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Evaluation of New Peak Detection Performance in MAM Using a Controlled Peptide Spike-in Strategy

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Introduction

The Multi Attribute Method (MAM) is widely adopted for monitoring of multiple product quality attributes (PQAs) in biotherapeutic products using LC/MS.[1] In addition to targeted monitoring of known critical quality attributes (CQAs), MAM workflows include New Peak Detection (NPD) to enable non targeted purity assessment.[2] NPD compares MS1 precursor ion features from a test sample against a reference standard to identify unexpected or emerging species. While highly powerful, implementation of NPD in regulated environments remains challenging due to the trade off between sensitivity and specificity. Lower detection thresholds increase the ability to detect low level impurities but also increase the risk of false positives arising from noise, adducts, or sample preparation variability. Regulatory guidance (USP <1060>, ICH Q2) emphasizes molecule specific optimization and robust performance characterization.[3] However, standardized approaches for evaluating NPD performance are limited. In this study, we introduce a controlled peptide spike in strategy to systematically evaluate the sensitivity, specificity, and robustness of an NPD workflow implemented within a MAM software environment.

Experimental

A tryptic digest of the NIST mAb was used as the reference (0.5 µg on column). A PRTC peptide mixture was spiked into the NISTmAb digest at 0.5–4 pmol, corresponding to approximately 0.2–1.5% of the total digest. LC/MS analyses were performed under standardized peptide mapping conditions, with data acquired in positive ion mode on an Agilent 6230C TOF mass spectrometer. Full separation and acquisition parameters are provided in Table 1.

Table 1. LC/MS acquisition parameters.

LC Conditions	
Column	AdvanceBio Peptide Mapping C18, 2.1 x 150 mm, (p/n 653750-902)
Solvent A	Water with 0.1% FA
Solvent B	ACN with 0.1% FA
Gradient	0 min, 2% B 75 min, 36% B 75.5 to 77 min, 80%B 79 min, 35% B 79.5 to 80 min, 80% B 80.5 min, 2%B 84 min, 2%B
Flow rate	0.4 mL/min
Injection volume	4 ul
Column temperature	40 °C

Experimental

MS Conditions (G6230C)	
Ion Source	Agilent Dual AJS ESI source
Polarity	Positive
MS Scan Range	m/z 200 to 3000
Acquisition Rate	1 spectra/sec
Fragmentor	45 V
Skimmer	35 V
Data Storage	Profile
Drying Gas Flow	13 L/min
Nebulizer Pressure	35 psi
Sheath Gas Flow	12 L/min
Sheath Gas Temperature	300 °C
Capillary Voltage	3000 V
Nozzle Voltage	0 V
Gas Temperature	300 °C

Data processing:

Non-targeted MS data were processed using MAM software with an internally developed NPD algorithm. Differential analysis of MS1 features was performed between spiked samples and the NIST mAb reference using mass accuracy and retention time tolerances. Peak intensity thresholds were evaluated to balance true-positive detection and artifact control, while fold-change thresholds were applied as predefined criteria for feature classification. An overview of the workflow is shown in Figure 1.

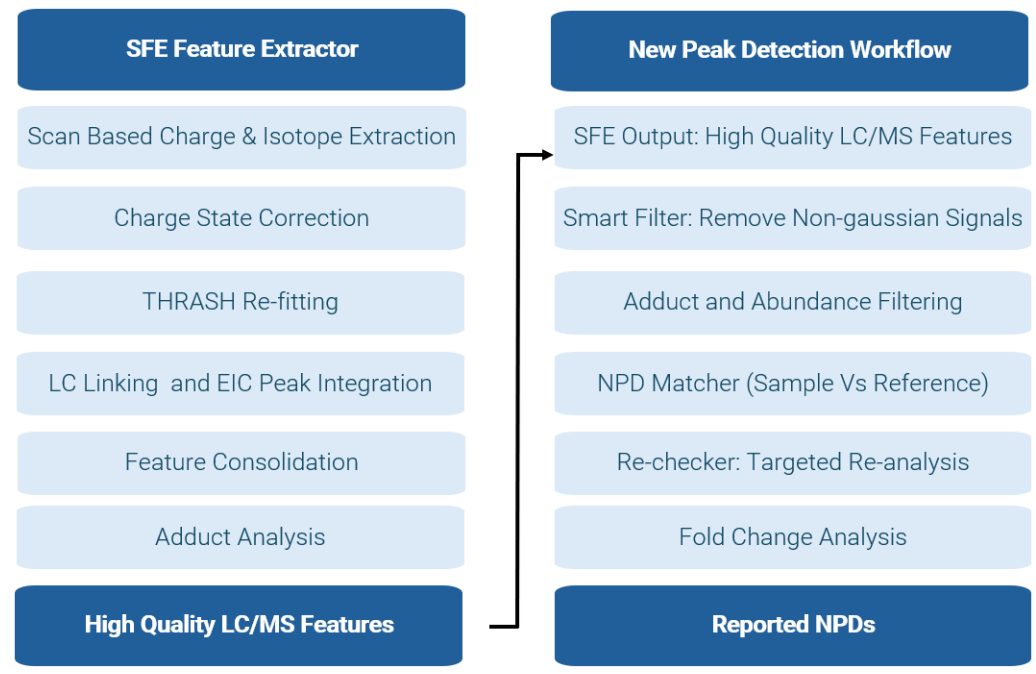


Figure 1. Untargeted SFE extracts and refines high quality LC/MS features from MS1 data using charge, isotope, LC linking, and adduct analysis. These features are processed by the NPD workflow using smart filtering, sample to reference matching, targeted re-analysis, and fold change criteria to report new peaks.

NPD Parameter Optimization

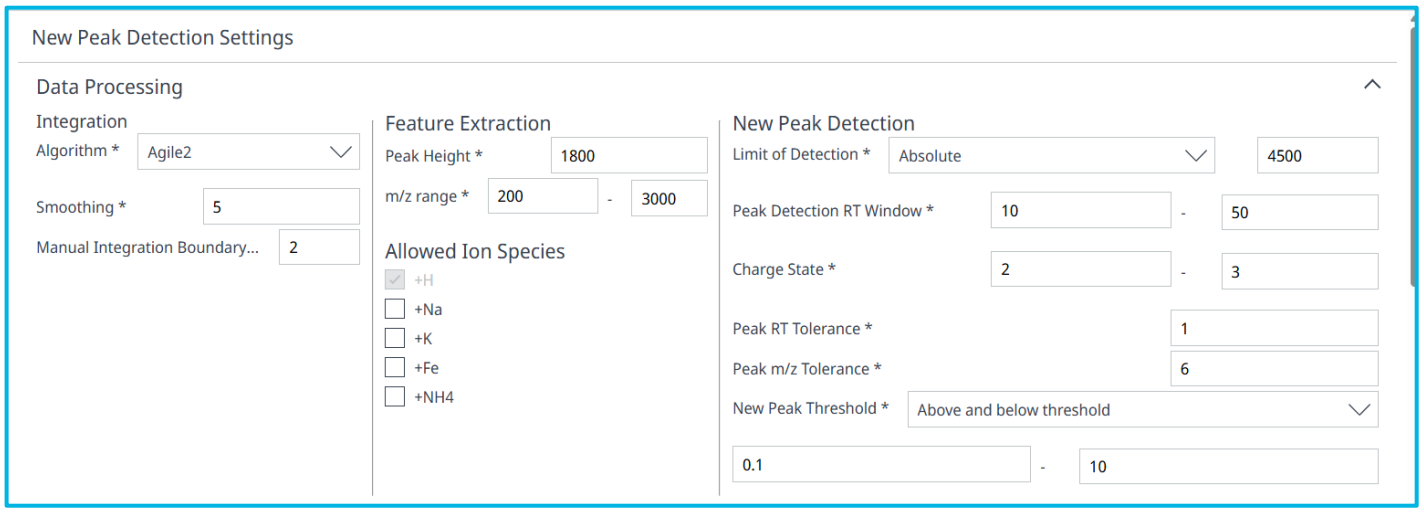


Figure 2. Key NPD parameters were optimized to balance sensitivity for low-level impurity detection with control of false positive reporting.

NPD Results Visualization and Review Interface

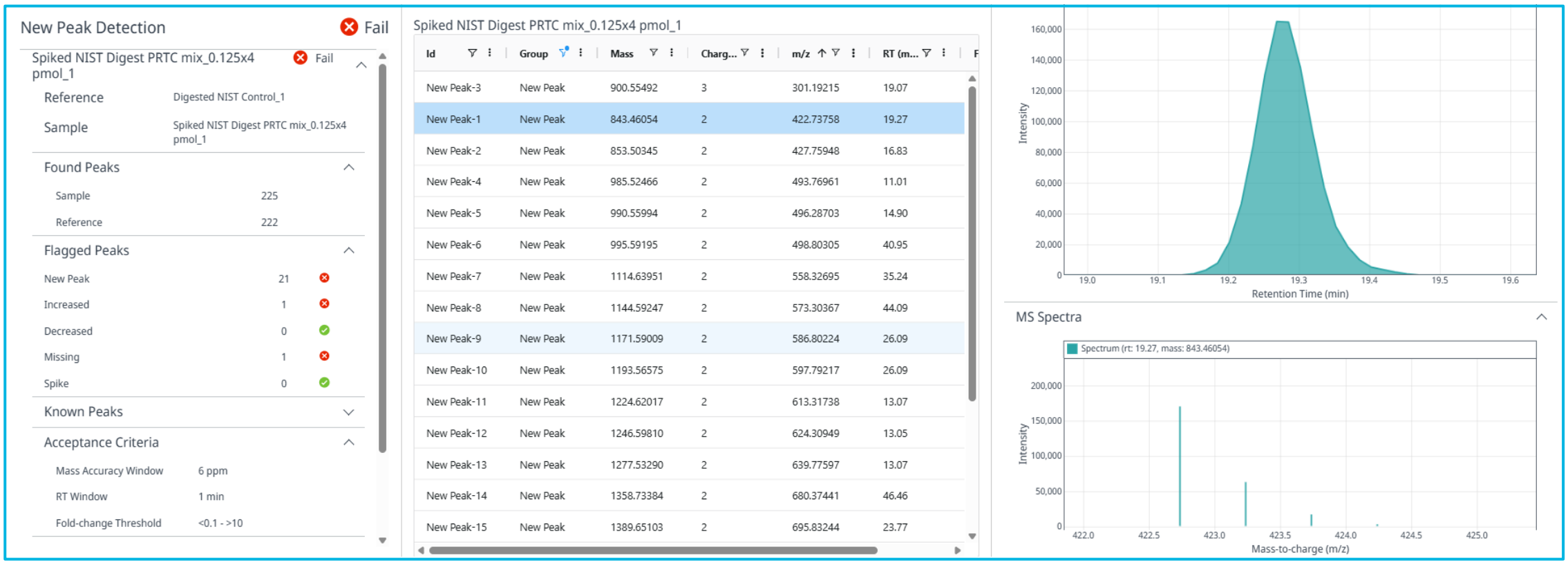


Figure 3. (Left) Sample overview highlighting flagged relative to the reference and evaluated against acceptance criteria. (Middle) Feature table summarizing m/z, retention time, charge state, and neutral mass for detected peaks.(Right) EIC and MS spectral views enabling confirmation of feature quality and differentiation of true peptide signals from artifacts..

Impact of LOD Threshold on NPD Sensitivity

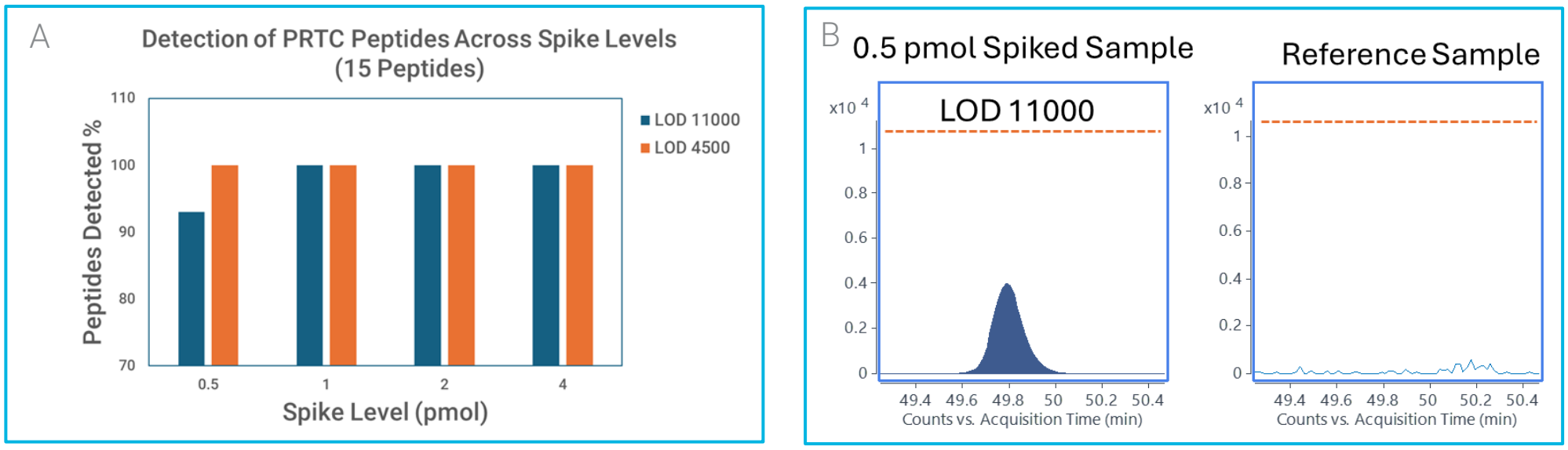


Figure 4. (A) Detection of 15 PRTC peptides across spike levels demonstrates consistent recovery at an LOD of 4,500, while a more stringent threshold reduces detection at lower concentrations. (B) Extracted ion chromatograms (EICs) of peptide ELASGLSFPVGFK* at 0.5 pmol show a detectable signal in the sample that falls below the LOD threshold of 11,000, resulting in missed detection, while no signal is observed in the reference matrix.

Results and Discussion

Specificity Assessment of NPD Using Replicate Reference Samples

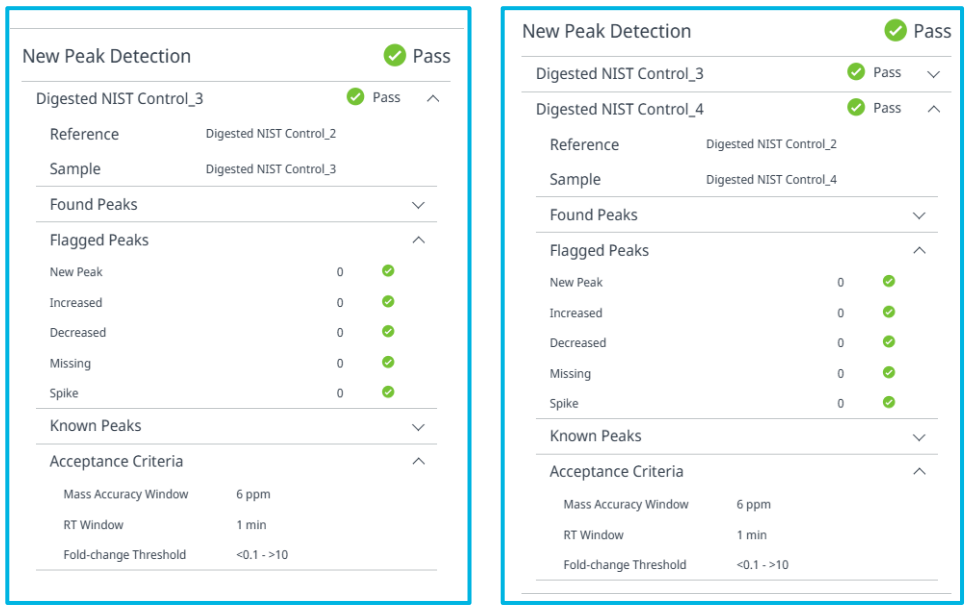
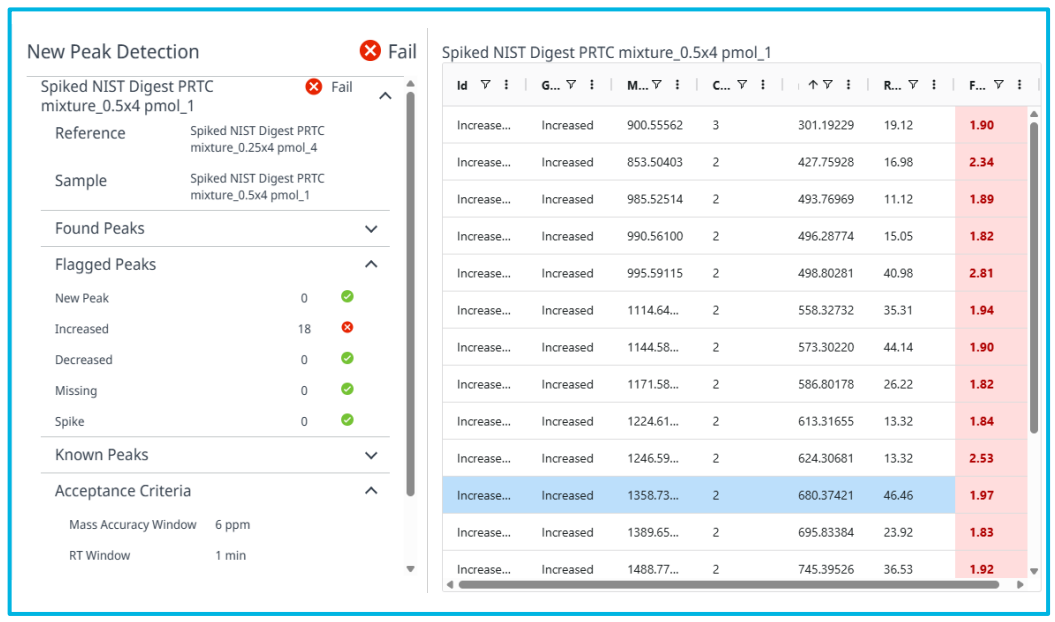


Figure 5. Comparison of replicate injections against a reference sample resulted in zero flagged features (New, Increased, Decreased, or Missing), demonstrating high specificity under optimized NPD parameter setting.

Fold-change validation: 2 pmol vs 1 pmol spiked samples.



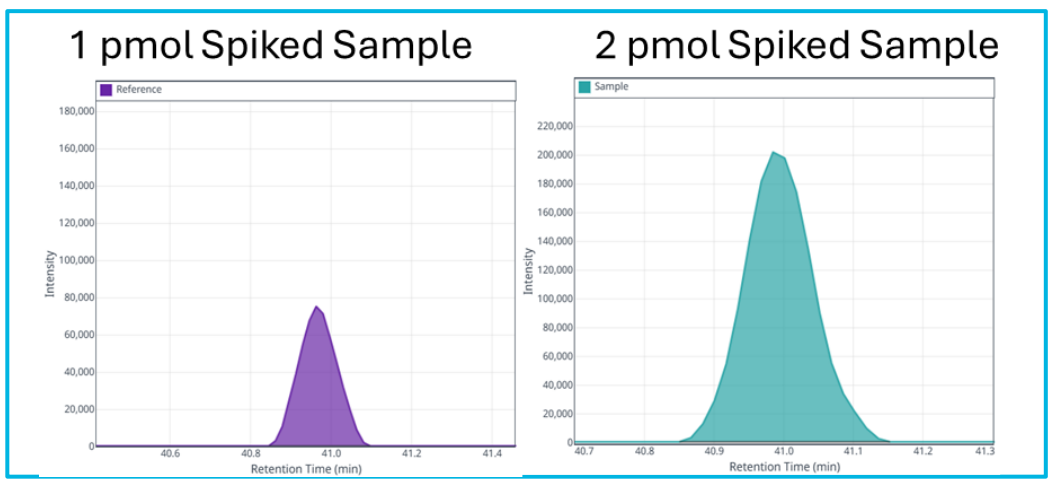
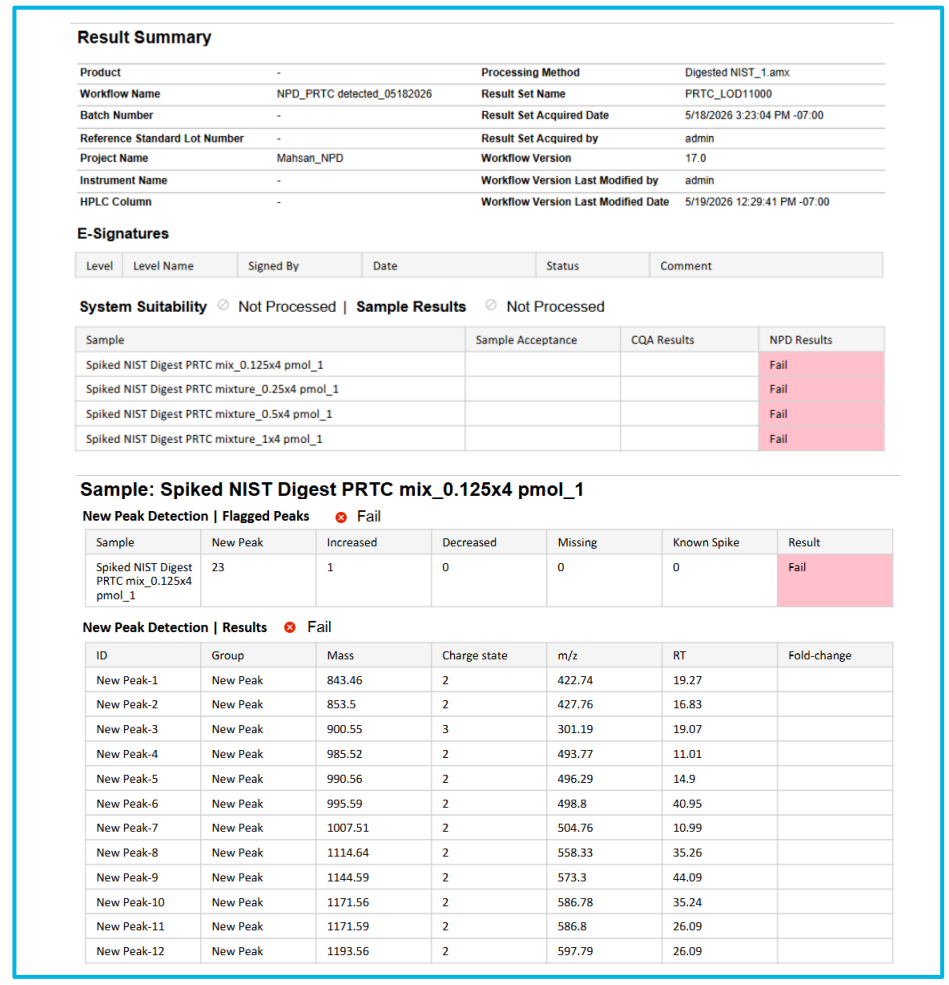


Figure 6. (Top panel) Table of features identified as increased in the 2 pmol sample relative to the 1 pmol sample. (Bottom panel) EICs for a representative peptide (m/z 680.3742) at 1 pmol and 2 pmol demonstrate a proportional increase in signal intensity. The observed fold change values are consistent with the expected ~2 increase, confirming accurate relative quantitation by the NPD algorithm.

NPD reporting and result summary in MAM



Result Summary

Product	-	**Processing Method**	Digested NIST_1.amx
Workflow Name	NPD_PRTC detected_05182026	**Result Set Name**	PRTC_LOD11000
Batch Number	-	**Result Set Acquired Date**	5/18/2026 3:23:04 PM -07:00
Reference Standard Lot Number	-	**Result Set Acquired by**	admin
Project Name	Mahsan_NPD	**Workflow Version**	17.0
Instrument Name	-	**Workflow Version Last Modified by**	admin
HPLC Column	-	**Workflow Version Last Modified Date**	5/19/2026 12:29:41 PM -07:00

E-Signatures

Level	Level Name	Signed By	Date	Status	Comment

System Suitability Not Processed | **Sample Results** Not Processed

Sample	Sample Acceptance	CQA Results	NPD Results
Spiked NIST Digest PRTC mix_0.125x4 pmol_1			Fail
Spiked NIST Digest PRTC mixture_0.25x4 pmol_1			Fail
Spiked NIST Digest PRTC mixture_0.5x4 pmol_1			Fail
Spiked NIST Digest PRTC mixture_1x4 pmol_1			Fail

Sample: Spiked NIST Digest PRTC mix_0.125x4 pmol_1

New Peak Detection | Flagged Peaks Fail

Sample	New Peak	Increased	Decreased	Missing	Known Spike	Result
Spiked NIST Digest PRTC mix_0.125x4 pmol_1	23	1	0	0	0	Fail

New Peak Detection | Results Fail

ID	Group	Mass	Charge state	m/z	RT	Fold-change
New Peak-1	New Peak	843.46	2	422.74	19.27	
New Peak-2	New Peak	853.5	2	427.76	16.83	
New Peak-3	New Peak	900.55	3	301.19	19.07	
New Peak-4	New Peak	985.52	2	493.77	11.01	
New Peak-5	New Peak	990.56	2	496.29	14.9	
New Peak-6	New Peak	995.59	2	498.8	40.95	
New Peak-7	New Peak	1007.51	2	504.76	10.99	
New Peak-8	New Peak	1114.64	2	558.33	35.26	
New Peak-9	New Peak	1144.59	2	573.3	44.09	
New Peak-10	New Peak	1171.56	2	586.78	35.24	
New Peak-11	New Peak	1171.59	2	586.8	26.09	
New Peak-12	New Peak	1193.56	2	597.79	26.09	

Figure 7. Structured NPD reporting provides summary results, flagged features, and supporting data for review and pass/fail determination.

Conclusions

- Controlled spike-in strategy enables systematic evaluation of NPD performance in MAM.
- Optimized LOD settings improve sensitivity, while higher thresholds can lead to false negatives.
- The workflow demonstrates high specificity with minimal false positives under optimized conditions.
- NPD preserves accurate fold-change measurements, supporting reliable trend analysis.
- Integrated reporting enables efficient review and decision-making for routine applications.

References

[1]Rogers, R.S. et al., Anal. Chem., 2015, 87, 1058–1065.
[2]Ren, D. et al., mAbs, 2018, 10, 859–870.
[3]USP <1060>; ICH Q2(R2)

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