Supplemental Information

Development and validation of a mass spectrometry based analytical method to quantify the ratio among Hemagglutinin Trimers in quadrivalent influenza nanoparticle vaccine - FluMos-v1

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Experimental Section

Materials and reagents. LC-MS grade water and acetonitrile were purchased from J. T. Baker (Center Valley, PA); formic acid was from Thermo Fisher Scientific (Tempe, AZ); guanidine HCl, was purchased from G Bioscience (St. Louis, MO); trypsin was purchased from Promega (Madison, WI); HATs and FluMos v1 cages were prepared in-house.

Sample preparation. For trypsin digestion, each sample was first denatured at 37 °C for 3 hrs using 6 M guanidine HCl, which was then diluted 6 times to 1 M guanidine using 50 mM ammonium bicarbonate solution (pH 7.8) as diluent. Protein was then incubated with trypsin at the mass ratio of 4:1 (trypsin: protein) at 37 °C overnight. A final concentration of 0.1% formic acid was added to terminate the digestion. Peptide standards were prepared by mixing the equal amount of tryptic digest of H1, H3 and HBy together with different mass ratio to HBv tryptic digest at 0.2, 0.5, 10, 1.5 and 2.0 without pentamer. For accuracy testing samples, equal amounts of undigested HATs H1, H3 and HBy mixed with HAT HBv at different mass ratio. Pentamer was then added at the mass of ¼ of total HATs followed by gentle vertexing to form FluMos v1 cage, which were then digested by trypsin. Quality control (QC) samples were prepared by mixing equal mass of H1, H3, HBy, HBv and pentamer and vertexing to form FluMos v1 cage which was further denatured and digested by trypsin. Clinical FluMos-v1 products lots were processed directly by denaturing with 6M guanidine HCl and digesting with trypsin.

Size-exclusion chromatogram coupled with fluorescence detection (SEC-FLR) analysis. Separation of FluMos v1 was carried out with an Acquity H-class Bio UPLC system (Waters, Milford, MA) operated by Empower 3 software. Samples were injected onto a Acquity BEH SEC column (200 Å, 1.7 µm, 4.6 mm x 50 mm) with mobile phase (2x phosphate buffer saline) delivered at 0.4 mL/min for 10 min. FLR detection was set with excitation and emission wavelengths at 278 nm and 330 nm, respectively.

LC-MS/MS analysis. The Acquity H-class Bio UPLC system (Waters Corporation, Milford, MA) operated using MassLynx v4.1 coupled with Q Exactive HF mass spectrometer (Thermo-Fisher Scientific, Waltham, MA) operated through Xcalibur software was used for the LC-MS/MS analysis. Mobile phases A and B consisted of 0.1% (v/v) formic acid in water and acetonitrile, respectively, delivered at a flow rate of 0.2 mL/min. Samples were injected onto a BEH C18 column (300 Å, 1.7 µm, 2.1 mm x 50 mm) heated at 65 °C. The UPLC gradient was (min-%B) 0-5%, 1-5%, 11-25%, 12-95%, 15-95%, 16-5% and 20-5%. The mass spectrometer was operated in positive ionization mode using parallel reaction monitoring (PRM) method. Two peptides were selected for HATs H1, H3 and HBy and one peptide for HBv with the parameters listed in Table 1 to monitor. The capillary voltage was 3.5 kV, and the desolvation temperature was 250 °C.

Calculation - Preparation of standard curves. The detected peptide peak areas of selected MS/MS fragment ions of each H1 peptide quantified from LC-MS/MS data by processing through XCalibur software were normalized against the peak area of selected fragment ion of HBv peptide in the same run. H1 peptide standard curves were prepared by plotting the normalized peak area of selected fragments against the premixed mass ratio of H1:HBv. The normalized peak areas of selected peptide fragments of testing samples were back calculated to the mass ratio of H1:HBv according to the standard curve. The average value of H1:HBv ratio was then obtained from two H1 peptides. The similar approach was applied to achieve the mass ratios of H3 and HBy to HBv, respectively. The default ratio of HBv was set as 1. The percentage of each HAT was calculated from the ratio of each HAT by with dividing the sum of all four HAT ratios.

Table S1. Types of Hemagglutinin (HA) strain components in the quadrivalent influenza nanoparticle vaccine (FluMos-v1) and nomenclature followed.

Flu Strain	HA Trimer	HA Trimer abbreviation
H1 (A/Idaho/07/2018)	HAT-H1-ID18	H1
H3_A1b_131K (A/Perth/1008/2019)	HAT-H3-PE19	H3
HBv (vic-B/Colorado/06/2017)	HAT-BV-CO17	HBv
Hby (Yam-B/Phuket/3073/2013)	HAT-BY-PH13	HBy

Table S2. Amino acid sequences of proteins used in this study (peptides selected for PRM are highlighted)

Pentamer: I53_dn5A

MGKYDGSKLRIGILHARWNAEIILALVLGALKRLQEFGVKRENIIIETVPGSFELP YGSKLFVEKQKRLGKPLDAIIPIGVLIKGSTMHFEYICDSTTHQLMKLNFELGIPVI FGVLTCLTDEQAEARAGLIEGKMHNHGEDWGAAAVEMATKFNLEHHHHHH

HAT1: I53_dn5B HAT-H1-ID18_H1 (A/Idaho/07/2018)

DTLCIGYHANNSTDTVDTVLEKNVTVTHSVNLLEDKHNGKLCKLRGVAPLHLGKCNIA GWILGNPECESLSTARSWSYIVETSNSDNGTCFPGDFINYEELREQLSSVSSFERFEIFPKTS SWPNHDSNKGVTAACPHAGAKSFYKNLIWLVKKGNSYPKLNQTYINDKGKEVLVLWGI HHPPTTADQQSLYQNADAYVFVGTSRYSKKFKPEIATRPKVRDQEGRMNYYWTLVEPG DKITFEATGNLVVPRYAFTMERNAGSGIIISDTPVHDCNTTCQTPEGAINTSLPFQNVHPIT IGKCPKYVKSTKLRLATGLR**NVPSIQSR**GLFGAIAGFIEGGWTGMVDGWYGYHHQNEQ GSGYAADLKSTQNAIDKITNKVNSVIEKMNTQFTAVGKEFNHLEKRIENLNKKVDDGFL DIWTYNAELLVLLENER**TLDYHDSNVK**NLYEKVRNQLKNNAKEIGNGCFEFYHKCDNT CMESVKNGTYDYPKYSEEAKLNREKIDGVSAEEAELAYLLGELAYKLGEYRIAIRAYRIA LKRDPNNAEAWYNLGNAYYKQGRYREAIEYYQKALELDPNNAEAWYNLGNAYYERG EYEEAIEYYRKALRLDPNNADAMQNLLNAKMREEGGWELQHHHHHH

HAT2: I53_dn5B HAT-H3-PE19_ H3_A1b_131K (A/Perth/1008/2019)

QKIPGNDNSTATLCLGHHAVPNGTIVKTITNDRIEVTNATELVQNSSIGEICDSPHQILDGG NCTLIDALLGDPQCDGFQNKKWDLFVERSRAYSNCFPYDVPDYASLRSLVASSGTLEFK NESFNWAGVKQNGTSSACIRGSSSSFFSRLNWLTHLNYTYPALNVTMPNKEQFDKLYIW GVHHPGTDKDQIFLYARSSGR**ITVSTR**RSQQAVIPNIGFRPRIRDIPSRISIYWTIVKPGDIL LINSTGNLIAPRGYFKIRSGKSSIMRSDAPIGKCKSECITPNGSIPNDKPFQNVNRITYGACP RYVKQSTLKLATGMRNVPEKQTRGIFGAIAGFIENGWEGMMDGWYGFRHQNSEGRGQ AADLKSTQAAIDQINGKLNRLIGKTNEKFHQIEK**EFSEVEGR**VQDLEKYVEDTKIDLWS

YNAELLVALENQHTIDLTDSEMNKLFEKTKKQLRENAEDMGNGCFKIYHKCDNACIGSI
RNGTYDHNVYRDEALNNRFQIKGVGGSAEEAELAYLLGELAYKLGEYRIAIRAYRIALK
RDPNNAEAWYNLGNAYYKQGRYREAIEYYQKALELDPNNAEAWYNLGNAYYERGEY
EEAIEYYRKALRLDPNNADAMQNLLNAKMREEGGWELQHHHHHH

HAT3: I53_dn5B HAT-BV-CO17_ HBv (vic-B/Colorado/06/2017)

DRICTGITSSNSPHVVKTATQGEVNVTGVIPLTTTPTKSHFANLKGTETRGKLCPKCLNCT
DLDVALGRPKCTGKIPSARVSILHEVRPVTSGCFPIMHDRTKIRQLPNLLRGYEHVRLSTH
NVINAEGAPGGPYKIGTSGSCPNITNGNGFFATMAWAVPDKNKTATNPLTIEVPYVCTEG
EDQITVWGFHSDNETQMAKLYGDSKPQKFTSSANGVTTHYVSQIGGFPNQTEDGGLPQS
GRIVVDYMVQKSGKTGTITYQR**GILLPQK**VWCASGRSKVIKGSLPLIGEADCLHEKYGG
LNKSKPYYTGEHAKAIGNCPIWVKTPLKLANGTKYRPPAKLLKERGFFGAIAGFLEGGW
EGMIAGWHGYTSHGAHGVAVAADLKSTQEAINKITKNLNSLSELEVKNLQRLSGAMDE
LHNEILELDEKVDDLRADTISSQIELAVLLSNEGIIINSEDEHLLALERKLKKMLGPSAVEIG
NGCFETKHKCNQTCLDKIAAGTFDAGEFSLPTFDSLNITAASAEEAELAYLLGELAYKLG
EYRIAIRAYRIALKRDPNNAEAWYNLGNAYYKQGRYREAIEYYQKALELDPNNAEAWY
NLGNAYYERGEYEEAIEYYRKALRLDPNNADAMQNLLNAKMREEGGWELQHHHHHH

HAT4: I53_dn5B HAT-BY-PH13_ Hby (Yam-B/Phuket/3073/2013)

DRICTGITSSNSPHVVKTATQGEVNVTGVIPLTTTPTKSYFANLKGTRTRGKLCPDCLNCT
DLDVALGRPMCVGTTPSAKASILHEVRPVTSGCFPIMHDRTKIRQLPNLLRGYEKIR**LST
QNVIDAEK**APGGPYRLGTSGSCPNATSKIGFFATMAWAVPKDNYKNATNPLTVEVPYIC
TEGEDQITVWGFHSDNKTQMK**SLYGDSNPQK**FTSSANGVTTHYVSQIGDFPDQTEDGG
LPQSGRIVVDYMMQKPGKTGTIVYQRGVLLPQKVWCASGRSKVIKGSLPLIGEADCLHE
EYGGLNKSKPYYTGKHAKAIGNCPIWVKTPLKLANGTKYRPPAKLLKERGFFGAIAGFL
EGGWEGMIAGWHGYTSHGAHGVAVAADLKSTQEAINKITKNLNSLSELEVKNLQRLSG
AMDELHNEILELDEKVDDLRADTISSQIELAVLLSNEGIIINSEDEHLLALERKLKKMLGPS
AVDIGNGCFETKHKCNQTCLDRIAAGTFNAGEFSLPTFDSLNITAASAEEAELAYLLGEL
AYKLGEYRIAIRAYRIALKRDPNNAEAWYNLGNAYYKQGRYREAIEYYQKALELDPNN
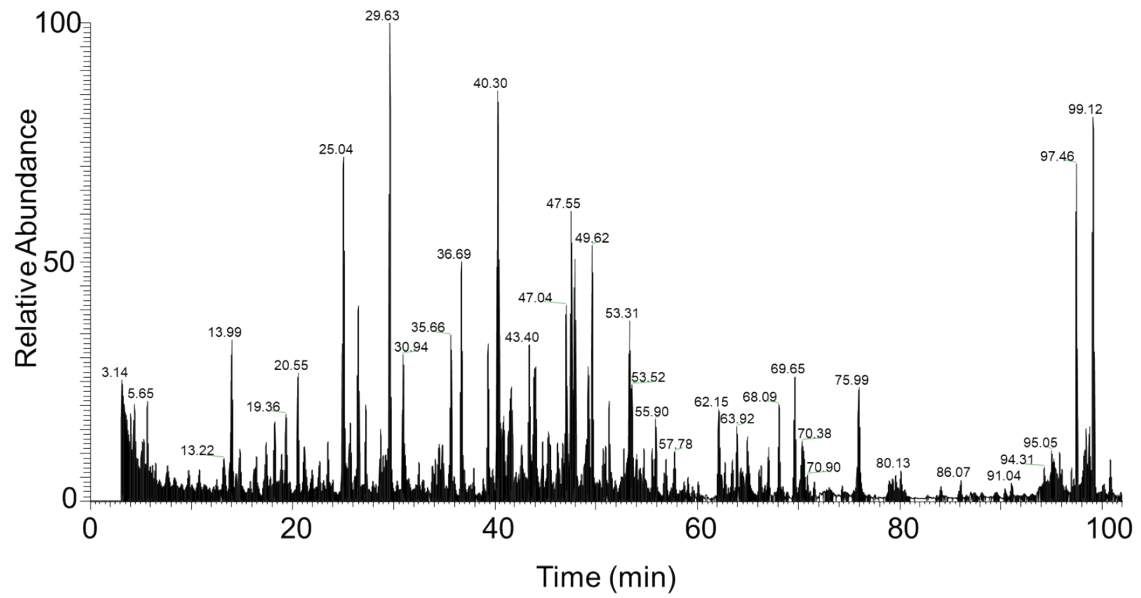AEAWYNLGNAYYERGEYEEAIEYYRKALRLDPNNADAMQNLLNAKMREEGGWELQH
HHHHH

Figure S1. Total ion chromatography (TIC) of tryptic digest of FluMos-v1 using 6 M guanidine denaturation for 30 min. High abundant peaks eluted after 80 min were observed, which are large peptides with missed cleavages.
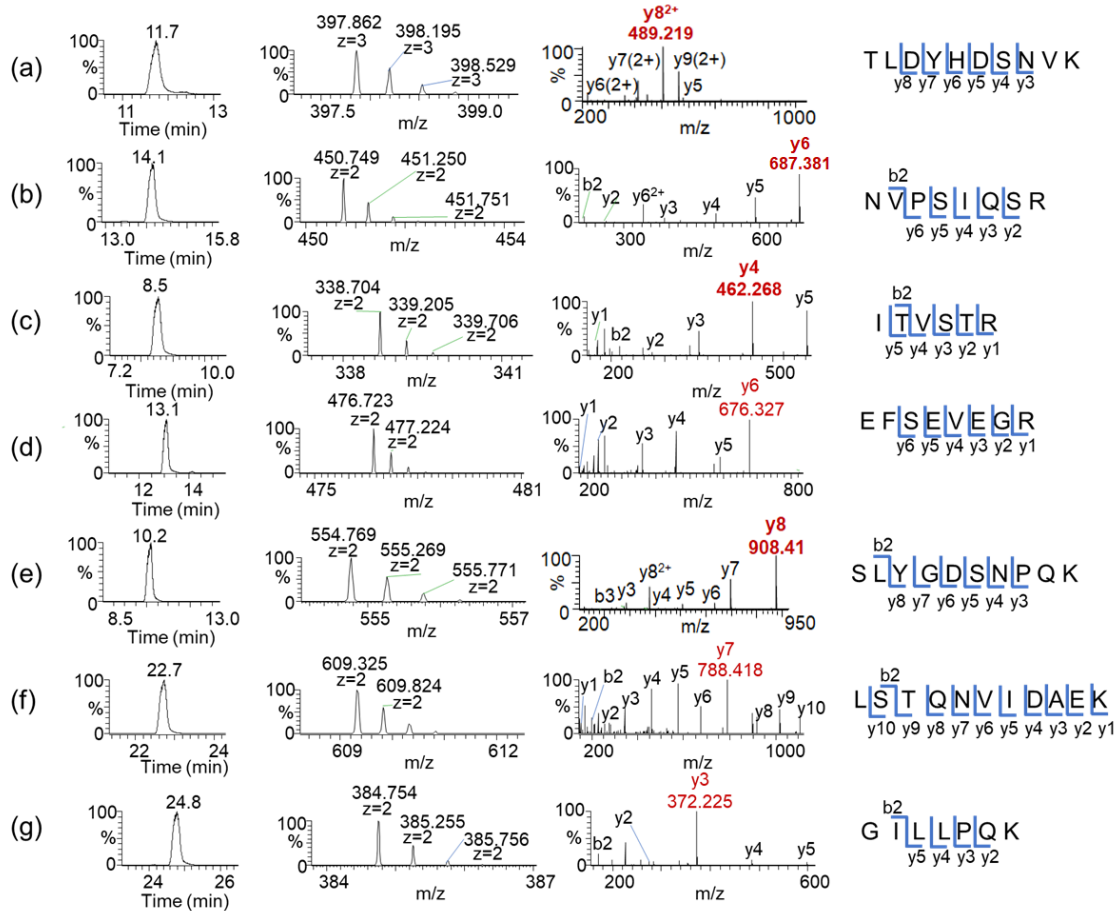
Figure S2. Signature peptides of H1, H3, HBy and HBv selected for quantification from 40 min LC-MS/MS run. XICs (left), MS full scan spectra (middle), and MS/MS spectra (right). H1 peptides (a) T434-K443 eluted at 11.7 min, with full scan m/z 397.862 and (b) N320-R327 eluted at 14.1 min, with full scan m/z 450.749. H3 peptides (c) I202-K270 eluted at 8.5 min with full scan m/z 338.704, and (d) E398-R405 eluted at 13.1 min, with full scan m/z 476.723. HBy peptides (e) S202-K211 eluted at 10.2 min, with full scan m/z 554.769, and (f) L119-K129 eluted at 22.7 min with full scan m/z 609.325. HBv peptides G264-K270 eluted at 24.8 min with full scan m/z 384.8. Good MS/MS sequence coverage was observed for all peptides.
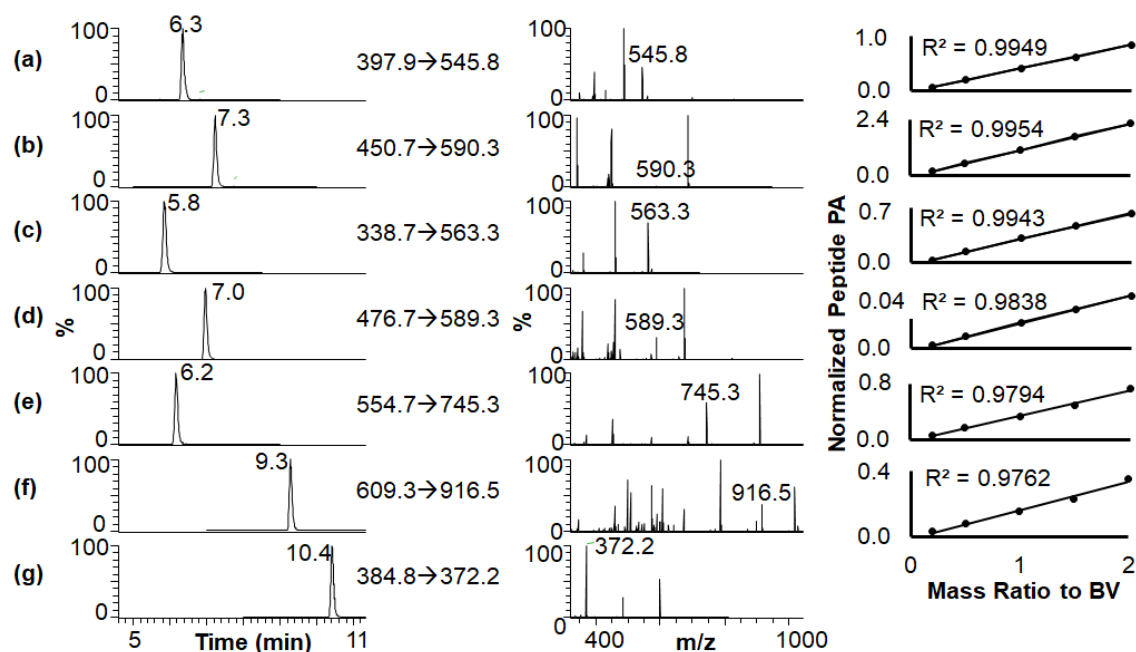
Figure S3. The extracted ion chromatograms of the selected MS/MS fragment ion (20 min LC run) of each HAT peptide are in the left, MS/MS spectra of each HAT peptide in the middle and the corresponding linear standard curves of normalized peptide peak area (PA) and ratio of each HAT to HBv on the right (refer to Table S8 for the selected PRM parameters) For peptide standard curves, y-axis is each peptide PA of H1, H3 and HBy divided by HBv peptide PA in the same run, and x-axis is the mass ratio between H1, H3 and HBy to HBv.

Table S3. Limit of Detection (LOD) and Limit of Quantification (LOQ) of ratio of peptides with standard HBv peptide.

Standard error method	H1		H3		HBy	
	Peptide 1	Peptide 2	Peptide 1	Peptide 2	Peptide 1	Peptide 2
Limit of Detection (LOD) of peptide ratio	0.041	0.063	0.081	0.046	0.255	0.275
Limit of Quantification (LOQ) of peptide ratio	0.125	0.191	0.246	0.139	0.772	0.834

Table S4. Accuracy for percentage of H1, H3, HBy and HBv analyzed by analyst 1 on day 1

H1, H3 & HBy	H1		H3		HBy		HBv		
Theoretical (%)	Each (%)	Accuracy (%)	Each (%)	Accuracy (%)	Each (%)	Accuracy (%)	Theoretical (%)	Each (%)	Accuracy (%)
12.5	13.3	106.5	14.1	112.6	14.0	111.9	62.5	58.6	93.8
20.0	22.7	113.4	19.0	95.2	19.8	99.0	40.0	38.5	96.2
25.0	27.7	110.6	22.0	88.0	25.3	101.1	25.0	25.1	100.3
27.3	29.7	109.0	24.0	87.9	26.7	98.0	18.2	19.6	107.7
28.6	32.5	113.8	24.5	85.9	28.0	98.1	14.3	14.9	104.4

Table S5. Accuracy for percentage of H1, H3, HBy and HBv analyzed by analyst 2 on day 2

H1, H3 & HBy	H1		H3		HBy		HBv		
Theoretical (%)	Each (%)	Accuracy (%)	Each (%)	Accuracy (%)	Each (%)	Accuracy (%)	Theoretical (%)	Each (%)	Accuracy (%)
12.5	12.7	101.5	12.9	103.1	11.6	93.0	62.5	62.8	100.5
20.0	22.5	112.6	19.2	96.0	18.8	93.8	40.0	39.5	98.8
25.0	27.4	109.5	26.0	103.8	23.4	93.8	25.0	23.2	93.0
27.3	30.7	112.4	27.2	99.7	25.0	91.8	18.2	17.1	94.2
28.6	31.5	110.1	28.3	99.0	26.4	92.3	14.3	13.9	97.2

Table S6. Accuracy for percentage of H1, H3, HBy and HBv analyzed by analyst 2 on day 3

H1, H3 & HBy	H1		H3		HBy		HBv		
Theoretical (%)	Each (%)	Accuracy (%)	Each (%)	Accuracy (%)	Each (%)	Accuracy (%)	Theoretical (%)	Each (%)	Accuracy (%)
12.5	13.0	104.0	11.0	87.9	10.9	87.2	62.5	65.1	104.2
20.0	21.8	109.1	18.7	93.3	18.5	92.3	40.0	41.1	102.7
25.0	27.4	109.7	23.6	94.5	24.3	97.3	25.0	24.6	98.5
27.3	30.0	110.0	26.0	95.2	27.0	98.8	18.2	17.1	94.0
28.6	34.4	120.5	26.2	91.7	26.0	91.2	14.3	13.3	93.2

Table S7. Accuracy measurement of each HAT percentage for 5 individually prepared QC samples

Theoretical (%)	Replicates	H1		H3		HBy		HBv	
		Each (%)	Accuracy (%)	Each (%)	Accuracy (%)	Each (%)	Accuracy (%)	Each (%)	Accuracy (%)
25.0	1	28.0	111.8	22.3	89.1	24.8	99.1	25.0	100.0
	2	27.7	110.8	22.2	88.8	24.4	97.5	25.7	103.0
	3	27.5	109.9	22.8	91.0	24.4	97.8	25.3	101.3
	4	27.0	108.0	22.3	89.4	24.5	97.9	26.2	104.7
	5	27.7	110.8	21.5	86.1	25.1	100.3	25.7	102.8
Average (n=5)		27.6		22.2		24.6		25.6	
RSD% (n=5)		1.3		2.0		1.2		1.7	

Table S8. The parameters of second fragment ion based PRM method for monitoring HAT peptides. The precursor ion m/z, charge states and collision energy (CE), second fragment ion range, and the corresponding peptide retention times were included.

HATs	Signature Peptides	Precursor Ions			Fragment ion (m/z)	RT (±5 min)
		m/z	z	CE (eV)		
H1	T^{434}LDYHDSNVK^{443}	397.9	3	15	545.8 (y9^{2+})	6.3
	N^{320}VPSIQSR^{327}	450.7	2	15	590.3 (y5)	7.3
H3	I^{202}TVSTR^{207}	338.7	2	20	563.3 (y5)	5.8
	E^{398}FSEVEGR^{405}	476.7	2	20	589.3 (y5)	7.0
HBy	S^{202}LYGDSNPQK^{211}	554.8	2	20	745.3 (y7)	6.2
	L^{119}STQNVIDAEK^{129}	609.3	2	20	916.5 (y8)	9.3
Internal standard						
HBv	G^{264}ILLPQK^{270}	384.8	2	20	372.2 (y3)	10.4

Table S9. Accuracy for percentage of H1, H3, HBy and HBv quantified based on second fragment ion from PRM data (88.9-115.6%)

H1, H3 & HBy	H1		H3		HBy		HBv		
Theoretical (%)	Each (%)	Accuracy (%)	Each (%)	Accuracy (%)	Each (%)	Accuracy (%)	Theoretical (%)	Each (%)	Accuracy (%)
12.5	14.2	113.4	13.8	110.7	13.7	109.9	62.5	58.6	93.7
20.0	23.6	118.2	19.3	96.4	19.7	98.7	40.0	39.1	97.6
25.0	27.3	109.0	22.5	90.1	25.7	102.8	25.0	25.7	102.9
27.3	29.5	108.2	24.5	89.8	27.2	99.7	18.2	19.9	109.4
28.6	33.0	115.6	25.4	88.9	28.6	100.1	14.3	15.4	107.5

Table S10. Good intermediate precisions for one sample run by 2 analysts in 3 days (total 3 runs)

Analyst	Day	H1%	H3%	HBy%	HBv%
1	1	27.0	20.3	25.2	27.5
2	2	27.1	22.4	23.1	27.3
2	3	28.2	21.4	22.1	28.2
Ave.% (n=3)		27.4	21.4	23.5	27.7
RSD% (n=3)		2.4	4.9	6.7	1.7