Sartorius	10/5/23
100µL - 1000µL Pipette	O39512B	12/31/23	Eppendorf Research Plus	6/20/23
500µL - 5000µL Pipette	J18397D	2/29/24	Eppendorf Research Plus	8/25/23
0.5µL - 10µL Pipette	N27646F	12/31/23	Eppendorf Research Plus	6/20/23
2µL - 20µL Pipette	G24188D	12/31/23	Eppendorf Research Plus	6/20/23
20µL - 200µL Pipette	N41555G	2/29/24	Eppendorf Research Plus	8/25/23
Calibrated Timer	221117644	1/5/24	FisherBrand	Not Applicable
Centrifuge	41650138	Not Applicable	Fisher Scientific accuSpin Micro 17	Not Applicable
Water Bath	300004011	5/31/24	Fisher Scientific Isotemp	5/26/23
E-Gel Power Snap Electrophoresis Device	2848022040147	Not Applicable	Invitrogen	Not Applicable
E-Gel Power Snap Camera	2848122060090	Not Applicable	Invitrogen	Not Applicable

TABLE 3: SUPPLIES		
Supply	**Supplier**	**Part Number**
epT.I.P.S. Pipette Tips	Eppendorf	0030071557
Small Square Polystyrene Weigh Boats, White, 20mL	Cole-Parmer	01017-05
2.0mL Microcentrifuge Tubes	FisherBrand	05-408-146
15mL Screw Cap Tube 120 x 17mm, Polypropylene with Print	Sarstedt	62.554.100
5mL Centrifuge Tube Rack	Bel-Art	F18513-4640

6. VALIDATION PROCEDURE:

6.1. NICKase Assay

6.1.1. Prepared standards utilizing the table below:

TABLE 4: STANDARD PREPARATIONS			
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**	1.18[1] of DNase I	1998.82[1]
Stock Solution B	**$0.2x10^{-2}$**	10 of 0.2	990
Stock Solution C	**$0.2x10^{-4}$**	10 of $0.2x10^{-2}$	990
100% Limit Std.	**$0.2x10^{-5}$**	100 of $0.2x10^{-4}$	900

[1]Refer to Step 6.1.1.1. for calculation to determine the volume of DNase 1 Enzyme.

6.1.1.1. **Note:** DNase (Endonuclease) Standard preparation is dependent on DNase 1 Enzyme concentration (found on the reagent container). The volume of DNase 1 enzyme used will be determined using the following equation (may be scaled as needed):

$$Volume\ of\ DNase\ 1\ (\mu L) = \frac{\left(0.2\frac{Units}{\mu L}\right) \times \left(Final\ Volume\ (\mu L)\right)}{DNase\ 1\ Enayme\ Concentration\ \left(\frac{Units}{\mu L}\right)}$$

6.1.2. Prepared each sample utilizing the table below:

TABLE 5: SAMPLE SOLUTION AND SPIKE RECOVERY PREPARATION			
Validation Sample Stock Solution Preparation			
Sample ID	**Sample Weight (g)**	**DNAse Free Tris Base (g)**	**DNase 1 Buffer Volume (µL)**
MES Sample Stock Solution	0.0210	0.0102	1000
Validation Sample Spike Solution Preparation			
Sample ID	**Volume of *Sample Stock Solution* (µL)**	**DNase 1 Buffer Volume (µL)**	**Amount of *Stock Solution C* (µL)**
0% Spike Sample Solution Rep. 1	100	900	0
0% Spike Sample Solution Rep. 2	100	900	0
0% Spike Sample Solution Rep. 3	100	900	0
100% Spike Sample Solution Rep. 1	100	800	100
100% Spike Sample Solution Rep. 2	100	800	100
100% Spike Sample Solution Rep. 3	100	800	100

6.1.3. Diluted Substrate prior to preparing reaction mix, as follows:

TABLE 6: SUBSTRATE PREPARATION		
DNase: Endonuclease Substrate Preparation		
Final Concentration (µg/µL)	**Volume of pBR 322 DNA Substrate (µL)**	**Volume of TE Buffer (µL)**
0.1	8	12

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

TABLE 7: REACTION MIX PREPARATION	
Endonuclease Reaction Mix	
Amount	**Solution**
(Y+1)x 1µL: 20µL	Diluted pBR 332 DNA Substrate
(Y+1) x 1µL: 20µL	DNase 10X Reaction Buffer (Endonuclease)
(Y+1) x 3µL: 60µL	Sterile Water

6.1.5. Labeled an appropriate number of microcentrifuge tubes and added previously prepared solutions to each of the tubes as follows:

TABLE 8: DIGESTION PREPARATIONS						
	Blank	**Test Solution**	**100% Spike Sample**	**System Suit. (Stock Solution C)**	**100% Limit Std.**	**Control**
Tube #	**1**	**2-5**	**6-7**	**8**	**9**	**10**
Reaction Mix (µL)	5	5	5	5	5	5
DNase 1 Buffer (µL)	5	-	-	-	-	5
Test Solution (µL)	-	5	5	-	-	-
Control Enzyme[1] (µL)	-	-	-	5[1]	5[1]	-

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

6.1.6. Mixed thoroughly and immediately placed the Control onto ice or into a temperature monitored refrigerator.

6.1.7. Incubated all others at 37°C for 4 hours.

6.1.8. Cooled tubes on ice or in a temperature monitored refrigerator for approximately 5 minutes. Centrifuged all tubes for 1 minute. To each tube, added 4 microliters of Gel Loading Buffer. Vortexed thoroughly. Centrifuged for 1 minute.

6.2. Electrophoresis

6.2.1. Utilized 1% Agarose E-GEL cassettes.

6.2.2. Removed the gel from package and gently removed the comb from the E-Gel Cassette.

6.2.2.1. **Note**: Loaded the gel within 15 minutes of opening the package and ran gel within one min of loading the samples

6.2.3. Inserted the gel cassette into the E-Gel Power Snap Electrophoresis Device, starting from the right edge.

6.2.4. Loaded the entire sample into to the well.

TABLE 9: GEL TEMPLATE	
Suggested Well Setup	
Lane ID	Sample ID
M	Blank
1	100% Level Limit Std.
2	System Suit. (Stock Solution C)
3	0% Spike Replicate 1
4	0% Spike Replicate 2
5	0% Spike Replicate 3
6	100% Spike Replicate 1
7	100% Spike Replicate 2
8	100% Spike Replicate 3
9	Control
10	DNAse Buffer

6.2.5. Loaded all empty wells with 14µL with DNAse Buffer.
6.2.6. Set up the run by selecting the E-Gel Protocol on the E-Gel Power Snap Electrophoresis Device. Ensure the run time is 10 min.
6.2.7. Ran the gel by pressing "Start Run"

6.3. Photograph
6.3.1. Connected the E-Gel Power Snap Camera to the Electrophoreses unit.
6.3.2. Pressed Capture in the home screen view.
6.3.2.1. **Note**: Allowed the gel to cool for 5-10 minutes before image capture to enhance sensitivity.
6.3.3. Exported image to a USB thumb drive.

7. VALIDATION PERFORMANCE SUMMARY:

TABLE 10: VALIDATION SUMMARY

Acceptance Criteria	Product	Results	Final Disposition
System Suitability • 0% (Blank) lane demonstrates substrate degradation equal to or more pronounced than the control lane. • The 100% Level standard lane shows substrate degradation more pronounced than the 0% (Blank). • The Stock Solution C lane level shows degradation beyond nicking of the plasmid, this is evident through being the lane with the furthest traveled substrate and/or depleted supercoiled and relaxed state bands. Specificity • The 100% Level Spike should demonstrate more pronounced degradation than the 0% spiked sample solution. • The 0% spike sample solution should demonstrate degradation equal to or more pronounced than the control. Limit of Detection • Report the NICKing activity level of detectability in DNase unit per gram. NMT 1 Unit/g is acceptable.	MES hydrate	System Suitability • Pass • Pass • Pass Specificity • Pass • Pass Limit of Detection • 1 Unit/g	Meets Requirements

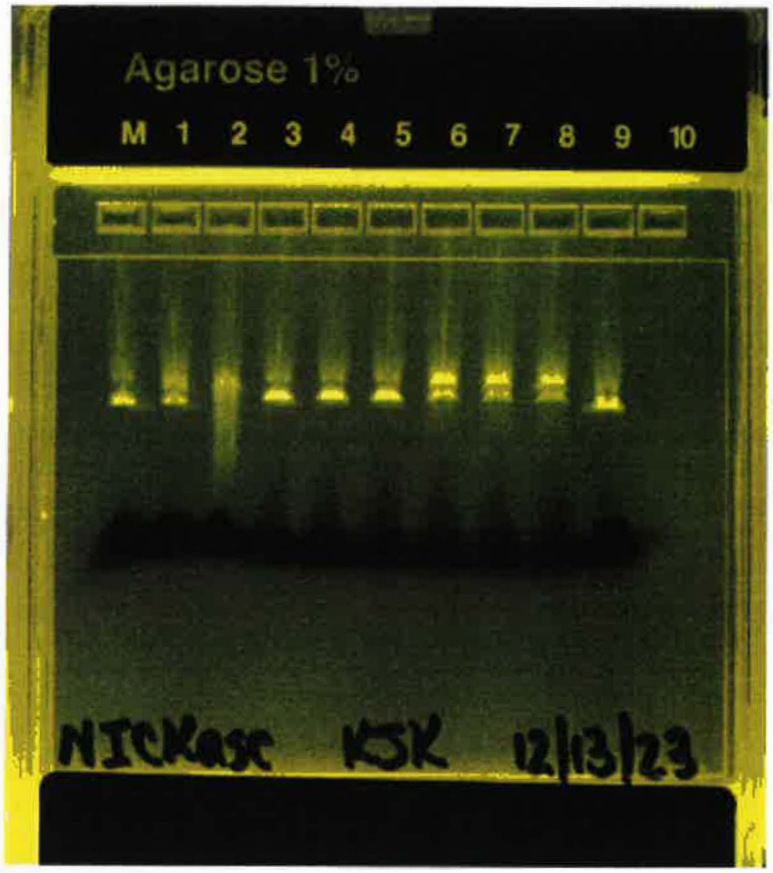
8. VALIDATION RESULTS:

8.1. MES Hydrate

8.1.1. Sample lot: ME3200-187-0819

8.1.2. Notebook Reference: MV12 pp14-17

8.1.3. Specification: NMT 1 Unit/gram (Reported as "None Detected").



REVIEWED BY: INITIAL: _____

DATE: 12/19/23

NICKase - MES Monohydrate

Well ID #	Sample	Well ID #	Sample
M	Blank	6	100% Spike Sample Solution Replicate 1
1	100% Level Limit Standard ($0.2x10^{-5}$ Units/µL DNase 1)	7	100% Spike Sample Solution Replicate 2
2	System Suitability (Stock Solution C) ($0.2x10^{-4}$ Units/µL DNase 1)	8	100% Spike Sample Solution Replicate 3
3	0% Spike Sample Solution Replicate 1	9	Control
4	0% Spike Sample Solution Replicate 2	10	Sterile Water
5	0% Spike Sample Solution Replicate 3		

8.1.4. System Suitability, Specificity, and Limit of Detection were evaluated for MES Hydrate. Results are summarized in Table 12.

TABLE 11: MES HYDRATE VALIDATION RESULTS	
System Suitability	
Parameter	**Result**
0% (Blank) lane demonstrates substrate degradation equal to or more pronounced than control lane.	Pass
The 100% level standard lane shows substrate degradation more pronounced than the 0% (Blank).	Pass
The Stock Solution C lane level shows degradation beyond nicking of the plasmid, this is evident through being the lane with the furthest traveled substrate and/or depleted supercoiled and relaxed state bands.	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. NMT 1 Unit/g is acceptable. $\frac{Units}{g} = \frac{1 \times 10^{-5} Unit}{(0.005mL \times 0.002 \frac{g}{mL})}$	1 Unit/g

8.1.5. Conclusion

8.1.5.1. The method DNase (NICKase) Assay is considered validated and suitable for use for MES Hydrate at the BioSpectra Bangor, PA facility. All acceptance criteria for system suitability, specificity, and Limit of Detection were met. The Limit of Detection was determined to be 1 Unit/gram.