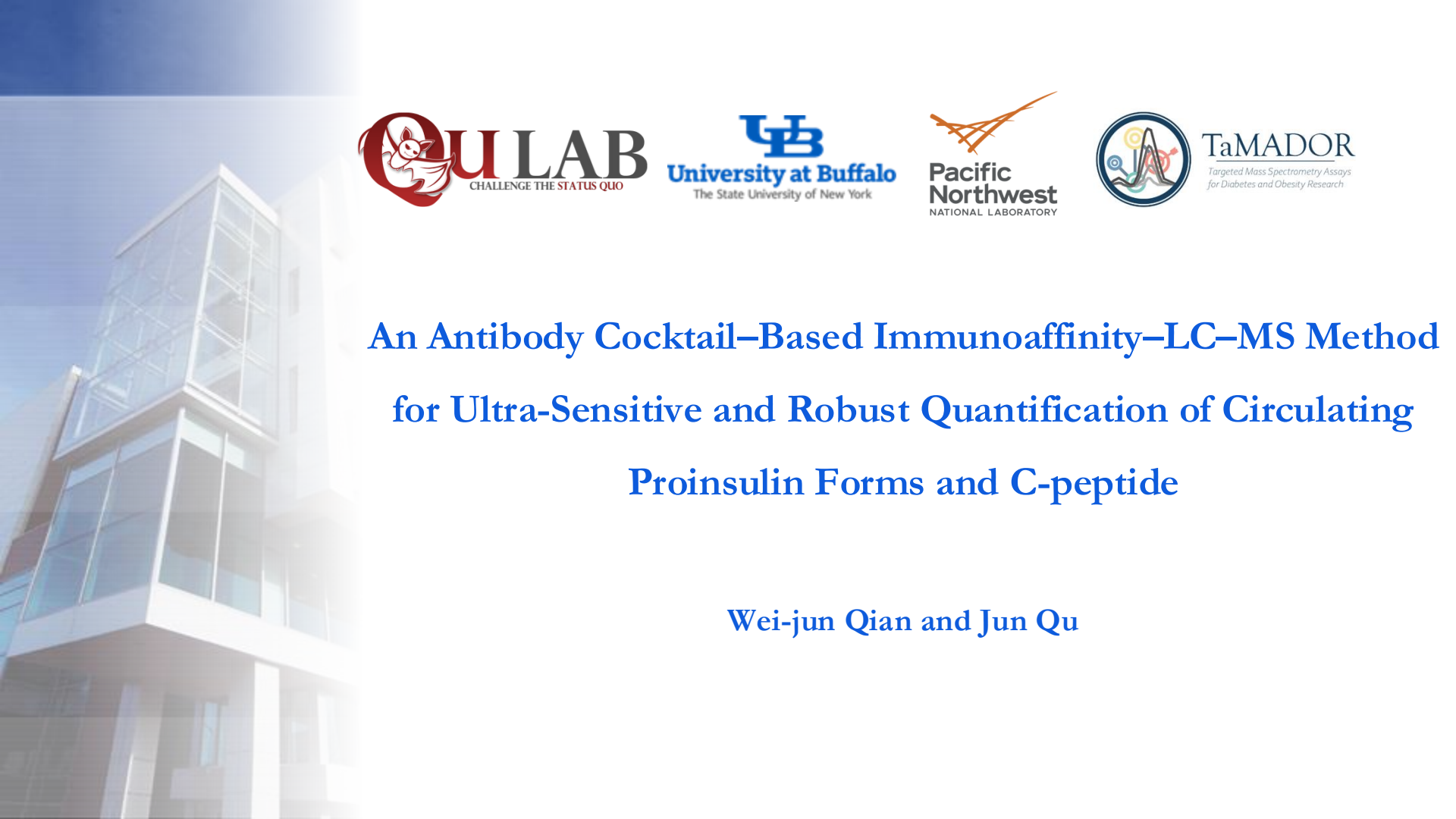
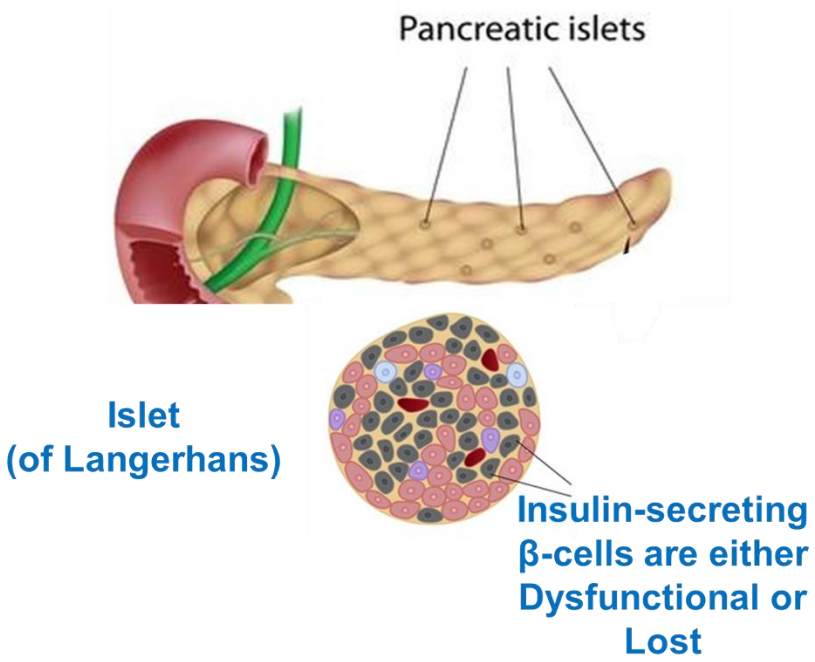
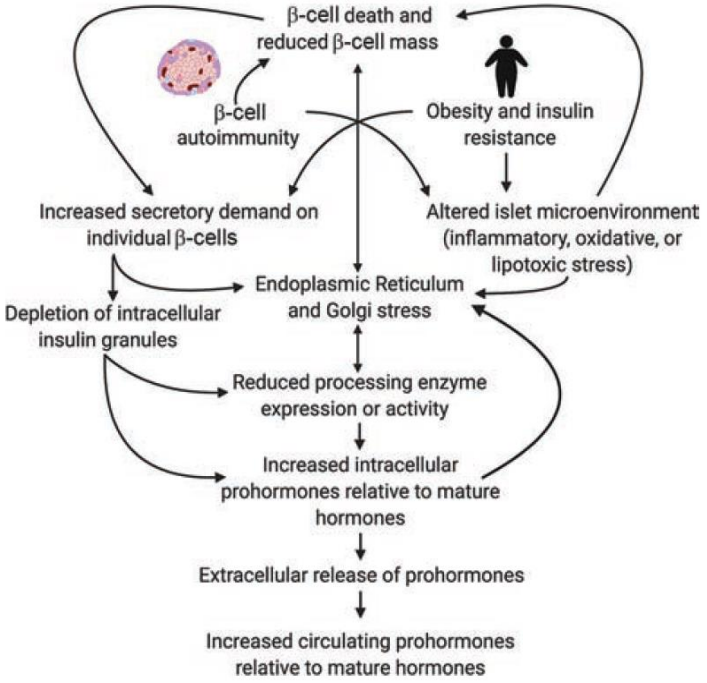


An Antibody Cocktail–Based Immunoaffinity–LC–MS Method for Ultra-Sensitive and Robust Quantification of Circulating Proinsulin Forms and C-peptide

Wei-jun Qian and Jun Qu

Impaired proinsulin processing is a hallmark for β-cell dysfunction in diabetes

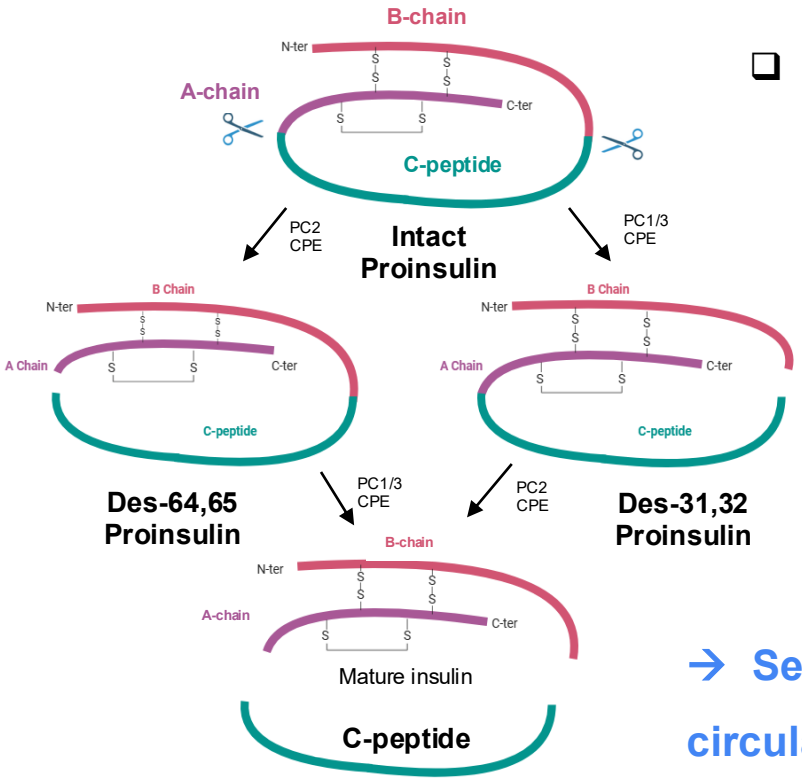




- **~70-90% loss of β-cell function at T1D diagnosis.**
- **~40-50% loss at T2D diagnosis.**

Diabetes 2021;70:1038–1050

Proinsulin forms and altered proinsulin processing



- **Altered processing of proinsulin could be an important indicator of β-cell functions**
  - β-Cell Stress and Secretory Overload;
  - Impaired Convertase Activity;
  - ER Stress and Misfolding;
  - Selective β-Cell Loss

→ **Selectively quantifying specific proinsulin forms in circulation is critical**

Transl Res. 2019, 213: 90–99.
Diabetologia, 2023, 66: 2283–2291
Diabetes Care, 2023,1, 46(12): 2129-2136

Challenges in measuring proinsulin forms in circulation

Low levels in circulation

Forms	Concentration in circulation
Proinsulin	10-200 pg/mL (healthy subjects)
Des-31,32	Not previously quantified, but speculated to have comparable levels as proinsulin
Des-64,65	Never detected in circulation before, might has much lower levels than Des-31,32
C-peptide	500-3000 pg/mL (healthy)

J Proteome Res, 2021, 20, 4507−4517
Diabetes Care 1998;21(1):127–133

Challenges in developing proinsulin form quantification assay

Severe cross-reactivity in most proinsulin ELISA kits used in clinical investigations

Interference	Mercodia (Cat. 10-1118-01)	ALPCO (Cat. 80-PINHUT-CH01)
Des-31,32	**95%**	**100%**
Des-64,65	**84%**	**100%**
Insulin	<0.03%	<0.6%
C-peptide	<0.006%	<0.1%

→ A method with high sensitivity and high selectivity is needed

5

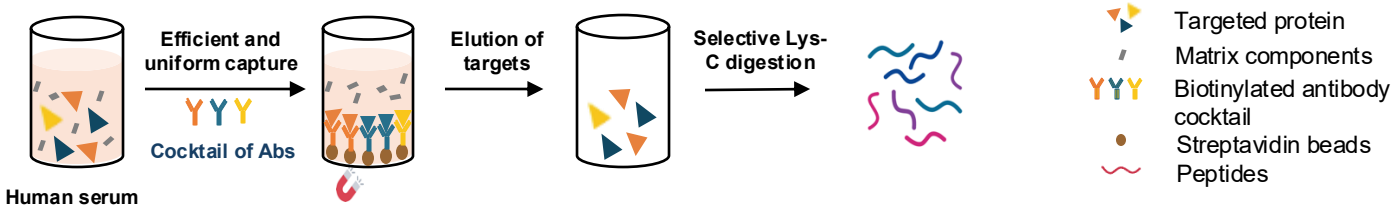
Insufficient sensitivity in current LC-MS assays

Reference	Sample preparation method	Instrumentation	Capability
J Chromatogr B, 2022, 1211, e123482	• 100 µL plasma • PPT + SPE + online trap	• Micro-flow LC (3 µL/min) • Waters Xevo TQ-S	• Des-31,32: **not quantitative**; • C-peptide and insulin: quantitative; • Proinsulin: **Undetectable in healthy subjects** and some patients
Clin Chem 2021, 67, 854-862	• 250 µL plasma • PPT + SPE	• High-flow LC • Thermo QE-Plus (high-resolution)	• Insulin and C-peptide: **semi-quan** • Proinsulin and des-31,32 were identified in patients • **Undetectable proinsulin in healthy subjects** • **Des-64,65 was not detected**
Forensic Toxicol, 2017, 35: 106-113	• 100 µL plasma • PPT + immuno-enrichment + online trap	• Nano-flow LC (350 nL/min) • Thermo QE (high-resolution)	• LOD (LOQ) for proinsulin **50 pg/mL**; • Proinsulin only detectable in 30% samples

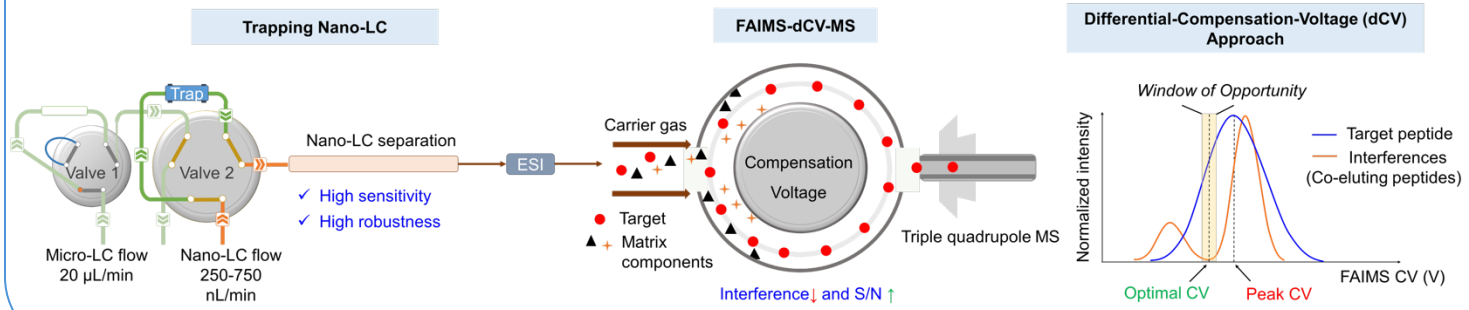
An ultra-sensitive LC-MS-based strategy

Patent Pending

Efficient and uniform capture of proinsulin forms with an optimized antibody cocktail

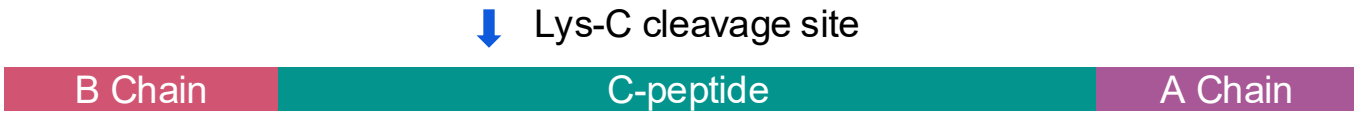


Highly sensitive quantification with trapping-nano-LC-FAIMS/dCV-MS system



To produce selective surrogate peptides for specific forms

- Intact amino acid sequence



C-peptide: EAEDLQVGQVELGGGPGAGSLQPLALEGSLQ

Proinsulin: FVN…TPKTRREAEDLQVGQVELGGGPGAGSLQPLALEGSLQKRRGIV…YCN

Des-31,32: EAEDLQVGQVELGGGPGAGSLQPLALEGSLQKRRGIV…YCN

Des-64,65: FVN…TPKTRREAEDLQVGQVELGGGPGAGSLQPLALEGSLQ

To produce selective surrogate peptides for specific forms

- Lys-C generated surrogate peptides: idea by Wei-jun Qian

C-peptide:	**EAEDLQVGQVELGGGPGAGSLQPLALEGSLQ**
Proinsulin:	**TRREAEDLQVGQVELGGGPGAGSLQPLALEGSLQK**
Des-31,32:	**EAEDLQVGQVELGGGPGAGSLQPLALEGSLQK**
Des-64,65:	**TRREAEDLQVGQVELGGGPGAGSLQPLALEGSLQ**

Quantitative bias by pan-antibody capture across different forms

- Intact amino acid sequence

C-peptide: EAEDLQVGQVELGGGPGAGSLQPLALEGSLQ

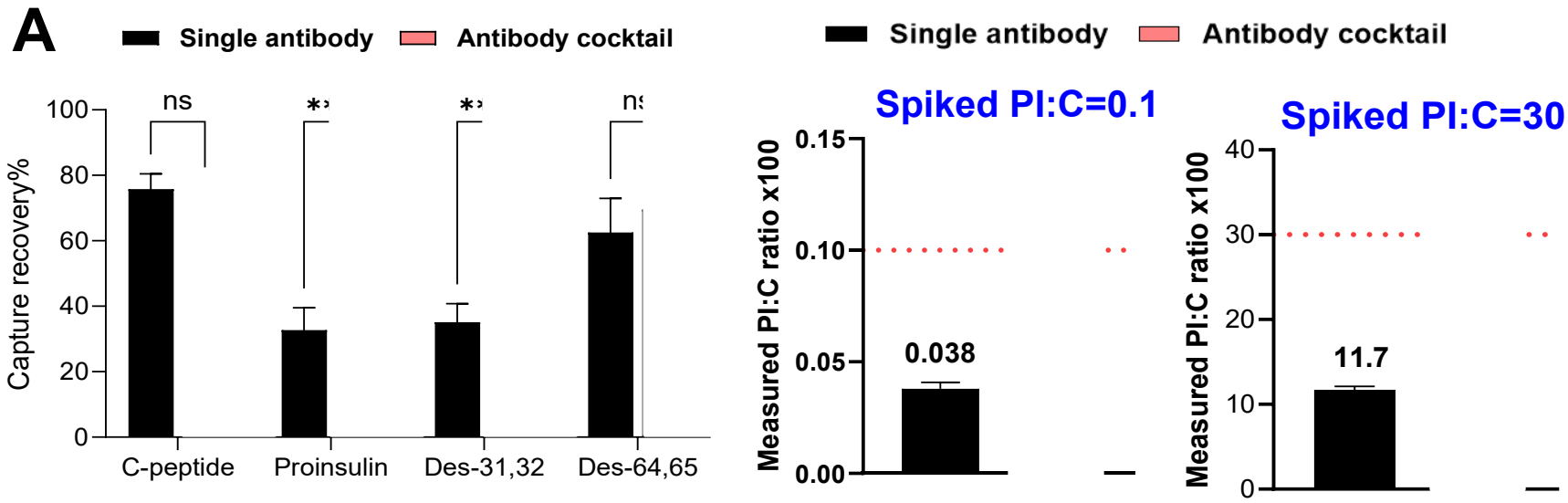
Proinsulin: FVN…TPKTRREAEDLQVGQVELGGGPGAGSLQPLALEGSLQKRRGIV…YCN

Des-31,32: EAEDLQVGQVELGGGPGAGSLQPLALEGSLQKRRGIV…YCN

Des-64,65: FVN…TPKTRREAEDLQVGQVELGGGPGAGSLQPLALEGSLQ

Anti-C-peptide antibody (pan mAb)

Quantitative bias by pan-antibody capture across different forms



- PI:C ratio: molar ratio of proinsulin and C-peptide, multiplied by 100;
- Healthy PI:C=1~2; Diabetes PI:C= 2~10 in different publications.

Anal. Chem. 2025, in press;
doi.org/10.1021/acs.analchem.5c03256

Antibody-cocktail: improvement of capturing coverage

Patent Pending

C-peptide: EAEDLQVGQVELGGGPGAGSLQPLALEGSLQ

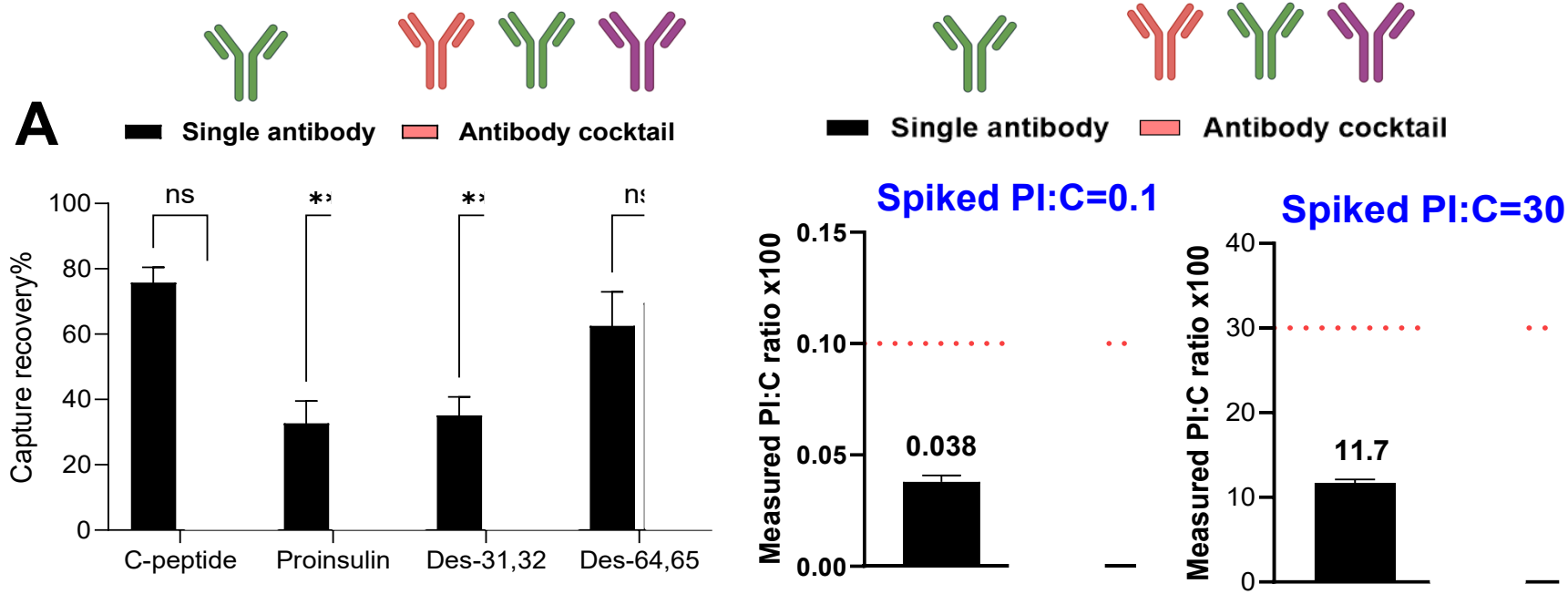
Proinsulin: FVN…TPKTRREAEDLQVGQVELGGGPGAGSLQPLALEGSLQKRRGIV…YCN

Des-31,32: EAEDLQVGQVELGGGPGAGSLQPLALEGSLQKRRGIV…YCN

Des-64,65: FVN…TPKTRREAEDLQVGQVELGGGPGAGSLQPLALEGSLQ

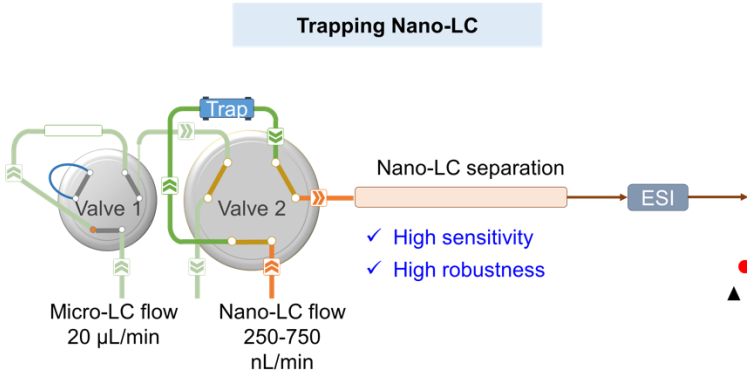
Uniform immunocapture with an antibody cocktail

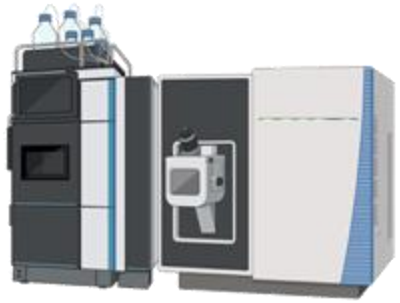
(Clone C-pep-01+5E4/3+7F8)



Anal. Chem. 2025, in press;
doi.org/10.1021/acs.analchem.5c03256

Trapping nano-LC FAIMS-dCV-MS system





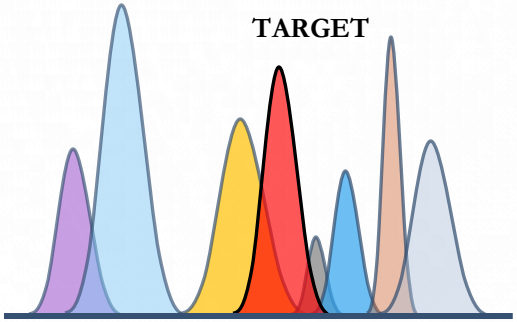
Large ID trap:

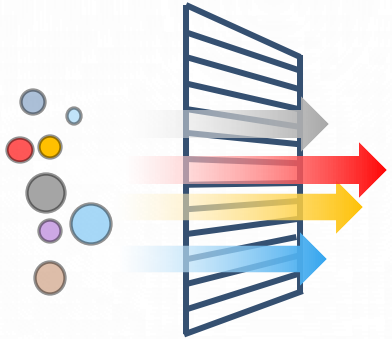
- ✓ Exceptional robustness;
- ✓ High sensitivity;
- ✓ Selective trap and delivery: remove matrix components;
- ✓ Rapid loading;
- ✓ Reproducible gradient delivery

Dynamic trapping/nano flows

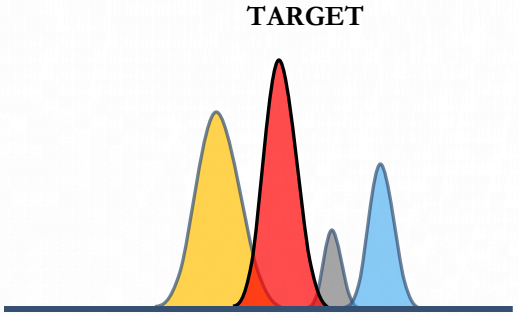
- ✓ Rapid mass transfer without band broadening;
- ✓ High throughput: cycle time~15 min;
- ✓ High ESI response
- ✓ Exceptional robustness

FAIMS: separation mechanism orthogonal to LC or MS

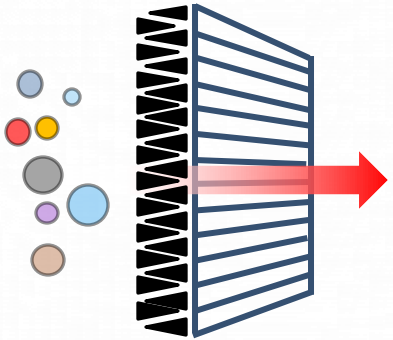




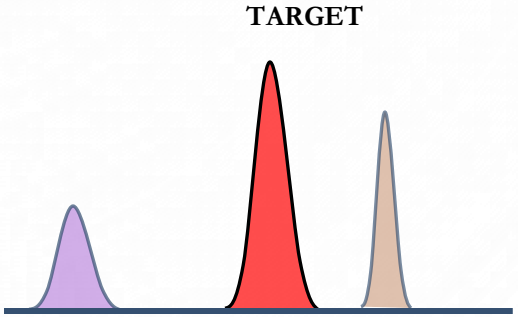
Non-orthogonal
separation mechanisms



No S/N improvement



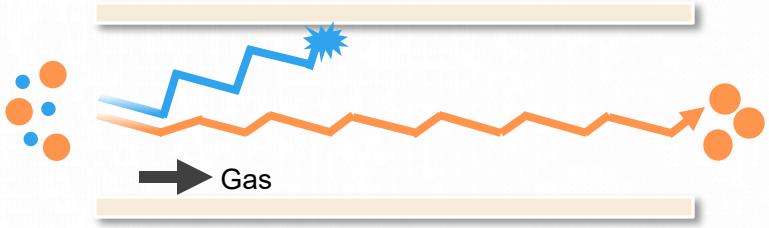
Orthogonal separation mechanisms



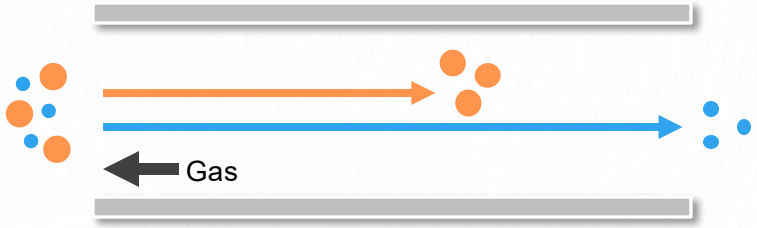
S/N improvement

Field Asymmetric Ion Mobility Spectrometry (FAIMS) VS conventional IMS

FAIMS: differential ion mobility



Conventional ion mobility spectrometry



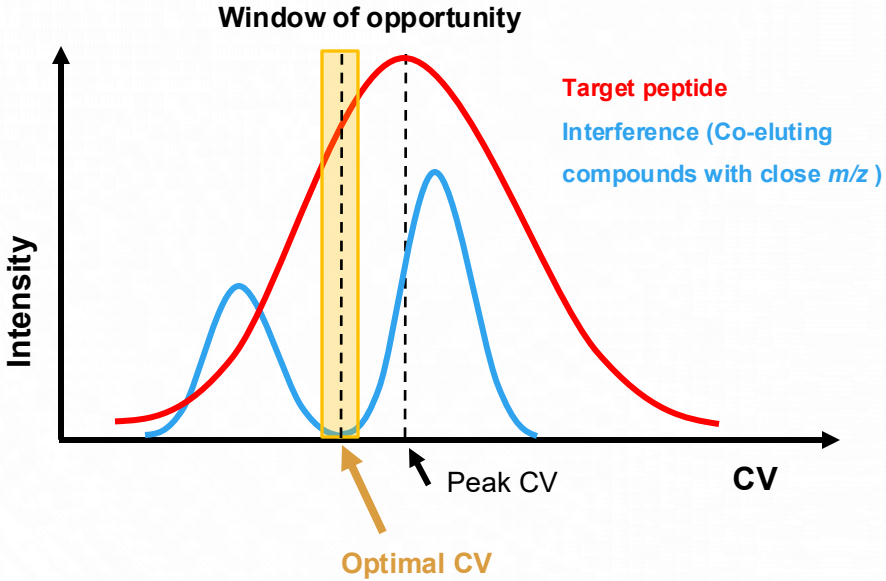
Two advantages of FAIMS compared with conventional IMS

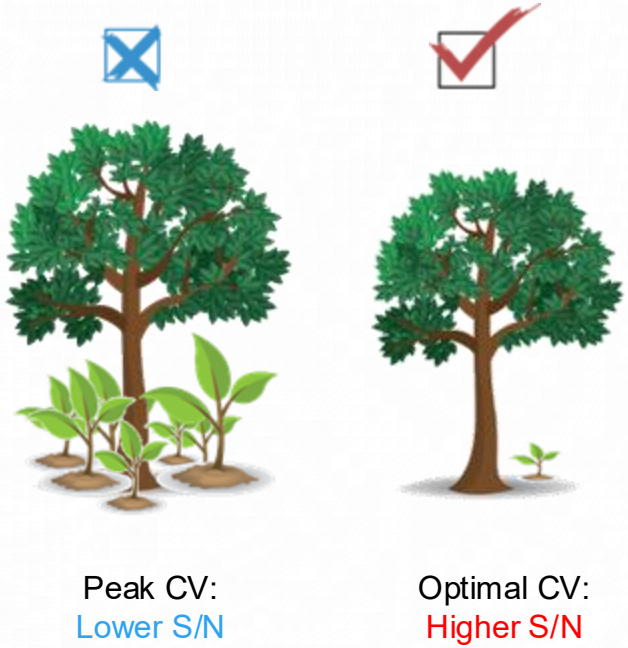
- **Continuous filter**, allows uninterrupted sampling of the ion stream (100% ion time utilization)
- **More orthogonal to *m/z***, which can significantly improve the separation of analytes

FAIMS-dCV

Take full advantage of the extra selectivity by FAIMS in targeted protein quantification

Anal. Chem. 2024, 96, 32, 13140–13149

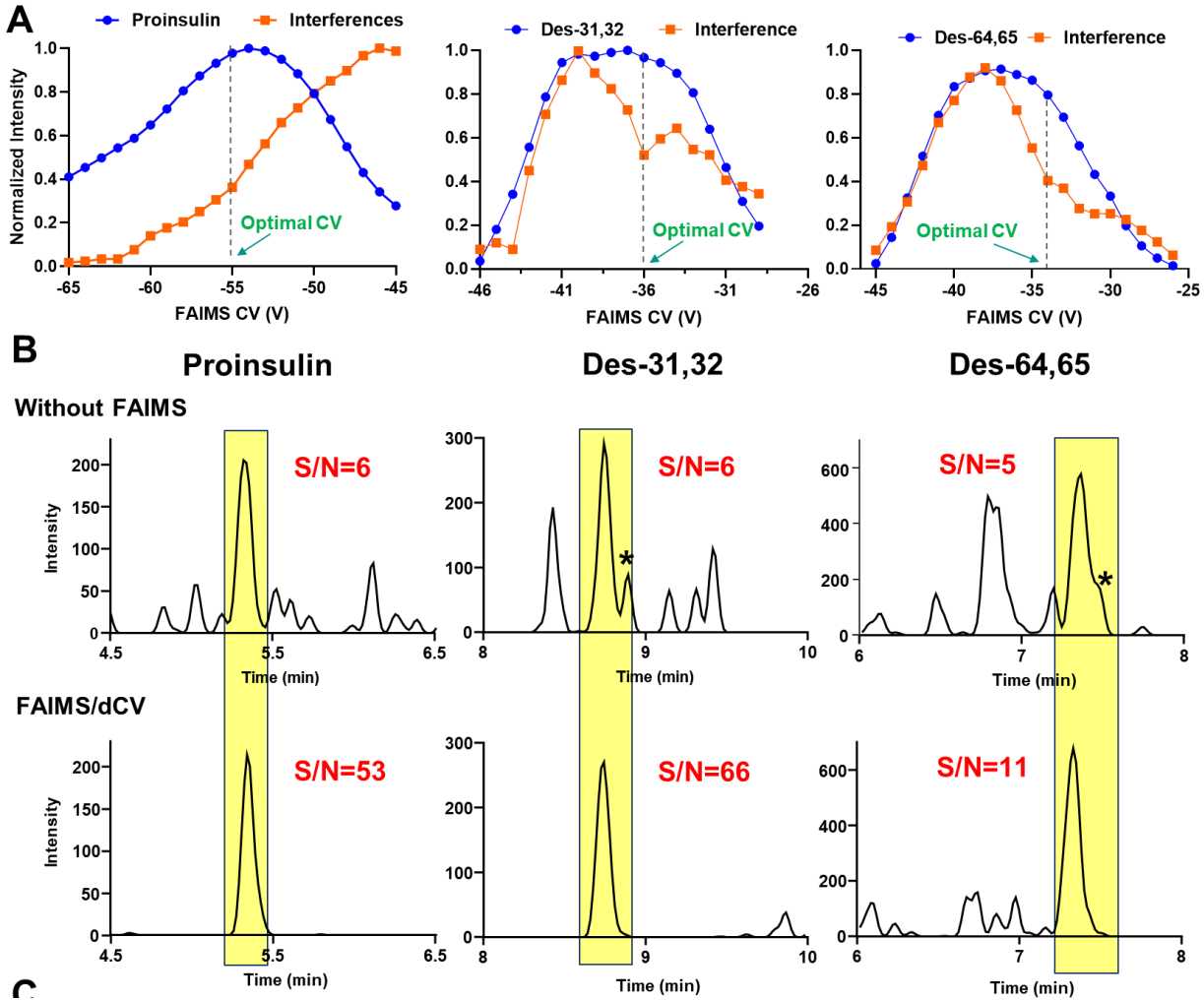




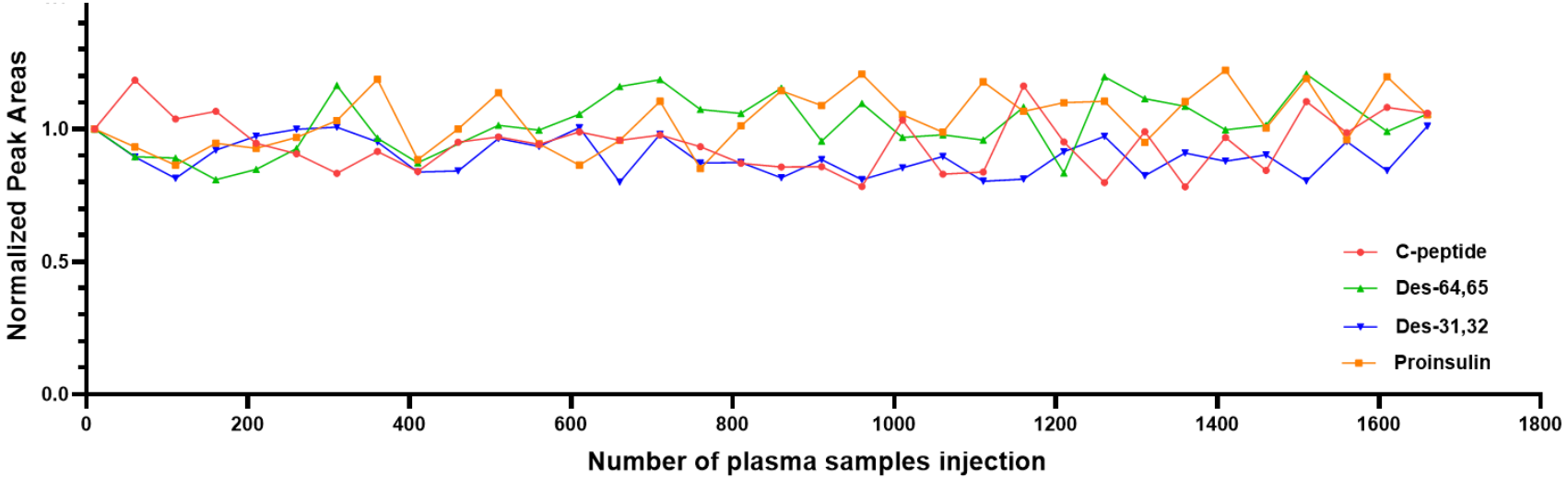
Peak CV:
Lower S/N

Optimal CV:
Higher S/N

A novel different CV (dCV) method for ultra-sensitive and selective protein quantification in plasma/tissues

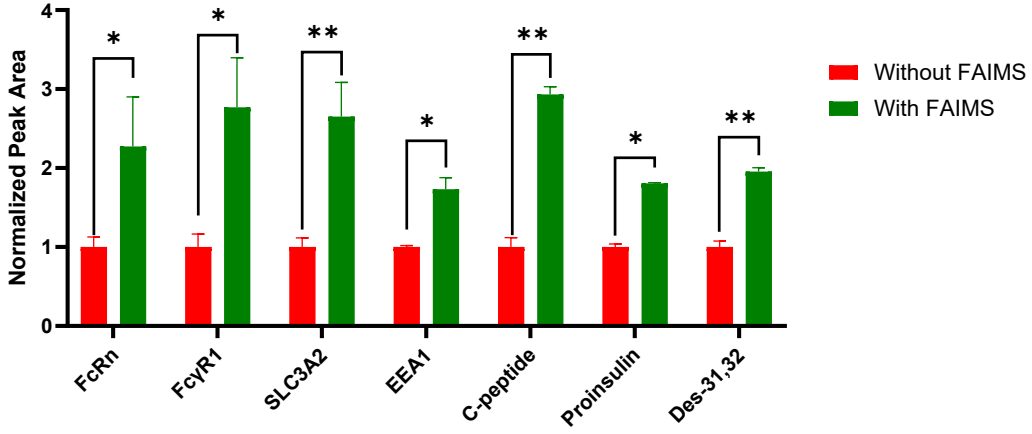

A
Proinsulin
Interferences
Normalized Intensity
Optimal CV
FAIMS CV (V)
Des-31,32
Interference
Optimal CV
FAIMS CV (V)
Des-64,65
Interference
Optimal CV
FAIMS CV (V)
B
Proinsulin
Des-31,32
Des-64,65
Without FAIMS
S/N=6
S/N=6
S/N=5
Intensity
Time (min)
FAIMS/dCV
S/N=53
S/N=66
S/N=11
Intensity
Time (min)

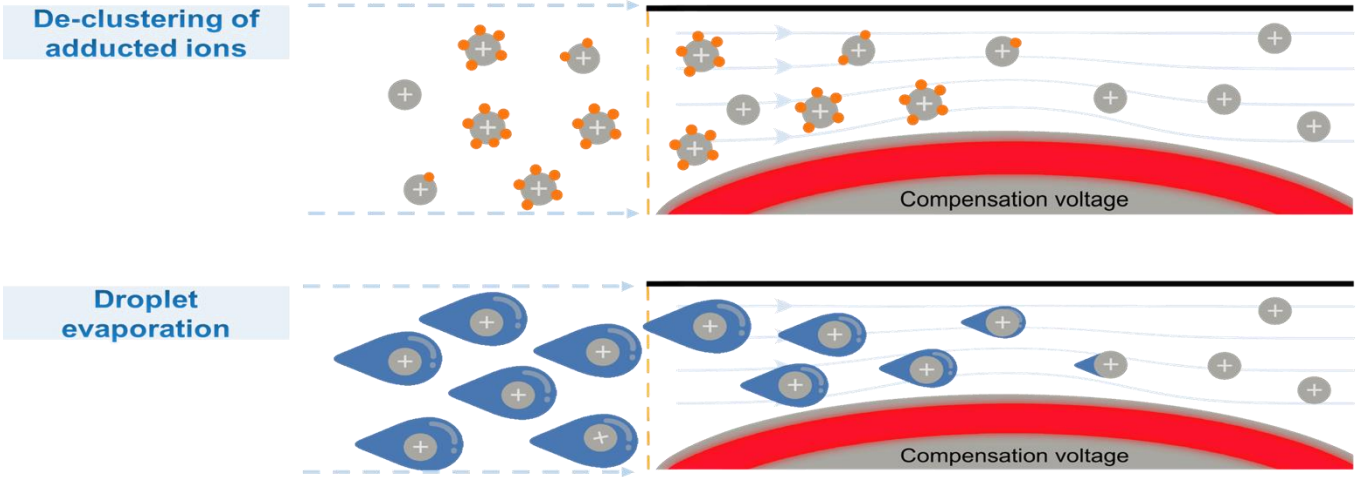
Exceptional robustness



No need for instrument cleaning/maintenance for >4 months

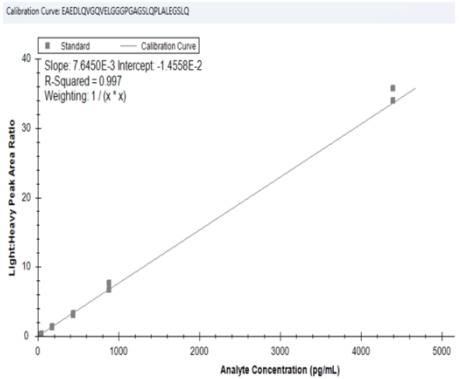
FAIMS significantly improves the intensities of peptide ions under nano-spray



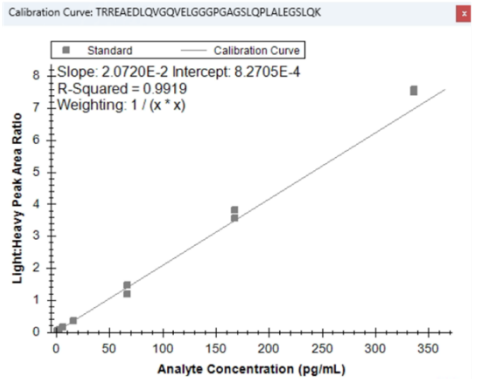


Calibration curves in surrogate matrix (equine serum)

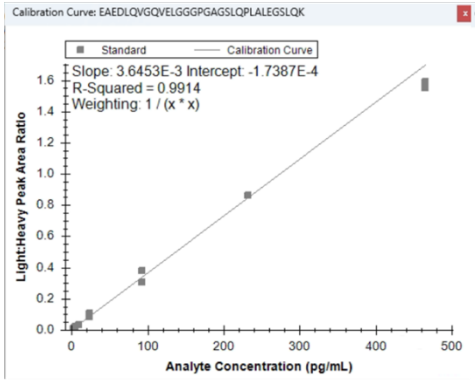
C-peptide



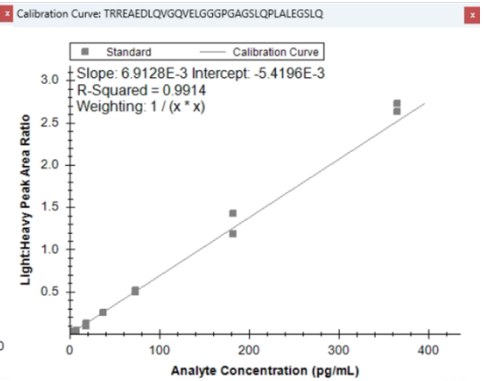
Proinsulin



Des-31,32



Des-64,65



The range of concentration:

C-peptide: 8.8 – 4400 pg/mL;

Proinsulin: 1.7 – 340 pg/mL;

Des-31,32: 2.3 – 460 pg/mL;

Des-64,65: 3.6 – 360 pg/mL.

Amino acid analysis was analyzed on standards.

Validation

Validation results for quantification of the four targets in serum

Analyte	Linearity range (pg/mL)	QC accuracy (%)/CV% (n=3)			
		[a]LOQ	[b]Low-QC	[b]Mid-QC	[b]High-QC
C-peptide	8.8- 4400	97.5/16.6	96.0/4.3	94.1/4.9	107.1/6.7
Proinsulin	1.7 – 337	100.2/9.6	97.9/4.2	88.8/1.0	105.1/3.2
Des-31,32	2.3 – 468	93.3/9.9	98.7/13.8	106.3/9.6	108.4/6.9
Des-64,65	3.6 – 365	87.0/1.2	106.7/11.6	105.6/14.3	98.5/3.6

[a]The validated LOQs for C-peptide, Proinsulin, Des-31,32 and Des-64,65 are respectively 8.8, 1.7, 2.3 and 3.6 pg/mL in serum.
[b]The levels of High, Mid, and Low QC in serum: C-peptide 3520.0, 440.0, and 22.0pg/mL; Proinsulin at 272.0, 34.0 and 4.3pg/mL; Des-64,65 at 292.0,36.5 and 4.6 pg/mL; Des-31,32 at 372.0, 46.5 and 5.8pg/mL

Anal. Chem. 2025, in press;
doi.org/10.1021/acs.analchem.5c03256

Application in measuring a clinical cohort of 80 subjects

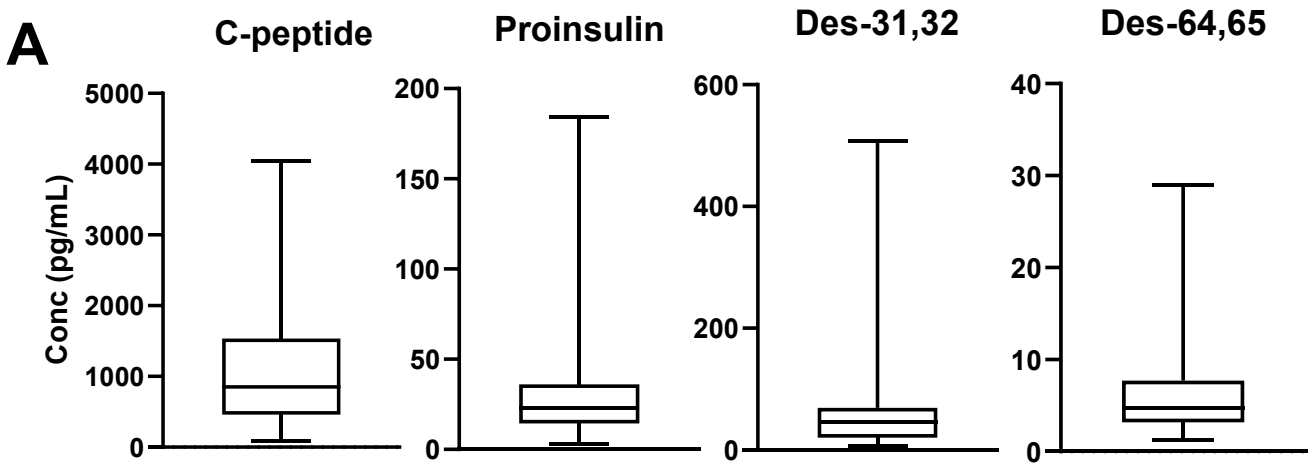
The demographic characteristics of the four groups of clinical samples (N=20/group)

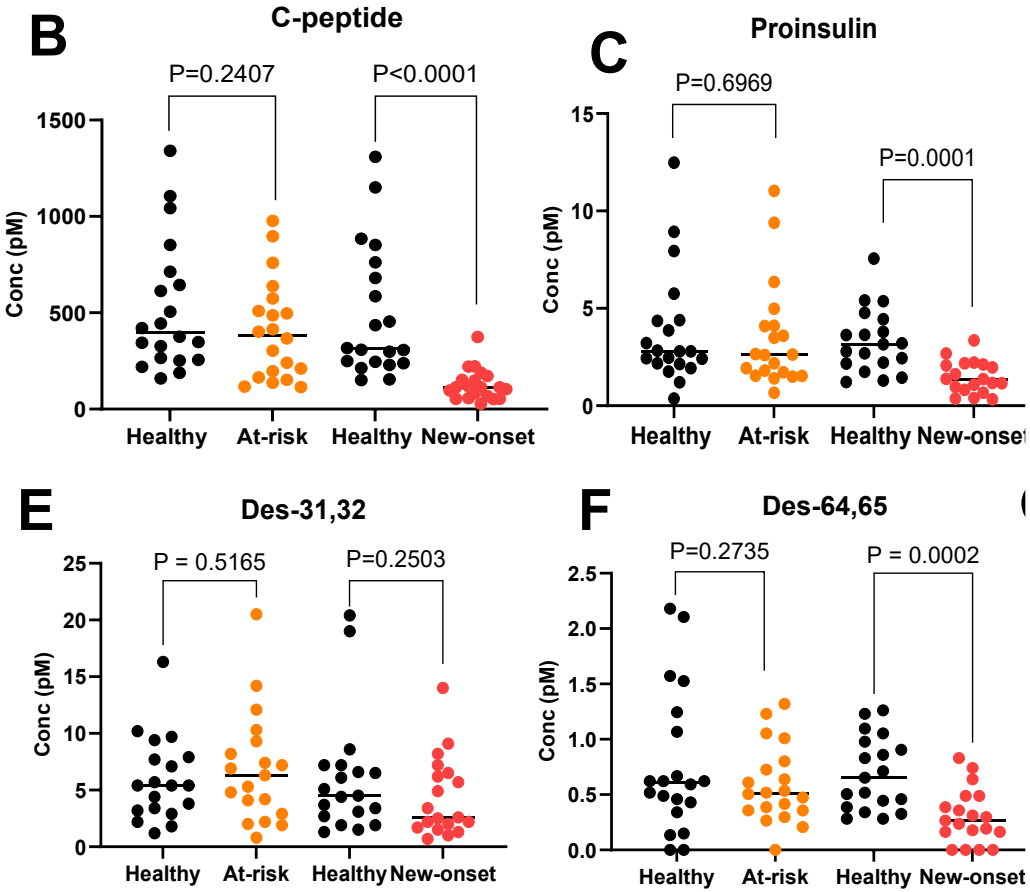
	At-risk	Healthy Control#1	New onset T1D	Healthy Control#2
Gender: Female/Male, N	6/13	6/13	8/12	8/12
Age (yr): mean (SD)	13 (10.0)	13 (9.8)	12.4 (5.9)	12.5 (6.0)
BMI (kg/m^2): mean (SD)	20 (6.8)	20 (5.8)	19.7 (4.7)	20.2 (4.7)

Application in measuring a clinical cohort of 80 subjects

Proinsulin and Des-31,32 were quantified in all samples;

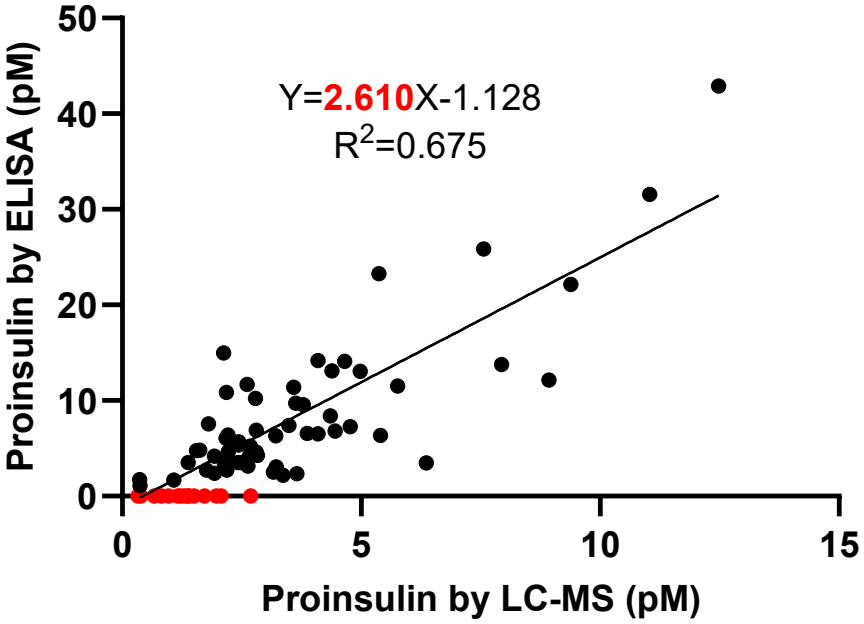
Des-64,65 was quantified in ~70% samples

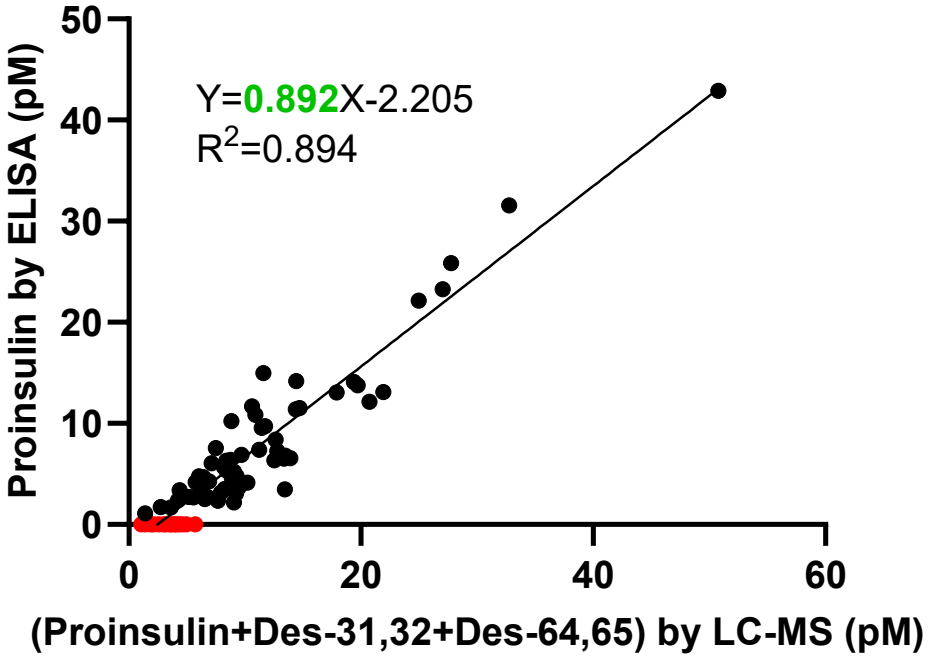




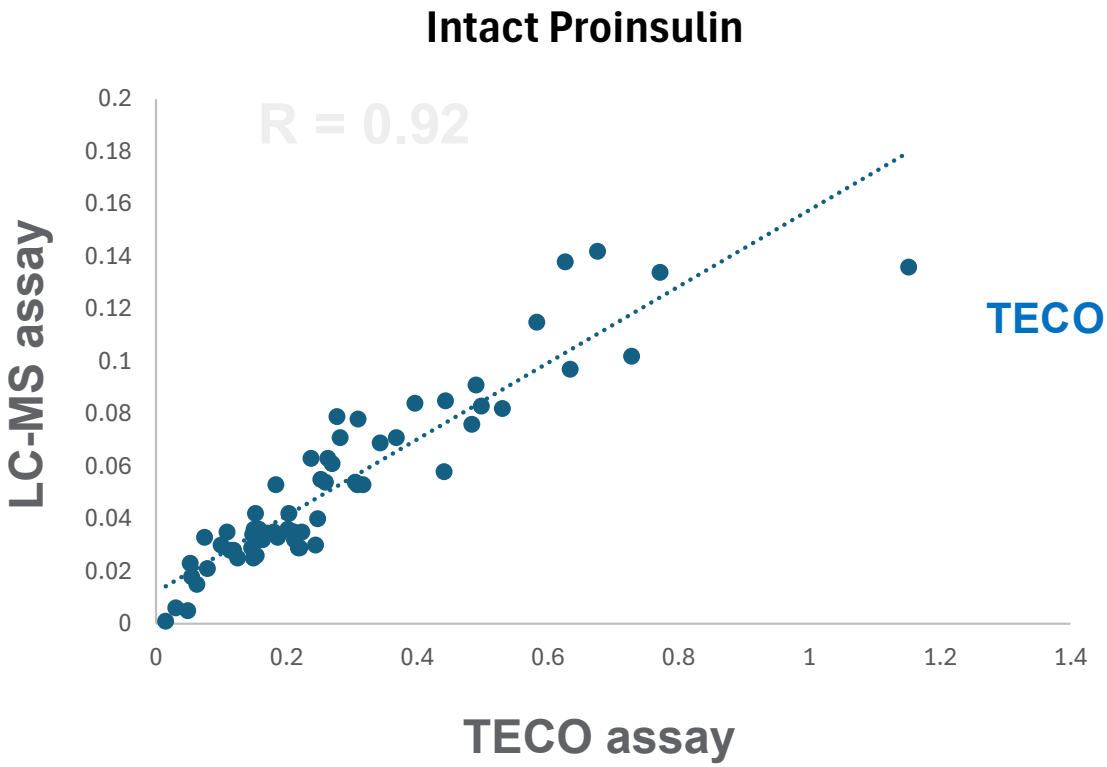
Anal. Chem. 2025, in press;
doi.org/10.1021/acs.analchem.5c03256

LC-MS vs. ELISA (Mercodia 10-1118-01)



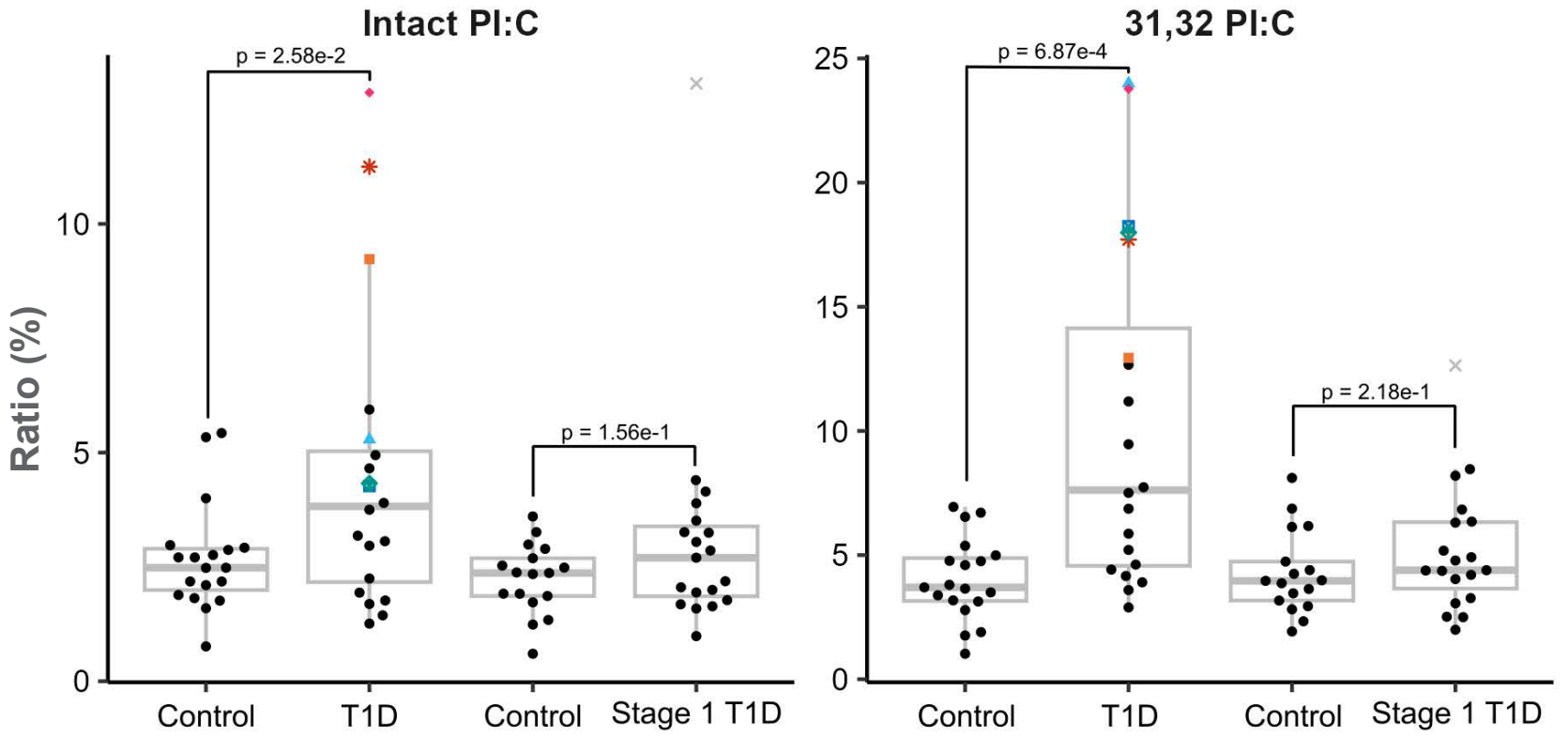


LC-MS vs. ELISA (TECO intact proinsulin assay)

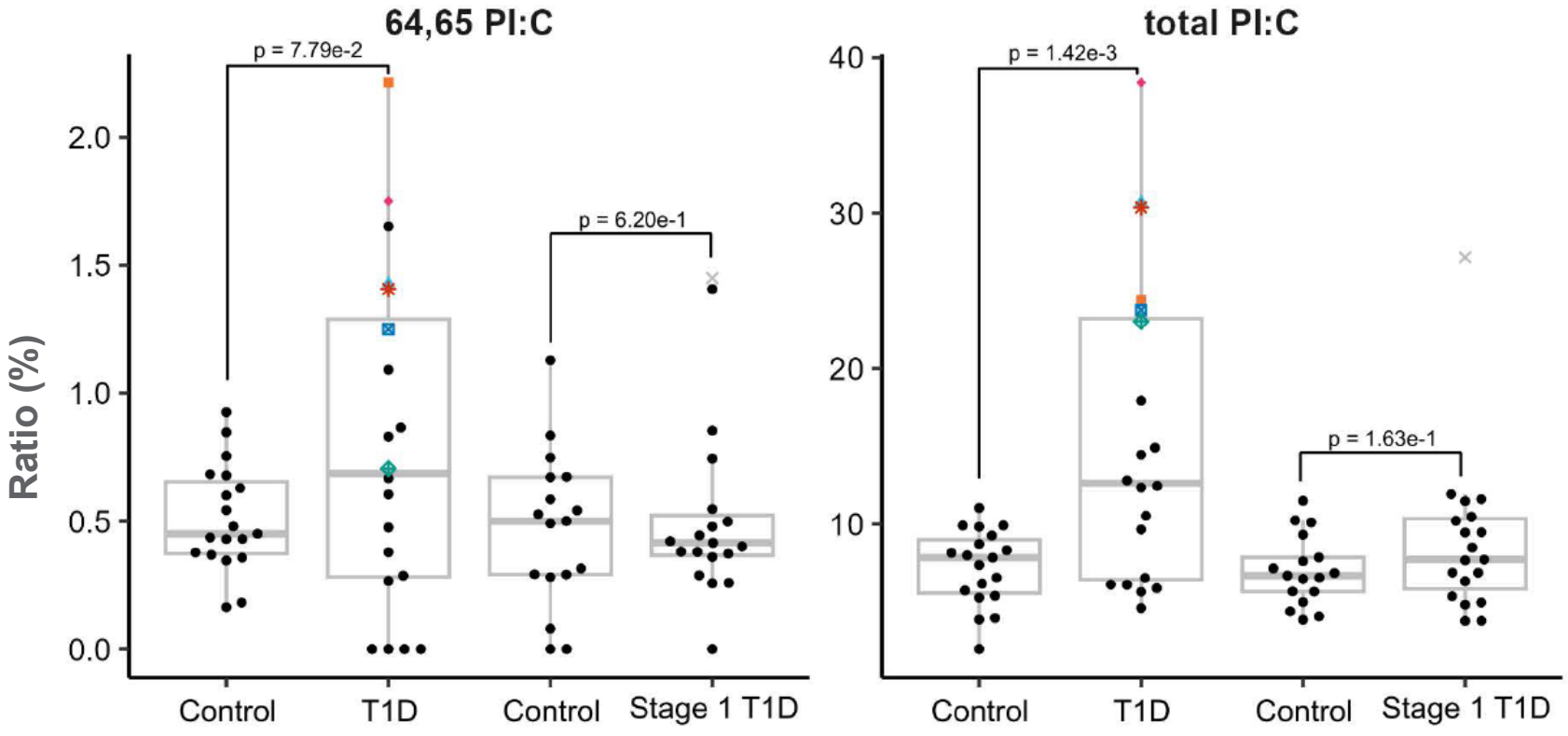


TECO Intact Proinsulin assay

Specific Proinsulin Form to C-peptide Ratio



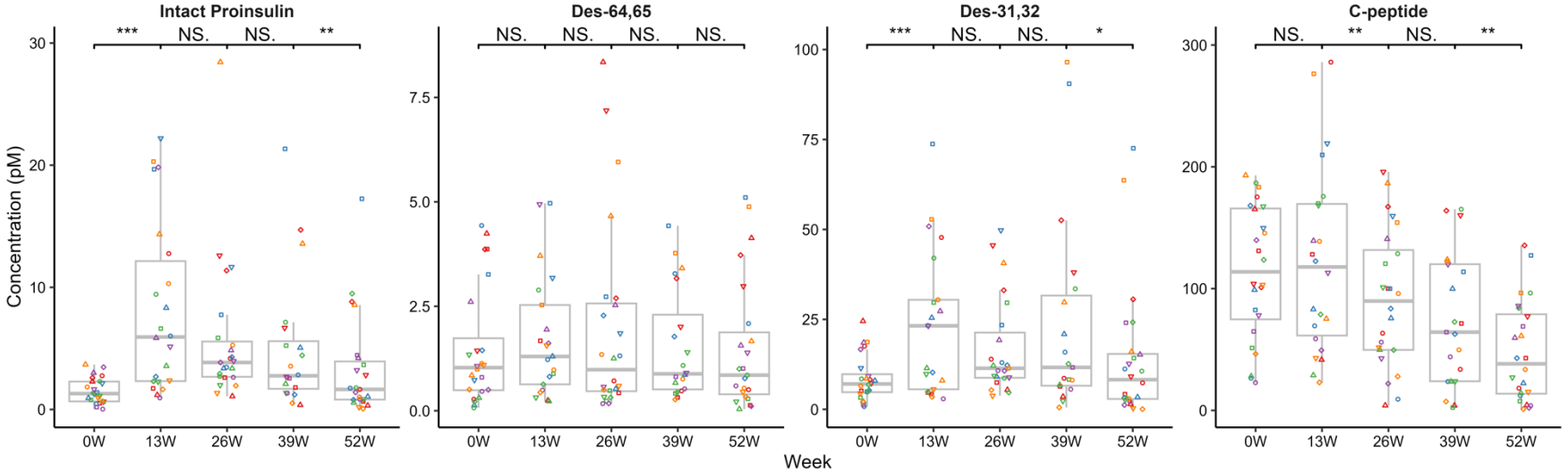
Specific Proinsulin Form to C-peptide Ratio



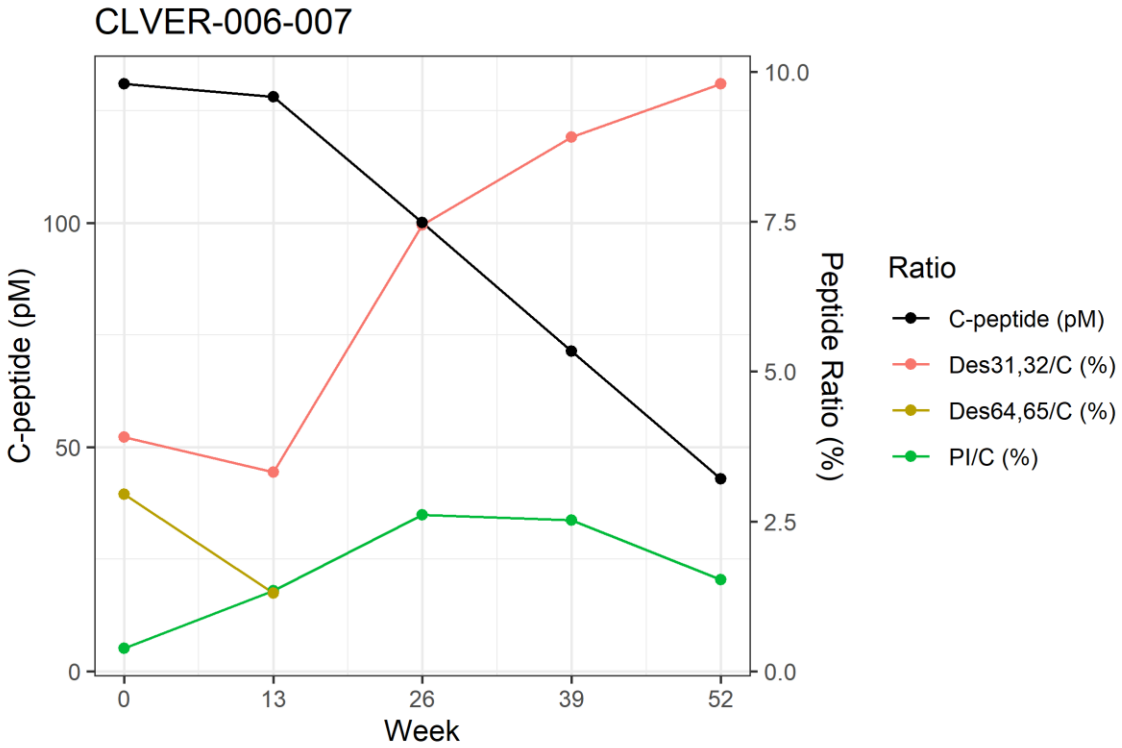
2nd Application:
Longitudinal Samples after Clinical T1D Diagnosis (Hybrid closed loop arm from the CLVer Trial)

Forlenza G.P., et al, JAMA, 2023.

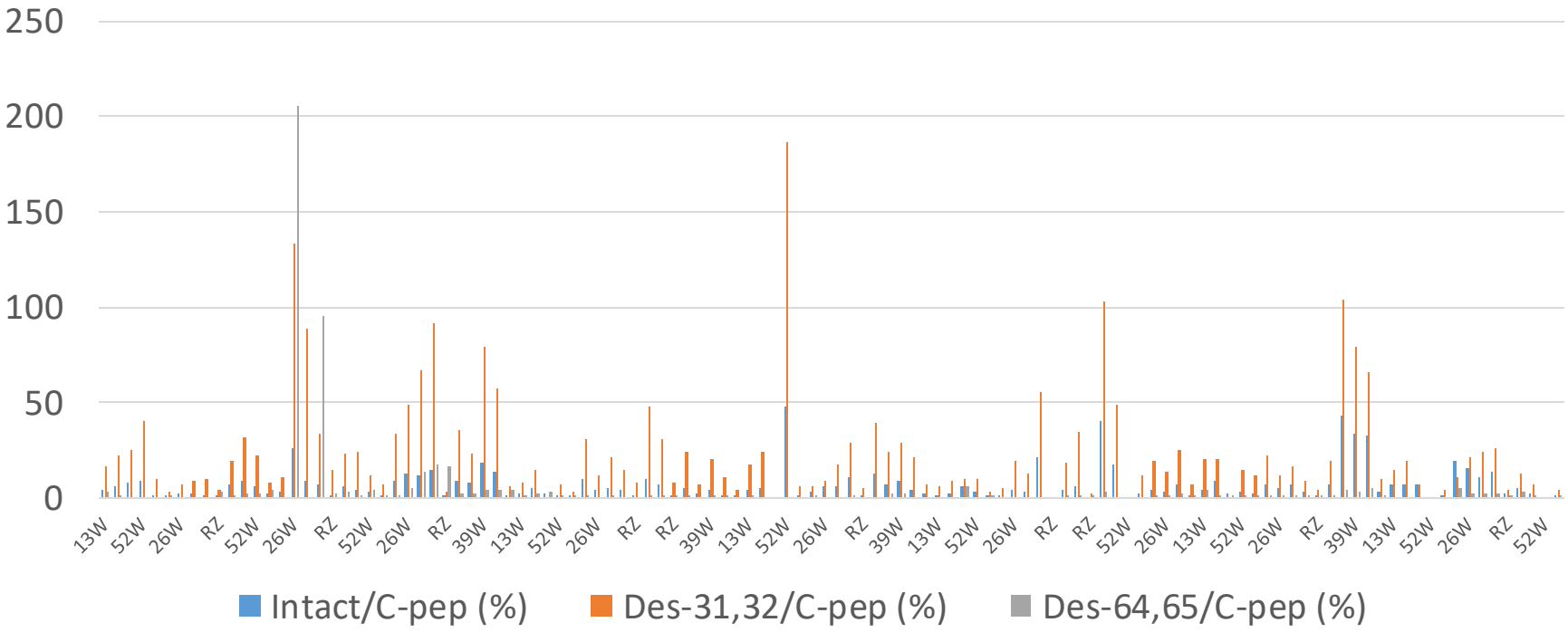
Concentrations (pM)



- **25 patients followed at 13W, 26W, 39W, and 52w after T1D diagnosis.**



Des-31, 32 is the most abundant intermediate form in T1D



Summary

- **The first method for accurate measurement of intact proinsulin, des-31,32 proinsulin, des-64,65 proinsulin, and C-peptide in circulation**

✓ Achieved ultra-high sensitivity (2-5 pg/mL) with excellent robustness and reasonable throughput (15 min/cycle).

✓ Enabled profiling of proinsulin processing *in vivo* with unprecedented detail

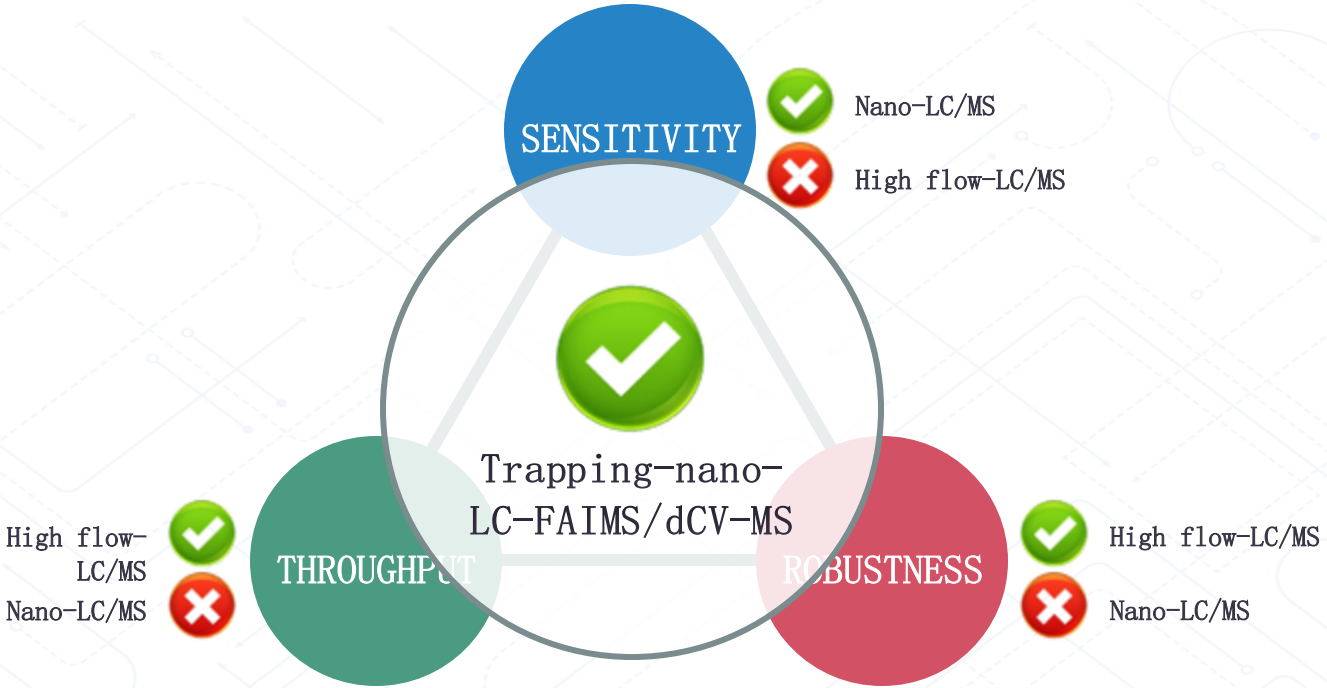
✓ Showed superior sensitivity and selectivity to a total proinsulin ELISA method.

- **Novel observations:**

✓ All forms of proinsulin and their ratios to C-peptide are abnormally elevated in the T1D setting, consistent with β-cell processing dysfunction.

✓ The three forms of proinsulin showed unique patterns, which may reflect different types of β-cell stress and disease subtypes/outcomes.

✓ Among the three proinsulin forms, the **des-31,32 proinsulin-to-C-peptide ratio** appeared to be the most robust discriminator of T1D.


SENSITIVITY
Nano-LC/MS
High flow-LC/MS
Trapping-nano-LC-FAIMS/dCV-MS
THROUGHPUT
High flow-LC/MS
Nano-LC/MS
ROBUSTNESS
High flow-LC/MS
Nano-LC/MS

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Summary

- **Potential clinical application of the assay:**

✓ Staging, early detection and risk stratification;

✓ Refining the classification and phenotypes of the T1D;

✓ Monitor disease progress and therapeutic response;

✓ Characterization of β-cell dysfunction (*e.g.* detect subclinical β-cell dysfunction) and monitor β-cell-centric treatment effects.

- **Limitation of the assay**

✓ Signature peptide-based assay may not represent the exact molecular form of the targets;

✓ Unknown format or variants (e.g., truncation or modifications) containing the SP could interfere with the assay.

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TaMADOR
Targeted Mass Spectrometry Assays for Diabetes and Obesity Research

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