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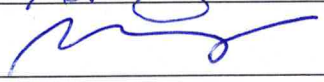
Operation Management	**Baozhong Zhao**	**17Mar2025**	
Quality Assurance	**Xibo Li**	**17Mar2025**	

Changes from previous version:

Section	Summary of Changes	Change Control Number
ALL	1. New document	

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1. PURPOSE

The purpose of this SOP is to document the procedure for the validation of the testing method to quantify the impurity levels in D0003 S-Probes by mass spectroscopy.

2. SCOPE

S-probes belonging to customer D0003 are a group of DNA oligonucleotide with 3' terminal thiophosphate with a length ranging from 40 to 90mers. During the chemical manufacturing process, the thiophosphate tends to desulfurize to generate a -16 Da impurity and dephosphorylate to generate a -96 Da impurity. The two impurities both render the S-Probe inactive in the customer's application. It's therefore necessary to have testing methods to accurately quantify the levels of both impurities to guide the manufacturing process. It has been known that even state-of-the-art chromatographic methods are incapable of baseline resolving these impurities from the Full-Length Product. Thus, a method based solely on mass spectrometry has been developed such that even a low-resolution modern mass spectrometer is able to resolve and quantify the undesired -16 Da and -96 Da oligonucleotide impurities.

3. INTERNAL REFERENCES

Document ID	Title
QUA001	Quality Manual
QUA006	Record Retention

4. EXTERNAL REFERENCES

Document ID	Title
ICH Q2 (R1)	Validation of Analytical Procedures: Text and Methodology

5. RESPONSIBILITIES

Job Function and/or Department	Responsibility
QC Scientists	All QC scientists must be trained on this SOP prior to performing the experiment

6. DEFINITION

Term	Definition
EC	Extinction coefficient
MW	Molecular weight
RMSD	Root Mean Square Deviation

7. METHOD DESIGN

7.1. To verify ESI MS can resolve the -16 Da and -96 Da impurities which may be present in the synthesis of the 3'-thiophosphate S-Probes, three model S-Probes of 48, 70 and 91 nucleotides in length were synthesized. Also synthesized were their corresponding impurity reference standards: the 3'-phosphate (-16 Da desulfurized impurity designated by the suffix "PO" after the model compound sequence ID) and the dephosphorylated impurity (-96 Da designated by the suffix "N-PTO" after the model compound sequence ID). The model compounds and their corresponding impurity standards are all brought to 10 OD/mL. (Note, the extinction coefficients for the model compounds and their impurities are identical, so these 10 OD/mL solutions are equimolar). The -16 Da and -96 Da impurity standards are separately spiked into three samples each of their corresponding model compound at 0.25, 0.50 and 0.75 relative percent molar ratio, yielding 10 samples in total for analysis for each model compound (the unadulterated model compound and the samples spiked to 0.25, 0.50 and 0.75 relative molar ratio in triplicate).

8. EQUIPMENT

8.1. Thermo Fisher Vanquish HPLC

8.2. Thermo Fisher LTQ-XL

8.3. Thermo Fisher Nanodrop One or Nanodrop One C

9. MATERIALS

9.1. Model Compound S20339-1

9.1.1. Sequence: GGG TTC CCT AAG GGT TGG ACG CGT GCC CCT TCA GAG AGT GGA GGA AAA* (Phos).

9.1.2. MW: 15034.75.

9.1.3. EC: 470,200

9.2. Model Compound S20339-28

9.2.1. Sequence: CGG TAC TGA ACA GGC GAT TCA AGC CGA CAG TAA CAG AGG CAG AAG AGT TGG ATT CTG TTG AGT TCA TGC C*(Phos).

9.2.2. MW: 21823.17.

9.2.3. EC 692,600

9.3. Model Compound S20339-63

9.3.1. Sequence: GGG TTC CCT AAG GGT TGG ACG CGA CAG GAT GAG CCT TGG AAT GTC GGA AAT AGG GCT TGT GAT CCA TTA ACC ACC GGA AAA GAT GGT TTT C*(Phos).

9.3.2. MW: 28393.39.

9.3.3. EC 889,800

9.4. Impurity Standard S20339-1 PO

9.4.1. Sequence: GGG TTC CCT AAG GGT TGG ACG CGT GCC CCT TCA GAG AGT GGA GGA AAA (Phos).

9.4.2. MW: 15018.68.

9.4.3. EC: 470,200

9.5. Impurity Standard S20339-28 PO

9.5.1. Sequence: CGG TAC TGA ACA GGC GAT TCA AGC CGA CAG TAA CAG AGG CAG AAG AGT TGG ATT CTG TTG AGT TCA TGC C(Phos).

9.5.2. MW: 21807.11.

9.5.3. EC 692,600

9.6. Impurity Standard S20339-63 PO

9.6.1. Sequence: GGG TTC CCT AAG GGT TGG ACG CGA CAG GAT GAG CCT TGG AAT GTC GGA AAT AGG GCT TGT GAT CCA TTA ACC ACC GGA AAA GAT GGT TTT C(Phos).

9.6.2. MW: 28377.32.

9.6.3. EC 889,800

9.7. Impurity Standard S20339-1 N-PTO

9.7.1. Sequence: GGG TTC CCT AAG GGT TGG ACG CGT GCC CCT TCA GAG AGT GGA GGA AAA.

9.7.2. MW: 14938.7.

9.7.3. EC: 470,200

9.8. Impurity Standard S20339-28 N-PTO

9.8.1. Sequence: CGG TAC TGA ACA GGC GAT TCA AGC CGA CAG TAA CAG AGG CAG AAG AGT TGG ATT CTG TTG AGT TCA TGC C.

9.8.2. MW: 21727.13.

9.8.3. EC 692,600

9.9. Impurity Standard S20339-63 N-PTO

9.9.1. Sequence: GGG TTC CCT AAG GGT TGG ACG CGA CAG GAT GAG CCT TGG AAT GTC GGA AAT AGG GCT TGT GAT CCA TTA ACC ACC GGA AAA GAT GGT TTT C.

9.9.2. MW: 28297.34.

9.9.3. EC 889,800

9.10. Prepare three spike samples (with relative molar ratio of impurity-to-parent being 0.25, 0.50, 0.75). For each spike sample, prepare three replicates, diluting with DI water.

9.10.1. Make 3 mL 10 OD of each model compound and 1 mL of their corresponding impurity standards at 10 OD/mL using the Nanodrop

9.10.2. For 0.25 relative molar percent add 25 uL of both impurity standards to 100 uL of the model compound

9.10.2.1. Repeat 3x to have three samples

9.10.3. For 0.50 relative molar percent add 50 uL of both impurity standards to 100 uL of the model compound

9.10.3.1. Repeat 3x to have three samples

9.10.4. For 0.75 relative molar percent add 75 uL of both impurity standards to 100 uL of the model compound

9.10.4.1. Repeat 3x to have three samples

9.10.5. Make a single pure sample of each model compound with 100 uL.

9.10.6. Make a second set of samples of model compound S20339-63 at 0.5 relative molar percent of S20339-63 PO and S20339-63 N-PTO in triplicate for intermediate precision analysis. Include a single pure sample of 100 uL of S20339-63.

9.10.7. Repeat 9.10.1-9.10.5 for the second and the third model compounds. Including the samples for intermediate precision analysis, there will be a total of 34 samples.

10. EXPERIMENT

10.1. Run 10 uL water blank followed by three 10 uL injections of the first model compound.

10.1.1. Verify the blank doesn't show interference to target molecular weights.

10.2. Run the spiked samples

10.3. Repeat with two other model compounds and their impurity spikes

10.4. Repeat one model compound and its spikes on a second LTQ equipment by a second analyst.

11. DATA ANALYSIS

11.1. Verify resolution of -16 Da and -96 Da peaks from the spiked model compound main peaks

11.2. Verify target masses are within acceptance criteria: MW ±0.05% of theoretical value

11.3. Determine the RMSD for each spiked sample using the %Relative Intensity as determined by the deconvolution algorithm.

11.4. For each relative molar ratio of (0.25, 0.50. 0.75), add in the contribution of the model compound background impurity (-96 Da or -16 Da peaks observed in model compound) to the spiked ratio.

$$\mathrm{RMSD} = \sqrt{\frac{\sum_{i=1}^{N} (x_i - \hat{x}_i)^2}{N}}$$

11.5. Using the RMSD equation determine the difference between the observed peak intensities and the theoretical values.

11.5.1. Acceptable criteria: ≤15% from theoretical values.

11.6. Precision: Measure peak ratio reproducibility and reliability within the same run (among three injections).

11.6.1. Acceptable criteria: peak ratio %RMSD ≤15%

11.7. Intermediate Precision: Measure the peak ratio reproducibility from run to run and equipment to equipment

11.7.1. Acceptable criteria: peak ratio %RMSD ≤15%

11.8. Robustness: Measure the impact of minor change of mass spectrometer parameters on peak ratio. Parameters to be evaluated: sample injection volume (5uL,15uL), sample concentration (5OD/mL, 15OD/mL),

11.8.1. Acceptable criteria: peak ratio %RMSD ≤15%

11.9. ACCEPTANCE CRITERIA FOR OUTGOING S-PROBES: Verify in triplicate that the sum of the %Relative peaks intensity of the -16 Da and -96 Da peaks is less than 0.425 relative peak intensity (15% less than the target value of 0.5).

11.10. Solution Stability: Measure the sample stability of SST at 4 ± 2°C for 24 and 48 hours

11.10.1. Acceptance criteria: < 10% increase in total -16 Da and -96 Da peaks in sample.