

Supplementary Materials: Design of a Quantitative LC-MS Method for Residual Toxins Adenylate Cyclase Toxin (ACT), Dermonecrotic Toxin (DNT) and Tracheal Cytotoxin (TCT) in *Bordetella pertussis* Vaccines

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Supplement S1. Ph. Eur. 1356 Limits for Residual Toxins ACT, DNT and TCT per dose of acellular Pertussis vaccine.

| Toxin | Abbreviation | Molecule Type | Theoretical Molecular Weight | EU Limit per Dose | Limit in Moles per Dose |
|-------------------------|--------------|---------------|------------------------------|-------------------|-------------------------|
| Adenylate Cyclase Toxin | ACT | Protein | 177 414 g/mol | 500 ng | 2.8 pmol |
| Dermonecrotic Toxin | DNT | Protein | 160 644 g/mol | Absence | Not Defined |
| Tracheal Cytotoxin | TCT | Glycopeptide | 921 g/mol | 1.84 ng | 2.0 pmol |

Supplement S2. Concatenated (QconCAT) protein construct containing ACT and DNT peptide sequences flanked by six flanking residues from the endogenous protein sequences and a C-terminal poly-histidine tag. Surrogate peptides with matching AQUA peptides denoted with underline. The QconCAT construct was expressed as a native recombinant protein in *E. coli* and purified using a C-terminal His-tag as per the described protocol in Scott *et al.* [1].

MGSSGTTSNVLRNIENAVGSARDDVLIGTELADRITGDAQA
 NVLRGAGGADAHWGQRALQGAQAVAAQRLVHAIAGRDTIR
 INAGADQLWFARQGNDLEREVRKASALGVDYYDNVRNVEN
 VIDEMPRKLDWEIAKFHLAATWIVPFYREIFFSTQDRSYR
 VDVWRSVLKELPALIGASGLRLSRSLLEYVKARYEIIYYLLN
 RVPHPLAIPAPMRNDDLVSIAATYDRAVIAYLKVRHYKVL
 SQPGLIARGIENHNRLQEVRAYIGDLSPVNDVLYRAGYDLD
 IAYLGKDVPGGGSTRITRHDEPVPIRRLLVAGFVSATTVGGR
 NQAFLR...HHHHHH

ACT
DNT

Supplement S3. Tune and inlet settings for the LC-MRM method on a Water Acquity H-class analytical UPLC and Xevo™ TQ-S mass spectrometer. Mobile phases consist of 0.1% FA in Water (i.e. line C) and 0.1% FA in ACN (i.e. line D).

ES+ Source | Fluidics | Diagnostics

Source Fitted: ESI:1

Voltages

Capillary (kV): 3.12 | 3.00

Cone (V): 123 | 30

Source Offset (V): 50

Temperatures

Desolvation Temp (°C): 600 | 600

Gas Flow

Desolvation (L/Hr): 993 | 1000

Cone (L/Hr): 155 | 150

Nebuliser (Bar): 5.6 | 7.0

Analysers

LM Resolution 1: 3.00

HM Resolution 1: 15.00

Ion Energy 1: 0.2

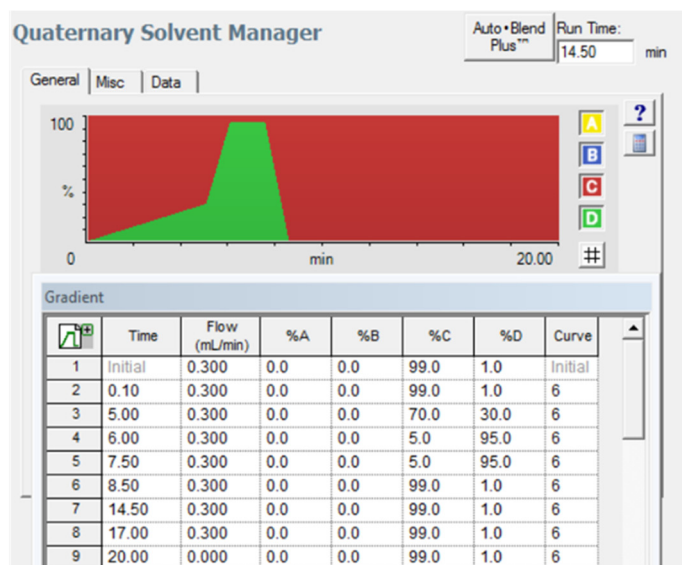
LM Resolution 2: 3.00

HM Resolution 2: 15.00

Ion Energy 2: 0.9

Collision Gas Flow (mL/Min): 0.14 | 0.15

Collision: 30



Sample Manager FTN

Run Time: 14.50 min

General | Data | Dilution | Events

Solvents

Wash Solvent Name: ACN/H2O (60:40 : v/v)

Purge Solvent Name: 10% Methanol

Pre-Inject Wash: 1 sec

Post-Inject Wash: 6 sec

Temperature Control

Column: 55.0 °C Alarm Band: ±5.0 °C

Sample: 8.0 °C ±2.0 °C

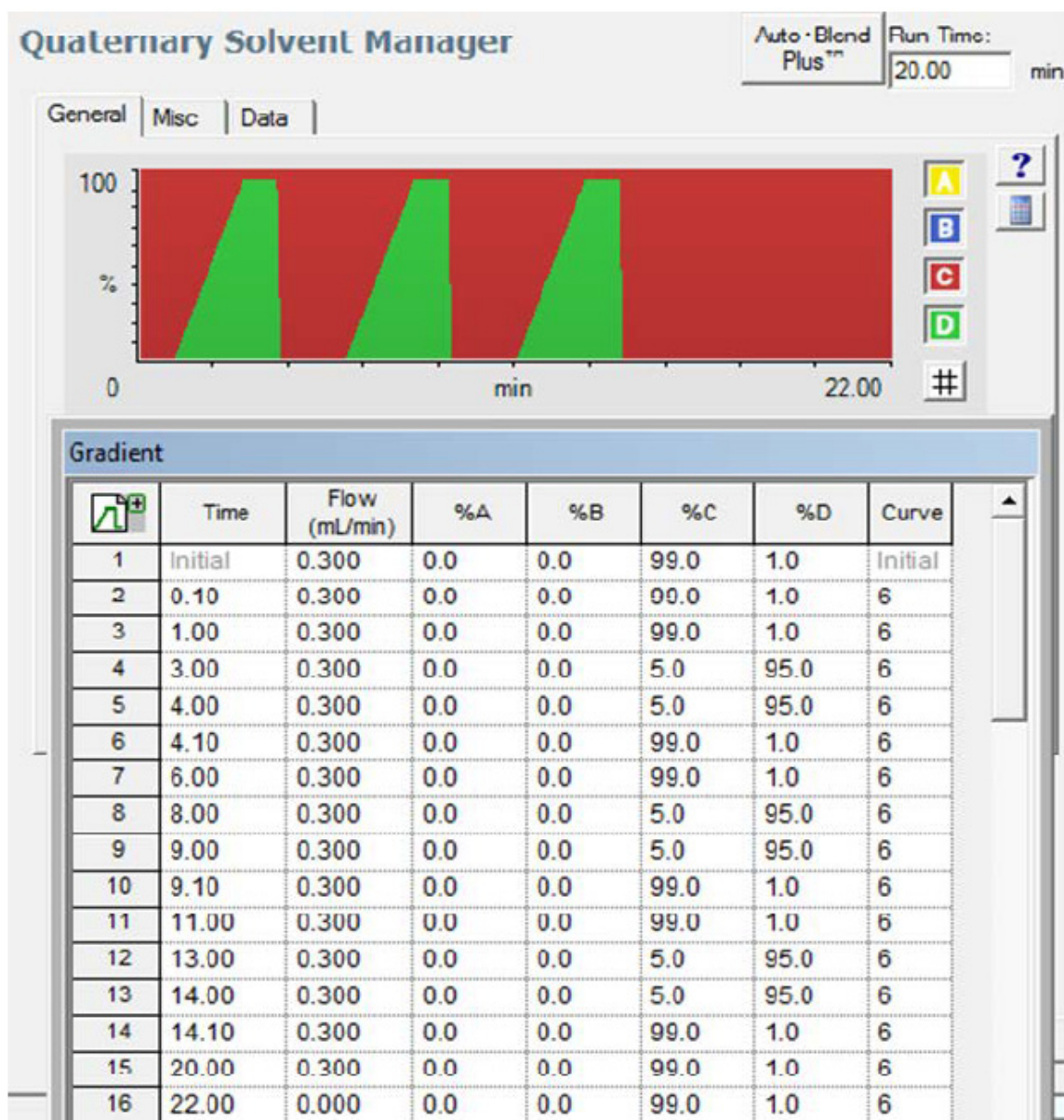
Loop Offline: ☐

Automatic min

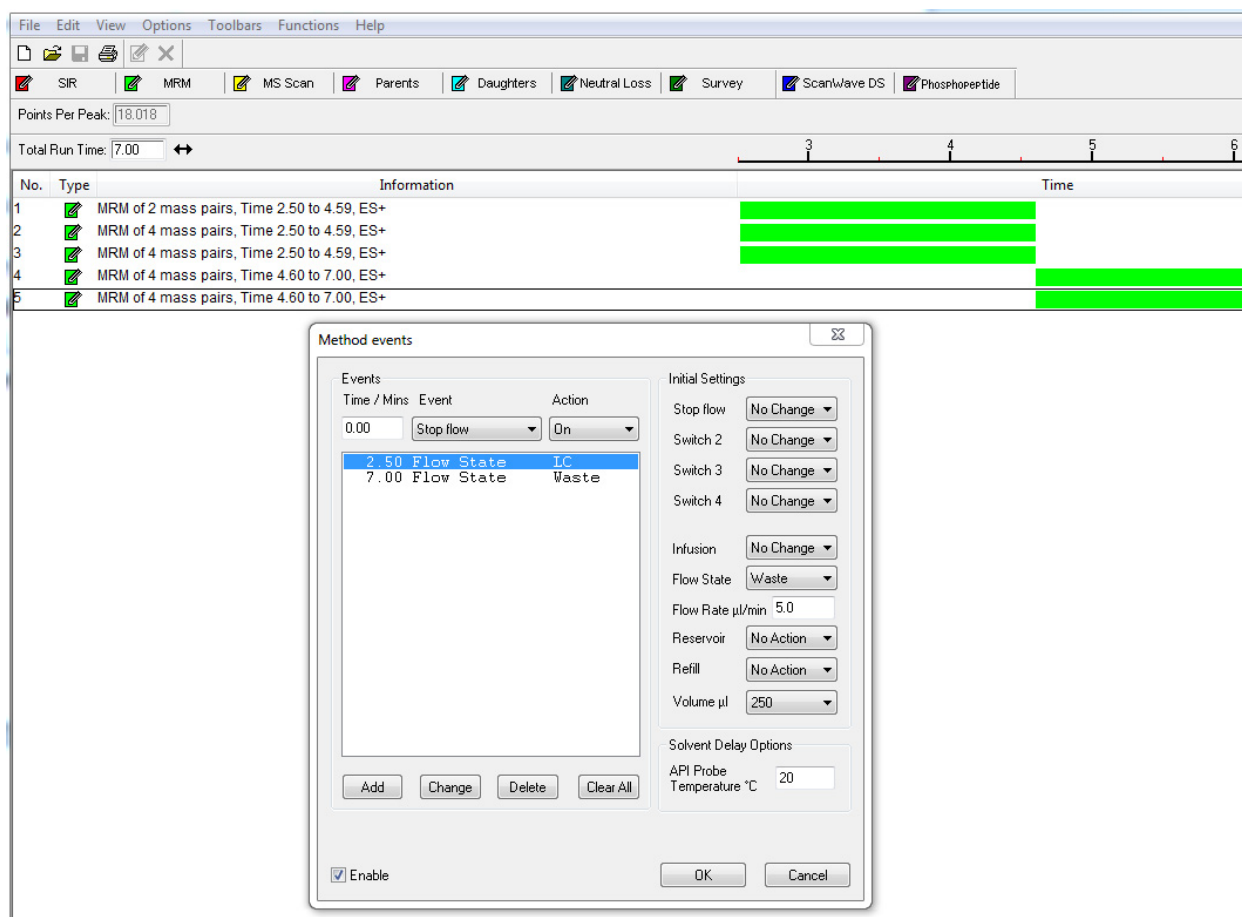
Load Ahead: ☐

Active Preheater: Use Console Configuration

Supplement S4. Triple sawtooth LC gradient for column cleaning between samples. Mobile phases consist of 0.1% FA in Water (i.e., line C) and 0.1% FA in ACN (i.e., line D).



Supplement S5. Optimized MS method for multiple reaction monitoring (MRM) analysis of ACT, DNT and TCT analysis on the Xevo™ TQ-S.

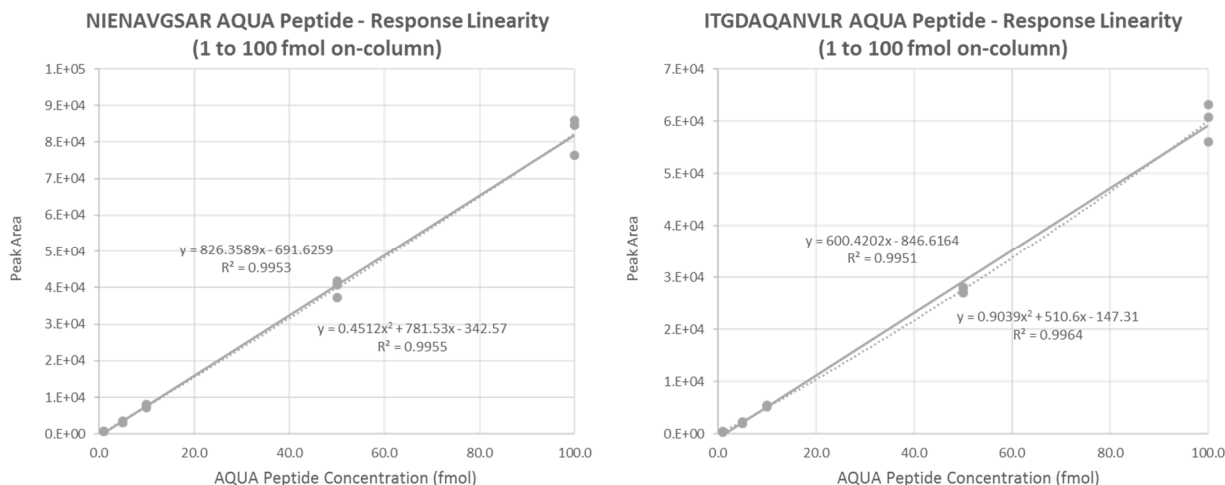


| MRM Function | Peptide | Fragment | Transition | Description | Precursor (m/z) | Fragment (m/z) | Cone (V) | Collision Energy (V) |
|--------------|---------------------|----------------|------------|-------------|-----------------|----------------|----------|----------------------|
| 1 | TCT | Loss of GlcNAc | 1 | TCT | 922.3888 | 719.3099 | 35 | 31 |
| | | | | | 461.6985 | 719.3099 | 35 | 11 |
| 2 | NIENAVGSAR (ACT) | y8 | 1 | Native | 515.7674 | 803.4006 | 35 | 19 |
| | | | | Heavy AQUA | 520.7716 | 813.4089 | 35 | 19 |
| | | y7 | 2 | Native | 515.7674 | 674.3580 | 35 | 22 |
| | | | | Heavy AQUA | 520.7716 | 684.3663 | 35 | 22 |
| 3 | ITGDAQANVLR (ACT) | y9 | 1 | Native | 579.3173 | 943.4956 | 35 | 22 |
| | | | | Heavy AQUA | 584.3214 | 953.5038 | 35 | 22 |
| | | y5 | 2 | Native | 579.3173 | 572.3515 | 35 | 22 |
| | | | | Heavy AQUA | 584.3214 | 582.3597 | 35 | 22 |
| 4 | ELPALIGASGLR (DNT) | y6 | 1 | Native | 598.8535 | 560.3151 | 35 | 21 |
| | | | | Heavy AQUA | 603.8576 | 570.3234 | 35 | 21 |
| | | y7 | 2 | Native | 598.8535 | 673.3991 | 35 | 24 |
| | | | | Heavy AQUA | 603.8576 | 683.4074 | 35 | 24 |
| 5 | NDDLVSIAATYDR (DNT) | y8 | 1 | Native | 726.8519 | 896.4472 | 35 | 24 |
| | | | | Heavy AQUA | 731.8560 | 906.4555 | 35 | 24 |
| | | b3 | 2 | Native | 726.8519 | 345.1041 | 35 | 29 |
| | | | | Heavy AQUA | 731.8560 | 345.1041 | 35 | 29 |

Supplement S6. TCT fragment ions from high-energy collision induced dissociation (HCD) on a Q-Exactive HF Orbitrap mass spectrometer.

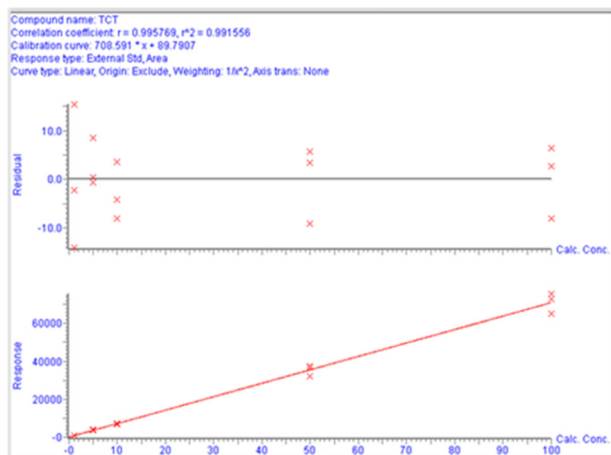
| Precursor (m/z) | Fragment (m/z) | Description | Ion Type | Chemical Formula |
|-----------------|------------------------------|--|-----------|--|
| 922.3893 (+) | 922.3853 | Intact TCT molecular ion [M+H] ⁺ | Precursor | [C ₃₇ H ₆₀ O ₂₀ N ₇] ⁺ |
| | 719.3073 | Loss of GlcNAc | Peptide | [C ₂₉ H ₄₇ O ₁₅ N ₆] ⁺ |
| | 701.2969 | Product from loss of GlcNAc + oxygen | Peptide | [C ₂₉ H ₄₇ O ₁₄ N ₆] ⁺ |
| | 534.2393 | Product of glycan cleavage (loss of 1-6-anhydro sugar GlcNAc + MurNAc) | Peptide | [C ₂₁ H ₃₆ O ₁₁ N ₅] ⁺ |
| | 516.2292 | Product of glycan cleavage | Peptide | [C ₂₁ H ₃₄ O ₁₀ N ₅] ⁺ |
| | 445.1918 | Peptide fragment | Peptide | [C ₁₈ H ₂₉ O ₉ N ₄] ⁺ |
| | 391.1813 | Peptide fragment (y-ion) | Peptide | [C ₁₅ H ₂₇ O ₈ N ₄] ⁺ |
| | 302.1339 | Peptide fragment | Peptide | [C ₁₂ H ₂₀ O ₆ N ₃] ⁺ |
| | 262.1392 | Peptide fragment (Ala-DAP) y ₂ ion | Peptide | [C ₁₀ H ₂₀ O ₅ N ₃] ⁺ |
| | 204.0862 | Intact GlcNAc oxonium ion | Glycan | [C ₈ H ₁₄ O ₅ N] ⁺ |
| 461.6985 (++) | 173.0918 | HexNAc fragment ion | Glycan | [C ₇ H ₁₁ O ₄ N] ⁺ |
| | 138.0547 | Oxonium fragment ion | Glycan | [C ₇ H ₈ O ₂ N] ⁺ |
| | 126.0549 | Oxonium fragment ion | Glycan | [C ₆ H ₈ O ₂ N] ⁺ |
| | 239.1021, 329.1336, 630.2602 | Consistent with fragment ions (MS/MS) from TCT peptidoglycan homologue in <i>Neisseria gonorrhoeae</i> (2) | N/Ap | Not defined |

Supplement S7. ACT AQUA peptide dose linearity with linear (solid line) and quadratic (dotted line) fits from 1 to 100 fmol on-column.

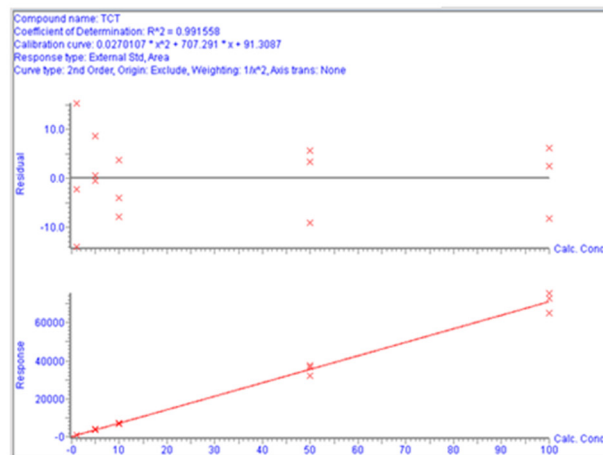


Supplement S8. External TCT calibration curves using the singly and doubly charged fragment ion transitions (922.40 > 719.31 and 461.70 > 719.31) for MRM analysis.

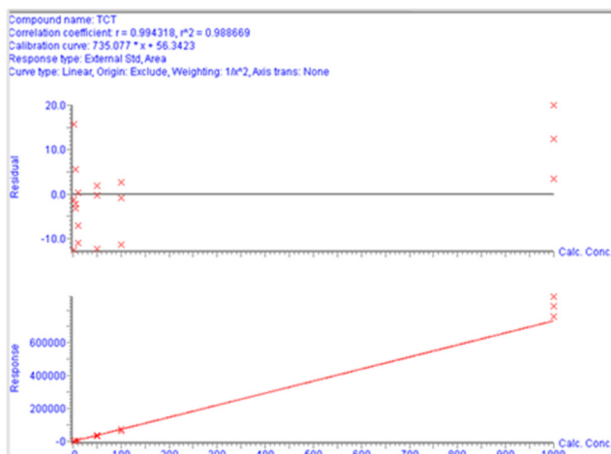
a) Linear – 5.31 to 531 pg



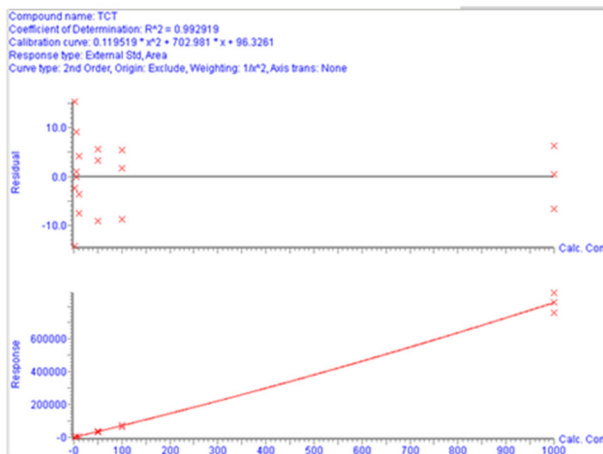
b) Quadratic – 5.31 to 531 pg



c) Linear – 5.31 to 5310 pg



d) Quadratic – 5.31 to 5310 pg



Supplement S9. TCT signal response from a multi-sample external calibration curve analyzed by MRM. Signal response from 922.40 > 719.31 and 461.70 > 719.31 transitions denoted by TCT 1+ and TCT 2+, respectively.

| TCT (pg) | TCT 1+ | | TCT 2+ | | TCT 1+ & TCT 2+ | | Ratio of TCT 2+ / TCT 1+ | |
|-----------------|--------------------------------|-------------|--------------------------------|-------------|--------------------------------|-------------|---------------------------------|--------------|
| | Average Peak Area (n=3) | % CV | Average Peak Area (n=3) | % CV | Average Peak Area (n=3) | % CV | Average Ratio | % CV |
| 5.31 | 1.91E+02 | 13.9% | 5.98E+02 | 14.5% | 7.89E+02 | 13.8% | 3.14 | 10.2% |
| 26.6 | 9.45E+02 | 17.9% | 2.79E+03 | 8.3% | 3.74E+03 | 4.7% | 3.04 | 24.6% |
| 53.1 | 1.72E+03 | 4.3% | 5.26E+03 | 8.7% | 6.98E+03 | 6.1% | 3.07 | 11.1% |
| 266 | 8.74E+03 | 3.7% | 2.68E+04 | 11.8% | 3.55E+04 | 8.0% | 3.07 | 15.1% |
| 531 | 1.70E+04 | 1.4% | 5.42E+04 | 9.5% | 7.12E+04 | 7.5% | 3.19 | 8.6% |
| 5314 | 2.85E+05 | 5.8% | 5.38E+05 | 8.6% | 8.23E+05 | 7.4% | 1.88 | 5.0% |

References

1. Scott KB, Turko IV, Phinney KW. Chapter Eleven - QconCAT: Internal Standard for Protein Quantification. In *Kelman Z, editor. Methods in Enzymology [Internet]*. Academic Press; 2016 [cited 2020 Mar 27]. p. 289–303. (Isotope Labeling of Biomolecules—Applications; vol. 566).
2. Martin SA, Rosenthal RS, Biemann K. Fast atom bombardment mass spectrometry and tandem mass spectrometry of biologically active peptidoglycan monomers from *Neisseria gonorrhoeae*. *J. Biol. Chem.* **1987**;262, 7514–7522.