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HEPES TESTING METHODS

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1. PURPOSE:

- 1.1. To provide the Quality Control (QC) Laboratory personnel with procedures for testing of HEPES raw materials (RM), in-process (IP), finished goods (FG), and stability.

2. SCOPE:

- 2.1. Applies to the testing HEPES in the QC Laboratory. Methods include testing for all types of HEPES sold by BioSpectra; only the specific tests required for requested type must be tested for.

3. RESPONSIBILITIES:

- 3.1. The Executive Director of Quality Control is responsible for the control, training, maintenance and implementation of this procedure.
- 3.2. The QC Analysts are responsible for compliance with the terms of this procedure. This includes notifying the Quality Control Manager and Quality Assurance Managers, or designees, if any analyses fail to meet their respective specifications.

4. REFERENCES:

- 4.1. *ACS, Reagent Chemicals*, current edition.
- 4.2. [Analytical Method of Analysis: ICP-MS for HEPES](#)
- 4.3. [Analytical Method Validation Protocol: HEPES Identity via HPLC](#)
- 4.4. [Analytical Method: Residual Solvents on the GC-FID](#)
- 4.5. *Current USP*
- 4.6. [Balance SOP](#)
- 4.7. [Bangor Portable Turbidimeter and Calibration SOP](#)
- 4.8. [Determination of ICH Q3D Elemental Impurities DCN: 20-003408](#)
- 4.9. [DNase \(Endonuclease\) Assay](#)
- 4.10. [DNase \(Exonuclease\) Assay](#)
- 4.11. [Endosafe nexgen-PTS Endotoxin Reader SOP](#)
- 4.12. [Laboratory Notebooks](#)
- 4.13. [Lambda 25 UV/Vis Operation and Calibration](#)
- 4.14. [Metrohm Titrando 907 Auto-Titrator SOP](#)
- 4.15. [Muffle Furnace SOP and Calibration](#)
- 4.16. [NexION 350X ICP-MS SOP](#)
- 4.17. [Protease Assay](#)
- 4.18. [RNase \(Ribonuclease\) Assay](#)
- 4.19. [Spectrum Two UATR SOP](#)
- 4.20. [Standardization of Titrants](#)
- 4.21. [XL200 pH/mV/Conductivity Meter SOP](#)
- 4.22. [Analytical Method of Analysis: HEPES via ICP-MS](#)

5. EQUIPMENT:

- 5.1. PerkinElmer Lambda 25 UV/Vis Spectrophotometer
- 5.2. Optically matched set of UV quartz cells, 10 mm path length
- 5.3. Analytical Balance
- 5.4. Endosafe nexgen-PTS Endotoxin Reader
- 5.5. Perkin Elmer Flexar HPLC

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- 5.6. PerkinElmer Spectrum Two UATR
- 5.7. PerkinElmer NexION 350X ICP-MS
- 5.8. XL200 pH/Conductivity Meter or equivalent pH / mv / Conductivity Meter
- 5.9. Muffle Furnace
- 5.10. Blue M Oven, or equivalent
- 5.11. Metrohm 907 Auto-Titrator
- 5.12. Hach 2100Q Portable Turbidimeter, or equivalent
- 5.13. Shimadzu GC-2010, FID detector

6. PROCEDURE:

6.1. MOTHER LIQUOR ABSORBANCE Refer to Batch Record:

- 6.1.1. Prepare 10 mL of a 1:1 dilution by pipetting 5 mL of purified water and 5 mL of the Mother Liquor into an LOD vial or small beaker.
- 6.1.2. Swirl to homogenize the solution.
- 6.1.3. Refer to Lambda 25 UV/Vis Operation and Calibration to determine the absorbance of the sample. Record results at specified wavelengths in the appropriate laboratory documentation and Batch Record.

6.2. MOTHER LIQUOR ASSAY Monitor:

- 6.2.1. Standardize Metrohm pH electrode as per Metrohm Titrando 907 Auto-Titrator SOP,
- 6.2.2. Standardize or perform a daily check of 0.1N NaOH as per Standardization of Titrants.
- 6.2.3. Accurately weigh 0.8g of sample and transfer to a beaker.
- 6.2.4. Dissolve in 50 mL of purified water. Determine the Assay concentration using the Metrohm Auto titrator.

$$\% \text{ HEPES} = \frac{(\text{mL} \times \text{N of NaOH}) (23.831)}{\text{Sample Weight (g)}}$$

- 6.2.5. Record results in the appropriate laboratory documentation and Batch Record.

6.3. ABSORBANCE (0.1 M) Refer to Summary Sheet:

- 6.3.1. Accurately weigh 0.60g of sample.
- 6.3.2. Transfer accurately weighed sample to a graduated cylinder and dilute to 25 mL with purified water.
- 6.3.3. Swirl to dissolve completely.
- 6.3.4. Refer to Lambda 25 UV/Vis Operation and Calibration to determine the Absorbance of the sample.

6.4. ABSORBANCE (0.05 M) Refer to Summary Sheet:

- 6.4.1. Accurately weigh 0.30g of sample.
- 6.4.2. Transfer accurately weighed sample to a graduated cylinder and dilute to 25mL with purified water.
- 6.4.3. Swirl to dissolve completely.
- 6.4.4. Refer to Lambda 25 UV/Vis Operation and Calibration to determine the Absorbance of the sample.

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6.5. ABSORBANCE (1M) Refer to Summary Sheet:

- 6.5.1. Accurately weigh 6.0g of sample.
- 6.5.2. Transfer accurately weighed sample to a graduated cylinder and dilute to 25mL with purified water.
- 6.5.3. Swirl to dissolve completely.
- 6.5.4. Refer to Lambda 25 UV/Vis Operation and Calibration to determine the Absorbance of the sample.

6.6. APPEARANCE AND COLOR Refer to Summary Sheet:

- 6.6.1. Place 25-50g of sample in a clean, dry glass beaker.
- 6.6.2. In an area with sufficient lighting, view the sample from all angles.
- 6.6.3. The sample should be white in color and characteristic of powder.
If the sample does not conform to these specifications, notify a supervisor immediately.

6.7. APPEARANCE OF SOLUTION (1% Water) Refer to Summary Sheet:

- 6.7.1. Prepare a 1% solution of the sample.
 - 6.7.1.1. Weigh 1.0g of sample and transfer to a 100mL volumetric flask.
 - 6.7.1.2. Dissolve of sample in purified water, and dilute to 100mL with purified water.
 - 6.7.1.3. Swirl to dissolve completely.
- 6.7.2. Solution must be clear and colorless when compared to a clear and colorless reference standard.

6.8. ASSAY AND pKa Refer to Summary Sheet:

- 6.8.1. Standardize Metrohm pH electrode as per Metrohm Titrando 907 Auto-Titrator SOP
 - 6.8.1.1. For pKa ensure that the slope of standardization is 99.3-101.0%.
- 6.8.2. Standardize 0.1N NaOH as per Standardization of Titrants.
- 6.8.3. Accurately weigh 0.8g of sample dried per LOD method, and transfer to a beaker.
 - 6.8.3.1. Raw Material may be analyzed as-is. Refer to the assay requirement of the code being tested.
- 6.8.4. Dissolve in 50 mL of purified water. Determine the Assay concentration using the Metrohm Auto titrator.
- 6.8.5. The pKa should be reported on the Assay printout from the Metrohm Auto titrator.

$$\% \text{ HEPES} = \frac{(\text{mL} \times \text{N of NaOH}) (23.831)}{\text{Sample Weight (g)}}$$

6.9. CHLORIDE Refer to Summary Sheet:

- 6.9.1. Weigh 2.0 g of sample and dissolve sample in 40 mL of purified water in a Nessler Color Comparison Tube. If necessary, neutralize the solution with nitric acid to litmus.
- 6.9.2. Pipette 0.141 mL of 0.02 N HCl and dilute to 40 mL with purified water in a Nessler Color Comparison Tube.
- 6.9.3. Add to each solution 1 mL of concentrated nitric acid and 1 mL of 0.1N silver nitrate. Dilute to 50 mL with purified water. Cover with parafilm and mix by inversion.
- 6.9.4. After 5 minutes, the turbidity of the sample prep does not exceed that produced by the standard when viewed against a dark background.

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- 6.9.5. If a visible difference in the turbidity is not observed, then utilize the Turbidimeter to measure the turbidity of the standard and the sample solutions. Follow Bangor Portable Turbidimeter SOP and Calibration.

6.10. **ENDOTOXINS**

Refer to Summary Sheet:

- 6.10.1. Accurately weigh 0.100g of sample into a sterile tube. Add 170 μ L of 1N NaOH. Dilute to 10 mL with LAL reagent water, dissolve, and mix thoroughly for a final concentration of 0.0100g/mL.

- 6.10.2. Refer to Endosafe nexgen-PTS Endotoxin Reader SOP for further instrument instructions and sample analysis.

6.11. **ENZYME ACTIVITY**

None Detected:

- 6.11.1. DNase, RNase, and Protease as per SOP.

6.12. **HEAVY METALS**

Refer to Summary Sheet:

- 6.12.1. Refer Section 5.26.

6.13. **IDENTIFICATION TEST (UATR)**

Passes Test:

- 6.13.1. Follow Spectrum Two UATR SOP.

6.14. **IDENTIFICATION (TLC equivalent)**

Refer to Summary Sheet:

- 6.14.1. Instrument Method Parameters: Perkin Elmer Flexar HPLC, column C18(2)-3.9x150mm

Parameter	Setting
Flow Type	Isocratic
Mobile Phase	0.1% Ammonia Formate pH (9-10)
Flow Rate	1.0mL/min
Injection Volume	1 μ L
Detector	UV at 195nm
Detector Temperature	Ambient
Column Temperature	Ambient
Run Time	5 minutes

- 6.14.2. Identity Standard Solution (2mg/mL HEPES CRS):

- 6.14.2.1. Accurately weigh 20 +/- mg of HEPES Reference Standard and transfer aliquot quantitatively to a 10mL volumetric flask. Dissolve in diluent. Dilute to volume with diluent and mix thoroughly.

- 6.14.3. Identity Sample Solution (2mg/mL HEPES):

- 6.14.3.1. Accurately weigh 20 +/- mg of HEPES sample and transfer aliquot quantitatively to a 10mL volumetric flask. Dissolve in diluent. Dilute to volume with diluent and mix thoroughly.

- 6.14.4. Analyze the standard against the sample utilizing the Instrument method parameters listed above in section 5.13.1. Defined peak for HEPES must be detectable and integrated properly. Retention time of the primary peak of the standard HEPES should correspond the primary peak in the sample HEPES.

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6.15. INSOLUBLE MATTER**Refer to Summary Sheet:**

- 6.15.1. Accurately weigh 20.00g of sample and transfer to a 600 mL beaker.
- 6.15.2. Add 200 mL of purified water. If necessary, utilize a Teflon encapsulated magnetic stirring bar and electric stir plate to dissolve the sample.
- 6.15.3. Dry a filter crucible and filter paper at 105°C ± 2°C for 1 hour. Cool in ambient air for 15 minutes and weigh.
- 6.15.4. Filter sample solution through the filter crucible using a suitable vacuum pump.
- 6.15.5. Rinse sample vessel and filter crucible with 100 mL of purified water.
- 6.15.6. Dry the filter crucible and filter paper at 105°C ± 2°C for 1 hour. Cool in ambient air for 15 minutes and weigh.

$$\% \text{ Insolubles} = \frac{\text{residue weight (g)}}{\text{sample weight (g)}} \times 100$$

6.16. LOSS ON DRYING (LOD)**Refer to Summary Sheet:**

- 6.16.1. Dry a Loss On Drying (LOD) vial in an oven at 105°C ± 2°C for 30 minutes. Cool for 15 minutes in a desiccator, weigh on the analytical balance, and record results.
- 6.16.2. Tare the dried vial and weigh 1 – 2 g of sample and record the weight.
- 6.16.3. Dry for 3 hours at 105°C ± 2°C. Cool for 15 minutes in a desiccator.
- 6.16.4. Reweigh and calculate the % LOD.
- 6.16.5. Retain sample for Assay, dried basis.

$$\% \text{ LOD} = \frac{\text{initial sample weight (g)} - \text{final sample weight (g)}}{\text{initial sample weight (g)}} \times 100$$

6.17. MICROBIAL CONTENT**Refer to Summary Sheet:**

- 6.17.1. Prepare ~20-30g of sample in a sterile vessel to send to MPL Laboratories for analysis.

6.18. pH of a 5% SOLUTION**Refer to Summary Sheet:**

- 6.18.1. Prepare a 5% solution of the sample.
 - 6.18.1.1. Accurately weigh 5.0g of sample.
 - 6.18.1.2. Transfer accurately weighed sample to a beaker and dissolve in 100mL of purified water.
 - 6.18.1.3. Swirl to dissolve completely.
- 6.18.2. Follow the appropriate SOP to measure and record the pH at 25 ± 2°C.

6.19. pH of a 1% SOLUTION**Refer to Summary Sheet:**

- 6.19.1. Prepare a 1% solution of the sample.
 - 6.19.1.1. Accurately weigh 1.0g of sample.
 - 6.19.1.2. Transfer accurately weighed sample to a beaker and dissolve in 100mL of purified water.
 - 6.19.1.3. Swirl to dissolve completely.
- 6.19.2. Follow the appropriate SOP to measure and record the pH at 25 ± 2°C.

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6.20. RESIDUAL SOLVENTS (Methanol)**Refer to Summary Sheet:****6.20.1. Calibration and System Suitability:**

6.20.1.1. Calibrate the GC-FID instrument using calibration standard levels 1,2,3,4 and 5 and diluent blank (Standard 0ppm) by pipetting 10mL of each standard to a headspace vial. Crimp to seal, mix thoroughly.

6.20.1.2. System Suitability Requirements: r^2 of NLT 0.95 is required for each solvent of interest.

6.20.1.3. Solution Preparation:

6.20.1.3.1. 0ppm (Blank): Purified water or equivalent

6.20.1.3.2. 10,000ppm Residual Solvent Stock Solution:

6.20.1.3.2.1. Prepare a 10,000ppm solution of Methanol in purified water by weighing approximately 0.50g of standard directly into a 50mL volumetric flask. Mix thoroughly. Calculate actual concentration based off CoA/purity.

6.20.1.3.3. Calibration Standard Preparation: Dilute (Refer to Table)mL of the 10,000ppm Methanol Stock Solution to 100mL with water. Mix thoroughly. Calculate (ppm) and report into laboratory notebook for each standard solution. Input the data into the calibration table of the method in the LabSolutions software.

Calibration Level	10,000 Methanol Stock (mL)	Final Volume (mL)
1	1.50	100
2	2.40	100
3	3.00	100
4	3.60	100
5	4.50	100

6.20.2. Sample Preparation:

6.20.2.1. Weigh 1.0g of sample to a head space vial. Add 10mL of purified water into a 20mL headspace vial. Dissolve, crimp to seal and mix thoroughly.

6.20.3. Refer to Analytical Method: Residual Solvents on the GC-FID for instrument parameters and sample analysis.

6.21. RESIDUE ON IGNITION/SULFATED ASH**Refer to Summary Sheet:**

6.21.1. Turn on muffle furnace and allow temperature to stabilize at 600°C. Follow Muffle Furnace SOP and Calibration for operation of the muffle furnace.

6.21.2. Utilize forceps to insert and remove crucible into the furnace.

6.21.3. Ignite the quartz crucible at $600 \pm 50^\circ\text{C}$ for 30 minutes. Cool in a desiccator for one hour and 30 minutes and weigh.

6.21.4. Weigh 1.0 g sample in the previously ignited quartz crucible. Moisten the sample with 0.5 mL of sulfuric acid.

6.21.5. Volatize the sample until the sample is thoroughly charred. Heat the sample slowly, so that the sample does not boil over and sample is not lost.

6.21.5.1. The rate of heating should be such that from $\frac{1}{2}$ to 1 hour is required to volatilize the sample.

6.21.5.2. Continue to heat the sample until all the excess sulfuric acid has been volatilized.

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- 6.21.6. Ignite the quartz crucible in a muffle furnace at $600 \pm 50^{\circ}\text{C}$ for 15 minutes or until all carbon has been removed.
- 6.21.7. Gently remove the ignited crucible with forceps from the furnace.
- 6.21.8. Inspect the crucible for cracks, chips, or signs of damage such as discoloration- the muffle furnace insulation is made of rough ceramics and metal, care must be taken to not crack, chip, or rub the crucible against the lining.
- 6.21.9. Cool in a desiccator for an hour and a half and reweigh.

$$\% ROI = \frac{\text{Residue weight (g)}}{\text{Sample weight (g)}} * 100$$

6.22. SULFATE**Refer to Summary Sheet:****6.22.1. Sample Preparation:**

- 6.22.1.1. Weigh out 2.0 g of sample and transfer to a 50 mL Nessler Color Comparison Tube. Dissolve in 40 mL purified water. If necessary, neutralize the solution with hydrochloric acid to litmus.

6.22.2. 50 ppm Standard Preparation:

- 6.22.2.1. Prepare a standard solution by pipetting 0.1 mL of 0.020 N H_2SO_4 in a 50 mL Nessler Color Comparison Tube. Dilute to 40 mL with purified water.

6.22.3. Procedure:

- 6.22.3.1. To both solutions add 1 mL of 3 N HCl and 3 mL of Barium Chloride TS. Dilute to 50 mL with purified water.
- 6.22.3.2. Cover with parafilm and mix by inversion.
- 6.22.3.3. Compare turbidity 10 minutes after addition of the barium chloride to the sample and standard solutions.
- 6.22.4. Any turbidity produced in the sample solution should not exceed that produced by the standard when viewed from above against a black surface.
- 6.22.5. If turbidity of the sample solution exceeds that of the standard, notify the QC Manager immediately.

6.23. SOLUBILITY (1%)**Refer to Summary Sheet:**

- 6.23.1. Weigh 1.0g of sample into a clean glass beaker.
- 6.23.2. Add 100mL of purified water and swirl to dissolve.
- 6.23.3. View sample from all angles under sufficient lighting. Solution should be clear and complete.
- 6.23.3.1. Note any insoluble matter present, if any, and refer to material TUPP and insoluble matter specification for disposition.

6.24. SOLUBILITY (5%)**Refer to Summary Sheet:**

- 6.24.1. Weigh 5.0g of sample into a clean glass beaker.
- 6.24.2. Add 100mL of purified water and swirl to dissolve.
- 6.24.3. View sample from all angles under sufficient lighting. Solution should be clear and complete.
- 6.24.3.1. Note any insoluble matter present, if any, and refer to material TUPP and insoluble matter specification for disposition.

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6.25. SOLUBILITY (0.05M)**Refer to Summary Sheet:**

- 6.25.1. Weigh 1.19g of sample into a clean glass beaker.
- 6.25.2. Add 100mL of purified water and swirl to dissolve.
- 6.25.3. View sample from all angles under sufficient lighting. Solution should be clear and complete.
 - 6.25.3.1. Note any insoluble matter present, if any, and refer to material TUPP and insoluble matter specification for disposition.

6.26. TRACE METALS (As, Ca, Cd, Co, Cu, Cr, Fe, Pb, Mg, Mn, Ni, K, Zn)**Refer to Summary Sheet:**

- 6.26.1. For quantitative metal requirements refer to the method DCN: 20-003520. Refer to the NexION 350X ICP-MS SOP for instrument parameters, standard calibration and sample analysis.

6.27. WATER (BY KARL FISCHER TITRATION)**Refer to Summary Sheet.:**

- 6.27.1. Standardize Composite 5 as per Standardization of Titrants.
- 6.27.2. Grind the sample in a dry mortar into a fine powder utilizing a pestle.
- 6.27.3. Immediately weigh 2.0g of sample into the glass weighing spoon and tare it.
- 6.27.4. Transfer the sample to the KF vessel by removing the rubber septum and adding the sample into the titration vessel.
 - 6.27.4.1. Do not leave the rubber septum open for long periods of time as this will allow moisture to enter the titration vessel.
- 6.27.5. Return the weighing spoon to the balance, making sure not to lose any sample that was left behind. Once the weight stabilizes, record the sample weight and transfer to instrument.
- 6.27.6. Check to make sure there is no residual sample stuck to the sides of the titration vessel.
 - 6.27.6.1. If there is any sample stuck to the side, stop the stir bead from spinning before swirling the vessel to rinse the sides.
- 6.27.7. Once the method begins, check to ensure the sample is fully dissolved before the titration begins (i.e. before the stir command completes).
- 6.27.8. The moisture content will then be determined by the Metrohm Auto Titrando 907.

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

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