Supplemental materials for manuscript Kushnir MM, Rockwood AL, Roberts WL, Abraham D, Hoofnagle AN, MeikleAW "Measurement of Thyroglobulin by LC-MS/MS in Serum and Plasma in Presence of Anti-Thyroglobulin Autoantibodies".

Detailed Methods

Preparation of reagents, standards, and quality control samples.

Calibration standards containing Tg were purchased from Beckman Coulter (Fullerton, CA); Tg concentrations in the standards were 0, 0.6, 6, 60, 150 ng/mL (0, 0.91, 9.1, 91 and 227 pmol/L). Rabbit polyclonal anti-Tg antibody was purchased from Covance (Princeton, NJ) and diluted to 30 ng/μL with 0.1% BSA. Serum quality control samples were pooled human serum samples and contained 2, 6.5, and 170 ng/mL (3, 9.8, 258 pmol/L) of Tg (as quantified by LC-MS/MS). Working internal standard (IS) of "winged" peptide, sequence PVPESKVIFDANAPV*AVRSKVPDS (V* [13C5; 15N]; mass shift 6 Da, RS synthesis Louisville, KY) was prepared at concentration 10 pg/μL (3.95 fmol/μL) in 20% acetonitrile in water. Trypsin (purity 99%, activity 15,000 BAEE units/mg protein), formic acid (FA), dithiothreitol (DTT) and sodium deoxycholate (DOC) were purchased from Sigma-Aldrich (St. Louis, MO). All other reagents were of highest purity commercially available. Solvents were of HPLC grade, purchased from JT Baker (Phillipsburg, NJ).

Conjugation of antibody to magnetic beads.

Custom (affinity purified) polyclonal rabbit anti-peptide antibody (Covance, Princeton, NJ) was conjugated to Tosyl activated magnetic beads (DynaBeads M280, Life Technologies, Carlsbad, CA) according to the manufacturer's recommendations. Briefly, the beads were washed with PBS and re-suspended in one molar ammonium sulfate solution containing 20 μg of antibody per milligram of beads. Beads were incubated at 37°C for 20 hours, washed and incubated for 1 hour with blocking buffer containing 0.5% BSA, and reconstituted to a concentration of 20 μg/μL (antibody content on the beads was approximately 0.2 μg/μL).

Sample preparation.

Sample preparation was performed on a liquid handler (epMotion, Eppendorf, Hamburg, Germany). To a 500 µL aliquot of serum or plasma sample 10 µL (30 ng/µL) of rabbit anti-Tg antibody was added and the samples were incubated for one hour at ambient temperature. After the incubation, 350 µL of saturated ammonium sulfate solution was added to the samples, and Tg was precipitated along with immunoglobulins (IG). The samples were centrifuged for 5 min at 15000g and the supernatants were discarded. The precipitates were reconstituted with 300 µL of water; and 10µL of IS, 10 µL of 20 mM DTT, and 30 µL of 5% DOC were added; the samples were incubated at 60°C for 30 min. Proteins in the Tg-containing fraction were denatured with DOC (30 µL of 5% solution), reduced with DTT (10 µL of 20 mM solution) and incubated at 60°C for 30 min. After the incubation, 400 µL of 25 mM ammonium bicarbonate and 10 µL of trypsin (4 µg/µL) were added to the samples, and the samples were incubated for 4 hours at 37°C. Experiments showed that concentration of DOC had a critical effect on the extent of denaturing and recovery of the targeted peptide; the lack of DOC led to incomplete digestion, while excess resulted in increased sample viscosity, which affected the IAE. The denaturing, reduction and digestion conditions were optimized to ensure rapid, complete, and reproducible digestion of Tg. Preliminary experiments showed no effect of the cysteines alkylation on the recovery of the targeted peptide (data not shown); therefore cysteines were not alkylated in this method.

Magnetic beads were processed using a Magnetic Stand-96 (Life Technologies, Carlsbad, CA). After the digestion, 5 µL of the magnetic beads suspension was added and the samples were incubated with agitation at 20°C for 8 h to allow antibody capture of the targeted peptide from the digest. The beads were washed with PBS (pH 7.4) three times, and the targeted peptide and the IS were eluted with 75 µL of 25 mM glycine (pH 2). The elutions were transferred into a 96-well plate, and 40 µL aliquots were injected on 2D LC-MS/MS. The overall time required for the sample preparation in a 96-well plate format was approximately 20 hours.

Instrumental analysis.

The 2D HPLC separation was performed on an HPLC system consisting of series 1260 and 1290 pumps (Agilent Technologies, Santa Clara, CA). A Zorbax XDB-CN 50x2.1, 5 µm HPLC

column was used for the 1st dimension separation with gradient of mobile phases A 98% to 87%A in 1.3 min (A, 10 mM FA in water; B, 10 mM FA in acetonitrile); the 2nd dimension separation was on a Poroshell 120EC-C18, 100x3, 2.7 μm column (both columns from Agilent Technologies) using gradient of the same mobile phases 87 to 75%A in 2 min. The sample compartment of the autosampler was set at 5°C, and the LC separation was performed at 30°C.

Samples were injected in 1st column with effluent directed to waste, between 2.2 and 2.8 min position of the switching valve was changed and the peak of interest was eluted from the 1st to the 2nd column; after that switching valve was turned to the original position the analytical separation was taking place on 2nd column, while the 1st column was reconditioning.

Quantitative analysis was performed on an API 5500 triple-quadrupole mass spectrometer with a V-spray ionization source operated in a positive ion, multiple reaction monitoring (MRM) mode. Mass transitions monitored in the method were *m/z* 636.36/1059.56, 636.86/1060.56, 636.36/912.49 and 636.36/541.35 for the VIFDANAPVAVR peptide, and *m/z* 639.34/1065.56, 639.84/1066.56, 639.34/918.48, 639.34/547.34, for the IS. The instrument settings were adjusted to maximize the sensitivity and the specificity of detection. The heating gas temperature was 450°C. The settings for the nebulizing gas (air), collision and curtain gas (nitrogen) were 40, 9, and 40. The optimized declustering potential, collision energy, collision cell exit potential, and entrance potential were 100, 30, 30, and 10V. The dwell time for the mass transitions was 35 ms. The Q1 quadrupole was set to high resolution and Q3 quadrupole was set to unit resolution; 0.5 and 0.7 Da at half height, respectively. The total analysis time per sample was 6.5 min. The data were processed using software (Analyst 1.5.2).

Total protein concentration was measured by spectrophotometric method using a NanoDrop™ 8000 (Thermo Scientific, Wilmington, DE); concentration of IgG and albumin were measured on an automated immunoassay analyzer (BN II, Dade Behring, Newark, DE) and Modular Analytics (Roche Diagnostics, Indianapolis, IN), respectively. The Tg-AAb test was performed on an automated immunoassay analyzer (IMMULITE 2000, Siemens, Tarrytown, NY).

Method validation

Method validation consisted of the evaluation of the imprecision, sensitivity, linearity, accuracy, recovery, carryover, ion suppression, and the establishment of reference intervals for Tg (1-5). Serum pools used during the method validation were prepared from remaining aliquots of patient samples submitted to ARUP laboratories for testing. All studies with human samples were approved by the Institutional Review Board of the University of Utah. An assessment of within and between run imprecision was performed by analyzing pools of human serum samples supplemented with Tg (AbD Serotec, Martinsri, Germany). Concentrations of Tg were 2, 6, 14 and 398 ng/mL (3, 9.1, 21.2, 603 pmol/L); the samples were analyzed in three replicates over five days; in addition three quality control samples (QC) samples were analyzed in routine runs over 20 days.

Limits of detection quantification of the method were evaluated by analyzing serum samples containing progressively lower concentration of Tg. Five samples (range 0.26 - 3.9 ng/mL) were prepared by serial dilution of serum pool containing 3.9 ng/mL (5.9 pmol/L) with a serum pool not containing Tg, the samples were analyzed in duplicate over two days. LOQ (upper limit of linearity) were determined as the lowest (highest) concentrations at which accuracy was within ±15%, imprecision was <15% and a ratio of the mass transitions maintained at ±30%. LOD was the lowest concentration at which chromatographic peaks were present in all mass transitions and signal to noise ratio was >5.

Linearity of the method was evaluated by analyzing seven samples prepared by mixing in different proportions two serum pools containing 5 and 1045 ng/mL (3.3 and 886 pmol/L) of Tg. The samples were analyzed in duplicate over two days. Blank samples were injected after high standards to evaluate carryover potential of the method.

The method was compared with the Access^{TM} Beckman Coulter DxI800 Tg IA performed at ARUP Laboratories. Two types of samples were used for the comparison: samples that tested negative for Tg-AAb (n=73, and samples that tested positive for Tg-AAb (n=113). Samples with concentration of Tg-AAb below 20 IU/mL were considered as Tg-AAb negative. In the Tg-AAb positive samples concentration of Tg-AAb was 20-3000 IU/mL; Tg recovery was

5-97%. Ratio of the concentrations determined from multiple mass transitions was used for evaluation of the specificity of analysis (Kushnir et.al, 2005).

Experiments for evaluation of the digest efficiency were performed by monitoring quantity of the VIFDANAPVAVR peptide produced during digestion of plasma sample containing 50 ng/mL of Tg [the digestion was performed at 37°C and was stopped at different time points from beginning of the digestion (0.5h, 1h, 2h, 4h, 8h, 16h, 24h)]. Following the digestion, isotope labeled internal standard (VIFDANAPV*AVR) was added to the samples and the VIFDANAPVAVR peptide and the internal standard were enriched from the digests (as described in section Materials and Methods). Optimal yield of the peptide was observed at digestion time of 4 hours.

Magnetic bead enrichment recovery was determined by performing affinity enrichment of a pool of digested serum samples containing 80 ng/mL of Tg. To three samples IS was added before, and to another three samples after the enrichment. Difference between the observed concentrations of Tg in the pre- and post-enrichment spiked samples gave a measure of the recovery.

Sample dilution (using Tg-negative serum) was evaluated by analyzing a serum sample containing over 3000 ng/mL of Tg (determined with LC-MS/MS) analyzed with dilution (5, 10, 20, 30, and 50 fold).

Effect of lipemia, hemolysis and icterus was evaluated by analyzing pools of ‘normal’ serum, lipemic, hemolized and ichteric samples ‘as is’, and mixed in ratio 1:1 (‘normal’ serum/ lipemic; ‘normal’ serum/ hemolized; and ‘normal’ serum/ icteric) and concentrations were compared with concentrations measured in the individual samples.

Ion suppression was evaluated using the post column infusion method (Matuzevsky et al., 2003). A set of patient samples free of Tg was analyzed by the method, while a solution of VIFDANAPVAVR peptide (100 ng/mL prepared in mix of 49%water/ 50% acetonitrile/ 1% formic acid, flow rate 5 µL/min) was infused into the effluent of the analytical column. The chromatograms were inspected for signs of ion suppression.

Blood from five volunteers was collected in potassium EDTA, sodium heparin, serum and serum separation tubes and the samples were analyzed by the method. Concentrations observed in the samples were compared within each individual.

Storage stability of thyroglobulin was evaluated with purpose of finding acceptable storage conditions. Aliquots of human serum pool were stored at room temperature, at 4°C, and at -20°C; the tubes were placed into a -70°C freezer after 1, 2, 3, 4, 8, 14, 21 and 28 days of storage and analyzed in a single batch.

Differences in concentrations among the groups in the experiments for evaluation of different types of collection tubes; problem samples (lypemia, hemolysis and icterus); and data of the experiments on assessment of the storage stability were evaluated using ANOVA.

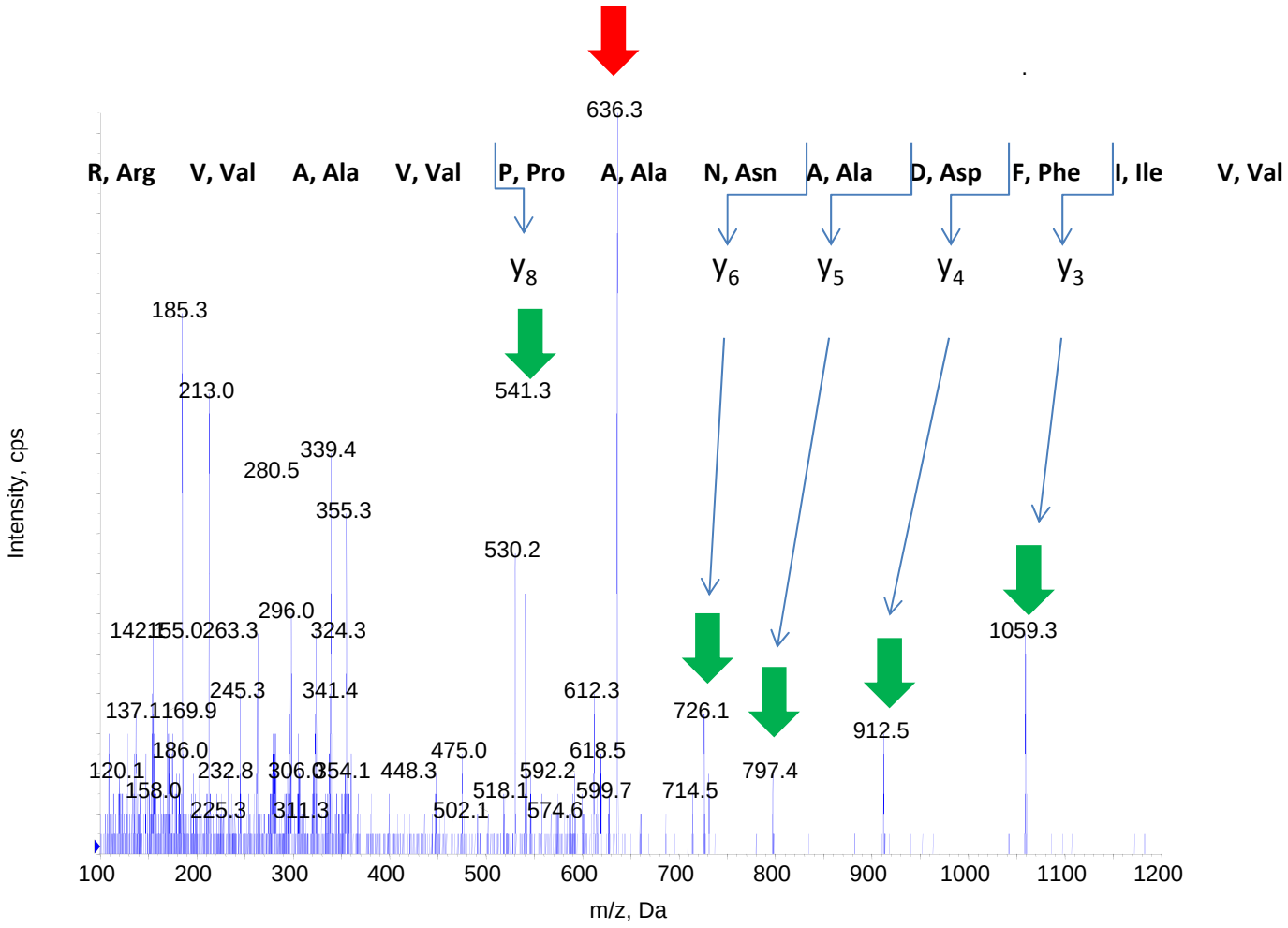
Reference interval study for Tg was performed with samples from self-reported healthy adult volunteers (25 men and 25 women) and 140 samples from children ages 1 – 17 (1, 4, 7, 10, 13, and 16 year old, 10 samples form boys and 10 samples form girls of the above ages). The volunteers were without chronic diseases, and not on thyroid medications; the blood was collected in serum separation tubes, serum was separated, and the samples were stored at -70°C.

References

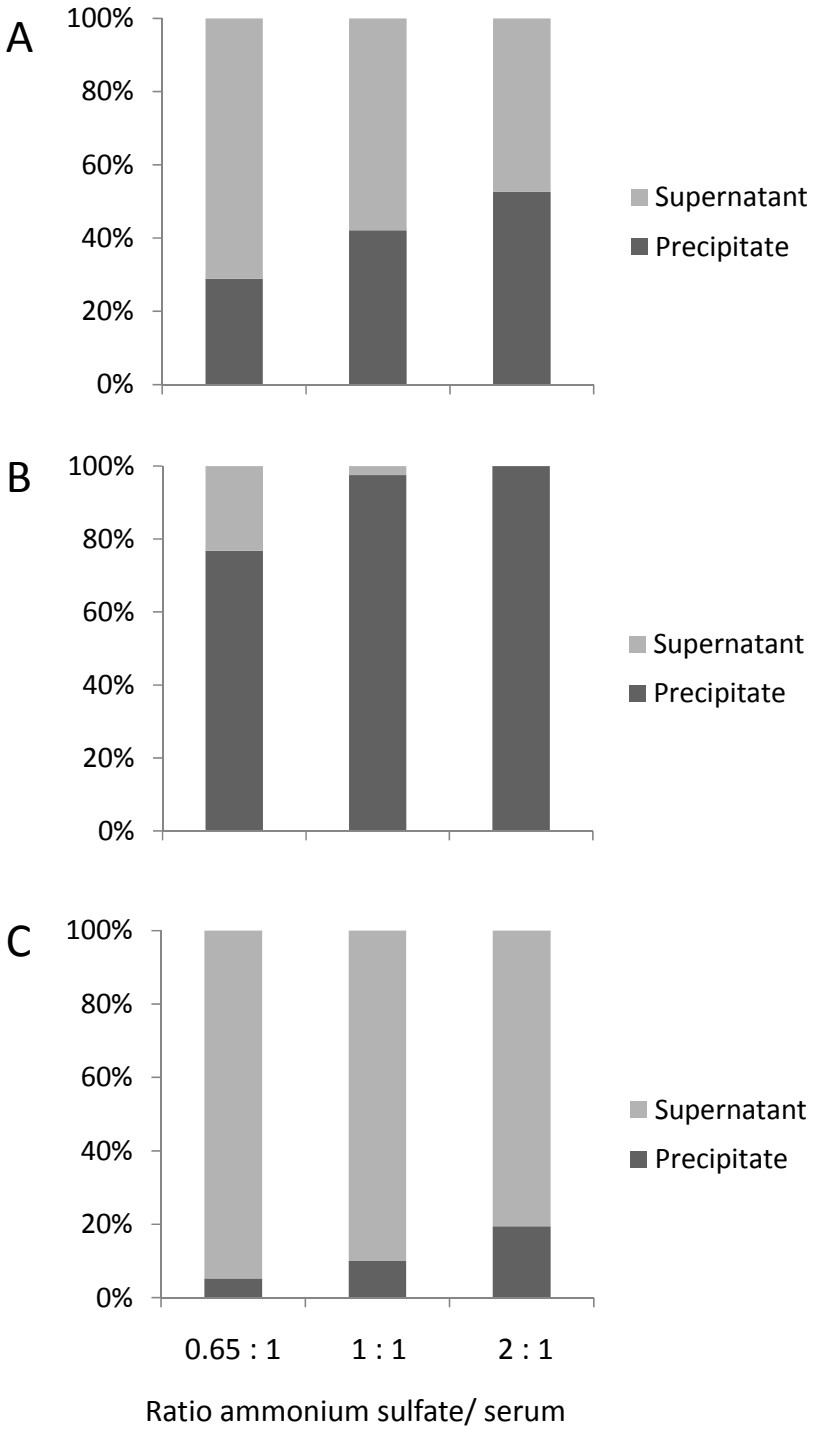
1. CLSI Document EP5-A2. Evaluation of Precision Performance of Quantitative Measurement Methods, Approved Guideline, Second Edition, 2004.
2. CLSI Document EP6-A. Evaluation of the Linearity of Quantitative Measurement Procedures, Approved Guideline, First Edition, 2003.
3. CLSI Document EP9-A2. Method Comparison and Bias Estimation Using Patient Samples, Approved Guideline, Second Edition, 2002.
4. CLSI Document GP10-A. Assessment of the Clinical Accuracy of Laboratory Tests, Approved Guideline, First Edition, 1995.
5. CLSI Document C28-A2. How to Define and Determine Reference Intervals, Approved Guideline, Second Edition, 2000.

Supplemental Table 1. Tg concentrations in lypemic, hemolized and icteric samples analyzed "as is" and mixed in ratio 1:1 with "normal" serum sample (all samples were analyzed in duplicate).

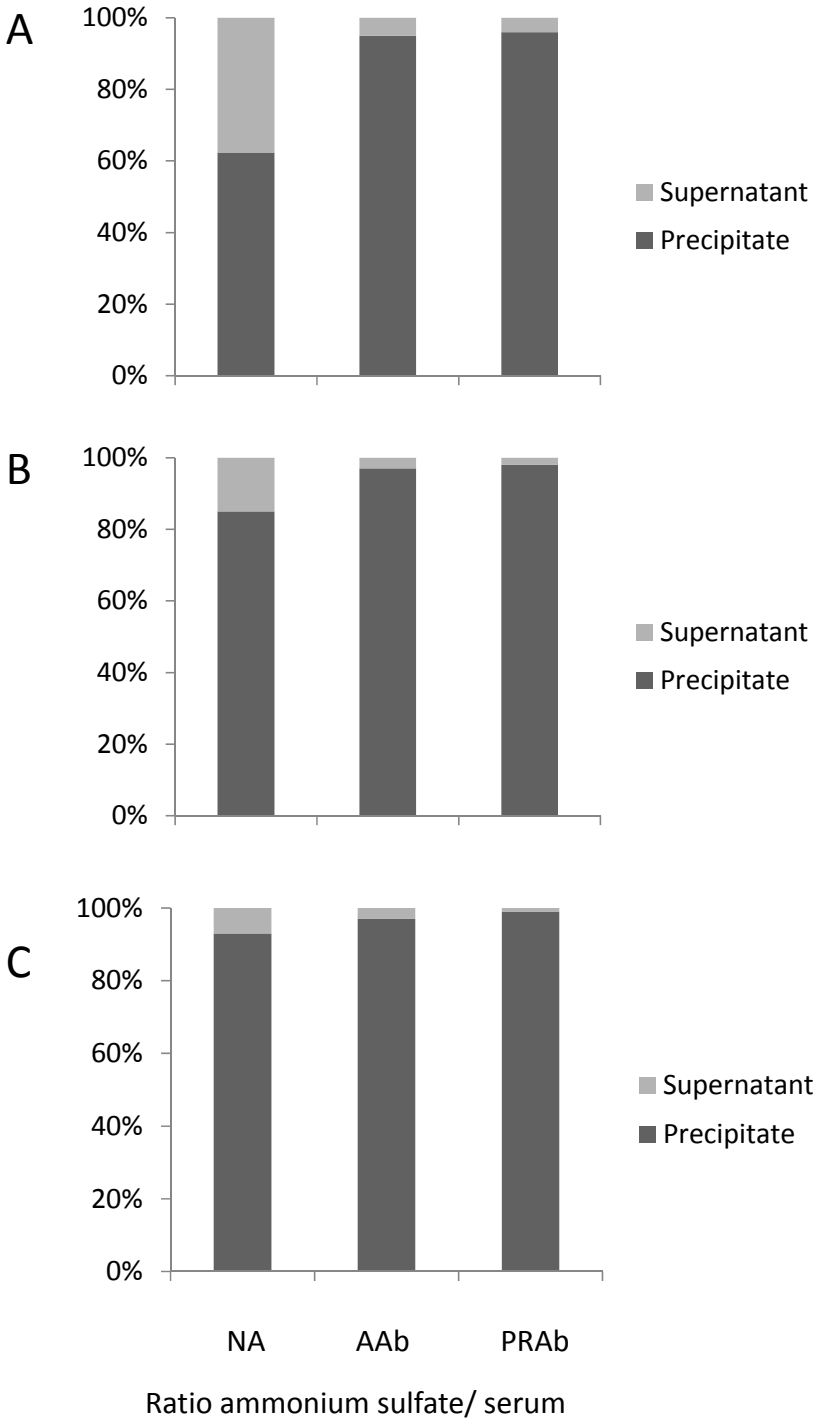
Sample	Observed concentration, ng/mL	Expected concentration, ng/mL	Deviation, %
"Normal" serum	19.3	na	na
Lipemic samples pool	0		
Mix 1 to 1 ("Normal" serum and Lipemic samples pool)	9.6	9.7	-0.8%
Hemolyzed samples pool	1.6		
Mix 1 to 1 ("Normal" serum and Hemolyzed samples pool)	10.3	10.4	-1.4%
Icteric samples pool	0		
Mix 1 to 1 ("Normal" serum and Icteric samples pool)	9.5	9.7	-1.2%



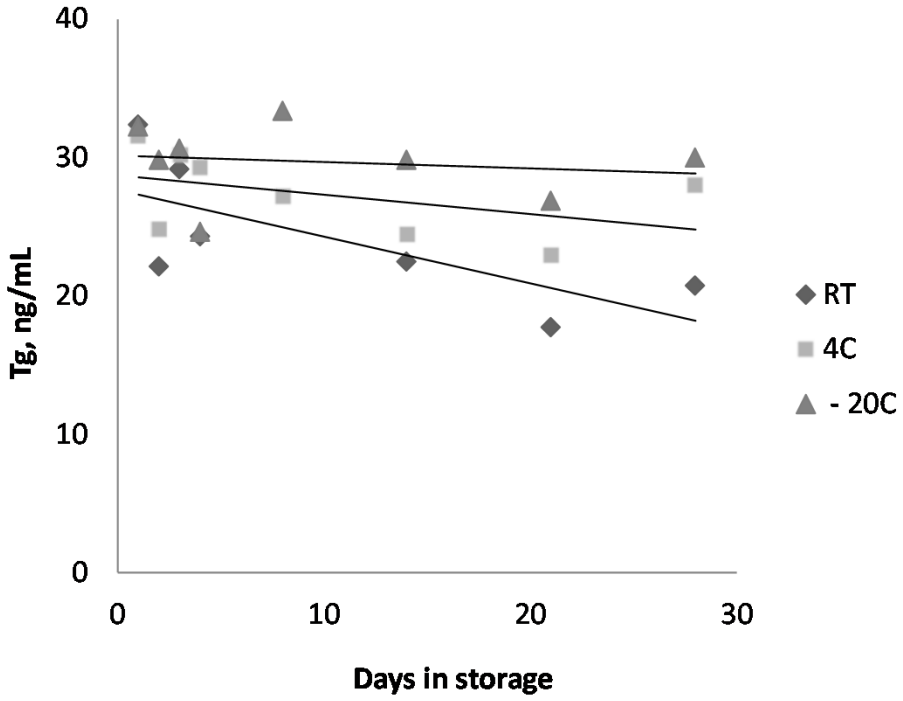
Supplemental Figure 1. Product ion mass spectrum of thyroglobulin-specific peptide, VIFDANAPVAVR. Red arrow points on double-charged parent ion; green arrows point on the major product ions.



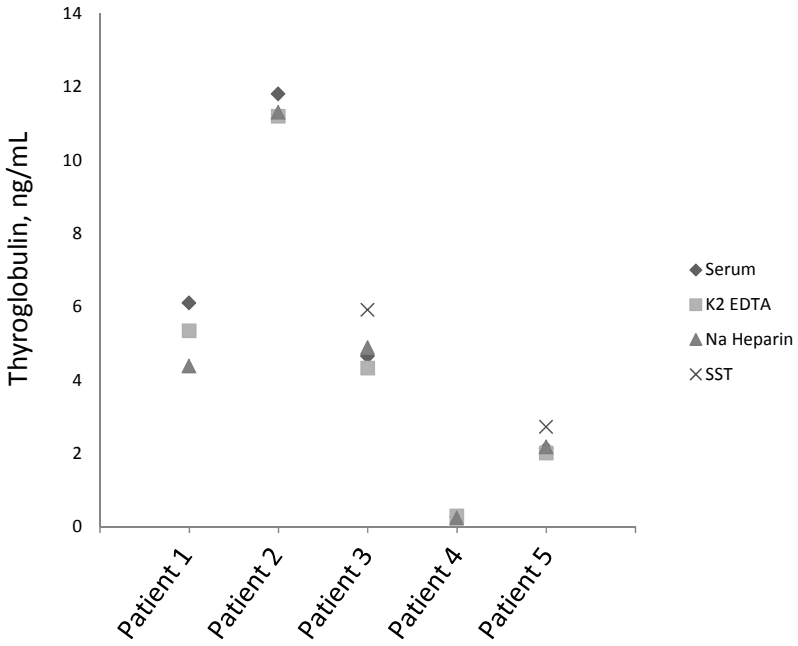
Supplemental Figure 2. Percent distribution of total proteins (A), IgG (B) and albumin (C) between the supernatants and the precipitates.



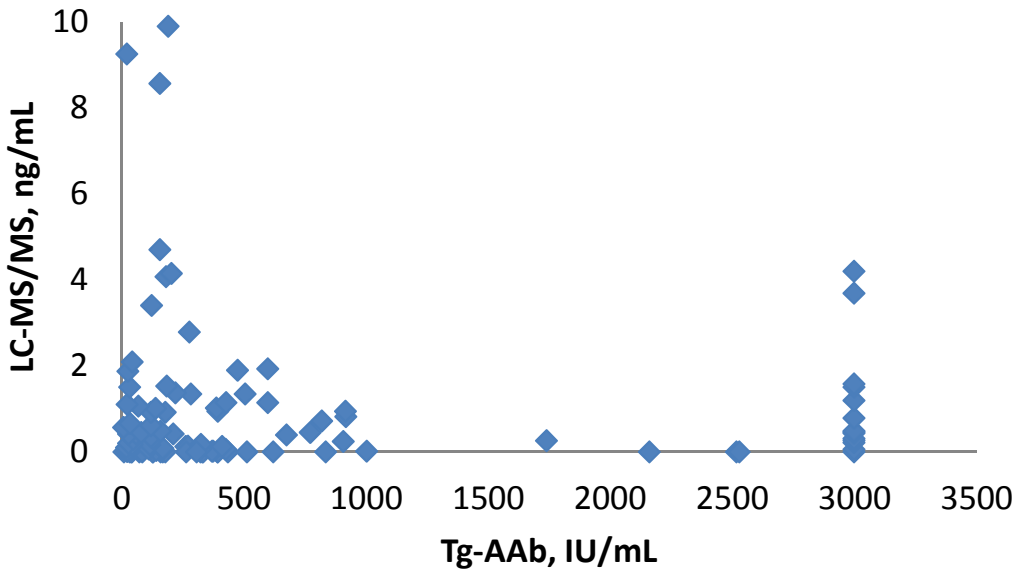
Supplemental Figure 3. Percent distribution of thyroglobulin between the supernatants and the precipitates in samples. Concentration of thyroglobulin in the sample 200 ng/mL; ratio of ammonium sulfate/serum 0.65:1 (A), 1:1 (B) and 2:1 (C). NA – no antibody, AAb – sample contains Tg-autoantibody, PRAb – polyclonal rabbit antibody added to the sample.



Supplemental Figure 4. Evaluation of thyroglobulin stability in sample transport tubes. Serum samples were stored in polypropylene sample transport tubes (Sarstedt, Nümbrecht, Germany) at room temperature (RT), 4 C and -20 C.



Supplemental Figure 5. Thyroglobulin concentration in samples collected in collected in serum, potassium EDTA, sodium heparin, and serum separation tubes (SST).



Supplemental Figure 6. Association between Tg-AAb concentration and concentration of Tg in Tg-AAb positive samples.