MP 542

Analyzing the HYE Species Mix: A Comprehensive Reference Standard for Mass Spectrometry Quality Control and Method Development

Marco Harms[1], Stephan Michalik[2], Christian Hentschker[2], Jana Richter[3], Anna Pashkova[3] ,Tabiwang N Arrey[3], Eugen Damoc[3], Kristin Surmann[1,2]
[1]ProTec Diagnostics GmbH, Greifswald, Germany, [2]Department of Functional Genomics, Center for Functional Genomics of Microbes, University Medicine Greifswald, Germany, [3]Thermo Fisher Scientific GmbH, Bremen, Germany

Introduction

Reliable and reproducible mass spectrometry (MS) performance is essential to obtain accurate proteomic data.
However, existing reference materials often lack standardization and fail to represent the diversity of biological samples, especially regarding the differences between eukaryotic and prokaryotic proteins.
To address this, the HYE Species Mix was developed as a tri-species protein standard combining human, yeast, and *Escherichia coli* proteins. It provides a versatile benchmark for method development, sensitivity testing, and quality control across a broad range of MS platforms and workflows. By offering defined complexity and two distinct formulations, the mix supports comprehensive evaluation of dynamic range, quantification robustness, and instrument performance.
Developed in collaboration between University Medicine Greifswald and ProTec Diagnostics GmbH, the HYE Species Mix was validated using a Thermo Scientific™ Orbitrap™ Astral™ mass spectrometer, ensuring reproducibility and comparability in MS-based proteomics.

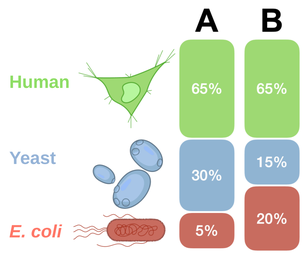
Composition and Analytical Workflow

Composition

The HYE Species Mix is available in two formulations with specific species ratios:

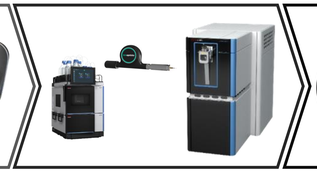
- Mix A (65% human, 30% yeast, 5% *E. coli*)
- Mix B (65% human, 15% yeast, 20% *E. coli*)

This design allows users to evaluate quantitative MS performance effectively. Standardized preparation ensures reproducibility and reliability in MS benchmarking[1].

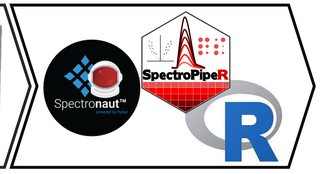


ProTec Diagnostics HYE Species Mix

Vanquish™ Neo UHPLC, Aurora™ Ultimate, Orbitrap Astral MS, FAIMS



Spectronaut™ (Biognosys AG)



Can be ordered at www.protec-diagnostics.com or use barcode →

- DIA: MS1 Orbitrap, MS2 Astral
- 30/50 samples per day (SPD)
- DirectDIA+ mode
- Analysis via SpectroPipeR package[2]

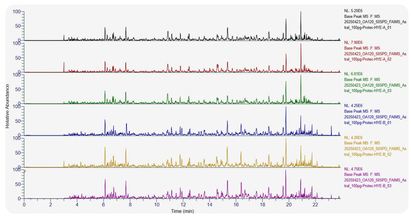
Material and Methods

Parameters	Low Load	High Load
LC Vanquish Neo Direct injection; 25 cm x 75 µm ID Aurora Ultimate TS (IonOpticks)	• Load range: **100 pg – 2 ng** • 20 min; 1–40% B • 28 min (50 SPD)	• Load range: **10 ng – 1µg** • 37 min; 4–45% B • 48 min (30 SPD)
MS Orbitrap Astral MS Orbitrap 240k Astral DIA; m/z 150 – 2000, Cycle 0.6 s	✓ FAIMS used • <u>MS1 (Orbitrap Analyzer):</u> m/z 400–800; AGC 500%; IT 100 ms • <u>MS2 (Astral Analyzer):</u> 10–20 Th windows; AGC 800%; IT 20–60 ms	– No FAIMS used • <u>MS1 (Orbitrap Analyzer):</u> m/z 380–980; AGC 500%; IT 5 ms • <u>MS2 (Astral Analyzer):</u> 2–5 Th windows; AGC 100%; IT 3–10 ms
Data Processing	Spectronaut v19.5 DirectDIA UniProt FASTA (no isoforms), normalized on Human FASTA, 6 files (2 x 3 replicates) were processed together for each load	

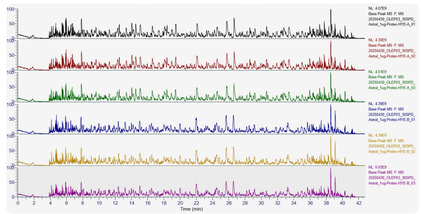
Results and Performance

BPCs of HYE Species Mix triplicates, measured with low and high load with Orbitrap Astral MS

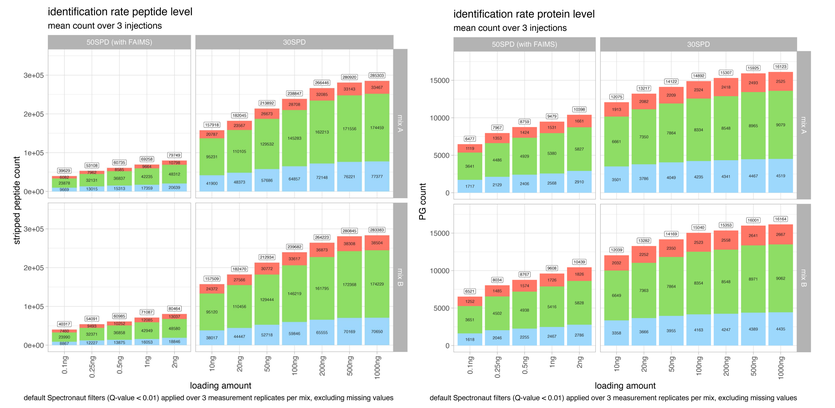
Low load: 0.1 ng, 50 SPD



High load: 1000 ng, 30 SPD



Peptide identification (left) and protein identification (right) over the sample load range 0,1 ng – 1µg of the HYE Species Mix.



default Spectronaut filters (Q-value < 0.01) applied over 3 measurement replicates per mix, excluding missing values

default Spectronaut filters (Q-value < 0.01) applied over 3 measurement replicates per mix, excluding missing values

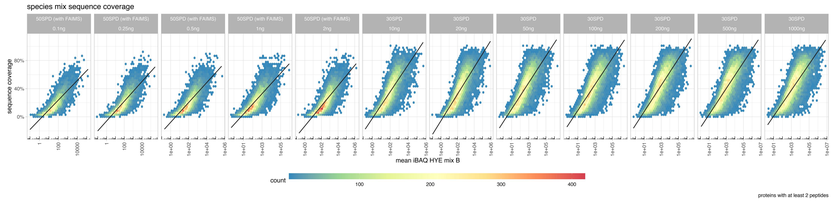
Performance benchmarking was carried out using our HYE Species Mix under both Low Load and High Load conditions to rigorously assess sensitivity, dynamic range, linearity, and reproducibility across a wide range of sample inputs.

Thanks to the optimized peptide digest and the well-balanced species composition of the mix, in combination with the advanced capabilities of the Orbitrap Astral mass spectrometer, exceptional depth of proteome coverage and consistent quantification accuracy were achieved, from 1000 ng down to 100 pg.

- ➢ At 1000 ng load, more than 16,000 protein groups were identified (>9,000 human, >4,500 yeast, >2,500 *E. coli*).
- ➢ At 10 ng, over 12,000 protein groups and nearly 160,000 peptides were detected.
- ➢ Even at 100 pg, over 6,500 protein groups and >40,000 peptides were still identified.

- ✓ Highly reproducible peptide separation and retention times across all loads
- ✓ Improved sensitivity and reduced background for low load samples in FAIMS mode
- ✓ Excellent linearity and minimal variability across replicates
- ✓ High stability of protein identification over four orders of magnitude sample loads

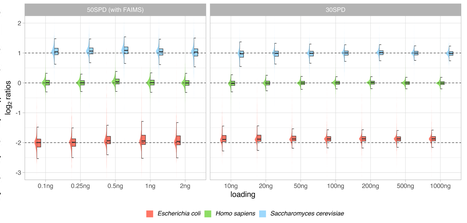
High performance over several orders of magnitude.



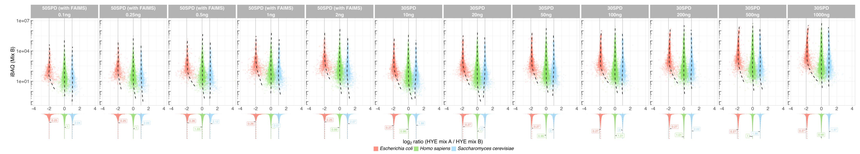
Quantitative Benchmarking

Robust HYE Species Mix A/B protein ratios across the sample load range of 0,1 ng – 1 µg (MS1 level ratios: 0,1 ng - 2 ng; MS2 level ratios: 10 ng - 1000 ng).

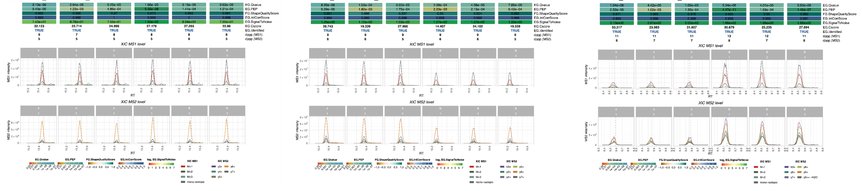
The HYE Species Mix enables precise quantitative benchmarking across all tested conditions. The defined ratios of human, yeast, and *E. coli* proteins were reliably reflected in the data, demonstrating the mix's suitability for assessing quantitative performance. MS1-level quantification is used for sample loadings of 2 ng or less, while MS2-level quantification applies for loadings of 10 ng or more. Even at low sample amounts, the observed protein intensities closely matched the expected proportions, confirming both the robustness of the analytical workflow and the accuracy of quantification down to individual protein levels.



proteins with at least 2 peptides



Quantitative accuracy of HYE Species Mix A/B ratios by selected peptides from human, yeast and *E. coli* (50SPD with FAIMS, 10ng loading).



Conclusion

The HYE Species Mix of ProTec Diagnostics, with its defined composition and dual formulation:

- ✓ Versatile and reliable standard for MS-based proteomics
- ✓ Sensitive detection and robust quantification across a broad dynamic range
- ✓ Evaluation of instrument performance and workflow robustness
- ✓ Quantitative benchmarking

With the HYE Species Mix, ProTec Diagnostics offers a ready-to-use, well-characterized benchmark standard that streamlines method development, enables confident quantification, and supports reliable performance monitoring in any MS-based proteomics workflow.

Conflict of Interest

The HYE Species Mix was developed in close collaboration between the University Medicine Greifswald and the start-up ProTec Diagnostics, where Marco Harms and Kristin Surmann are employed, ProTec Diagnostics distributes the described species mix to the market. The authors declare that the results presented are scientifically valid and have not been influenced by any financial or commercial interests.
Anna Pashkova, Jana Richter, Tabiwang N. Arrey, and Eugen Damoc are employees of Thermo Fisher Scientific, the manufacturer of the LCMS instrumentation used in this study.

References

[1] Navarro P, Kuharev J, Gillet LC, Bernhardt OM, MacLean B, Röst HL, Tate SA, Tsou CC, Reiter L, Distler U, Rosenberger G, Perez-Riverol Y, Nesvizhskii AI, Aebersold R, Tenzer S., *A multicenter study benchmarks software tools for label-free proteome quantification,* 2016, Nat Biotechnol. DOI: 10.1038/nbt.3685
[2] Michalik S, Hammer E, Steil L, Salazar MG, Hentschker C, Surmann K, Busch LM, Sura T, Völker U., *SpectroPipeR-a streamlining post Spectronaut® DIA-MS data analysis R package*. 2025. Bioinformatics. DOI: 10.1093/bioinformatics/btaf086