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MESH-4250 TEST METHODS

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1. PURPOSE:

1.1. To provide the testing methods for MES, Hydrate Product Code: MESH-4250.

2. REFERENCES:

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2.2. BSI-ATM-0123, MES Hydrate Testing Methods
2.3. BSI-SOP-0090, Lambda 25 UV/Vis Operation and Calibration
2.4. BSI-SOP-0096, RNase (Ribonuclease) Assay
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2.12. BSI-SPC-0352 v.1.0, MESH-4250 MES Hydrate Bio Pharma Grade Specifications

3. SPECIFICATIONS:

TABLE 1. MESH-4250 MES HYDRATE BIO PHARMA GRADE SPECIFICATIONS

ANALYSIS		SPECIFICATIONS
Absorbance (0.1M)	260nm	≤ 0.04 a.u.
	280nm	≤ 0.02 a.u.
Appearance		White Powder
Assay, Anhydrous Basis		≥ 99.5 %
Cytotoxicity (50% concentration)		Passes Test
Enzymes	DNase, Exonuclease	Passes Test
	NICKase, Endonuclease	Passes Test
	RNase	Passes Test
	Protease	Passes Test
Identification, IR		Conforms to Reference Standard
pH (1% solution)		3.5 – 4.1
Solubility (0.1M)		Clear and Colorless
Microbial Content	TAMC	≤ 100 CFU/g
	TYMC	≤ 100 CFU/g

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ANALYSIS		SPECIFICATIONS
Trace Metals	Aluminum (Al)	≤ 5 ppm
	Antimony (Sb)	≤ 5 ppm
	Arsenic (As)	≤ 3 ppm
	Barium (Ba)	≤ 5 ppm
	Bismuth (Bi)	≤ 5 ppm
	Cadmium (Cd)	≤ 5 ppm
	Calcium (Ca)	≤ 20 ppm
	Chromium (Cr)	≤ 5 ppm
	Cobalt (Co)	≤ 5 ppm
	Copper (Cu)	≤ 5 ppm
	Iron (Fe)	≤ 5 ppm
	Lead (Pb)	≤ 5 ppm
	Lithium (Li)	≤ 5 ppm
	Manganese (Mn)	≤ 5 ppm
	Magnesium (Mg)	≤ 5 ppm
	Molybdenum (Mo)	≤ 5 ppm
	Nickel (Ni)	≤ 5 ppm
	Sodium (Na)	≤ 20 ppm
	Zinc (Zn)	≤ 5 ppm
Water, KF		5.0 – 8.9 %

4. PROCEDURES:

4.1. ABSORBANCE (0.1M)

4.1.1. Weigh 0.53 g of sample and accurately transfer the weighed sample to a graduated cylinder and Q.S. to 25 mL with purified water.
4.1.2. Swirl to dissolve completely.
4.1.3. Refer to the Lambda 25 UV/Vis Spectrophotometer to determine the Absorbance of the sample.
4.1.3.1. Measure the sample at the following wavelengths: 260 nm and 280 nm.

4.2. APPEARANCE

4.2.1. Weigh a suitable amount of the sample into a clean, dry glass beaker.
4.2.2. In an area with sufficient lighting, view the sample from all sides.
4.2.3. The sample should be white in color and characteristic of crystals.

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4.3. **ASSAY (ANHYDROUS BASIS)**

4.3.1. Standardize 0.1 N sodium hydroxide in accordance with Standardization of Titrants utilizing the Metrohm Auto Titrator.
4.3.2. Accurately weigh 0.8 g of sample (measured as-is) and transfer to a suitable beaker.
4.3.3. Add 50 mL of purified water and stir to dissolve.
4.3.4. Titrate to the potentiometric endpoint with 0.1N sodium hydroxide.
4.3.5. Submerge the probe in storage solution after analysis is completed to condition the glass electrode. To calculate assay on the anhydrous basis, use below equation:

$$\%\ MES, Hydrate\ (as-is, anhydrous\ basis) = \frac{(mL \times N\ of\ NaOH)(19.524)}{Sample\ Weight\ (g)}$$

$$\%\ Mes, Hydrate\ (anhydrous) = \frac{As-Is\ Assay\ \%}{(100 - KF\ Value)} * 100$$

4.4. **CYTOTOXICITY (50% CONCENTRATION)**

4.4.1. Cytotoxicity at the 50% Dilution Concentration analysis will be performed by an outside testing laboratory.
4.4.1.1. Request the following: Modified MTT Cytotoxicity Test Protocol as a GMP Compliance Study from the Approved Contract Laboratory.
4.4.1.2. Package and send NLT 10 g of sample to Approved Contract Laboratory.
4.4.2. Analyses to be reported:
4.4.2.1. MTT Cytotoxicity Test at the 50% test article dilution.
4.4.2.2. Specification Required on Report to Pass: No Cytotoxic Potential
4.4.2.3. Specification on Report that Does Not Pass: Cytotoxic Potential

4.5. **ENZYME ACTIVITY**

4.5.1. Reference RNase, DNase, and Protease per procedures referenced in section 2 for reagent preparation, instrument use and setup.
4.5.2. **RNase**
4.5.2.1. Assay
4.5.2.2. Prepare each sample (2% or 0.02 g/mL) utilizing the table below:

Sample Solution Preparation	
Sample Weight (g)	**RNase Buffer Volume (µL)**
0.02	1000

4.5.2.3. Prepare standards utilizing the table below:

RNase (Ribonuclease) Standard Preparation			
Standard ID	**Final Concentration (Unit/µL)**	**Volume of RNase Solution[1] (µL)**	**Volume of RNase Buffer (µL)**
Stock Solution A	1×10^{-3}	2.5µL of RNase Solution[2]	1997.5[2]
Stock Solution B	0.2×10^{-3}	200µL of Stock Solution A	800
100% Limit Standard	0.2×10^{-4}	100µL of Stock Solution B	900

[1]10 mg/mL
[2]Volumes will vary based on the concentration reported on the RNase vendor's C of A.

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4.5.2.4. **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):

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

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

RNase Reaction Mix	
Amount	**Solution**
(Y + 1) x 1µL	RNA Millennium Marker
(Y + 1) x 1µL	RNase 10x Reaction Buffer
(Y + 1) x 3µL	Sterile Water

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

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

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

4.5.2.7. Mix thoroughly and immediately place the Control onto ice or into a temperature monitored refrigerator.
4.5.2.8. Incubate all others at 37ºC for 4 hours.
4.5.2.9. Cool tubes on ice or in a temperature monitored refrigerator for approximately 5 minutes.
4.5.2.10. Centrifuge all tubes for 1 minute.
4.5.2.11. To each tube, add 4 microliters of Gel Loading Buffer.
4.5.2.12. Vortex thoroughly.
4.5.2.13. Centrifuge for 1 minute.
4.5.2.14. Electrophoresis
4.5.2.14.1. Utilize 2% Agarose E-GEL cassettes.
4.5.2.14.2. Remove the E-GEL cassette from its packaging, carefully remove the well comb, and inspect under sufficient lighting.
4.5.2.14.2.1. Inspect for any damage to the wells and any defects.
4.5.2.14.2.2. **Note:** Load the gel within 15 minutes of opening the package and run the gel within one minute of loading the samples.

4.5.2.15. Insert the gel cassette into the E-Gel Power Snap Electrophoresis Device starting from the right edge.
4.5.2.16. Load the entire sample into the well.
4.5.2.17. Load all empty wells with 14µL of sterile water.
4.5.2.18. Set up the run by selecting the E-Gel Protocol 1 – 2% on the E-Gel Power Snap Electrophoresis Device.
4.5.2.19. Ensure the run time is 10 minutes
4.5.2.20. Run the gel by pressing "Start Run".

4.5.3. **DNase (Exonuclease)**

4.5.3.1. Assay

4.5.3.2. Prepare sample (2% or 0.02 g/mL) utilizing the table below:

Sample Solution Preparation	
Sample Weight (g)	**DNase Nuclease Buffer Volume (µL)**
0.02	1000

4.5.3.3. Prepare standards utilizing the table below.

DNase (Exonuclease) Standard Preparation			
Sample ID	**Final Concentration (Unit/µL)**	**Volume of Bal-31 Enzyme (µL)**	**Volume of DNase Nuclease Buffer (µL)**
100% Limit Standard	0.2×10^{-2}	2[1]	998[1]

[1]Volumes for a Bal-31 Nuclease Enzyme concentration of 1 Unit/µL.

4.5.3.3.1. **Note:** DNase (Exonuclease) Standard Preparation is dependent on Bal-31 Enzyme activity (found on the Certificate of Analysis or on the reagent container). The volume of Bal-31 Enzyme used will be determined using the following equation (may be scaled as needed):

$$\text{Volume of Bal-31 Enzyme (µL)} = \frac{\left(0.002\frac{Units}{\mu L}\right) \times \left(Final\ Volume\ (\mu L)\right)}{\text{Bal-31 Enzyme Activity } (\frac{Units}{\mu L})}$$

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

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

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

	Blank	Sample Solution	100% Limit Standard	Control
Tube Number	**1**	**2**	**3**	**4**
Reaction Mix (µL)	5	5	5	5
DNase Nuclease Buffer (µL)	5	-	-	5
Sample Solution (µL)	-	5	-	-
100% Limit Standard (µL)	-	-	5	-

4.5.3.6. Mix thoroughly and immediately place the Control onto ice or into a temperature monitored refrigerator.
4.5.3.7. Incubate all others at 37ºC for 4 hours.
4.5.3.8. Cool tubes on ice or in a temperature monitored refrigerator for approximately 5 minutes.
4.5.3.9. Centrifuge all tubes for 1 minute.
4.5.3.10. To each tube, add 4 microliters of Gel Loading Buffer.
4.5.3.11. Vortex thoroughly.
4.5.3.12. Centrifuge for 1 minute.
4.5.3.13. Electrophoresis
4.5.3.13.1. Utilize 1% Agarose E-GEL cassettes.
4.5.3.13.2. Remove the E-GEL cassette from its packaging, carefully remove the well comb, and inspect under sufficient lighting.
4.5.3.13.2.1. Inspect for any damage to the wells and any defects. If any damage is found,discard properly.
4.5.3.13.2.2. Note: Load the gel within 15 min of opening package and run gel within one min of loading the samples
4.5.3.14. Place the E-GEL cassette in the E-Gel Power Snap Electrophoresis Device Starting from the right edge.
4.5.3.15. Load the entire sample into the well
4.5.3.16. Load all empty wells with 14µL of Sterile Water.
4.5.3.17. Set up the run by selecting the E-Gel Protocol 1-2% on the E-Gel Power Snap Electrophoresis Device.
4.5.3.18. Ensure the run time is 15 min.
4.5.3.19. Run the gel by pressing “Start Run”

4.5.4. **DNase (NICKase)**
4.5.4.1. Assay
4.5.4.2. Prepare each sample utilizing the table below:

MES Hydrate Stock Solution Preparation			
Sample ID	**Sample Weight (g)**	**DNAse Free Tris Base (g)**	**DNase 1 Buffer Volume (µL)**
MES Sample Stock Solution	0.02	0.01	1000
Test Sample Solution	100	900	

4.5.4.3. Prepare standards utilizing the table below:

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**	X[1] of DNase I	2000
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]**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):

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

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

DNase: Endonuclease Substrate Preparation[1]		
Final Concentration (µg/ µL)	**Volume of pBR 322 DNA Substrate (µL)**	**Volume of TE Buffer (µL)**
0.1	8	12

[1] May be scaled as necessary.

4.5.4.5. Prepare a Reaction Mix, where Y represents the total number of tubes to be prepared, asfollows:

Endonuclease Reaction Mix	
Amount	**Solution**
(Y+1)x1µL	Diluted pBR 332 DNA Substrate
(Y+1)x1µL	DNase 10X Reaction Buffer (Endonuclease)
(Y+1)x3µL	Sterile Water

4.5.4.6. Label an appropriate number of microcentrifuge tubes and add previously preparedsolutions to each of the tubes as follows:

	Blank	**Test Solution**	**System Suit. (Stock Solution C)**	**100% Limit Std.**	**Control**
Tube #	**1**	**2**	**4**	**5**	**6**
Reaction Mix (µL)	5	5	5	5	5
DNase 1 Buffer (µL)	5	-	-	-	5
Test Solution (µL)	-	5	-	-	-
Control Enzyme[1] (µL)	-	-	5[1]	5[1]	-

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

4.5.4.7. Mix thoroughly and immediately place the Control onto ice or into a temperature monitored refrigerator.

4.5.4.8. Incubate all others at 37ºC for 4 hours.

4.5.4.9. Cool tubes on ice or in a temperature monitored refrigerator for approximately 5 minutes. Centrifuge all tubes for 1 minute. To each tube, add 4 microliters of Gel Loading Buffer. Vortex thoroughly. Centrifuge for 1 minute.

4.5.4.10. Electrophoresis

4.5.4.10.1. Utilize 1% Agarose E-GEL cassettes.

4.5.4.10.2. Remove the E-GEL cassette from its packaging, carefully remove the well comb, andinspect under sufficient lighting.

4.5.4.10.2.1. Inspect for any damage to the wells and any defects.

4.5.4.10.2.2. **Note:** Load the gel within 15 minutes of opening the package and run gel within one min of loading the samples.

4.5.4.11. Place the E-GEL cassette into the E-Gel Power Snap Electrophoresis device starting from the right edge.

4.5.4.12. Load the entire sample into the well.

4.5.4.13. Load all empty wells with 14µL of DNAse buffer.

4.5.4.14. Set up the run by selecting the E-Gel Protocol 1-2% on the E-Gel Power Snap Electrophoresis Device.

4.5.4.15. Ensure the run time is set to 10 min.

4.5.4.16. Run the gel by pressing “Start Run”

4.5.5. **Protease**

4.5.5.1. Assay:

4.5.5.2. Prepare sample (typically 2% or 0.02 g/mL) utilizing the table below (Solution may be scaled as needed):

Sample Solution Preparation	
Weight	**Purified Water Volume**
0.03 g	1.5 mL

4.5.5.3. Prepare standards utilizing the table below:

Protease System Suitability And Standards Preparation				
Standard Solution ID	Final Conc. Of Proteinase K (Unit/mL)	Proteinase K in Sample (Unit/g)	*PKWSS* Solution Volume (µL)	Purified Water Volume (µL)
Calibration Standard 1	0.004	0.004	20 µL of *(PKWSS)*	980
Calibration Standard 2	0.010	0.010	50 µL of *(PKWSS)*	950
Calibration Standard 3	0.020	0.020	100 µL of *(PKWSS)*	900
Calibration Standard 4	0.050	0.050	250 µL of *(PKWSS)*	750
Calibration Standard 5	0.100	0.100	500 µL of *(PKWSS)*	500

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

Protease Reaction Mix	
Amount	**Solution**
(Y+1) x 0.2 mL	Protease Substrate Solution
(Y+1) x 0.05 mL	Protease 10X Reaction Buffer

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

	Blank	Test Solution	0.004 Unit/g	0.01 Unit/g	0.02 Unit/g	0.05 Unit/g	0.1 Unit/g
Tube #:	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Purified Water (µL):	250	-	250	250	250	250	250
Test Solution (µL):	-	250	-	-	-	-	-
Control Enzyme[1] (µL):	-	-	5[1]	5[1]	5[1]	5[1]	5[1]
Reaction Mix (µL):	250	250	250	250	250	250	250

[1]Appropriately diluted Proteinase K

4.5.5.5.1. Mix thoroughly.

4.5.5.5.2. Ensure the water bath is at least ¾ full. Incubate at 37ºC for 16-18 hours.

4.5.5.5.3. Cool tubes in a temperature monitored refrigerator or on ice for approximately5 minutes. Centrifuge for 5-10 seconds. Add to each tube 0.67 mL of 10% TCA and mix thoroughly.

4.5.5.5.4. Cool in a temperature monitored refrigerator or on ice for 30-60 minutes. Centrifuge for 5-10 seconds. Rotate tubes 180º in the centrifuge. Centrifuge for 1 minute.

4.5.5.5.5. Carefully remove 0.65 mL of the supernatant and add it to 0.65 mL of 0.5 NNaOH and mix thoroughly. Absorbance measurements: Within 1 hour, zero the spectrophotometer at 440 nm with water in matched 1cm pathlength cuvettes.

4.5.5.5.6. Open the Win Lab software and open the Protease Quant. Method.

4.5.5.5.7. Calibrate the UV/Vis Spectrophotometer by ensuring that the Blank is assigned as “Blank”, the Calibration Standards 1 through 5 as assigned as “Standard” and all Samples are assigned as “Sample” in the “Type” column on the “Sample info” window of the UV WinLab Software.

4.5.5.5.8. Input the Calibration standards Proteinase K concentration in Units/g into the “Concentration” column on the “Sample info” window of the UV WinLab software.

4.5.5.5.9. Perform the absorbance of the blank at 440nm against purified water and record the value. The value should not exceed 0.10 Absorbance Units (a.u.). If it does exceed 0.10 a.u., make a new Substrate Solution with fresh Buffer for Substrate and repeat the Assay.

4.5.5.5.10. Measure the absorbance of all the Standards and Samples at 440nm against purified Water.

4.6. IDENTIFICATION (IR) (AS-IS)

4.6.1. Follow Spectrum Two UATR SOP for instrument use and setup.
4.6.2. Analyze sample as-is.
4.6.3. Place the prepared solid sample on the UATR crystal using a static free scoop.
4.6.4. Align the swinging arm with the crystal and apply force by turning the green arm clockwise.
4.6.5. Press “Scan” on the top Toolbar. The program will preview the sample. Turn the green arm until the Force Gauge is approximately 125, or until the noise has subsided.
4.6.6. Once the Force Gauge is adjusted, press “Scan”.
4.6.7. Once the scan is complete, release the swinging arm by turning it counterclockwise.
4.6.8. Clean the UATR crystal and the swinging arm with methanol and a KimWipe.

4.7. MICROBIAL

4.7.1. Microbial analysis will be performed by an outside testing laboratory
4.7.1.1. Package and send NLT 35 g of sample to Approved Contract Laboratory
4.7.2. Analyses:
4.7.2.1. Total Aerobic Microbial Count (TAMC)
4.7.2.2. Total Yeast Microbial Count (TYMC)

4.8. pH OF A 1% SOLUTION

4.8.1. Weigh 1.0 g of sample. Transfer to a suitable beaker.
4.8.2. Add 100 mL of purified water and stir to mix.
4.8.3. Follow the appropriate SOP for calibration and pH measurement.

4.9. SOLUBILITY (0.1M)

4.9.1. Weigh 0.53 g of sample and quantitatively transfer the aliquot to a 25-mL volumetric flask and dissolve in ~15-20 mL of purified water.
4.9.2. Q.S. to 25 mL with purified water. Scale as required.
4.9.3. View sample from all sides under sufficient light noting any apparent color or undissolved particulate. Solution should be clear (complete) and colorless to pass test.

4.10. TRACE ELEMENTS

4.10.1. Refer to Analytical Method: Determination of Elemental Impurities in MES Hydrate, DCN: BSI-ATM-0115 and NexION 350X ICP-MS SOP, DCN: BSI-SOP-0303 for reagent preparation, instrument use and setup.
4.10.2. Sample Preparation
4.10.2.1. Samples are stable for 24 hours.
4.10.2.2. Weigh approximately 100 mg of the sample into a 50 mL Digitube®.
4.10.2.3. Transfer approximately 10 mL of deionized water and swirled to dissolve sample.
4.10.2.4. Add 3.75 mL of Acid Digestion Mixture and swirl solution periodically to react and mix thoroughly.

4.10.2.5. Add deionized water to approximately 45 mL and then transfer 1.0 mL of Internal Standard/Complexing Solution.
4.10.2.6. Dilute to a final volume of 50 mL with deionized water and mix thoroughly.

4.11. WATER BY KARL FISCHER

4.11.1. Perform a standardization of the titrant (Composite 5) as per Standardization of Titrants.
4.11.2. Immediately weigh 0.1 g of as-is sample into the glass weighing spoon and tare it.
4.11.3. Transfer the sample to the KF vessel by removing the rubber septum and adding the sample into the titration vessel.
4.11.3.1. Do not leave the rubber septum open for long periods of time as this will allow moisture to enter the titration vessel.
4.11.4. Return the weighing spoon to the balance, making sure not to lose any sample that was left behind. Once the weight stabilizes, transfer the sample weight to the auto-titrator software.
4.11.5. Check to make sure there is no residual sample stuck to the sides of the titration vessel.
4.11.6. Ensure the sample is fully dissolved before the titration begins (i.e. before the stir command completes).
4.11.7. The moisture content will be determined by the Metrohm Auto Titrando 907, using the following equation:

$$\%\ Moisture = \frac{(mL\ of\ Composite\ 5)(\frac{mg}{mL} of\ Composite\ 5)(0.1)}{Sample\ Weight\ (g)}$$