Supplementary Information

Details of experimental procedures

Materials and Chemicals. Human Immunoglobulin G (IgG) standard and one pooled serum sample were purchased from Sigma-Aldrich (St. Louis, MO). Ten serum samples were collected from healthy patients by the UC Davis Medical center, following protocols approved by the UC Davis Medical Center IRB. The serum samples were stored at -80°C prior to sample preparation. Sequencing grade modified trypsin and Dithiothreitol (DTT) were purchased from Promega (Madison, WI). Iodoacetamide (IAA) was purchased from Sigma-Aldrich (St. Louis, MO).

Sample preparation. Accurate amount of IgG standards were weighed using a micro balance (Mettler Toledo, XP26), and dissolved in 50 mM NH_4HCO_3 to a concentration of 5 mg/mL (IgG stock solution). Sonication was applied for 1 h for IgG proteins to dissolve. For trypsin digestion, 20 µL of the IgG stock solution (100 µg IgG protein) was reconstituted in 50 mM NH_4HCO_3 (freshly made) to a total volume of 100 µL. Proteins were reduced using 2 µL of 550 mM DTT in a 60°C water bath for 50 min, and alkylated using 4 µL of 450 mM IAA at room temperature in the dark for 30 min. Then, 1 µg of trypsin in 2 µL of 50 mM NH_4HCO_3 was added and proteins were digested in a 37°C water bath for 18 h. For the tryptic digestion of serum samples, 100 µL of 50 mM NH_4HCO_3 was added to 2 µL of serum. Reduction, alkylation and trypsin digestion were performed as described above for the IgG standard. The resulting peptide samples were used directly for mass spectrometry (MS) analysis without any sample cleanup.

UPLC-ESI-QqQ analysis. The peptide samples were analyzed using an Agilent 1290 infinity LC system coupled to an Agilent 6490 triple quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA). An Agilent Eclipse plus C18 (RRHD 1.8 µm, 2.1x100 mm) was used for UPLC separation.

The IgG standard sample was diluted serially in nano pure water to obtain a calibration curve for protein quantitation prior to injection. No dilution was performed for the serum samples. 1.0 µL of sample was injected for each run. Three replicate injections were performed for each IgG standard solutions to evaluate the instrument repeatability. One nano-pure water blank was run after every three sample runs to evaluate potential carry over.

The LC separation was performed using a binary gradient at 0.5 mL/min flow rate: solvent A of 3% acetonitrile, 0.1% formic acid; solvent B of 90% acetonitrile, 0.1% formic acid in nano-pure water (v/v). The 10-minute gradient was as follows: 0 min at 2.0% B; 2.5 min at 5.0% B; 7.0 min at 26.5% B; the column was washed at 100% B from 7.1 min to 8.6 min, and reequilibrated at 2.0% B from 8.7 min to 10 min.

The MS was operated in the positive mode. Q1 and Q3 were operated at unit resolution. The optimal parameters used were as follows: drying gas (N_2) temperature and sheath gas (N_2) temperature 300°C, drying gas flow rate 11 L/min, sheath gas flow rate 12 L/min, nebulizer pressure 30 psi, capillary voltage 1800 V, fragmentor voltage 280 V, RF voltage amplitude of high pressure and low pressure ion funnel were 100 V and 60 V, respectively.

The dynamic MRM mode was used, in which transitions were monitored only when the target analyte was eluted. The cycle time was fixed at 500 ms, while the dwell time depended on the number of concurrent transitions monitored. The maximum number of concurrent

transitions was 15, and the minimum and maximum dwell time were therefore 32.25 ms and 499.20 ms, respectively.

The MRM result was analyzed using Agilent MassHunter Quantitative Analysis B.4.0 software. The peak area was integrated by the software, and used for quantitation. The limit of detection (LOD) and limit of quantitation (LOQ) are defined as S/N ≥ 3 and 6, respectively.

Nano-LC-Chip-QTOF analysis. The peptide samples were profiled using an Agilent 1200 series HPLC-Chip system coupled to an Agilent 6520 QTOF (Agilent Technologies, Santa Clara, CA). The microfluidic chip was consisted of two C18 columns (300 Å, 5 µm): one for enrichment (4 mm, 40 nL), one for separation (43 mm x 75 µm).

No dilution was performed for the tryptic peptide samples. 3.0 µL was injected for each sample run. The LC separation was performed using a binary gradient at 0.3 µL/min flow rate: solvent A of 3% acetonitrile, 0.1% formic acid; solvent B of 90% acetonitrile, 0.1% formic acid in nano-pure water (v/v). The 60-minute gradient used was as follows: 0 min at 3.0% B; 5 min at 8% B; 40 min at 35% B; 45 min at 45% B; the column was washed at 97% B from 48 min to 50 min, and reequilibrated at 3% B from 50.1 min to 60 min.

The MS was operated in the positive mode with the parameters as follows: drying gas temperature 325°C, drying gas flow rate 5 L/min, fragmentor voltage 175 V, skimmer voltage 65 V. The acquisition rate was 7.99 spectra/s and 3 spectra/s for the MS scan and MS2 scan, respectively. The collision energy used for the tandem experiment was calculated on the basis of the m/z value using the relationship

$$CE\ (eV) = 1.8\ \frac{m}{z} \times 100\ - 2.4 \qquad \text{eq (1)}$$

Data analysis. The tandem data was analyzed using the online Global Proteome Machine (GPM) to evaluate the protein content in the samples.[40] Briefly, tandem results were exported as Mascot Generic files (MGF) using MassHunter Qualitative Analysis 4.0 software (Agilent Technologies). Results were searched against the SwissProt human protein database with the X! Tandem algorithm. A MS tolerance of ±10 ppm and a MS/MS tolerance of ±100 ppm were used. Up to one missed cleavage site was allowed for trypsin digestion. Carbamidomethylation to cysteine was selected as a complete modification, while oxidation of methionine and deamination of glutamine were selected as a potential modification.

Peptides for IgG protein quantitation were selected based on the peptide profile. Glycopeptide identification was performed using in-house software, GPFinder. Briefly, carbohydrate oxonium ions, such as, m/z 204.08 (HexNAc), m/z 366.14 ($Hex_1HexNAc_1$), m/z 292.09 (Neu5Ac) and m/z 657.24 ($Hex_1HexNAc_1Neu5Ac_1$) were used as diagnostic fragments for glycopeptides. The glycopeptide composition was assigned on the basis of their exact mass and the fragmentation pattern.

Table SI-1

Table SI-1. Inter-day repeatability of the tryptic digestion of an IgG standard. Relative standard deviations are based on triplicates performed on different days. The two "common peptide" are found in all the subclasses. The peptides for each individual subclass are provided. When multiple peptides are provided, the one with the lowest RSD was used for quantitation.

Protein	Sequence	Average Resp. (Ion Counts)	RSD (%)
Common peptide	NQVSLTCLVK	59,204	14.0%
	DTLMISR	988,877	2.9%
IgG1	TTPPVLDSDGSFFLYSK	30,592	18.9%
	FNWYVDGVEVHNAK	8,208	3.7%
	GPSVFPLAPSSK	415,139	4.2%
IgG2	CCVECPPCPAPPVAGPSVFLFPPKPK	29,305	8.3%
IgG3	WYVDGVEVHNAK	5,270	5.6%
	TPEVTCVVVDVSHEDPEVQFK	959	20.3%
IgG4	TTPPVLDSDGSFFLYSR	476	3.7%

Table SI-2

Table SI-2. IgG concentrations obtained in serum samples. The total IgG concentration was calculated using the linear regression curve in Figure SI-3, $y=1.16x10^3 x + 1.12x10^3$, $R^2 =1.000$. The average IgG concentration of these serum samples was 10.6 mg/mL, RSD=29.3%.

Sample	Age (Years)	IgG Conc.* (mg/mL)
Pooled serum	Unknown	10.1
Serum Control 1	35	8.1
Serum Control 2	57	15.1
Serum Control 3	35	13.5
Serum Control 4	57	10.0
Serum Control 5	54	14.1
Serum Control 6	47	5.9
Serum Control 7	62	7.6
Serum Control 8	54	8.5
Serum Control 9	25	10.0
Serum Control 10	55	14.3

Figure SI-1

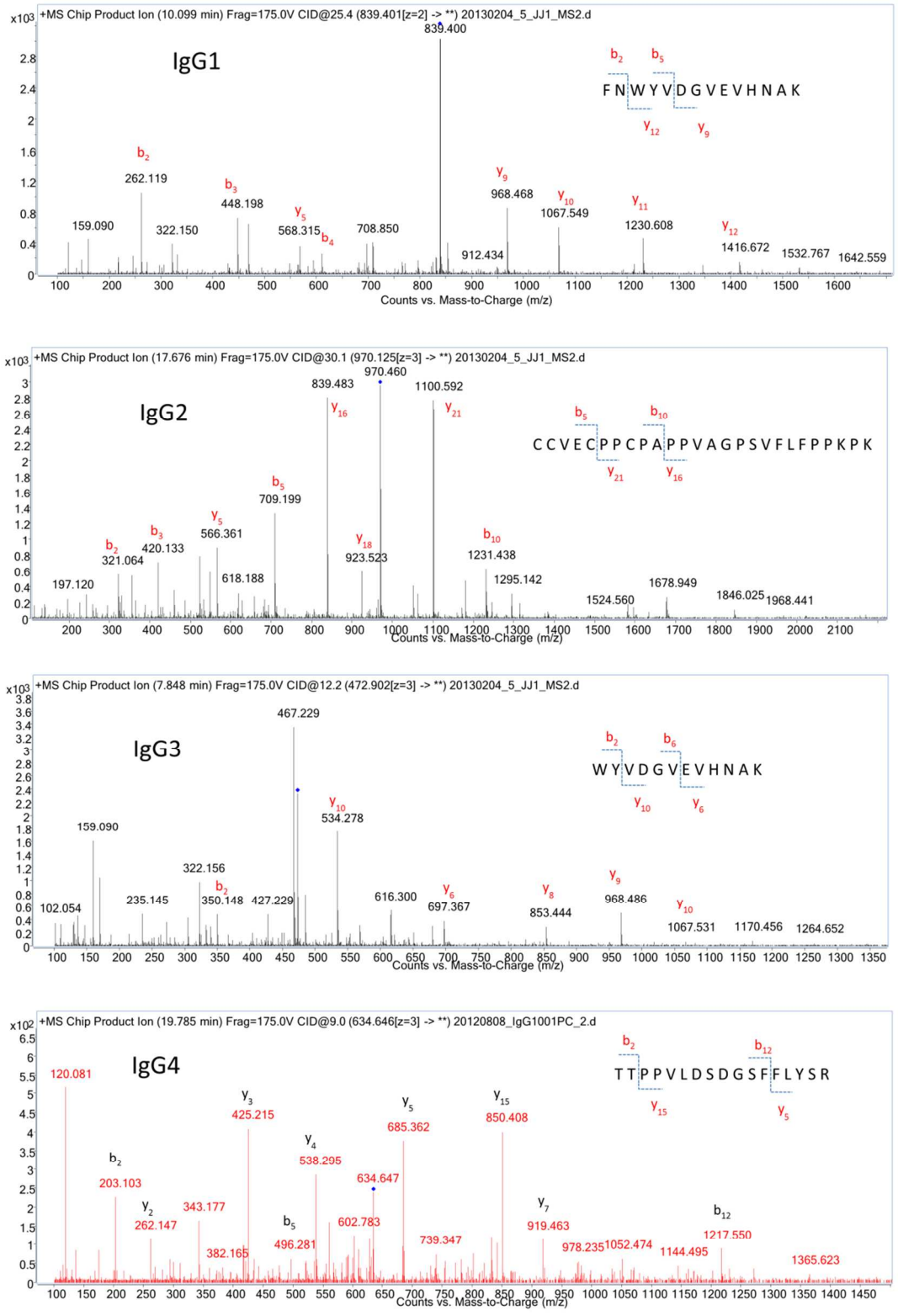

Figure SI-1. Tandem spectra of the unique peptides of the four IgG subclasses as obtained by Q-TOF mass spectrometry. The tryptic peptides (inset) were used for quantitation in the MRM experiments. The tandem mass spectra show that these peptides generate mostly y and b-type ions. The MRM transitions were selected according to the most abundant y or b fragment ion.

Figure SI-2

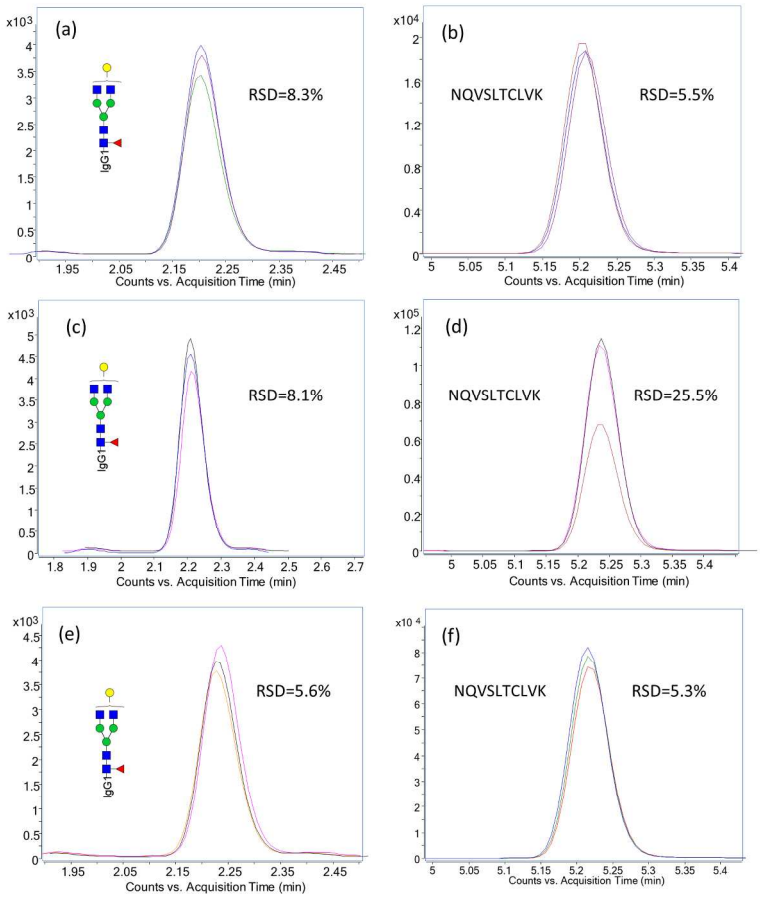

Figure SI-2. Repeatability of the intra-day and inter-day instrumental response and the intra-day enzyme digestion. Overlayed extracted ion chromatograms are depicted for the IgG1 glycopeptide $Hex_4HexNAc_4Fuc_1$-EEQYNSTYR (left panels) and the peptide NQVSLTCLVK (right panels). To evaluate the intra-day instrument stability, three replicate LC-MS analyses of the tryptic glycopeptide and the peptide were performed on the same day (a and b). For these experiments RSD values were 8.3% and 5.5%, respectively. Similar experiments were performed on three separate days to determine day-to-day variations. The results are shown as overlayed chromatograms in (c) and (d). Values were higher corresponding to 25.5% for one peptide but lower for the glycopeptides (8.1%). The large variation in this peptide was later determined to be due to deamination of the peptide, which was diminished in the single day analysis (b). To evaluate digest replicates in the same day, three samples were digested and analyzed on the same day. The results show low RSD for both the glycopeptide (e) and the peptide (f), RSD 5.6% and 5.3%, respectively.

Figure SI-3.

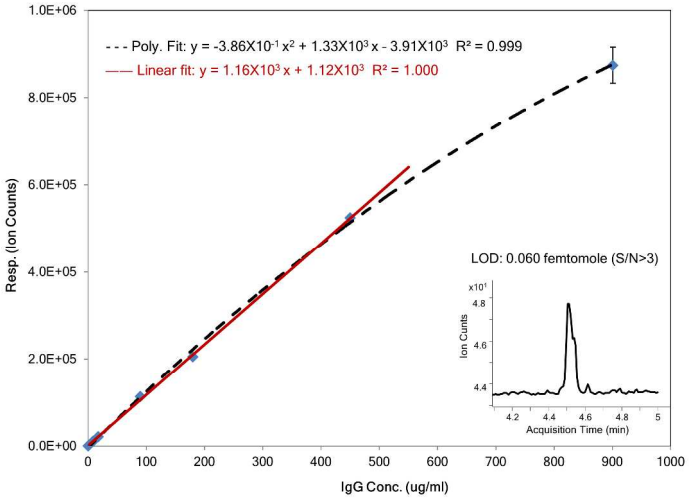

Figure SI-3. Peptide calibration curve for total IgG quantitation. The peptide sequence is DTLMISR. The IgG concentrations plotted were from 0.901 µg/ml to 9.01x10^2 µg/ml. (Dashed line) The response can be fitted to a quadratic equation, y = − 0.386 x^2 + 1.33x10^3 x – 3.91x10^3, R^2=0.999. The dynamic rang was over 1000. (Solid line) The linear fit generated an equation, y=1.16x10^3 x + 1.12x10^3, R^2=1.000. The linear range was around 500. The limit of detection for IgG content was 0.060 femtomoles (or 9.01x10^{-3} µg/ml, S/N>3). For each concentration, there are three replicate injections to evaluate the instrument inter-day repeatability. The result shows a RSD <5%.

Figure SI-4.

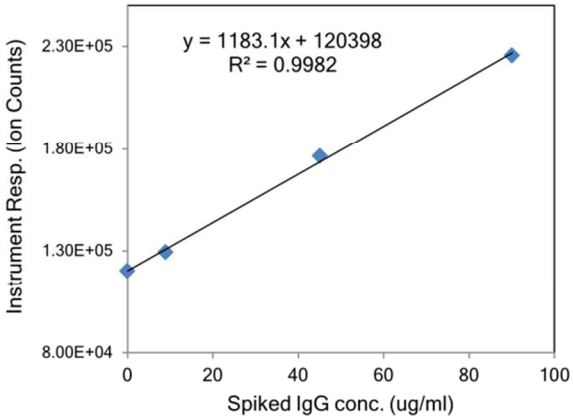

Figure SI-4. Standard addition reveals limited matrix effects in serum. IgG standards were spiked into serum to evaluate effect of the serum matrix. The IgG concentration in the diluted sample was determined to be 1.02 x 10^2 µg/ml (the value at the x-intercept), which corresponded to 11.5±0.5mg/ml in serum.

Figure SI-5.

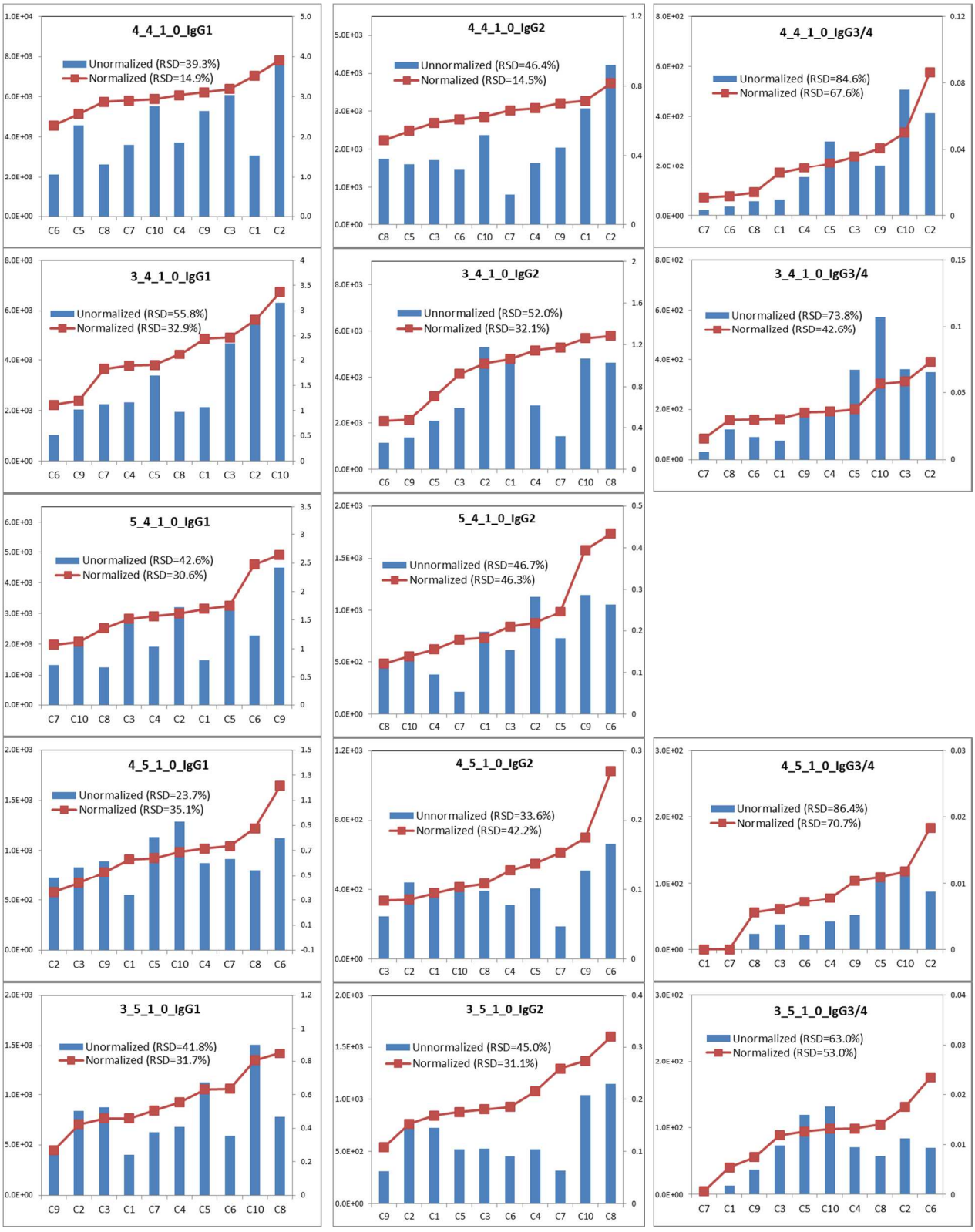
4_4_1_0_IgG1
Unormalized (RSD=39.3%)
Normalized (RSD=14.9%)
C6 C5 C8 C7 C10 C4 C9 C3 C1 C2
4_4_1_0_IgG2
Unnormalized (RSD=46.4%)
Normalized (RSD=14.5%)
C8 C5 C3 C6 C10 C7 C4 C9 C1 C2
4_4_1_0_IgG3/4
Unormalized (RSD=84.6%)
Normalized (RSD=67.6%)
C7 C6 C8 C1 C4 C5 C3 C9 C10 C2
3_4_1_0_IgG1
Unormalized (RSD=55.8%)
Normalized (RSD=32.9%)
C6 C9 C7 C4 C5 C8 C1 C3 C2 C10
3_4_1_0_IgG2
Unnormalized (RSD=52.0%)
Normalized (RSD=32.1%)
C6 C9 C5 C3 C2 C1 C4 C7 C10 C8
3_4_1_0_IgG3/4
Unormalized (RSD=73.8%)
Normalized (RSD=42.6%)
C7 C8 C6 C1 C9 C4 C5 C10 C3 C2
5_4_1_0_IgG1
Unormalized (RSD=42.6%)
Normalized (RSD=30.6%)
C7 C10 C8 C3 C4 C2 C1 C5 C6 C9
5_4_1_0_IgG2
Unnormalized (RSD=46.7%)
Normalized (RSD=46.3%)
C8 C10 C4 C7 C1 C3 C2 C5 C9 C6
4_5_1_0_IgG1
Unormalized (RSD=23.7%)
Normalized (RSD=35.1%)
C2 C3 C9 C1 C5 C10 C4 C7 C8 C6
4_5_1_0_IgG2
Unnormalized (RSD=33.6%)
Normalized (RSD=42.2%)
C3 C2 C1 C10 C8 C4 C5 C7 C9 C6
4_5_1_0_IgG3/4
Unormalized (RSD=86.4%)
Normalized (RSD=70.7%)
C1 C7 C8 C3 C6 C4 C9 C5 C10 C2
3_5_1_0_IgG1
Unormalized (RSD=41.8%)
Normalized (RSD=31.7%)
C9 C2 C3 C1 C7 C4 C5 C6 C10 C8
3_5_1_0_IgG2
Unnormalized (RSD=45.0%)
Normalized (RSD=31.1%)
C9 C2 C1 C5 C3 C6 C4 C7 C10 C8
3_5_1_0_IgG3/4
Unormalized (RSD=63.0%)
Normalized (RSD=53.0%)
C7 C1 C9 C3 C5 C10 C4 C8 C2 C6

Figure SI-5 (continued).

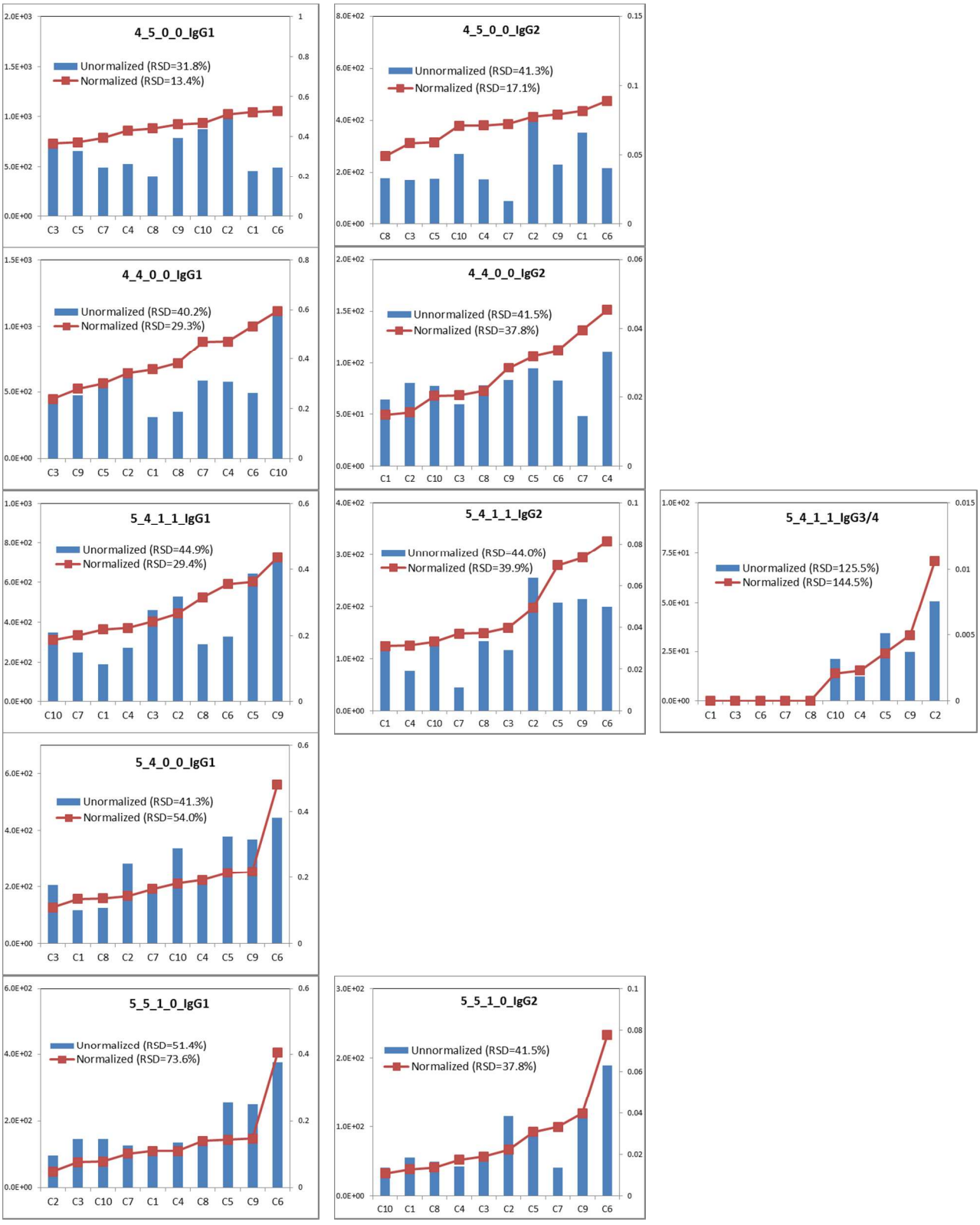

Figure SI-5 (continued).

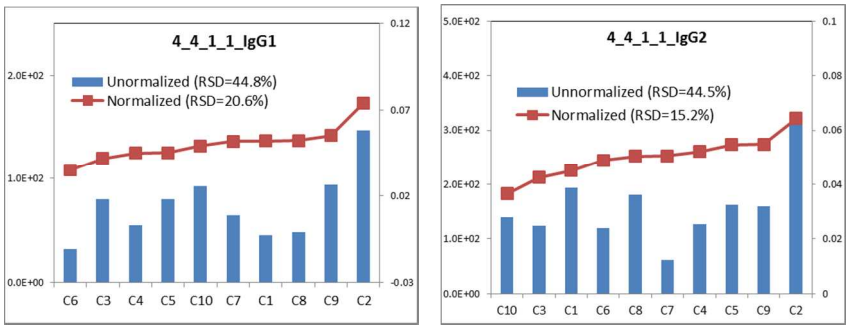

Figure SI-5. Normalized (red squares) and unnormalized (blue bars) glycopeptide abundances of 26 IgG glycopeptides monitored in the sera of individuals. Normalization was performed using equation 2. For each glycoform, the normalized trends in abundances do not directly match the unnormalized ones. The results suggest the protein abundances play a big role in the intensities of individual glycoforms.