Stable isotope dimethyl labeling coupled to selected reaction monitoring enhances throughput by multiplexing relative quantitation of targeted proteins

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Supplemental Abstract

In this study we present a proof-of-concept for targeted relative protein quantitation workflow using chemical labeling in the form of dimethylation, coupled with selected-reaction-monitoring (SRM). We first demonstrate close to complete isotope incorporation for all peptides tested. The accuracy, reproducibility, and linear dynamic range of quantitation are further assessed based on known ratios of non-human standard proteins spiked into human cerebrospinal fluid (CSF) as a model complex matrix. Reliable quantitation is observed with high reproducibility (CV <20%) for analyte concentrations present at a dynamic range of 4 orders of magnitude lower than that of the background proteins. Accurate (error <15%) relative quantitation of 45 major human plasma proteins is also shown. Dimethyl-SRM was further examined by comparing the relative quantitation of eight proteins in human CSF with the relative quantitation obtained using synthetic heavy peptides coupled to stable isotope dilution–SRM (SID-SRM). Comparison between the two methods reveals that the correlation between dimethyl-SRM and SID-SRM is within 0.3-39% variation, demonstrating the accuracy of relative quantitation using dimethyl-SRM. Dimethyl labeling coupled with SRM provides a fast, convenient and cost-effective alternative for relative quantitation of a large number of candidate proteins/peptides.

Supplemental experimental procedures

Protein digestion and reductive amination (Stable isotope dimethyl labeling)

All protein samples were denatured with 8M urea in 50mM NH_4HCO_3 buffer (pH 8) for 1h before being subjected to reduction, alkylation and digestion by LysC followed by trypsin.[1] 10µg of digested and desalted samples was dissolved in 100 µL of 100 mM TEAB buffer. CH_2O (10uL, 4%, "light") or 4% CD_2O (4µl, 4%, "intermediate") or $^{13}CD_2O$ (10µl, 4%, "heavy") was added followed by 10µL of 600mM $NaBH_3CN$ (light and intermediate) or 10µL of 600mM $NaBD_3CN$ (heavy). The mixture was incubated for 2h at room temperature, and the reaction was quenched by adding 16µL of 1% ammonia and 8µL formic acid.[2] The three differentially labeled samples were pooled and again desalted using the *Oasis*® HLB µElution 96 well plate (Oasis, Millipore). The labeled samples are ready for SRM analysis without fractionation.

Peptide selection criteria

Only peptides without methionine residues, no ragged ends and no PTM motifs (e.g., NXT/S, for possible N-glycosylation) were considered. Peptide lengths were chosen between 7-17 amino acids and no miscleavage was allowed.[3]

SRM transition selection in MRMPilot

MRMPilot predicts the peptide charge states based on the number of basic residues in a peptide, and commonly uses b- and y- ion fragments with *m*/*z* values greater than the precursor ion *m*/*z* as Q3 masses for the peptide Q1/Q3 ion pair. SRM acquisition methods were constructed by using predicted collision energy (CE). The default values for de-clustering potential (DP) and collision cell exit potential (CXP) for all SRM ion pairs were 110V and 15V, respectively. As each peptide was monitored in light-, intermediate- and heavy-labeled channels, this resulted in nine transitions per peptide in most of the experiments presented in this manuscript. Since a typical SRM peak has a width of approximately 30s, each peak comprised a minimum of 10 data points.

Synthetic peptides and peptide optimization

The seven selected peptides were mixed in a final concentration of 500fmol/ul in 30% acetonitrile/0.1 formic acid for infusion at a flow rate of 300nl/min using a PicoPlus 11 syringe pump (Harvard apparatus, MA). Infused peptide solutions were analyzed by nanoelectrospray using a QTRAP4000. The most intense charge state was determined by ramping the DP voltages during the Q1 scans. DP voltages were tuned to ensure maximum efficiency of ion transfer of the precursor ion (Q1) into the MS instrument and achieve the maximum SRM signal. Using the tuned Q1 parameters for each peptide, the dominant CID fragment ions generated from each peptide were determined by ramping collision energy of 5-120V in 2V increments. All MS parameters except DP and CE were detailed in the 'NanoLC-SRM analyses' section.

SRM data analysis

SRM data were processed using MultiQuant 2.2 (AB Sciex) with the MQ4 algorithm for peak integration. Quantitative ratios were calculated on the basis of the peak area after integration. A 1min retention time window and expected retention time was chosen as 'group'. A two-point smoothing with a peak splitting factor of two was set. The default MultiQuant values for noise percentage and base-line subtraction window were used. Linear regression of all calibration curves was performed using a standard 1/x (x=concentration ratio) weighing option to aid in covering a wide dynamic range. The peptides reported in the studies were checked for background interference and S/N >10 were set as the threshold in the data analysis.

Generation of response curve

Prior to SID-SRM experiment, we generated linear response curves (a dilution series ranging from estimated 1fmol to 2pmol) in order to assess LOQ (S/N >10) of all the targeted endogenous peptides in CSF samples. Linear correlation between the area ratios of endogenous and SIS peptides (i.e., endogenous/heavy) *vs.* known concentration of spiked-in SIS peptide is shown in Figure S-4. As shown in

Figure S-4, excellent linear response curves (R^2 >0.96) were obtained for five peptides representing the five proteins, chromatogranin-A, secretogranin, α-1 acid glycoprotein, α-2 macroglobulin and prostaglandin H2 D-isomerase. The two peptides, IVESYQIR (contactin-1) and ALDFAVGEYNK (Cystatin-C) were excluded due to low S/N level of endogenous peptides; while ELPEHTVK (vitamin D-binding protein) was excluded due to poor solubility of the synthetic peptides in designated buffer. The SIS peptide amount added to each of the six samples was identical, and close to the endogenous peptide level in CSF based on the SIS response curve results.

Supplemental figure S-1

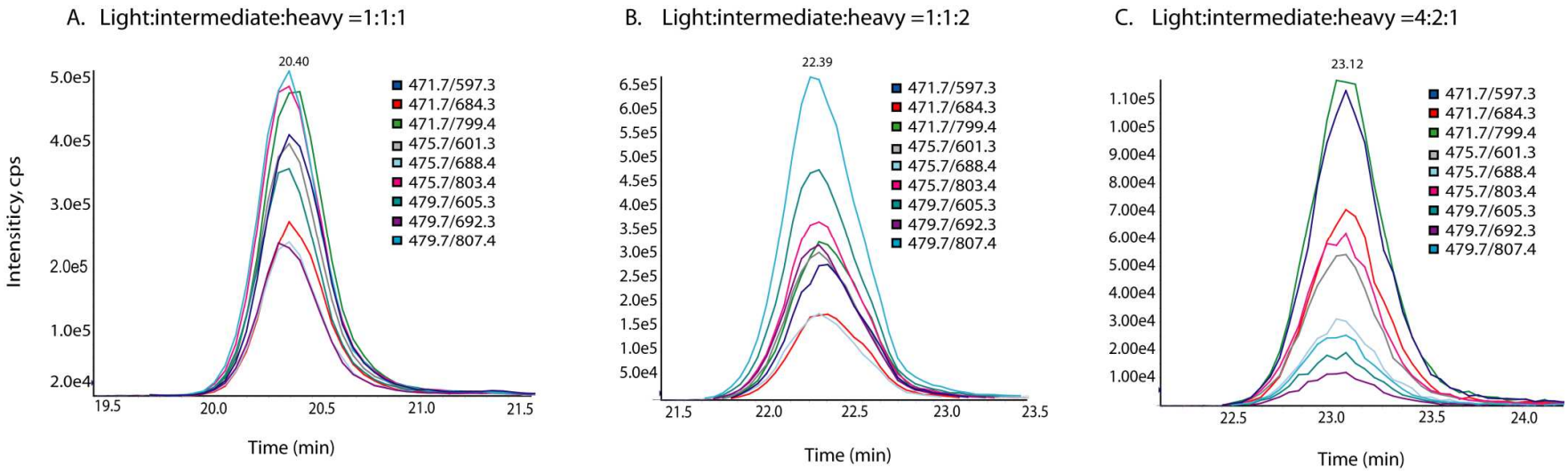

Figure S-1. Representative XICs of dimethyl triplets. BSA is labeled with dimethyl reagents in (light: intermediate: heavy) ratios of 1:1:1; 1:1:2 and 4:2:1 respectively. XICs for DDSPDLPK, a proteotypic peptide derived from BSA in (A) 1:1:1 ratio, (B) 1:1:2 and (C) 4:2:1 were shown. Three transitions were monitored per labeled peptide; each color representing one transition.

Supplemental figure S-2

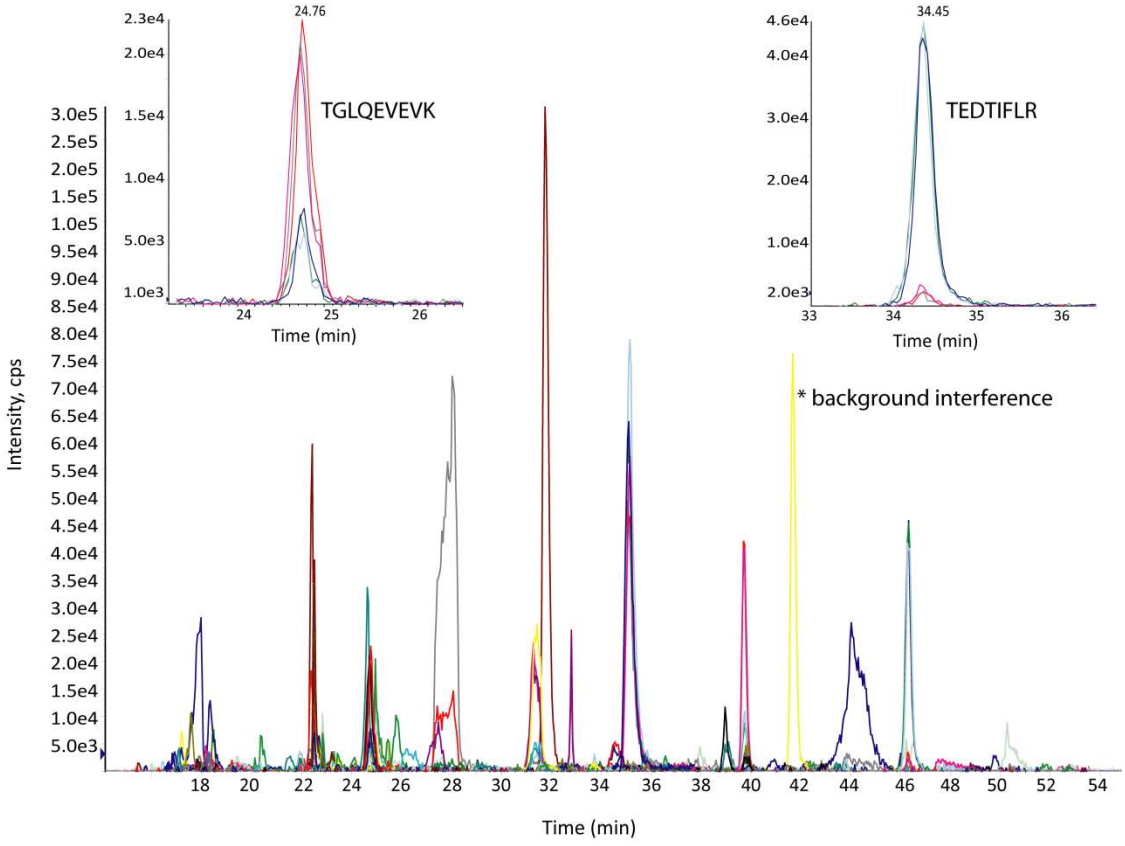

Figure S-2. XICs of all 30 peptides derived from test cerebrospinal fluid (CSF). 10 µg each of trypsinized CSF were labeled with light (D_0), intermediate (D_2) and heavy ($^{13}CD_2$) dimethyl reagents, separately and mixed in 1:1:1 ratio, followed by LC-SRM/MS analysis. Two transitions per labeled peptide were monitored, resulting in 180 transitions. Representative XICs for TGLQEVEK and TEDTIFLR in dimethyl triplets are shown as inserts. Background interference was observed at 42 min retention time.

Supplemental figure S-3

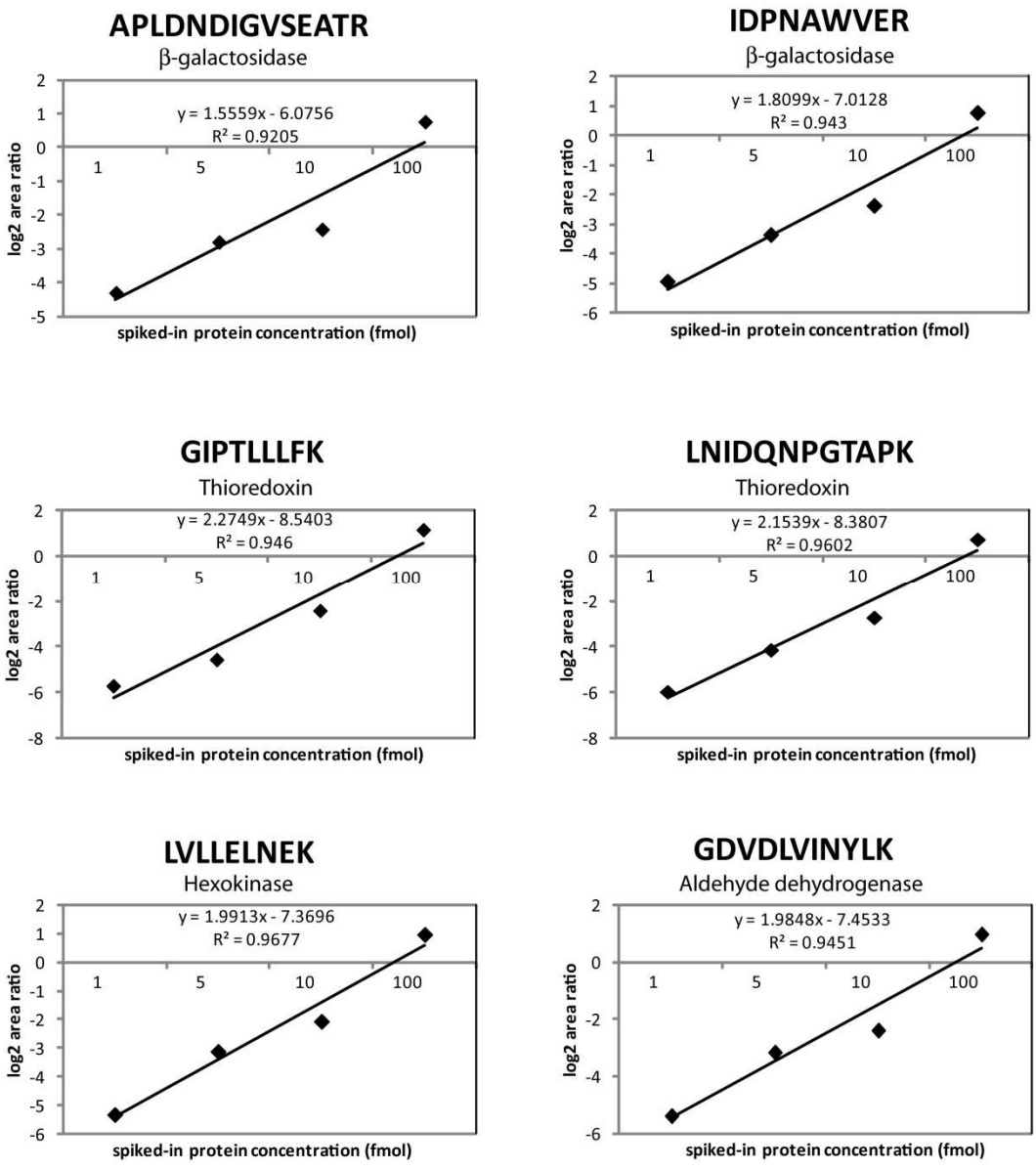

Figure S-3. Evaluation of limit of quantitation (LOQ) of dimethyl-SRM method. LOQ of dimethyl-SRM was investigated by spiking a mixture of five non-human proteins into human CSF at seven different concentrations per μg CSF. Log_2 area ratios are plotted against concentrations of spiked-in proteins.

Supplemental figure S-4

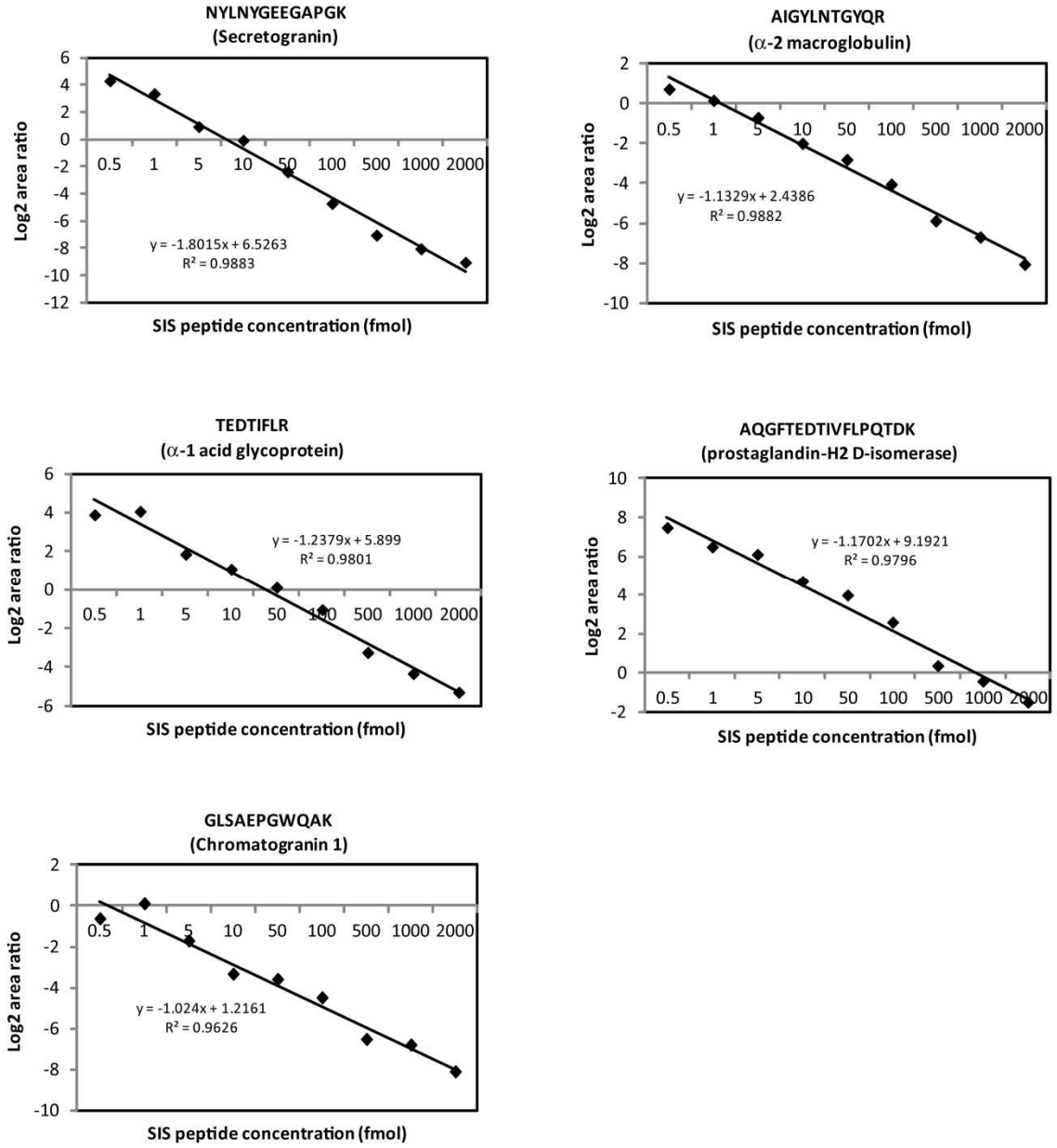

Figure S-4. Response curve for SID (stable isotope dilution) in the quantitation of CSF biomarkers. Linear correlation between the area ratios of endogenous and SIS peptides (endogenous/heavy) *vs.* known concentration of spiked-in SIS peptide is depicted.

Supplemental figure S-5

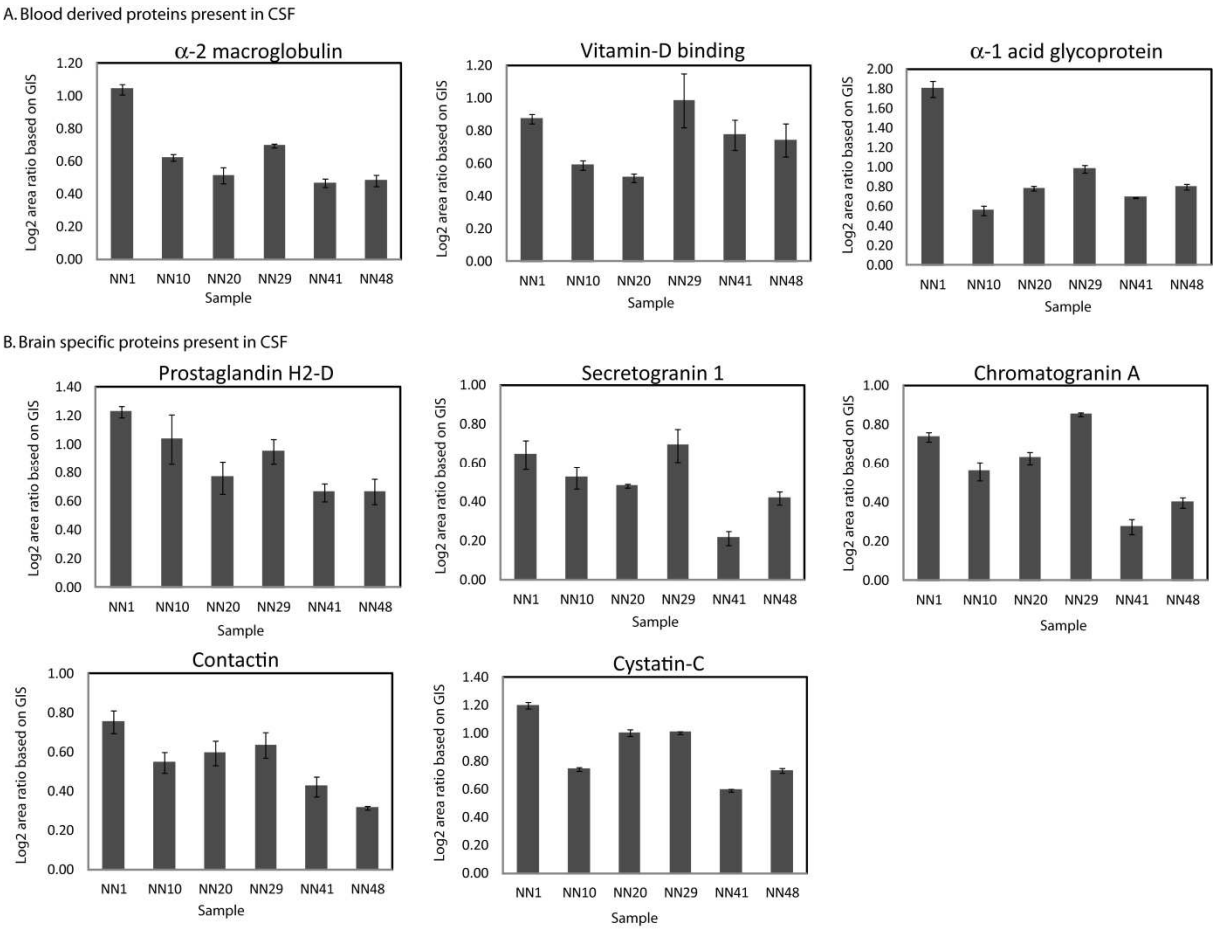

Figure S-5. Normalized quantity of targeted peptides across CSF samples. Analysis of CSF was performed as described in workflow (see Figure.3). After normalization by using XICs from the Global Internal Standard (GIS, labeled as Light) as denominator, the area ratios (i.e., I/L and H/L) for each peptide are plotted to show the levels of targeted peptides for all six CSF samples analyzed. The levels of (A) blood-derived proteins and (B) brain specific proteins are depicted.

Table S-1. Evaluation of stable isotope incorporation after dimethyl labeling. The excel file lists the dimethyl labeled peptides derived from BSA and their respective MS operating parameters generated by MRMPilot. The tryptic peptides were labeled with light (D_0), intermediate (D_2) and heavy ($^{13}CD_2$) dimethyl labels and mixed in a ratio of 1:1:1. Nine peptides were monitored across one LC-SRM run. Non-labeled peptides and their corresponding information are colored in red. The peptides in which non-labeled versions were detected are highlighted in grey.

Table S-2. Evaluation of linearity and accuracy of dimethyl-SRM. The excel file lists the dimethyl triplet labeled peptides derived from BSA and their respective optimized parameters generated by MRMPilot. The tryptic BSA peptide were labeled with light(D_0)-, intermediate(D_2)- and heavy(^{13}CD2)- dimethyl labels and then mixed in a ratio of 1:1:1, 1:1:2 and 4:2:1. Nine peptides were monitored across one LC-SRM. The list of targeted peptides, charge state, length of peptides, transitions, observed ratio (light/intermediate/heavy), retention time, width at 50% and signal to noise level are described. Observed area ratios are listed with % variation from expected area ratio (% error). The most intense transitions (highest S/N ratio) used for quantitative analysis are colored in bold black. XICs for DDSPDLPK, a proteotypic peptide derived from BSA in 1:1:1, 1:1:2 and 4:2:1 ratio are described in Figure S-1.

Table S-3. Evaluation of reproducibility of dimethyl-SRM using CSF sample. The excel file lists the proteins and peptides used for evaluating reproducibility of dimethyl-SRM, as well as Q1/Q3 masses generated by MRMPilot are tabulated. 180 transitions for 10 proteins were monitored. Peptides, in which background interference was detected, were discarded. Signal to noise level, retention time and the peak area ratios calculated by MultiQuant are listed. The area ratios across four replicas are reported with relative standard deviations. The outliner peptides are highlighted in bold red. Observed area ratios were listed with % variation (% error) from expected area ratio. Representative XIC is shown in Figure S-2.

Table S-4. Evaluation of limit of detection (LOD) and limit of quantitation (LOQ) in dimethyl-SRM. (A) Labeling and mixing of seven non-human proteins spiked in to human CSF sample. **(B)** The excel file list the targeted proteins, peptides and their corresponding Q1/Q3

masses generated by MRMPilot are described. Eight peptides derived from non-human proteins were chosen (peptides, in which background interference was detected, were discarded). One peptide from BSA was monitored as an internal standard. Signal to noise level, retention time and observed area ratio of each peptide are tabulated. For limit of detection (LOD), S/N >3 was chosen as a cutoff whereas S/N >10 was set for limit of quantitation (LOQ). Outlier ratios which are not considered for evaluation process (bold red). Discarded outlier peptides are in bold black. Log_2 area ratios are plotted against concentration of spiked-in proteins and shown in Figure S-3.

Table S-4A. Labeling and mixing of seven non-human proteins spiked in to human CSF sample.

Protein Mixture	Protein concentration Light-labeled (fmol)	Protein concentration Intermediate-labeled (fmol)	Protein concentration Heavy-labeled (fmol)	Standard protein concentration ratio
M1	0.1	0.5	50	1:5:500
M2	50	0.1	0.5	500:1:5
M3	0.5	50	0.1	5:500:1
M4	1	5	50	1:5:10
M5	50	1	5	10:1:5
M6	5	50	1	5:10:1
M7	10	100	50	1:10:5
M8	50	10	100	5:1:10
M9	100	50	10	10:5:1

Table S-5. Investigation of isotopic effect in dimethyl-SRM. The excel file lists the peptides used for calculation of retention time difference (ΔRT) between isotope species of each peptide are tabulated. The peptides which were observed with ΔRT >6s are highlighted in bold red. The outlier retention times are in bold black. For graph, refer to Figure. 2.

Table S-6. Dimethyl-SRM quantitation of 45 major plasma proteins. 44 major plasma proteins which were previously reported were chosen for this experiment.[4, 5] **(A)** The selected proteins, targeted peptide sequences and Q3 masses are listed. Regression analysis results such as slope, intercept and R^2 value are also described. **(B)** The excel file list the target proteins, peptides and the selected transition used for quantitation analysis. Regression analysis for each peptide is also described.

Table S-6A. Dimethyl SRM quantification of 44 human plasma proteins.

Index	Protein	Component Name	Q3	Light vs heavy (GIS)			Medium vs heavy (GIS)			error (%)	CV (%)
				Slope	Y-intercept	R^2	Slope	Y-intercept	R^2		
1	Afamin	DADPDTFFAK	y7+	0.972±0.029	0.064±0.134	0.998	1.021±0.022	0.043±0.104	0.999	5.14	4.68
2	Transthyretin	AADDTWEPFASGK	y9+	1.011±0.021	0.039±0.098	0.994	1.057±0.007	0.007±0.034	0.999	6.12	5.60
3	Interatrypsin inhibitor HC	AAISGENAGLVR	y9+	0.999±0.010	0.010±0.048	0.992	1.024±0.014	0.040±0.063	0.995	4.25	3.42
4	Angiotensionogen	ALQDQLVLVAAK	y3+	1.001±0.015	0.035±0.072	0.993	0.975±0.994	0.024±0.113	0.999	5.43	7.71
5	AntithrombinIII	DDLYVSDAFHK	y6+	0.997±0.068	0.112±0.319	0.999	1.062±0.044	0.002±0.206	0.999	6.99	7.46
6	Fibrinogen γ chain	DTVQIHDITGK	y7+	1.007±0.037	0.004±0.176	0.993	1.036±0.018	0.013±0.083	0.999	5.48	3.04
		DNC[CAM]C[CAM]ILDER*	***y4+***	***1.015±0.025***	***0.052±0.115***	***0.9998***	***0.981±0.010***	***0.015±0.050***	***1.000***	***4.48***	***4.30***
7	Transferrin	EGYYGYTGAFR*	y7+	0.998±0.012	0.004±0.058	0.997	1.026±0.018	0.02±0.0860	0.991	3.55	1.73
		EDPQTFYYAVAVVK	***y10+***	***0.917±0.009***	***0.016±0.120***	***0.990***	***0.88±0.054***	***0.112±0275***	***0.991***	***11.37***	***8.11***
8	Complement factor B	EELLPAQDIK	y8+	1.060±0.092	0.142±0.433	0.998	1.015±0.019	0.043±0.087	0.999	6.57	4.41
9	Prothrombin	ETAASLLQAGYK	y7+	1.024±0.006	0.006±0.027	0.999	1.017±0.010	0.025±0.045	0.997	3.82	3.26
10	Ceruloplasmin	EYTDASFTNR	y6+	1.013±0.050	0.065±0.235	0.999	0.988±0.039	0.071±0.182	0.995	4.70	4.91
		EVGPTNADPVC[CAM]LAK*	***y6+***	***0.962±0.016***	***0.026±0.076***	***0.999***	***0.962±0.032***	***0.069±0.156***	***0.991***	***8.18***	***4.70***
11	Vitronectin	FEDGVLDPDYPR	y5+	1.038±0.027	0.030±0.125	0.999	1.044±0.017	0.009±0.079	0.999	4.25	4.95
12	Fibrinogen α chain	GSESGIFTNTK	y8+	0.928±0.021	0.035±0.100	0.998	1.022±0.019	0.026±0.089	0.999	4.96	4.85
		VQHIQLLQK*	***y8+***	***1.002±0.016***	***0.044±0.076***	***0.999***	***0.959±0.010***	***0.002±0.051***	***0.999***	***4.78***	***4.45***
13	Plasminogen	LFLEPTR	y6+	0.981±0.026	0.029±0.124	0.998	1.054±0.007	0.011±0.033	1.000	4.23	3.11
14	α-2antiplasmin	LGNQEPGGQTALK	y8+	1.023±0.026	0.041±0.120	0.998	1.081±0.028	0.008±0.130	0.998	7.01	2.11
		LC[CAM]QDLGPGAFR*	***y7+***	***0.9762±0.034***	***0.002±0.165***	***0.999***	***0.946±0.005***	***0.002±0.025***	***0.999***	***3.68***	***3.84***
15	Albumin, serum	LVNEVTEFAK	y5+	0.983±0.002	0.003±0.010	0.997	1.042±0.018	0.011±0.087	0.999	4.68	1.87
16	Fibronogen β chain	QGFGNVATNTDGK	y7+	1.014±0.021	0.023±0.099	0.997	1.050±0.006	0.007±0.030	0.999	4.28	4.24
		EDGGGWWYNR*	***y7+***	***0.955±0.023***	***0.039±0.113***	***0.998***	***0.961±0.018***	***0.0332±0.091***	***0.999***	***3.99***	***6.01***
17	Apolipoprotein AIV	SLAPYAQDTQEK	y9+	0.979±0.029	0.009±0.135	0.998	1.068±0.020	0.014±0.093	0.999	6.10	6.43
18	Complement factor H	SPDVINGSPISQK	y8+	1.004±0.022	0.014±0.101	0.998	1.018±0.010	0.014±0.047	0.997	4.32	7.74
19	Gelsolin, isoform 1	TGAQELLR	y6+	1.043±0.032	0.000±0.052	0.997	0.887±0.068	0.005±0.321	0.998	9.26	10.80
20	Complement C3	TGLQEVEVK	y7+	1.103±0.007	0.026±0.032	0.999	1.018±0.021	0.041±0.100	0.999	6.15	5.30
		AVLYNYR*	***y6+***	***0.992±0.013***	***0.021±0.062***	***0.999***	***0.988±0.012***	***0.032±0.057***	***0.999***	***4.75***	***3.20***

21	Heparin cofactor II	TLEAQLTPR	y7+	0.994±0.015	0.013±0.071	0.993	1.059±0.016	0.023±0.075	0.993	3.69	4.04
22	Apolipoprotein CI lipoprotein	TPDVSSALDK	y9+	1.004±0.016	0.037±0.076	0.992	1.012±0.020	0.043±0.095	0.988	4.95	2.96
23	Kininogen1	TVGSDTFYSFK	y9+	1.023±0.002	0.002±0.008	1.000	1.037±0.006	0.007±0.029	0.999	2.66	4.58
		YFIDFVAR*	***y7+***	***1.0672±0.028***	***0.026±0.124***	***0.999***	***0.954±0.020***	***0.046±0.099***		***4.13***	***4.09***
24	Complement C4 β chain	VGDTLNLNLR	y9+	1.020±0.026	0.049±0.120	0.998	1.049±0.019	0.025±0.088	0.990	4.60	3.70
25	Haptoglobin β chain	VGYVSGWGR	y5+	0.998±0.020	0.008±0.093	0.999	1.022±0.006	0.006±0.029	0.999	3.39	3.13
26	Apolipoprotein CIII	DALSSVQESQVAQQAR*	y10+	0.980±0.014	0.025±0.066	0.994	1.014±0.015	0.026±0.069	0.994	5.42	3.94
		GWVTDGFSSLK***	***y8+***	***0.858±0.371***	***0.033±0.253***	***0.998***	***0.876±0.514***	***0.041±0.458***	***0.991***	***7.96***	***5.38***
27	Complement C4 γ chain	VEYGFQVK*	y6+	1.014±0.067	0.083±0.314	0.999	1.021±0.010	0.005±0.045	0.997	4.21	4.27
		ITQVLHFTK***	***y8+***	***1.065±0.015***	***0.1023±0.012***	***0.999***	***0.929±0.266***	***0.0481±0.026***	***0.999***	***5.14***	***4.24***
28	a1antichymotrypsin	EIGELYLPK	y7+	0.964±0.026	0.038±0.124	0.998	1.157±0.043	0.068±0.203	0.959	5.81	5.22
29	Apolipoprotein B-100	FPEVDVLTK	y8+	1.266±0.135	0.213±0.632	0.997	1.135±0.182	0.186±0.853	0.992	11.52	6.39
		LTISEQNIQR*	***y7+***	***0.938±0.026***	***0.022±0.131***	***0.997***	***0.849±0.020***	***0.002±0.109***	***0.991***	***9.69***	***10.68***
30	α-1bglycoprotein	LETPDFQLFK	y7+	0.982±0.008	0.021±0.038	0.998	1.024±0.036	0.063±0.171	1.000	4.63	7.19
31	Apolipoprotein E	LGPLVEQGR	y7+	1.040±0.006	0.017±0.026	0.999	1.016±0.089	0.003±0.133	0.998	3.01	6.30
32	Apolipoprotein AI	LLDNWDSVTSTFSK*	y9+	1.056±0.023	0.027±0.110	0.995	1.040±0.006	0.002±0.027	1.000	6.07	3.46
		ATEHLSTLSEK	***y8+***	***0.947±0.125***	***0.218±0.457***	***0.994***	***0.979±0.102***	***0.128±0.496***	***0.997***	***8.51***	***10.14***
33	α-2macroglobulin	LLIYAVLPTGDVIGDSAK	y11+	1.926±0.406	0.753±1.904	0.987	1.274±0.138	0.214±0.647	0.997	15.36	5.78
34	Apolipoprotein AII precursor	SPELQAEAK	y8+	0.902±0.089	0.088±0.416	0.997	1.076±0.020	0.045±0.092	1.000	10.27	5.68
35	Serum amyloid P component	VGEYSLYIGR	y7+	0.980±0.044	0.037±0.206	0.999	1.129±0.179	0.123±0.838	0.993	9.31	13.78
36	Coagulation factor XIIa HC	VVGGLVALR	y8+	1.029±0.047	0.013±0.223	0.999	1.097±0.084	0.044±0.393	0.998	5.92	9.14
37	Plasma retinolbinding protein	YWGVASFLQK	y6+	1.066±0.030	0.024±0.140	0.998	1.178±0.027	0.003±0.128	1.000	9.69	10.68
38	Zince α2 glycoprotein	EIPAWVPFDPAAQITK	y10+	1.022±0.004	0.020±0.020	1.000	1.055±0.035	0.022±0.162	1.000	6.14	4.30
39	α-1acid glycoprotein 1	EQLGEFYEALDC[CAM]LR**	y6+	0.729±0.113	0.200±0.531	0.993	1.007±0.083	0.109±0.391	0.998	10.11	5.99
40	Hemopexin	NFPSPVDAAFR	y9+	0.960±0.098	0.136±0.458	0.997	1.323±0.153	0.278±0.718	0.996	7.46	3.74
41	Clusterin	ELDESLQVAER	y7+	0.721±0.029	0.099±0.136	0.995	1.031±0.282	0.226±1.312	0.978	14.94	10.14
42	L-selectin	AEIEYLEK***	y7+	1.039±0.056	0.006±0.085	0.997	0.939±0.067	0.040±0.363	0.999	11.80	9.95
43	Complement Component C9	VVEESELAR*	y7+	0.399±0.923	0.128±1.187	0.968	1.052±0.012	0.011±0.235	0.996	12.44	8.38
44	β-2glycoprotein I	ATVVYQGER	y7+	1.115±0.041	0.030±0.191	0.999	1.064±0.082	0.087±0.385	0.998	12.90	6.08

*selected peptides based on Kim et al [4]

**peptide which has Q1(3+)
***quantation based on only 4 mixing experiments
**** quantitation based on only 3 mixing experiments
error (%) represents the percentage variation of observed ratios from expected ratio
CV (%) refers to the variation across the three technical replicates
The discarded peptides are in bold italic

Table S-6A. Dimethyl SRM quantification of 44 human plasma proteins. The accuracy (% error) and reproducibility (%CV) of the dimethyl-SRM method was evaluated using the 45 human plasma proteins which were previously reported by Kuzyk *et al.* [5] and Kim *et al.* [4]. The plasma was trypsinized and equally divided into 3 aliquots which were individually labeled with light (L)-, intermediate (M)- and heavy (H)-dimethyl labels. Labeled peptides were then mixed in five different mixing ratios of (L:M:H) 1:3:10, 3:1:10, 1:1:1, 3:10:1 and 10:3:1. The selected proteins, targeted peptide sequences and Q3 masses are listed. Regression analysis results such as slope, intercept and R^2 value are also described.

Table S-7. SID-SRM quantitation of CSF biomarker protein. The excel file lists the targeted proteins, target synthetic peptides, Q1/Q3 masses, SRM optimized parameters (i.e., DP, CXP and CE). The peak area ratios calculated by MultiQuant, retention time, width at 50% and signal to noise level of each transition are summarized. Technical reproducibility (%CV) and biological variation are also listed. The most intense transitions which are considered for quantitation are in bold black.

Table S-8. Dimethyl-SRM quantitation of CSF biomarker proteins. The excel file lists the targeted proteins, peptides, their corresponding charge states and selected transitions. Eight CSF proteins which were moderately abundant; representing potential biomarker candidates for multiple sclerosis (MS) patients were targeted. The peak areas, area ratios calculated by MultiQuant, retention time, width at 50% and signal to noise level of selected transitions are summarized. The average protein ratios across three technical replicas were listed with relative standard deviations. Technical and biological variation are also calculated and described as %CV. Outlier peptides are colored in bold black. The levels of blood-derived proteins and brain specific proteins across six different samples were depicted in Figure S-5.

Table S-9. Comparison between the two methods, dimethyl-SRM *vs.* SID-SRM

Targeted Proteins in CSF	Selected peptides	Selected transitions	spiked-in concentration SID-SRM (fmol)	# of Patients	SID-SRM area ratio	Technical variation %CV	Dimethy-SRM area ratio	Technical variation %CV	% Error dimethyl *vs* SID
Secretogranin-1	NYLNYGEEGAPGK	y9,y10,y11	20	NN1	0.57	2.71	0.74	0.08	17.72
				NN10	0.64	4.95	0.56	11.94	9.89
				NN20	0.61	5.91	0.55	4.89	6.95
				NN29*	**0.51**	**3.16**	**0.71**	**21.64**	**23.33**
				NN41	0.27	5.85	0.29	17.61	3.91
				NN48	0.39	9.98	0.44	12.12	7.31
α-2 macroglobulin	AIGYLNTGYQR	y6,y7,y9	5	NN1	1.25	7.31	1.03	2.28	13.28
				NN10*	**0.42**	**5.17**	**0.67**	**1.90**	**32.62**
				NN20	0.51	2.13	0.49	16.58	2.42
				NN29	0.60	4.93	0.66	2.81	6.23
				NN41*	**0.33**	**1.14**	**0.53**	**8.20**	**33.10**
				NN48	0.46	6.68	0.52	8.99	7.62
α-1 acid binding protein	TEDTIFLR	y5,y6,y7	50	NN1	1.57	1.92	1.71	0.92	5.73
				NN10	0.70	5.03	0.55	3.72	16.91
				NN20*	**0.54**	**8.64**	**0.88**	**1.55**	**33.79**
				NN29	0.86	5.03	1.10	6.88	16.78
				NN41	0.96	7.58	0.79	3.36	13.50
				NN48	0.67	7.78	0.83	3.00	15.84
Prostaglandin H2-D isomerase	AQGFTEDTIVFLPQTDK	y10,y11,y13	500**	NN1	1.00	6.98	1.08	2.27	5.37
				NN10	0.92	8.87	0.75	3.63	14.50
				NN20	0.69	9.21	0.69	8.51	0.18
				NN29	0.75	9.91	1.01	3.14	20.11
				NN41	0.60	2.96	0.53	7.24	8.80
				NN48	0.57	0.42	0.58	4.35	1.82
Contactin-1	IVESYQIR	y5,y6,y7	50	NN1	Low S/N	-	0.76	3.6	-
				NN10			0.57	6.6	
				NN20			0.62	5.1	
				NN29			0.64	4.9	
				NN41			0.39	4.5	
				NN48			0.33	17.5	
Vitamin-D binding protein	ELPEHTVK	y4,y5,y6	Poor solubility	NN1	Poor solubility	-	0.73	9.6	-
				NN10			0.72	7.6	
				NN20			0.51	12.3	
				NN29			1.23	21.3	
				NN41			0.69	21.4	
				NN48			0.93	21.7	
Chromatogranin-A	GLSAEPGWQAK	y6,y7,y8	Low S/N	NN1	Low S/N	-	0.74	3.0	-
				NN10			0.56	8.7	
				NN20			0.63	5.1	
				NN29			0.85	1.1	
				NN41			0.27	13.7	
				NN48			0.40	6.1	
Cystatin-C	ALDFAVGEYNK		Poor solubility	NN1	Poor solubility	-	1.20	2.2	-
				NN10			0.74	2.0	
				NN20			1.00	2.4	
				NN29			1.00	1.3	
				NN41			0.59	2.0	
				NN48			0.73	2.2	

Low S/N = signal to noise less than 10.
* More than 20% error between the two methods, SID-SRM and dimethyl-SRM.
**SIS peptide which was approximated 3 times lower than the endogenous peptide present in the analyte.

Supplemental reference:

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