Supplemental Materials

Quantifying Thyroglobulin, a Low-abundance Protein in Serum, by Immunoaffinity Peptide Enrichment and Tandem Mass Spectrometry.

Contents:
Supplemental Methods. Page 1.
Supplemental Figure 1. Page 3.
Supplemental Table 1. Page 4.

Supplemental Methods

Peptide isolation and identification Purified human thyroglobulin (Cortex Biochem, San Leandro, CA) was digested with three different digestion protocols. For Digestion 1, Tg was denatured in 3.5 mol/L urea in 100 mmol/L NH4HCO3, reduced with 5 mmol/L dithiothreitol (DTT; Bio-Rad, Hercules, CA), alkylated with 15 mmol/L iodoacetamide (Bio-Rad), and then digested for 18 h at 37°C with modified sequencing grade trypsin (Promega; Madison, WI; 100 µg Tg per mL; trypsin:Tg ratio 1:20 w:w). Digestion 2 used the same conditions as Digestion 1 without reduction and alkylation. Digestion 3 used the same conditions as Digestion 1 except that Tg was supplemented with normal human serum (2 mg Tg per 126 mg human serum proteins; 1.28 mg protein per mL).

The resulting peptides from each digestion were purified using a hydrophilic lipophilic binding (HLB) solid phase extraction cartridge (1 mL bed volume; Waters, Milford, MA). After washing twice with 1 mL 0.1% formic acid in water, peptides were eluted with 80% acetonitrile, 0.3% formic acid, dried down under nitrogen, and reconstituted in 100 µL of 5% acetonitrile, 0.3% formic acid in water.

Peptides were identified using two complementary techniques: i) microelectrospray ionization liquid chromatography-tandem mass spectrometry (LC-MS/MS) on a Thermo-Finnigan LTQ ion trap analyzer (Waltham, MA); and ii) off-line HPLC-plate spotting followed by matrix assisted laser desorption ionization-tandem time of flight mass spectrometry (MALDI-TOF-TOF) with an Applied Biosystems 4700 mass analyzer (Foster City, CA).

For ion trap data, database searching was performed using Sequest (Thermo Finnigan) (1). The human IPI database (version 3.07) was searched using a parameters file that specified no enzyme, average precursor mass tolerance of 3.6 Da, monoisotopic fragment mass tolerance of 0.5 Da, carboxymethylation of all cysteines (M+57), and variable oxidation of methionines (M+16). The results were then filtered to accept only tryptic digests, including partial digests.

For time-of-flight data, database searching was performed using Mascot (Matrix Science, Boston, MA) (2). The Swiss-Prot database (version 42.0) was searched locally using parameters that specified trypsin as the enzyme, up to 5 missed cleavages, monoisotopic precursor mass tolerance of 1.0 Da, monoisotopic fragment mass tolerance of 0.1 Da, carboxymethylation of all cysteines (M+57), and variable oxidation of methionines (M+16). The peptides used in the assay described in the text were unique in the non-redundant human genome database by BLAST searching of the peptide sequences against the translated database (www.ncbi.nlm.nih.gov/blast/Blast.cgi). Parameters were optimized for short sequences (Word Length of 2, Expect Value of 20,000, using the PAM30 alignment algorithm).

Liquid chromatography-tandem mass spectrometry Peptides (6 µL) were loaded onto a peptide trapping column (1 mm x 5mm, Acclaim PepMap100 C18; Dionex, Sunnyvale, CA) and washed

with 100% buffer A (2% acetonitrile, 0.1% formic acid in water) before being eluted onto a 0.15 x 150 mm 5 mm 200 Å C18AQ analytical column (Microm, Auburn, CA) with a linear gradient to 35% buffer B (98% acetonitrile, 0.1% formic acid) over 8 min at 1,000 nL/min using a Tempo 1D-plus nanoflow liquid chromatography system (Eksigent; distributed by Applied Biosystems).

Details of the chromatographic method:

Channel 1 (loading peptides onto trapping column and washing)

Solvent A 98% H2O, 2% ACN, 0.1%FA

Time (min)	Flow (nL/min)
0	1000
0.02	20000
3.02	20000
3.03	1000

Channel 2 (gradient development for delivery to mass spectrometer)

Solvent A 98% H2O, 2% ACN, 0.1%FA

Solvent B 98% ACN, 2% H2O, 0.1% FA

Flow Rate 1000nL/min

Time (min)	% Solvent A	% Solvent B
0	95	5
8	60	40
10	5	95
13	5	95
15	95	5
27	95	5

Peptides were analyzed in multiple reaction monitoring mode (MRM) with an Applied Biosystems API 4000 QTRAP using the transitions listed below. Peak areas for the transitions were summed for each peptide using Analyst (version 1.4.2). Endogenous peptide peak areas were normalized to the peak area of the internal standard peptide and averaged for duplicate injections.

Electrospray ion source parameters:

Parameter	Setting
Curtain Gas	20
IonSpray Voltage*	2600-3100
IonSource Gas 1*	15-27
Interface Heater Temperature	140
Collision Gas	High
Declustering Potential	110
Entrance Potential	10
Collision Cell Exit Potential	17
Detector	2000

* Spray voltage and source gas were optimized daily for ion spray stability.

Mass spectrometer settings for MRM:

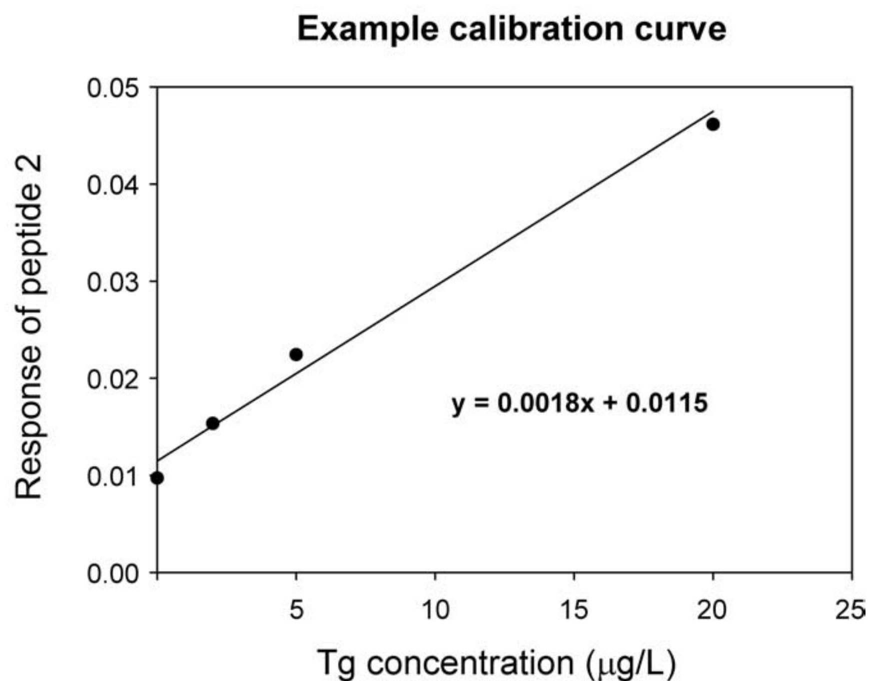
Peptide	Precursor Ion	Product Ion	Product Ion Designation	Dwell Time	Collision energy
Peptide #2	475.25	836.4	y7	50	26
Peptide #2	475.25	779.39	y6	50	24
Peptide #3	604.34	850.47	y8	50	33
Peptide #3	604.34	963.55	y9	50	33
Internal Std	639.75	1065.56	y10	50	34
Internal Std	639.75	547.35	y5	50	34
Peptide #1	636.36	1059.6	y10	50	34
Peptide #1	636.36	541.3	y5	50	34

References

1. Ducret A, Van Oostveen I, Eng JK, Yates JR, 3rd, Aebersold R. High Throughput Protein Characterization by Automated Reverse-Phase hromatography/Electrospray Tandem Mass Spectrometry. *Protein Sci* 1998;**7**:706-19.

2. Perkins DN, Pappin DJ, Creasy DM, Cottrell JS. Probability-Based Protein Identification by Searching Sequence Databases Using Mass Spectrometry Data. *Electrophoresis* 1999;**20**:3551-67.

Supplemental Figure 1



Example calibration curve. Four human serum calibrators with known Tg concentrations (0, 2, 5, and 20 μg/L) were processed and analyzed in parallel with other samples. The calibrators were fit using linear regression in Excel and the concentration of samples determined by interpolation using the regression equation.

Supplemental Table 1. Peptides detected in digested, purified Tg.[a]

		Linear Ion Trap (peak area)			MALDI-TOF-TOF (peak height)
Peptide	**m/z**[b]	**Digestion 1**	**Digestion 2**	**Digestion 3**	**Digestion 1**
VIFDANAPVAVR	1272.5	58,210,204	81,447,918	194,201	51,030
LGDQEFIK	950.1	55,335,595	53,930,998	34,267	8,802
SHGQDSPAVYLK	1302.4	47,082,888	123,119	5,659	5,368
FPLGESFLVAK	1208.4	44,489,374	65,300,983	111,541	11,170
GGADVASIHLLTAR	1381.6	42,916,756	41,942,023	118,664	17,907
FLQGDHFGTSPR	1362.5	40,413,027	64,930,010	55,123	39,950
FLAVQSVISGR	1177.4	37,065,560	55,773,419	0	18,100
FPDAFVTFSSFQR	1549.7	33,791,534	42,267,723	75,971	14,392
WESQLPQPR	1141.3	30,972,286	56,904,484	0	20,485
SQAIQVGTSWK	1205.4	22,604,066	3,943,502	3,572	4,616
KVPTFATPWPDFVPR	1759.1	22,157,262	13,653,736	55,741	6,121
TSGLLSSWK	979.1	17,249,237	3,423,684	0	1,571
LALQFTTNPK	1133.3	16,877,659	17,944,078	70,315	5,354
LRNEDLGLPPLFPPR	1735.0	16,390,867	15,413,817	98,462	5,648
AVLM#GGSALSPAAVISHER[c]	1883.2	12,210,274	7,198,321	4,955	4,510

[a] Purified thyroglobulin was digested and peptides were purified as detailed above in Supplemental Methods.
[b] Average mass/charge is listed for the MH^+ ion of each peptide.
[c] M# refers to oxidized methionine (increased mass of 16 Da).