



Translation and Implementation of the PeptiQuant™ Plus Human Plasma BAK-270

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Abstract

The large-scale quantitation of proteins in biomarker screening exercises is being increasingly employed in the proteomics field to discover, and ultimately validate, disease indicators. These evaluations are commonly performed in blood plasma samples by liquid chromatography (LC)-tandem mass spectrometry (MS/MS). To assist researchers' developments and applications, Cambridge Isotope Laboratories, Inc. (CIL) supplies PeptiQuant™ Plus biomarker assessment kits (BAKs) for plasma protein interrogations by bottom-up LC-MS/MS. As part of a joint collaboration with CIL and Université de Montpellier, the latest BAK-270 was translated to a Shimadzu LC-MS platform at the Université de Montpellier. This application note outlines that process, its results, and an overview into its clinical application at Montpellier Hospital.

Keywords

Human plasma, human serum, biomarkers, multiplex, LC-MRM

Introduction

Considerable efforts are being leveraged year over year to discover and validate protein biomarkers using MS-based quantitative approaches. The motivation for this lies in personalized or precision medicine whereby biomarkers (i.e., biological indicators) are sought for enhanced disease monitoring, companion diagnostics, and improved patient outcomes. Quantitation can be accomplished in a number of ways, with the use of targeted MS/MS (acquisition mode: multiple reaction monitoring, MRM) and stable isotope-labeled standards (SIS, be it peptides or proteins) being a popular implementation within a bottom-up proteomics regime. In its most basic orientation, an MRM with SIS peptide approach can quantify multiplexed panels of plasma proteins, many of which have putative or approved association(s) to disease and cancer. In this type of method operation, the labeled standards are constructed to

resemble the chemical structure of their endogenous (natural or NAT) analogue.

MS screening studies can be aided by commercialized kits that have been validated for their intended use. One type of kit is PeptiQuant Plus, with the biomarker assessment line designed for the precise and rapid quantitation of proteins (e.g., 270 using 270 peptides as surrogates) in undepleted and nonenriched human plasma.^{1,2} Favorable to the PeptiQuant kits is that they have been rigorously characterized according to the complete set of CPTAC (Clinical Proteomic Tumor Analysis Consortium) guidelines.³ To help facilitate standardization, the kits are supplied with key materials and tools for broad implementation. While the kits are already optimized to select LC-MS platforms, this may not be applicable to all users' experimental setups.

In this note, the procedure and test results for translating an off-the-shelf PeptiQuant Plus BAK-270* to the clinical proteomics laboratory at Montpellier Hospital is described. The full co-author list has experience in this type of translation having successfully adapted previous PeptiQuant kits to other platforms for alternate quantitative proteomic sample applications.⁴⁻⁸ The supplied materials in the BAK-270 were all utilized in the development and translation, with the final bottom-up LC-MRM/MS method ultimately applied to human plasma/serum samples. Note that while the defined translation here is for a Shimadzu platform (comprises Shim-pack GISS-HP HPLC column coupled to an 8060 triple quadrupole mass spectrometer), other LC-MS arrangements should be extendable.

*PeptiQuant is a trademark of MRM Proteomics Inc., the commercial product originator.

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Experimental

Materials

All chemicals were of the highest reagent grade and the solvents (for sample and eluent preparations) of LC-MS grade. The following lyophilized materials were supplied with the 100 sample BAK-270 (catalog no. **BAK-A6490-270-100**) and were stored at -80°C until use:

- SIS peptide mix ("S" on clear-capped vial, quantity 1)
- NAT peptide mix (clear-capped vial, quantity 1)
- TPCK-treated trypsin (purple-capped vial, quantity 1)
- Bovine serum albumin (green-capped vial, quantity 1)

CRYOcheck™ Normal Reference Plasma (Precision BioLogic) was used as a test matrix for optimizing the peptide mix targets. Also used in the evaluations were nonpathological, anonymized plasma (K₂-EDTA anticoagulant, n = 4) and serum (n = 3) samples from Montpellier's Neurological and Clinical Research Memory Center of cognitive/behavioral disorders (Biobank registry no. DC-2008-417).

Solution Preparations

The SIS and NAT peptide mixtures were solubilized with 682 and 65 µL, respectively, of solvent (0.1% formic acid, FA, in 30% acetonitrile, ACN). These were vortexed and centrifuged briefly then stored on ice or at -80°C until use. All reagents for the bottom-up sample preparations were prepared up-front (e.g., buffer, denaturant, reductant) using a protocol akin to that published previously. Trypsin was solubilized immediately prior to use and stored on ice until dispense.

Sample Preparations

Preparations were performed in 1.5 mL LoBind microcentrifuge tubes (Eppendorf). A 10 µL aliquot of undiluted plasma or serum was treated with a denaturant (9 M urea in 300 mM Tris pH 8.0), a reductant (20 mM dithiothreitol in 300 mM Tris pH 8.0), and an alkylating agent (100 mM iodoacetamide). Denaturation and reduction occurred simultaneously at 37°C for 30 min, while alkylation occurred afterward in the dark at ambient temperature for 30 min. After decreasing the concentration of urea to 0.5 M with 272 µL of 100 mM Tris (pH 8.0), proteolysis was initiated by the addition of 35 µL of TPCK-treated trypsin (1 mg/mL) at a 20:1 w:w substrate:enzyme ratio. Proteolysis proceeded for 18 h at 37°C, after which time a digest aliquot was combined with chilled solutions of FA (43 µL at 20%) and a SIS peptide mixture (10 µL) in a 96-well 1.2 mL polypropylene deepwell storage plate (Abgene™, Thermo Scientific).

After the SIS peptide addition, an aliquot was concentrated by solid phase extraction on AssayMAP C₁₈ cartridges using the AssayMAP Bravo (Agilent). Peptide samples were eluted with 30 µL of elution buffer (0.1% FA in 70% ACN) into a 96-well plate. The concentrated eluates were then frozen, lyophilized to dryness, and rehydrated in 60 µL of mobile phase A (composition: 0.1% FA in 2% ACN) for LC-MRM/MS. The concentration of the peptide mixture was estimated to be 1 µg/µL (based on an estimated 60 mg/mL total protein concentration). Samples were kept on ice until processing.

LC-MRM/MS

The LC-MS platform consisted of a reversed-phase HPLC column (150 × 2.1 mm, 3 µm particles; Shim-pack GISS-HP; Shimadzu) interfaced to a triple quadrupole (QqQ) mass spectrometer (8060, Shimadzu) via an ESI source (operated in the positive ion mode). The column was maintained in a thermostatted compartment (at 50°C) within a Nexera LC-40 system (Shimadzu). Peptide mixtures (from vials housed in a 4°C autosampler) were separated at a flow rate of 0.4 mL/min using the following ACN gradient (time in min, eluent B composition in %): 0, 2; 2, 7; 48, 30; 51, 45; 51.5, 80; 53.5, 80; 54, 2. Mobile phase B was 0.1% FA in 100% ACN. Each run was followed by an injection of 0.1% FA at the starting eluent conditions for a 4 min column equilibration.

All MRM transitions and corresponding collision energies (CEs) were empirically determined from tuning experiments using the SIS peptide mixture. The general and specific MS/MS parameters utilized in the optimized assays are listed in **Tables 1** and **2**, respectively. Monitoring the BAK quantifier panel with 3 min MRM detection windows provides minimum dwell times of 18 ms.

Table 1. Source and gas parameters used in the 8060 QqQ. Ultra-high-purity nitrogen served as the carrier gas in all settings and unit resolution (0.7 Da full-width-at-half maximum) was used in the quadrupoles.

Parameter	Value
Interface voltage	1.7 kV
Heat block temperature	400°C
Interface temperature	300°C
Desolvation line temperature	250°C
Nebulizing gas flow rate	3 L/min
Heating gas flow rate	10 L/min
Drying gas flow rate	10 L/min

Table 2. Specific MRM parameters for the SIS and NAT peptides monitored on the 8060 QqQ. Quantifier transitions are listed here only, with the peptide sequences detailed in the kit user's manual. **Note:** The SIS peptides are $^{13}\text{C}/^{15}\text{N}$ -labeled at their C-terminal L-Lys or L-Arg residue (equates to +6, +8, or +10 Da shift from NAT) and were synthesized by solid-phase chemistry using CIL's protected amino acids (e.g., **CNLM-8474-H** for L-Arg and **CNLM-4754-H** for L-Lys).

Protein	Peptide Product Ion Type	MRM Transition (Q1 → Q3 <i>m/z</i>)		Retention Time (min)		CE (V)
		NAT	SIS	Start	End	
60 kDa Heat shock protein mitochondrial	y_5^{2+}	372 → 573	376 → 581	10.0	13.0	14.0
72 kDa Type IV collagenase	y_8^{2+}	696 → 993	700 → 1001	12.0	15.0	24.0
78 kDa Glucose-regulated protein	y_{12}^{2+}	783 → 676	788 → 681	25.0	28.0	26.0
A disintegrin and metalloproteinase with thrombospondin motifs 2	y_6^{2+}	453 → 680	457 → 688	25.7	28.7	16.5
A disintegrin and metalloproteinase with thrombospondin motifs 9	y_5^{2+}	438 → 600	443 → 610	8.9	11.9	16.0
A disintegrin and metalloproteinase with thrombospondin motifs 20	y_2^{2+}	563 → 288	568 → 298	14.5	17.5	25.0
Actin, aortic smooth muscle	y_4^{3+}	597 → 475	601 → 485	30.0	33.0	17.0
Adhesion G protein-coupled receptor F5	y_8^{3+}	377 → 458	380 → 462	24.2	27.2	12.0
Adipocyte plasma membrane-associated protein	y_7^{2+}	555 → 883	560 → 893	16.6	19.6	19.5
Adiponectin	y_8^{2+}	886 → 864	890 → 872	13.1	16.1	34.0
ADM	y_5^{2+}	468 → 609	473 → 619	19.3	22.3	18.0
Afamin	y_7^{2+}	563 → 825	567 → 833	21.5	24.5	20.0
Alpha-1-acid glycoprotein 1	y_{13}^{3+}	570 → 704	573 → 708	21.5	24.0	18.5
Alpha-1-antichymotrypsin	y_7^{2+}	531 → 819	535 → 827	26.0	29.0	20.0
Alpha-1-antitrypsin	y_8^{2+}	508 → 415	512 → 419	26.0	29.0	17.0
Alpha-1B-glycoprotein	y_8^{2+}	619 → 498	623 → 502	35.5	38.0	21.5
Alpha-2-antiplasmin	y_8^{2+}	656 → 771	660 → 779	7.4	10.4	27.0
Alpha-2-HS-glycoprotein	y_5^{2+}	407 → 579	411 → 587	14.8	17.8	15.0
Alpha-2-macroglobulin	y_6^{2+}	628 → 738	633 → 748	17.0	19.9	26.0
Angiogenin	y_4^{2+}	579 → 455	583 → 463	16.6	19.6	29.5
Angiopietin-related protein 3	y_8^{3+}	416 → 510	419 → 514	26.1	29.1	14.0
Angiotensinogen	y_{10}^{2+}	634 → 542	638 → 546	28.4	30.9	20.0
Antithrombin-III	y_8^{3+}	437 → 483	439 → 487	19.4	21.9	14.0
Apolipoprotein A-I	y_{10}^{3+}	405 → 572	408 → 576	6.4	8.9	16.5
Apolipoprotein A-II	y_8^{2+}	486 → 443	490 → 447	5.5	8.0	21.5
Apolipoprotein A-IV	y_6^{2+}	704 → 631	708 → 639	17.3	19.8	28.5
Apolipoprotein B-100	y_8^{2+}	524 → 450	528 → 454	26.7	29.7	22.5
Apolipoprotein C-I	y_6^{2+}	601 → 739	605 → 747	26.1	28.6	23.0
Apolipoprotein C-II	y_6^{2+}	518 → 658	522 → 666	15.0	18.0	18.5
Apolipoprotein C-III	y_8^{2+}	598 → 854	602 → 862	28.7	31.7	21.0
Apolipoprotein C-IV	y_6^{2+}	536 → 717	541 → 727	19.9	22.9	23.0
Apolipoprotein D	y_8^{2+}	615 → 890	619 → 898	19.4	22.4	21.5
Apolipoprotein E	y_7^{2+}	484 → 399	489 → 404	13.0	16.0	18.5
Apolipoprotein F	b_6^{3+}	566 → 613	569 → 613	29.5	32.0	19.0
Apolipoprotein L1	y_5^{2+}	473 → 647	477 → 655	4.4	7.4	19.0
Apolipoprotein M	y_5^{2+}	409 → 599	414 → 609	23.0	26.0	16.0
Apolipoprotein(a)	y_7^{2+}	521 → 721	526 → 731	5.3	8.3	21.5
Aromatase	y_7^{2+}	626 → 893	631 → 903	41.0	44.0	22.0
Atrial natriuretic peptide receptor 1	y_8^{2+}	600 → 986	605 → 996	23.7	26.7	22.0
Attractin	y_6^{2+}	443 → 700	448 → 710	6.8	9.8	16.0
Autism susceptibility gene 2 protein	y_{10}^{2+}	561 → 938	565 → 946	8.8	11.8	22.0
B-cell scaffold protein with ankyrin repeats	y_8^{3+}	353 → 422	356 → 426	4.4	7.4	12.5
Beta-2-glycoprotein 1	y_5^{2+}	511 → 652	516 → 662	6.0	9.0	22.0
Beta-2-microglobulin	y_4^{3+}	374 → 459	377 → 467	6.0	8.5	15.0
Beta-Ala-His dipeptidase	y_5^{2+}	620 → 570	624 → 578	25.7	28.2	24.5
Beta-nerve growth factor	y_5^{2+}	375 → 547	379 → 555	3.0	6.0	14.0
Biotinidase	b_4^{3+}	360 → 451	363 → 451	15.2	17.7	14.5
C4b-binding protein alpha chain	y_5^{2+}	625 → 545	630 → 555	27.8	30.8	24.0
Cadherin-13	y_7^{2+}	638 → 705	643 → 715	7.6	10.6	31.5

Protein	Peptide Product Ion Type	MRM Transition (Q1 → Q3 <i>m/z</i>)		Retention Time (min)		CE (V)
		NAT	SIS	Start	End	
Cadherin-5	y_6^{2+}	553 → 560	557 → 568	4.3	7.3	21.5
Calcitonin	y_7^{3+}	543 → 585	546 → 593	22.0	25.0	16.5
Calcitonin gene-related peptide 1	y_7^{2+}	588 → 702	592 → 710	13.9	16.9	24.5
Calponin-1	y_5^{2+}	308 → 516	312 → 524	4.9	7.9	12.5
Carbonic anhydrase 1	y_7^{2+}	485 → 758	489 → 766	25.0	28.0	18.5
Carboxypeptidase B2	y_4^{2+}	447 → 524	452 → 534	16.9	19.9	23.0
Carboxypeptidase N catalytic chain	y_7^{2+}	491 → 391	496 → 396	14.8	17.8	15.5
Carboxypeptidase N subunit 2	y_6^{2+}	644 → 715	649 → 725	19.9	22.4	31.5
Cartilage acidic protein 1	y_7^{2+}	439 → 721	444 → 731	21.8	24.8	20.0
Cathelicidin antimicrobial peptide	y_6^{2+}	443 → 702	448 → 712	6.3	9.3	17.0
Cation-independent mannose-6-phosphate receptor	y_4^{3+}	404 → 457	407 → 467	8.2	11.2	13.5
CD40 ligand	y_6^{2+}	471 → 726	475 → 734	16.5	19.5	17.0
CD44 antigen	y_{11}^{3+}	462 → 612	466 → 617	26.0	29.0	17.0
CD5 antigen-like	y_5^{2+}	376 → 539	381 → 549	5.5	8.3	17.0
cDNA FLJ53327	y_5^{2+}	537 → 531	542 → 541	2.0	5.0	23.0
Ceruloplasmin	y_8^{3+}	394 → 452	396 → 456	5.3	7.8	16.0
Cholesteryl ester transfer protein	y_6^{2+}	844 → 728	849 → 738	51.1	54.1	39.0
Cholinesterase	y_8^{2+}	599 → 921	604 → 931	14.5	17.5	25.0
Chromogranin-A	y_6^{2+}	593 → 587	597 → 595	18.8	21.8	21.0
Claudin-5	y_6^{2+}	451 → 690	455 → 698	23.9	26.9	16.5
Clusterin	y_3^{2+}	644 → 375	649 → 385	16.7	19.2	21.5
Coagulation factor IX	y_5^{2+}	531 → 692	536 → 702	31.3	34.3	20.0
Coagulation factor V	y_8^{2+}	572 → 943	577 → 953	19.1	22.1	20.0
Coagulation factor VII	y_7^{2+}	647 → 979	651 → 987	28.0	31.0	24.5
Coagulation factor VIII	y_7^{3+}	375 → 437	378 → 442	5.5	8.5	13.0
Coagulation factor X	y_5^{2+}	561 → 649	566 → 659	26.1	28.6	21.0
Coagulation factor XI	y_4^{2+}	517 → 460	522 → 470	6.0	8.5	22.5
Coagulation factor XII	y_5^{2+}	464 → 573	469 → 583	7.5	10.0	26.5
Coagulation factor XIII A chain	b_4^{2+}	844 → 435	848 → 435	39.8	42.8	27.0
Coagulation factor XIII B chain	y_5^{3+}	369 → 627	372 → 637	3.6	6.1	18.5
Collagen alpha-1(I) chain	y_4^{2+}	441 → 457	446 → 467	13.8	16.8	17.0
Collagen alpha-1(III) chain	y_7^{2+}	490 → 641	494 → 649	3.0	6.0	25.5
Collagen alpha-1(XVIII) chain	y_7^{2+}	446 → 721	451 → 731	17.5	20.5	17.0
Collagen alpha-2(I) chain	y_5^{2+}	420 → 528	425 → 538	4.3	7.3	19.5
Complement C1q subcomponent subunit A	y_5^{2+}	381 → 593	386 → 603	12.9	15.9	19.0
Complement C1q subcomponent subunit B	y_5^{2+}	383 → 581	388 → 591	10.5	13.0	15.0
Complement C1q subcomponent subunit C	y_7^{2+}	542 → 809	547 → 819	23.3	26.3	23.0
Complement C1r subcomponent	y_5^{2+}	391 → 611	395 → 619	14.8	17.8	15.5
Complement C1r subcomponent-like protein	y_4^{2+}	492 → 550	497 → 560	6.3	9.3	24.5
Complement C1s subcomponent	y_4^{2+}	639 → 458	644 → 468	24.8	27.8	22.5
Complement C2	b_4^{3+}	358 → 469	360 → 469	12.9	15.9	12.5
Complement C3	y_6^{2+}	501 → 731	505 → 739	12.4	15.4	19.0
Complement C4	y_3^{2+}	771 → 373	775 → 381	18.1	20.6	35.5
Complement C5	y_5^{2+}	455 → 664	459 → 672	26.1	28.6	16.5
Complement component C6	y_4^{3+}	396 → 506	398 → 514	32.3	35.3	16.5
Complement component C7	y_9^{2+}	565 → 494	570 → 499	6.0	8.5	20.0
Complement component C8 alpha chain	y_7^{2+}	497 → 733	502 → 743	14.2	17.2	21.5
Complement component C8 beta chain	y_7^{3+}	354 → 430	357 → 434	8.5	11.0	12.5
Complement component C9	y_9^{2+}	621 → 521	625 → 525	34.6	37.6	21.5
Complement factor B	y_6^{2+}	578 → 671	582 → 679	19.9	22.9	20.5
Complement factor D	y_3^{3+}	379 → 405	382 → 415	1.9	4.9	18.0
Complement factor H	y_4^{3+}	398 → 442	401 → 450	2.3	5.3	22.5
Complement factor I	y_8^{2+}	596 → 946	600 → 954	31.9	34.9	21.0

Protein	Peptide Product Ion Type	MRM Transition (Q1 → Q3 <i>m/z</i>)		Retention Time (min)		CE (V)
		NAT	SIS	Start	End	
Corticosteroid-binding globulin	y_4^{2+}	883 → 520	887 → 528	34.8	37.8	36.0
C-reactive protein	y_4^{2+}	354 → 490	358 → 498	20.0	23.0	14.0
Creatine kinase B-type	y_6^{2+}	616 → 742	621 → 752	34.3	37.3	25.5
Creatine kinase M-type	y_5^{2+}	454 → 631	459 → 641	17.0	20.0	15.5
Cystatin-C	y_6^{2+}	613 → 709	617 → 717	23.0	26.0	21.5
Desmoplakin	y_4^{2+}	570 → 486	574 → 494	24.3	27.3	29.0
Dickkopf-related protein 1 and 2	y_3^{3+}	381 → 450	385 → 460	18.6	21.6	16.0
Di- <i>N</i> -acetylchitinase	y_4^{2+}	514 → 580	519 → 590	9.7	12.2	22.5
Elastin	y_{12}^{2+}	662 → 605	666 → 609	23.8	26.8	27.0
Endothelial lipase	y_7^{2+}	448 → 783	453 → 793	6.2	9.2	230
Endothelial protein C receptor	y_7^{2+}	516 → 817	521 → 827	35.8	38.8	19.5
Epidermal growth factor receptor	y_9^{2+}	604 → 548	609 → 553	33.2	36.2	25.0
E-selectin	b_3^{3+}	396 → 402	398 → 402	11.0	13.5	22.5
Extracellular matrix protein 1	y_8^{2+}	659 → 821	663 → 829	10.7	13.2	22.0
Fatty acid-binding protein heart	y_7^{2+}	454 → 707	459 → 717	18.4	21.4	17.5
Ferritin heavy chain	y_9^{3+}	432 → 541	434 → 545	17.9	20.9	14.0
Ferritin light chain	y_7^{2+}	804 → 913	809 → 923	36.3	39.3	34.5
Fetuin-B	y_6^{2+}	456 → 700	460 → 708	34.6	37.1	16.5
Fibrinogen alpha chain	y_7^{2+}	553 → 879	557 → 887	12.9	15.9	21.5
Fibrinogen beta chain	y_5^{3+}	709 → 600	713 → 610	26.3	28.8	29.5
Fibrinogen gamma chain	y_{11}^{3+}	497 → 600	501 → 605	16.6	19.6	16.0
Fibronectin	y_6^{3+}	622 → 734	625 → 744	12.4	15.4	21.5
Fibulin-1	y_6^{2+}	589 → 694	594 → 704	21.7	24.2	24.5
Ficolin-2	y_5^{3+}	463 → 617	466 → 625	18.1	20.6	14.0
Ficolin-3	y_7^{3+}	349 → 713	352 → 721	4.6	7.1	16.0
Follistatin-related protein 1	y_5^{2+}	454 → 645	458 → 653	5.6	8.6	14.5
Fructose-biphosphate aldolase B	y_{10}^{2+}	622 → 931	626 → 939	25.9	28.9	24.5
Galectin-3	y_3^{2+}	431 → 450	436 → 460	21.1	24.1	25.5
Galectin-3-binding protein	y_8^{2+}	678 → 870	682 → 878	37.7	40.2	33.5
Gamma-enolase	y_5^{3+}	620 → 664	623 → 674	42.7	45.7	24.5
Gelsolin	b_3^{2+}	660 → 200	664 → 200	23.9	26.9	29.0
Glial fibrillary acidic protein	y_6^{2+}	589 → 779	594 → 789	18.7	21.7	22.5
Glutamate receptor ionotropic NMDA 2A	y_4^{2+}	477 → 444	481 → 452	18.3	21.3	25.0
Glutamate receptor ionotropic NMDA 2B	y_7^{2+}	545 → 749	549 → 757	17.0	20.0	23.5
Glutathione S-transferase P	y_5^{2+}	376 → 537	380 → 545	13.2	16.2	15.0
Glutathione peroxidase 3	y_{12}^{2+}	777 → 649	781 → 653	24.9	27.4	26.5
Haptoglobin	y_9^{2+}	645 → 496	649 → 500	33.0	36.0	21.5
Heat shock protein beta-1	y_5^{2+}	582 → 589	587 → 599	32.3	35.3	24.5
Hemoglobin subunit alpha	y_4^{3+}	510 → 488	513 → 498	11.1	14.1	19.0
Hemopexin	y_9^{2+}	610 → 480	615 → 485	27.6	30.6	20.5
Heparin cofactor 2	y_6^{2+}	514 → 685	519 → 695	14.5	17.5	22.5
Hepatocyte growth factor-like protein	y_7^{2+}	594 → 891	599 → 901	28.9	31.9	30.0
Histidine-rich glycoprotein	y_3^{2+}	912 → 331	916 → 339	41.9	44.9	40.0
Hornerin	y_{13}^{3+}	583 → 637	586 → 642	3.5	6.5	25.5
Hyaluronan-binding protein 2	y_7^{2+}	493 → 788	497 → 796	14.3	17.3	17.5
Ig gamma-1 chain C region	y_4^{2+}	593 → 418	597 → 426	25.4	28.4	31.0
Ig mu chain C region	y_5^{2+}	388 → 571	393 → 581	21.9	24.4	14.5
Ig mu heavy chain disease protein and Ig mu chain C	y_5^{2+}	450 → 615	455 → 625	19.4	22.4	17.5
IgGfC-binding protein	y_9^{2+}	712 → 1007	717 → 1017	15.5	18.5	25.5
Immunoglobulin kappa variable 4-1	y_{13}^{3+}	606 → 770	609 → 774	20.6	23.6	18.0
Insulin-like growth factor I	y_{13}^{3+}	556 → 732	559 → 737	17.5	20.5	17.0
Insulin-like growth factor-binding protein 1	y_{12}^{3+}	510 → 673	514 → 678	19.0	22.0	15.0
Insulin-like growth factor-binding protein 2	y_7^{2+}	484 → 742	489 → 752	15.1	18.1	21.5

Protein	Peptide Product Ion Type	MRM Transition (Q1 → Q3 <i>m/z</i>)		Retention Time (min)		CE (V)
		NAT	SIS	Start	End	
Insulin-like growth factor-binding protein 3	y_6^{2+}	473 → 685	478 → 695	26.1	29.1	21.0
Insulin-like growth factor-binding protein complex acid labile subunit	b_3^{2+}	732 → 314	737 → 314	31.8	34.8	25.5
Inter-alpha-trypsin inhibitor heavy chain H1	y_7^{3+}	668 → 806	672 → 816	34.4	37.4	28.5
Inter-alpha-trypsin inhibitor heavy chain H2	y_7^{2+}	415 → 629	419 → 637	5.8	8.8	14.0
Inter-alpha-trypsin inhibitor heavy chain H4	y_8^{3+}	604 → 457	608 → 462	30.6	33.6	17.0
Intercellular adhesion molecule 1	y_8^{2+}	540 → 854	544 → 862	31.1	34.1	20.0
Interleukin-10	y_5^{3+}	394 → 560	397 → 568	7.5	10.5	12.0
Interleukin-6	y_7^{2+}	541 → 806	546 → 816	2.9	5.9	20.0
Interstitial collagenase	y_6^{2+}	877 → 706	881 → 714	44.7	47.7	39.0
Kallistatin	y_{11}^{3+}	429 → 593	431 → 597	21.6	24.6	14.0
Keratin type I cytoskeletal 10	y_{10}^{2+}	631 → 820	636 → 830	8.7	11.7	26.0
Keratin type I cytoskeletal 9	y_7^{2+}	530 → 846	535 → 856	20.0	23.0	18.0
Keratin-type II cytoskeletal 2 epidermal	y_6^{2+}	597 → 659	602 → 669	18.1	21.1	25.0
Kininogen-1	y_{17}^{3+}	713 → 637	716 → 640	31.6	34.6	18.5
Lactotransferrin	y_{11}^{2+}	768 → 602	772 → 606	31.8	34.8	29.5
Leucine-rich alpha-2-glycoprotein	y_6^{2+}	590 → 725	595 → 735	32.0	35.0	20.5
Lipopolysaccharide-binding protein	y_8^{2+}	624 → 920	629 → 930	35.0	38.0	24.0
L-selectin	y_6^{2+}	497 → 794	501 → 802	15.0	18.0	18.0
Lumican	y_9^{3+}	433 → 549	435 → 553	15.3	18.3	14.0
Lysozyme C	y_4^{2+}	394 → 531	399 → 541	21.9	24.9	14.5
Mannan-binding lectin serine protease 1	y_4^{2+}	816 → 504	820 → 512	27.6	30.6	37.0
Mannan-binding lectin serine protease 2A	y_5^{2+}	494 → 575	499 → 585	26.6	29.6	22.5
Mannose-binding protein C	y_6^{2+}	476 → 652	480 → 660	34.2	37.2	19.0
Matrix Gla protein	y_5^{2+}	638 → 615	643 → 625	12.0	15.0	28.0
Matrix metalloproteinase-9	y_4^{2+}	489 → 464	494 → 474	15.0	18.0	18.5
Melanotransferrin	y_7^{2+}	571 → 815	576 → 825	18.1	21.1	24.0
Metalloproteinase inhibitor 1	y_7^{2+}	617 → 717	622 → 727	23.3	26.3	21.5
Metalloproteinase inhibitor 2	y_5^{2+}	397 → 501	401 → 509	12.4	15.4	13.5
Metalloproteinase inhibitor 4	y_{11}^{2+}	650 → 551	654 → 555	7.3	10.3	20.5
Microtubule-associated protein tau	y_6^{2+}	557 → 686	561 → 694	9.2	12.2	19.5
Mucin-16	y_4^{2+}	532 → 504	537 → 514	18.3	21.3	20.0
Myelin basic protein	y_8^{2+}	488 → 819	492 → 827	5.3	8.3	18.5
Myeloblastin	y_{10}^{3+}	430 → 539	434 → 544	18.8	21.8	13.0
Myeloperoxidase	y_5^{2+}	456 → 666	461 → 676	28.0	31.0	16.5
<i>N</i> (G), <i>N</i> (G)-dimethylarginine dimethylaminohydrolase 1	y_9^{2+}	575 → 525	579 → 529	5.0	8.0	23.5
<i>N</i> -acetylmuramoyl-L-alanine amidase	y_4^{3+}	518 → 482	521 → 492	24.9	27.9	23.0
Natriuretic peptides B	y_6^{2+}	437 → 646	442 → 656	6.0	9.0	19.0
Neuropilin-2	y_4^{2+}	343 → 501	348 → 511	7.7	10.7	14.0
Neutrophil gelatinase-associated lipocalin	y_4^{2+}	361 → 508	366 → 518	12.3	15.3	13.5
Nucleoside diphosphate kinase A, B	y_6^{2+}	439 → 634	443 → 642	29.2	32.2	17.0
Occludin	y_8^{2+}	638 → 947	642 → 955	21.7	24.7	22.0
Osteopontin	y_5^{2+}	483 → 607	488 → 617	15.7	18.7	16.5
Oxidized low-density lipoprotein receptor 1	y_6^{2+}	437 → 631	442 → 641	5.5	8.5	20.0
Pappalysin-1	y_6^{2+}	532 → 717	536 → 725	22.0	25.0	20.0
Peroxisoredoxin-1	y_3^{2+}	447 → 409	452 → 419	13.0	16.0	16.0
Peroxisoredoxin-2	y_6^{2+}	431 → 692	435 → 700	28.0	31.0	16.5
Phosphatidylcholine-sterol acyltransferase	y_6^{2+}	692 → 669	697 → 679	19.3	22.3	25.0
Phosphatidylinositol-glycan-specific phospholipase D	y_4^{2+}	490 → 488	495 → 498	22.2	25.2	21.5
Phospholipid transfer protein	y_8^{2+}	664 → 514	669 → 519	9.6	12.6	22.0
Pigment epithelium-derived factor	y_{10}^{2+}	692 → 1142	696 → 1150	29.0	32.0	25.0
Plasma protease C1 inhibitor	y_8^{2+}	593 → 910	598 → 920	33.9	36.9	21.0
Plasma serine protease inhibitor	y_4^{3+}	524 → 514	527 → 524	38.1	41.1	24.0
Plasminogen	y_6^{2+}	570 → 699	574 → 707	18.0	21.0	20.0

Protein	Peptide Product Ion Type	MRM Transition (Q1 → Q3 m/z)		Retention Time (min)		CE (V)
		NAT	SIS	Start	End	
Plasminogen activator inhibitor 1	y_5^{2+}	553 → 504	557 → 512	8.2	11.2	19.5
Plastin-2	y_7^{2+}	499 → 885	503 → 893	32.8	35.8	18.0
Platelet endothelial cell adhesion molecule	y_8^{2+}	762 → 896	766 → 904	26.5	29.5	36.0
Platelet glycoprotein VI	y_3^{2+}	438 → 407	442 → 415	4.1	7.1	16.0
Platelet-activating factor acetylhydrolase	y_8^{3+}	576 → 886	578 → 894	25.9	28.9	19.5
Pregnancy zone protein	y_9^{2+}	552 → 990	556 → 998	19.6	22.6	19.5
Proenkephalin-A	y_5^{2+}	523 → 562	528 → 572	8.9	11.9	22.5
Prolactin	y_5^{2+}	383 → 652	387 → 660	9.2	12.2	15.0
Protein AMBP	y_8^{2+}	481 → 412	485 → 416	2.3	5.3	21.0
Protein deglycase DJ-1	y_5^{2+}	364 → 543	368 → 551	21.1	24.1	14.5
Protein S100-A12	y_6^{2+}	452 → 710	456 → 718	5.9	8.9	15.5
Protein S100-A9	y_5^{2+}	439 → 649	443 → 657	24.9	27.9	17.0
Protein S100-B	y_4^{2+}	423 → 460	427 → 468	2.6	5.6	16.5
Protein Z-dependent protease inhibitor	y_6^{2+}	635 → 692	640 → 702	34.2	37.2	24.0
Proteoglycan 4	y_8^{2+}	685 → 963	690 → 973	19.6	22.6	24.5
Prothrombin	y_6^{2+}	597 → 710	602 → 720	25.1	28.1	22.0
P-selectin	y_6^{2+}	489 → 691	493 → 699	22.2	25.2	20.5
Ras GTPase-activating protein nGAP	y_8^{2+}	714 → 882	719 → 892	5.5	8.5	25.5
Resistin	y_7^{2+}	616 → 763	621 → 773	29.0	32.0	30.5
Retinol-binding protein 4	y_8^{2+}	599 → 849	603 → 857	35.5	38.5	21.0
Serotransferrin	y_7^{2+}	489 → 735	493 → 743	15.0	18.0	17.5
Serum albumin	y_8^{2+}	575 → 937	579 → 945	19.5	22.5	21.0
Serum amyloid A-1 and A-2 proteins	y_4^{2+}	498 → 406	502 → 414	5.2	8.2	18.0
Serum amyloid A-4 protein	y_4^{2+}	447 → 445	452 → 455	2.9	5.9	22.0
Serum amyloid P-component	y_{11}^{2+}	697 → 591	701 → 595	12.4	15.4	24.0
Serum paraoxonase/arylesterase 1	y_8^{2+}	942 → 868	947 → 878	34.4	37.4	38.0
Serum paraoxonase/lactonase 3	y_6^{2+}	514 → 688	518 → 696	24.0	27.0	23.5
Sex hormone-binding globulin	y_6^{2+}	456 → 724	461 → 734	7.5	10.5	16.5
SPARC	y_{11}^{3+}	473 → 589	477 → 594	19.8	22.8	15.0
Spermine oxidase	y_{12}^{3+}	518 → 614	521 → 619	11.6	14.6	16.0
Sterile alpha motif domain-containing protein 9-like	y_7^{2+}	601 → 746	605 → 754	21.9	24.9	21.0
Stromelysin-1	y_6^{2+}	524 → 784	528 → 792	23.0	26.0	19.5
Target of Nesh-SH3	y_8^{2+}	548 → 820	552 → 828	20.6	23.6	20.5
TBC1 domain family member 10A	y_7^{2+}	560 → 422	564 → 426	14.0	17.0	20.0
Tenascin	y_7^{2+}	526 → 803	531 → 813	13.0	16.0	19.5
Tenascin-X	y_6^{2+}	723 → 720	728 → 730	29.2	32.2	27.0
Tetranectin	y_5^{2+}	773 → 473	777 → 481	19.9	22.9	35.5
Thrombomodulin	y_7^{3+}	634 → 617	637 → 627	51.5	54.5	25.0
Thrombospondin-1	y_4^{2+}	436 → 488	441 → 498	21.6	24.6	16.0
Thrombospondin-4	y_4^{3+}	416 → 518	418 → 526	25.2	28.2	16.5
Thyroglobulin	y_{12}^{2+}	703 → 586	708 → 591	23.7	26.7	22.5
Thyroxine-binding globulin	y_6^{3+}	289 → 348	292 → 352	10.0	13.0	12.0
Tissue factor pathway inhibitor	y_7^{2+}	545 → 780	549 → 788	20.8	23.8	19.5
Tissue-type plasminogen activator	y_7^{2+}	507 → 816	511 → 824	3.3	6.3	18.0
Transcription factor SOX-1	y_5^{3+}	658 → 545	661 → 555	26.2	29.2	28.5
Transferrin receptor protein 1	y_{11}^{3+}	558 → 620	561 → 625	22.2	25.2	19.0
Transthyretin	y_{11}^{3+}	456 → 611	459 → 616	26.4	29.4	15.5
Tumor necrosis factor receptor superfamily member 1A	y_5^{2+}	501 → 587	506 → 597	24.8	27.8	19.0
Tumor necrosis factor receptor superfamily member 1B	y_4^{2+}	475 → 478	479 → 486	11.9	14.9	17.0
Vascular cell adhesion protein 1	y_4^{2+}	580 → 432	584 → 440	12.6	15.6	30.5
Vascular endothelial growth factor B	y_8^{2+}	619 → 1039	624 → 1049	31.5	34.5	23.5
Vascular endothelial growth factor D	y_5^{2+}	425 → 622	429 → 630	6.1	9.1	19.5
Vascular non-inflammatory molecule 3	y_5^{2+}	381 → 531	385 → 539	2.6	5.6	15.0

Protein	Peptide Product Ion Type	MRM Transition (Q1 → Q3 m/z)		Retention Time (min)		CE (V)
		NAT	SIS	Start	End	
Vasorin	y_7^{2+}	575 → 746	580 → 756	13.9	16.9	23.5
Vitamin D-binding protein	y_5^{2+}	400 → 587	404 → 595	13.1	16.1	14.5
Vitamin K-dependent protein C	y_6^{2+}	433 → 752	438 → 762	12.5	15.5	19.5
Vitamin K-dependent protein S	y_6^{2+}	478 → 694	483 → 704	26.1	29.1	17.0
Vitamin K-dependent protein Z	y_8^{3+}	404 → 474	407 → 479	28.2	31.2	13.5
Vitronectin	y_5^{2+}	711 → 647	716 → 657	23.7	26.7	34.5
von Willebrand factor	y_9^{2+}	620 → 886	624 → 894	14.5	17.5	24.5
Xaa-Pro dipeptidase	y_4^{2+}	460 → 458	465 → 468	17.0	20.0	23.5
Zinc-alpha-2-glycoprotein	y_{14}^{2+}	892 → 770	896 → 774	43.0	46.0	28.5

Data Processing and Analysis

Data acquisitions were collected in LabSolutions software (version 5.99 SP2, Shimadzu), while analysis of the raw data was performed with Skyline software (version 20.2.0). The extracted ion chromatograms (XICs) were manually inspected in the Skyline files first to ensure correct peak selection and accurate integration before evaluating figures of merit in Excel.

Results and Discussion

The BAKs are designed to quantify a multiplexed panel of high-to-low abundance proteins (general concentration range: mg/mL to sub ng/mL) in undepleted and nonenriched biosamples. Before applying the 270-plex to target applications, preliminary optimization and method testing on Université de Montpellier's Shimadzu instrument platform must first be conducted. The following sections discuss these results and outlines a few insights into the current research applications being conducted at Montpellier Hospital.

Method Development

The specific MRM acquisition parameters for the 270-peptide panel were optimized by empirical measurement of the SIS peptide mix. This was accomplished by unscheduled LC-MRM. CE was programmed based on Skyline prediction then optimized by screening a voltage ramp from the predicted value. Stemming from this data, the three most abundant ion pairs (reflects a quantifier and two qualifiers in application) were compiled, along with their corresponding CE voltages, for each peptide. The selected product ions were predominately "y" and were chosen without regard to their precursor charge (double or triple). Identical collision energies were used for the NAT peptides because their physicochemical properties are the same.

Following the instrument optimizations, the SIS and NAT peptide mixes were evaluated by LC-MS/MS in unscheduled MRM mode. Slight modification to the LC gradient supplied in the kit manual constituted the final method, with all 270 peptides being detectable. The peptide extracted ion chromatograms (XICs) in the matrix-free analysis were observed to be symmetric and devoid of interference, while the LC gradient produced a relatively even distribution of peptides across the chromatographic space (Figure 1).

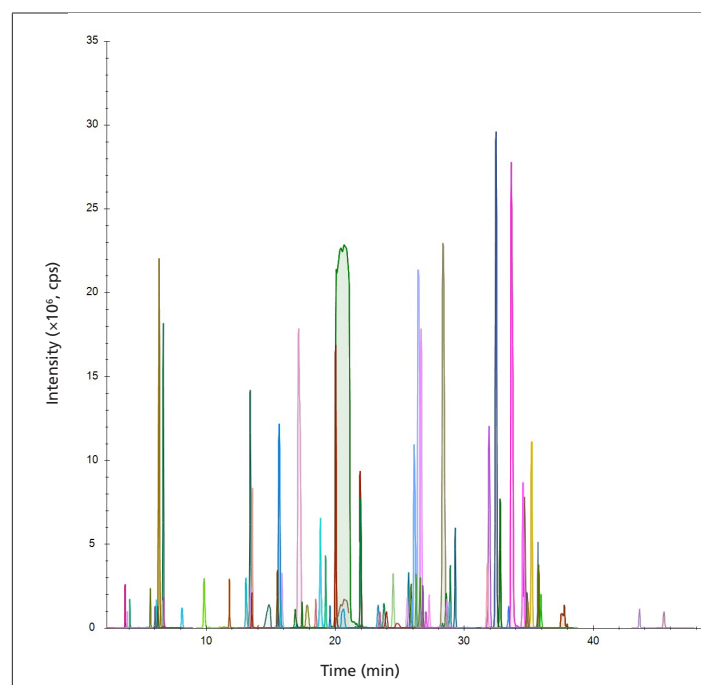


Figure 1. XIC trace of a panel of 270 target peptides measured by RPLC-MRM/MS on the Shimadzu platform (Shim-pack GISS-HP column, +ESI, 8060 QqQ).

Biosample Evaluation

To evaluate the method's fit-for-purpose, commercial plasma (CRYOcheck™) and biobanked plasma/serum (Montpellier Hospital) samples were evaluated by bottom-up LC-MRM/MS. General concordance was observed in type and number of peptides detected in the reference plasma and patient sample pools. In the sample pools, 213 interference-free peptides were observed. These peptides correspond to proteins of high (e.g., antithrombin-III, fibrinogen chains, haptoglobin), moderate (e.g., apolipoproteins, complement components and factors), and low (e.g., insulin-like growth factors, metalloproteinase inhibitors, creatine kinase M-type) abundance. **Figure 2** illustrates a representative set of peptide XICs for different protein abundance levels measured in human plasma by LC-MRM/MS.

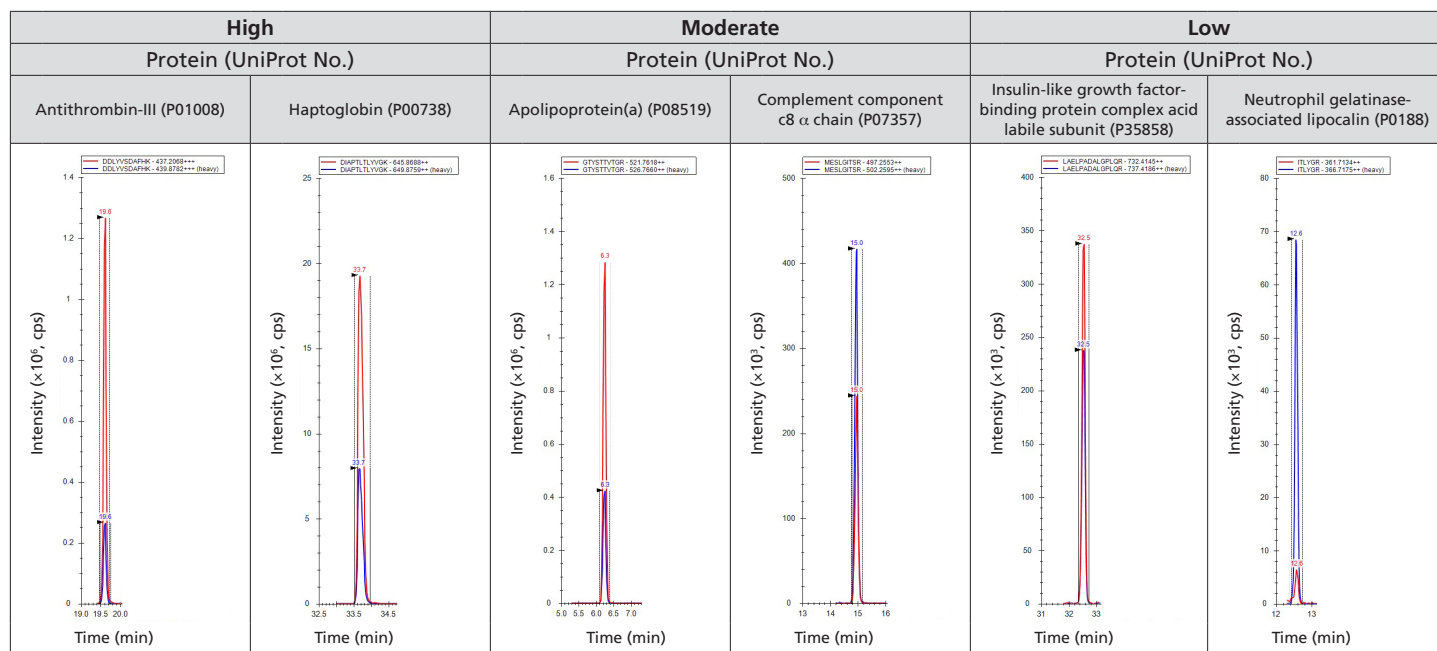


Figure 2. Peptide XICs measured in human plasma by LC-MRM/MS. The classification denotes protein abundance level. In the XIC overlays, the NAT signals are the red trace and SIS the blue.

The proteins cover a diverse breadth of molecular/biological processes (e.g., protease binding, inflammatory response) and pathways (e.g., blood coagulation), with numerous having putative or validated correlations to disease (e.g., neurodegeneration, ocular, cardiovascular). Included in the undetected 57 were several proteins of low abundance (e.g., DJ-1, occludin, tumor necrosis factor receptors). These may also not have been observed because the sample test pools originated from nonpathological patients. Overall, the developed method enabled the reproducible detection of a multiplexed panel of interference-free peptides that is appropriate for extension to study applications. The proteins span multiple orders of magnitude in concentration, which further highlights the suitability of this bottom-up proteomic method to quantify a broad range of targets in a highly complex plasma sample. It must be noted that the effectiveness of this method has additionally been successfully demonstrated in cerebrospinal fluid and could prove useful in other sample types, too.

Study Applications

Currently in progress at Montpellier Hospital is the application of the final BAK-270 proteomic method to a large-scale clinical trial of a COVID-19 patient cohort (study no. NCT04619693, title: "Biomarkers for Dexamethasone Response in Sars-Cov-2/COVID-19 Pneumonia"). Here, the 270-plex protein biomarker panel is being evaluated to distinguish dexamethasone responders versus nonresponders in SARS-CoV-2 hypoxemic pneumonia patients. Quantitation is accomplished by external standard addition with forward calibration curves.⁷ The outcome of this work should have a significant, and positive, impact in the treatment and follow-up of hospitalized patients. In other research activities, the BAK-270 is being used for high-throughput biomarkers discovery using targeted MS in alternate types of human biosamples (e.g., cerebrospinal fluid, perilymph, tears, saliva) and is often associated with machine learning. This approach allows the reliable classification of samples into disease and treatment groups and, eventually, may be applicable for diagnostics in the clinical laboratory.

Conclusions

The PeptiQuant Plus BAK-270 was successfully adapted to the Shimadzu LC-MS system for multiplexed protein interrogation of human plasma/serum samples. Initial empirical tuning of MS and MS/MS parameters with the SIS peptide mixture ensured that optimum detection of the target peptide panel was achieved. Minor optimization of the LC gradient was also employed, but may not be necessary, provided that the peptides are interference-free and of good chromatographic integrity. The final method is currently being applied at Montpellier Hospital to a large-scale clinical trial of COVID-19 patients, with additional study extensions in progress at Montpellier Hospital.

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Related Products

Catalog No.	Description	No. of Peptides	Unit Size	Optimized Instrument
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WFPK	PeptiQuant Plus Human Plasma Workflow QC Kit	35	1 or 2 runs	<ul style="list-style-type: none"> • 6490/6495 QqQ • Q Exactive Plus • QTRAP 6500
BAK-125	PeptiQuant Plus Human Plasma Biomarker Assessment Kit	125	20, 50, or 100 samples	<ul style="list-style-type: none"> • 6490/6495 QqQ • QTRAP 6500 • Q Exactive Plus • Xevo TQ-XS
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BAK-270	Expanded PeptiQuant Plus Human Plasma Biomarker Assessment Kit	270	100 samples	<ul style="list-style-type: none"> • 6490/6495 QqQ • QTRAP 6500 • Q Exactive Plus

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