Small Volume Retinol Binding Protein Measurement by Liquid Chromatography-Tandem Mass Spectrometry

Supplemental Material

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Specimen Collection and Handling
A minimum of 30 µL serum is required (red or gold top preferred). Lithium heparin plasma is also acceptable. EDTA plasma is not acceptable. Serum/plasma separation should be performed as soon as possible. Samples are stable up to 1 week at 4-8°C, or 1 month at -20°C.

Equipment
Rainin LTS™ single-channel pipette, 1-10µL, with 20µL tips
Rainin LTS™ single-channel pipette, 20-200µL, with 200µL tips
Rainin LTS™ single-channel pipette, 100-1000µL, with 1000 µL tips
Rainin 100-1200µL 8-channel repeater pipette
Rainin, 8-channel pipette, 200 µL
Eppendorf Repeater™ stream pipette
Waters ACQUITY Ultra Performance Liquid Chromatography (UPLC) system
Waters Xevo TQ-S triple quadrupole mass spectrometer with electrospray ionization source
Scientific Industries Vortex Genie 2 vortex mixer
Cole-Parmer multi-tube vortex mixer
Eppendorf ThermoMixer® C with standard non-heated lid
Texan™ reagent reservoir for multichannel pipettes with lid, non-sterile (Sigma Aldrich R1525)
Praxair Liquid Nitrogen Tank, 350 psi, (NI 4.8LC180-350)
Praxair Compressed argon gas tank (AR 6.0RS-K)

Supplies
Eppendorf™ LoBind Microcentrifuge Tubes: Protein, 1.5 mL (cat.# 022431081)
Eppendorf™ LoBind Microcentrifuge Tubes: Protein, 5 mL, safe-lock (cat.# 0030108302)
Conical tube, 15 mL, 17x120mm (Cole-Palmer, cat.# UX-06336-88)
Conical tube, 50 mL (Fisher, cat.# 05-539-7)
Polypropylene vial 50 mL (PerkinElmer, cat. # N8145124)
Greiner MASTERBLOCK®, storage microplate, 96 well, 2 mL (cat.# 780270)
Closure, Micro Mat, square, 96-wel (Agilent, cat.# SN800220)
3M™ Empore™ Sealing Tape Pad, Model 660, for 96 Well Plates (cat.# 98060404724)
Waters ACQUITY UPLC HSS T3 VanGuard Pre-column, 100Å, 1.8 µm, 2.1 mm X 5 mm (cat.# 186003976)
Waters ACQUITY UPLC HSS T3 Column, 100Å, 1.8 µm, 2.1 mm X 50 mm (cat.# 186003538)

Chemicals
Water, Optima™ LC/MS Grade (Fisher, cat.# W6-4)
Water, deionized (DI, Millipore >18Ω)
Acetonitrile, Optima™ LC/MS Grade (Fisher, cat.# A955-4)
Methanol, Optima™, LC/MS Grade (Fisher, cat.# A456-4)
Formic acid 88%, BAKER ANALYZED® ACS, J.T. Baker® (VWR, cat.# JT0128-1)

Formic acid 98-100% Suprapur® (Supelco, cat.# 111670)
Dimethylsulfoxide (Thermo Scientific, cat.# 85190)
Hydrochloric acid, 1N (Fisher, cat.# SA48-4)
Ammonium bicarbonate, BioUltra, ≥99.5% (Sigma-Aldrich, cat.# A6141)
Dithiothreitol (Bio-Rad. cat.# 1610610)
Iodoacetamide (Acros Organics, cat.# AC122270050)
Trypsin, TPCK Treated (Worthington, cat.# LS003740)
Trifluoroethanol, 99.8%, extra pure (Acros Organics, cat.# 139750250)
Fish serum, Cell Culture Grade (GenWay Biotech, cat.# GWB-AE89C9)
Peptide, sequence FSGTWYAMAK, lysine C13/N15-labeled (mass shift +8), 2 mg/mL in 5% ACN, 0.1% formic acid (New England Peptide)
Peptide, sequence YWGVASFLQK, lysine C13/N15-labeled (mass shift +8), 2 mg/mL in 5% ACN, 0.1% formic acid (New England Peptide)
Peptide, sequence LIVHNGYC(+57)DGR, arginine C13/N15-labeled (mass shift +10) with cysteine acetylation, 2 mg/mL in 5% ACN, 0.1% formic acid (New England Peptide)
Native Human Retinol Binding Protein (Bio-Rad, cat.# 7970-0504)
Commercial pooled human serum, off-the-clot (OTC, Golden West Biologicals)
Pooled population serum pool (prepared in-house with >400 leftover clinical samples)

Calibration

Initial five-point external calibrators curve were established using purified human RBP (purity and concentration determined by amino acid analysis) spiked into fish serum. Aliquots (40-50 µL) were stored in 500µL protein LoBind microcentrifuge tubes at - 20°C for 2 years. Subsequent batches are now generated using pooled human sera spiked with purified human RBP (to approximately 10 mg/dL, standard E below) and diluted with pooled human sera and fish serum as indicated. New standards are assayed against current standards on at least 3 separate days to establish set points.

Standard Preparation

Standard [a]	Standard E (mL)	Population Pool (mL)	Fish Serum (mL)	Est. Set Point (mg/dL)
STD E	5	0	0	10
STD D	2.5	2.5	0	7.0
STD C	0	5	0	5.6
STD B	0	2.5	2.5	2.3
STD A	0	0.5	4.5	0.5

[a] All standards were prepared starting from a serum population pool (STD C). The high calibrator (STD E) consisted of STD C spiked with purified RBP. Remaining standards consisted of dilutions of STD C (STD A and STD B) or a 1:1 mixture of C and E (STD D).

Quality Control Materials

Quality control materials are made from low and high concentration pooled human sera with target values of 1.0 mg/dL and 4.0 mg/dL, respectively. Aliquots (40-50 µL) are prepared in 500 µL LoBind microcentrifuge tubes and stored at -20°C for 2 years. New controls are assayed at least 10 times over at least 3 days to establish means.

Working Reagent Preparation

Internal Standard Peptide Mixture

Peptides (supplied at approximately 2 mg/mL in 5% ACN with 0.1% formic acid, verified concentration by AAA on certificate of analysis provided in µM) concentration. For a total volume of 10 mL, determine volume of each peptide stock required to reach final concentration 2 µM for peptide LIVHNGYC(+57)DGR-heavy (arginine C13,N15-labeled) and 3 µM for peptides YWGVASFLQK-heavy and FSGTWYAMAK-heavy. Add standards to a container and bring volume to 10 mL with 5% acetonitrile in H_2O. Aliquot (450 µL each) to 500 µL LoBind snap-cap microcentrifuge tubes. Working internal standard mix is stable at - 20°C for 2 years and 3 freeze-thaw cycles.

Ammonium Bicarbonate (100 mM)

In a 50 mL polypropylene vial, weigh 0.1976g (+/-0.010g) ammonium bicarbonate powder (open under hood). Bring to 25mL using deionized water. Vortex to mix. Store at room temperature for up to 12 hours.

Dithiothreitol (DTT), 0.5 M

Note: DTT is a skin and respiratory irritant and should be handled in a chemical fume hood.
In a 1.5 mL microcentrifuge tube, weigh approximately 18 mg of DTT. Add volume of 100 mM ammonium bicarbonate needed to reach 0.5 M DTT (actual number of milligrams x 12.966 µL per milligram). For example, if the tube contained 32.5mg DTT (32.5 X 12.96 = 421.2) add 421 µL of 100 mM AMBIC. Store at room temperature. Use immediately.

Hydrochloric Acid (1 mM)

In a 50 mL conical tube, add 5 mL deionized water. Add 10 µL 1M HCl. Bring to 10 mL using deionized water. Store at room temperature for up to 12 hours.

Iodoacetamide (0.5 M)

Warm iodoacetamide to room temperature before opening. In a 1.5 mL microcentrifuge tube, weigh out approximately 40 mg iodoacetamide. Record actual weight. Weighed aliquots can be stored at 2-8°C for 6 months protected from light. Add volume of 100 mM ammonium bicarbonate needed to reach 0.5 M (actual number of milligrams x 10.81 µL per milligram). Vortex to dissolve the iodoacetamide. Protect from light and use immediately.

Trypsin (2 mg/mL)

Warm Worthington trypsin to room temperature before opening. In a 5 mL Eppendorf LoBind tube, weigh approximately 4 mg trypsin powder. Record actual weight. Store weighed trypsin aliquots at 2-8°C for up to 2 months or -20°C for up to 1 year. Prepare 2 mg/mL solution by adding the appropriate volume of 1 mM HCl: (actual number of mg Trypsin) x (0.5 mL). Mix well by inversion (do not vortex).

Notes: Trypsin is a fluffy powder and is sensitive to static electricity. Use of N95 face mask is required during handling. Prior to using a new lot of trypsin, a test run of calibrators and controls digested with the new trypsin must be performed and compared to the old lot.

Diluent 1 (Sample Diluent Containing DTT and Heavy Labeled Peptides in ammonium bicarbonate)

Prepare diluent in excess of what is required for samples. In a 5 mL LoBind conical tube, combine:

- Internal Standard Peptide Mixture: (4 µL) x (number of samples + 5)
- 100 mM ammonium bicarbonate: (31 µL) x (number of samples + 5)
- 0.5 M DTT (1 µL) x (number of samples + 5)

Diluent 2 (TFE Diluent)

Prepare diluent in excess of what is required for samples. In a 100 mL glass bottle, combine:

- Deionized water: (433 µL) x (number of samples + 5)
- 100 mM ammonium bicarbonate: (167 µL) x (number of samples + 5)
- 0.5 M DTT: (1 µL) x (number of samples + 5)

Store up to 12 hours at room temperature in a well-sealed container.

Diluent 3 (Sample Injection Diluent, 98:2 water:acetonitrile and 0.1% formic acid)

In a 50 mL conical vial, combine:

- Water, LC/MS grade: 39.2mL
- Acetonitrile, LC/MS grade: 800 µL
- Formic acid, 88% (add in fume hood): 40 µL

Store up to 1 month at room temperature.

Chromatography Solvent and Wash Buffer Preparation

Mobile Phase A (97.9% Optima LC/MS water/2% DMSO/0.1% formic acid)

In a 1 L graduated cylinder, combine 20 mL DMSO (LC/MS grade) and 1000 µL formic acid (98%, Suprapure). Bring to 1 liter with water (LC/MS grade). Store at room temperature for up to 1 month.

Mobile Phase B (97.9% Optima LC/MS Methanol/2%DMSO/0.1% formic acid)

In a 1 L graduated cylinder, combine 20 mL DMSO (LC/MS grade) and 1000 µL formic acid (98%, Suprapure). Bring to 1 liter with methanol (LC/MS grade). Store at room temperature for up to 1 month.

Sample Manager Purge (Optima LC/MS grade water with 0.1% formic acid (>98%)

Combine water (LC/MS grade) with high purity formic acid (1000:1, water to formic acid). Store at room temperature for up to 6 months.

Needle Wash (Optima LC/MS grade acetonitrile with 0.1% formic acid)

Combine acetonitrile (LC/MS grade) with high purity formic acid (1000:1, acetonitrile to formic acid). Store at room temperature for up to 6 months.

Syringe Wash (50:50 Optima LC/MS grade acetonitrile:Optima LC/MS grade water)

Combine acetonitrile (LC/MS grade) with water (LC/MS grade) (50:50). Store at room temperature for up to 6 months.

Seal Wash (10% Optima Methanol in Optima Water)

Stable 6 months, room temperature.

Sample Preparation

Denaturation

1. Gather standards, controls and system suitability from the freezer and thaw.
2. Turn on and set Thermomixer to 65°C.
3. Obtain fresh DI water to make daily working reagents.
4. Prepare 100 mM AMBIC and 0.5 M DTT.
5. Forward or reverse pipette (be consistent) 5 µL of standard, control or patient serum into the appropriate wells of a MASTERBLOCK 96-Well Deep Well Microplate.
6. Prepare 100 mM AMBIC/IS pep mix/DTT. Add DTT and IS peptide immediately before use to limit peptide binding to vial and limit DTT oxidation.
7. Add 36 µL of 100 mM AMBIC/IS/DTT mix to each well. You will have very little dead volume to work with when pipetting. (Recommend use of Eppendorf Repeater Stream with 1mL [yellow] tip).
8. Cap with blue micromat and touch down in plate centrifuge to collect all liquid to bottom of well.
9. In chemical fume hood, prime tip 2-3 times with TFE, add 35 µL of TFE, cap with blue micromat mat. (Recommend use of Rainin 20-200 µL 8-channel pipet and reagent reservoir).
10. Mix immediately on multi-vortex for 5 - 10 seconds at speed 7.
11. Mix on the prepared Thermomixer at 65°C and 1400 rpm for 1 hour.
12. During the last 3-5 minutes of incubation, prepare 0.5 M iodoacetamide (light sensitive).

Alkylation

1. Remove plate from Thermomixer and let sit for 1-2 minutes, touch down in plate centrifuge to collect any condensation. Open in fume hood to release TFE vapor collected in wells.
2. Add 4 µL of 0.5 M iodoacetamide to each well, cap with blue micromat mat.
3. Touch down in plate centrifuge to pull all liquid to bottom of well.
4. Mix immediately on multi-vortex for 5 - 10 seconds at speed 7.
5. Incubate in dark place (i.e. a drawer, etc.) at room temperature for 30 minutes.
6. As soon as incubation has started, turn Thermomixer temperature down to 37°C with lid off as it takes approximately 15-20 minutes to cool down and reach the lower set temperature.
7. Meanwhile, prepare TFE dilution buffer and 1 mM HCl.

Digestion

1. Once incubation is complete, add 600 µL of TFE diluent (DI H20/AMBIC/DTT mix) to each well to dilute out TFE to ≤ 5% (v/v) to inactivate it and prevent denaturation of the Trypsin enzyme. (Recommend use of the Rainin 8-channel electronic 100-1200 µL pipet with reagent reservoir). Cap with blue micromat mat and mix on multi-vortex for 5-10 seconds at speed of 5-6, do not let solution touch mat. It is important to mix the samples and TFE diluent well before adding the trypsin.
2. Prepare trypsin and use immediately.
3. Add 18.5 µL of 2 mg/mL of trypsin to each well. (Recommend use of Eppendorf Repeater Stream with 0.5 mL [purple/brown] tip).
4. Cap with blue micromat mat.

5. Mix immediately on multi-vortex for 5 - 10 seconds on speed 5-6, *being careful not to allow solution to contact cap mat*.
6. Mix on a Thermomixer at 37°C and 1400 rpm for 90 minutes.
7. Meanwhile, prepare the instrument. Submit injection solvent and system suitability samples approximately 30-40 minutes before the end of digestion (see step 5 in section below). If using a separate injection plate, prepare now (step 1 in section below).
8. After the 90 minutes, touch down in plate centrifuge to remove condensation/liquid from cap mat.
9. In chemical fume hood, add 6 µL 88% formic acid to each well to stop digestion, changing tips between each well if pipetting down into liquid to prevent contamination. (Recommend use of Rainin 10µL 8-channel pipette and reagent reservoir - okay to re-use 88% formic acid if not contaminated).
10. Re-cap with blue micromat tightly, touch down plate in centrifuge then mix immediately on multi-vortex on speed 5-6 for 5 - 10 seconds. Do not let solution touch the cap mat.

Post-Digestion

1. Pipette into a new MASTERBLOCK 96-Well Deep Well Microplate, or unused wells of the digest plate, 145 µL of 98:2 H20:ACN/0.1%FA Final Injection Diluent into each well being used. (Recommend use of Rainin 20-200 µL 8-channel pipet and reagent reservoir).
2. Transfer 55µL of each digest to corresponding well into the new injection plate (55 µL of digest from A2 to well A2 on injection plate). (Recommend use of Rainin 20-200 µL 8-channel pipet and reagent reservoir).
3. Seal with 3M Empore Adhesive tape and vortex at speed 7 with multi-vortex mixer until well mixed, 5-10 seconds. If bubbles in wells persist, centrifuge in plate centrifuge for 1-2 minutes.
4. Pipette approximately 200 µL of injection diluent and add contents of system suitability tube to a glass injection vials with pre-slit lids and place in the blue 48-slot vial tray in locations A1, and A2 respectively.
5. Load plate into auto-sampler tray, make sure appropriate bed layout has been selected. The first injections are the injection diluent blank and system suitability followed by standards, injection diluent, controls, injection dilutent and patients. Injection volume is 20 µL.
6. Record the system suitability parameters on the run log and assess for acceptability before proceeding with the injection of standards, controls and patients.
7. If a separate plate was used for injection, seal the digestion plate with 3M Empore Adhesive tape and save refrigerated until the run has been reported.

Dilution Protocol

If a sample result is greater than the highest calibrator, then perform a 1:4 dilution of the sample in negative control on the next run.:

1. In a micro-centrifuge tube combine 5mcL patient sample with 15 µL negative control.
2. Mix well by vortex.
3. Test 5 mcL of the diluted sample.
4. Multiply the result by 4 and report up to 20.0 mg/dL or as >20.0 mg/dL, if necessary.

Liquid Chromatography Configuration

1. Column: Acquity UPLC TSS T3 2.1x50mm 1.8 micron with guard column
2. Run Time: 8.0 min

3. Injection volume: 20mcL

Chromatography Configuration

Parameter	Value
Column Temperature	50°C
Sample Temperature (Autosampler)	5°C
Pre-Inject Wash	0 seconds
Post-Inject Wash	6 seconds
Injection Type	N/A
Extension Loop Size	50 µL
Needle Size	15 µL
Syringe	100 µL
Injection Volume	20 µL

Chromatography Gradient

Time	Flow (mL/min)	%A [a]	%B [b]
0.00	0.3	98.0	2
0.50	0.3	98.0	2
5.00	0.3	23.0	77.0
5.10	0.6	2.0	98.0
6.10	0.6	98.0	2.0
7.90	0.3	98.0	2.0

[a] 97.9% Optima LC/MS water/2% DMSO/0.1% formic acid (made with 98% Suprapure FA).

[b] Solvent B: 97.9% Optima LC/MS Methanol/2%DMSO/0.1% formic acid (made with 98% Suprapure FA).

Mass Spectrometry Settings

Electrospray (MS Tune) Settings.

Parameter	Value
Capillary	1.0 kV
Desolvation Temperature	500 °C
Desolvation Gas	1000 L/Hr
Cone Gas	50 L/Hr

Selected Reaction Monitoring

Peptide	Transition	Cone (V)	Collision (V)
FSGTWYAMAK	581.4657>927.5978	30	16
FSGTWYAMAK (Heavy)	585.4757>935.4800	28	16
YWGVASFLQK	600.0157>849.6610	38	16
YWGVASFLQK (Heavy)	604.0157>857.6376	40	18
LIVHNGYC[+57]DGR	652.5096>841.4220	64	24
LIVHNGYC[+57]DGR (Heavy)	657.5096>851.4220	64	24

Quality Assurance

All patient results must have a minimum internal standard peak area of 1300 for FSG and 800 for LIV prior to resulting. Calibration curve R^2 must be greater than 0.95.

ASSAY VALIDATION

Assessment of fish serum as surrogate matrix. Fish (salmon) and human sera (single source and pooled) were spiked at 3 levels using purified RBP (100 mg/dL in DMSO). Non-spiked and spiked samples were analyzed 6 times per concentration per matrix. The difference in increment was assessed between matrices (acceptable +/- 20%).

Analytical measurement interval. Linearity was assessed in two overlapping mixing studies. In the first, a high serum pool was generated from pooled sera spiked with purified human RBP (to >20 mg/dL), and mixed in 10% increments (0-100%, v/v) with a low concentration pool (~4.5 mg/dL). In the second, the low concentration pool was diluted to 0.6 mg/dL with fish serum and mixed in 10% increments with a medium concentration pool (6.9 mg/dL). Both sets of 11 samples were analyzed 4 times per each sample. Recovery (based on mean result) and imprecision (%CV) were determined for each admixture. Imprecision was further evaluated below this range by analysis of 5 independent low-level RBP samples diluted with fish serum (deficient for RBP) to achieve goal concentrations of ~0.50 mg/dL (desired lower limit of quantification). Each was analyzed in replicates of 8 per day over 5 days (40 total results each).

Limit of detection. Limit of detection was estimated based on the approach described in reference 19. Blank matrix (salmon serum) was analyzed in 5 replicates to estimate the limit of blank (LOB). LOD was then defined as mean concentration in the blank sample plus 1.65 x the standard deviation of measurement in a low sample (0.05 mg/dL was used, corresponding to SD of Sample 5 in Supplemental Table 3).

Imprecision. Assay repeatability was assessed at 3 levels using low, mid, and high pools (pool concentrations were determined by LC-MS/MS, Supplemental Table 4). The mid-concentration pool was made by combining residual sera. The low pool (0.63 mg/dL) was generated from 1:5 dilution of the mid pool in fish serum and the high pool from addition of purified human RBP to the mid pool. The pools were analyzed in replicates of 5 on 5 separate days.

Interference. Interferences from hemolysis, hyperlipidemia, hypertriglyceridemia, and hyperbilirubinemia were assessed using Sun Diagnostics Assurance Interferences kits (INT-01 and INT-01P). Serum base pools free of pathological concentrations hemoglobin, triglycerides, total protein, and bilirubin were analyzed before and after adulteration in replicates of 10. Interference from kidney disease was assessed by mixing leftover serum samples with low (~0.6 mg/dL) and high (~10 mg/dL) creatinine concentrations at 25% increments (5 points) evaluated in quadruplicate. Interference by hemolysis was further assessed in two dose-response experiments. First, a patient pool with no detectable hemoglobin (based on spectrophotometry) was spiked with Assurance Interference prepared hemoglobin or saline. These specimens were mixed from 0-100% in 25% increments. Final hemoglobin concentrations ranged from 0-650 mg/dL (n=5 hemoglobin concentrations; 6 replicates/concentration). Second, the same control pool was spiked with saline or hemolysate made by washing and freeze/thawing red blood cells. These specimens were mixed from 0-100% in 25% increments. Final hemoglobin concentrations ranged from 0-650 mg/dL (n=5 hemoglobin concentrations; 6 replicates/concentration). When making the specimen with the highest concentration of hemoglobin, the volume of hemolysate (from Sun Diagnostics or made in-house) did not exceed 5% of the final volume.

Matrix effects and ion suppression. A potential for matrix effects was examined through mixing studies of 5 pairs of single sourced human sera (10 unique patients). Each pair was mixed in 25% increments and analyzed in triplicate. Ion suppression was further evaluated through post-column infusion experiment (T injection of internal standard mixture).

Carryover. A low patient near the LLMI was run 10 times sequentially (before), then run 10 more times alternating with a high patient sample (high, low, high, low...etc.).

Sample type and stability. Samples were collected from 3 healthy volunteers in serum (red top), serum separator (gold), and lithium heparin-anticoagulated plasma separator (lime green) tubes (BD Vacutainer® blood collection tubes, Franklin Lakes, NJ). Samples sat approximately 30 minutes after collection before centrifuging. Serum/plasma was removed from cells and aliquoted. The samples were then analyzed immediately, 6 times per sample for each tube type. Separate aliquots were analyzed in triplicate at 1, 2, 7, and 14 days post-collection after storage at room temperature, 4°C, and -20°C. Recovery relative to analysis at initial collection was determined. In addition, the influence of EDTA on trypsin was evaluated by replenishing the calcium (final concentration 4.4 mM) in specimens freshly collected into purple top tubes and comparing the results to specimens from the same volunteers freshly collected into red top tubes.

Digest (peptide) stability. Individual patient specimens (n=4) were digested in replicates of 5. After stopping the digestion, the plate was split into 6 plates (below). Each digestion was analyzed after six different treatments: diluted digest (in Diluent 3, see Standard Operating Procedure) re-injected immediately after first analysis, original diluted digest re-injected after being left on the autosampler overnight, diluted and undiluted digests stored refrigerated for 3 days, diluted and undiluted digests stored at -20°C for 8 days.

1) Original diluted digest (injected twice - autosampler stability),
2) An additional diluted digest - left on autosampler overnight and injected next day.
3) Diluted digest - stored refrigerated for 3 days
4) Undiluted digest - stored refrigerated for 3 days, then diluted and analyzed
5) Diluted digest - stored frozen at -20 for 8 days
6) Undiluted digest - stored frozen at -20 for 8 days, then diluted and analyzed

Method comparison. Method comparison was performed with the Siemens BN ProSpec® nephelometer using residual sera/plasma samples (n=89). The ProSpec has an imprecision (%CV) of 3.1% and 2.3% at 2.28 mg/dL and 5.99 mg/dL, respectively. The clinically reportable range of the assay is 1.0-8,600 mg/dL, which includes an on-board x5 dilution as needed.

Reference range. Residual, de-identified serum specimens from an ambulatory pediatric population were utilized to evaluate the distribution of RBP results in healthy children. Outliers were repeated before data was analyzed parametrically.

Supplemental Table 1. Assessment of Fish Serum as Surrogate Matrix

Peptide [a]	Spike Level [b]	Increment (mg/dL) [c]						Difference in Increment (%) [d]	
		Human, Pool		Human, Single		Fish Serum		Fish vs. Pool	Fish vs. Single
FSG	Unspiked	-	[3.0]	-	[1.7]	-	[0.1]	-	-
	Level 1	**2.5**	[5.6]	**2.4**	[4.1]	**2.5**	[2.6]	**-3.1%**	**2.9%**
	Level 2	**6.4**	[9.4]	**5.4**	[7.1]	**4.6**	[4.7]	**-28.3%**	**-14.9%**
	Level 3	**9.7**	[12.7]	**9.7**	[11.4]	**8.0**	[8.1]	**-17.2%**	**-17.5%**
LIV	Unspiked	-	[3.0]		[1.8]	-	[0.0]	-	-
	Level 1	**2.0**	[5.0]	**2.0**	[3.9]	**2.3**	[2.3]	**16.7%**	**12.4%**
	Level 2	**4.8**	[7.8]	**4.5**	[6.4]	**4.4**	[4.4]	**-8.2%**	**-2.7%**
	Level 3	**8.5**	[11.6]	**7.7**	[9.5]	**7.0**	[7.0]	**-17.7%**	**-8.4%**
Both	Unspiked	-	[3.0]	-	[1.8]	-	[0.1]	-	-
	Level 1	**2.2**	[5.3]	**2.2**	[4.0]	**2.4**	[2.4]	**5.5%**	**7.3%**
	Level 2	**5.6**	[8.6]	**4.9**	[6.7]	**4.5**	[4.5]	**-19.7%**	**-9.3%**
	Level 3	**9.1**	[12.1]	**8.7**	[10.5]	**7.5**	[7.6]	**-17.5%**	**-13.5%**

[a] Peptide used for quantification. The clinical assay measures RBP as an average of the two concentrations derived from separate measurements of the FSGTWYAMAK and LIVHNGYCDGR peptides ("Both").

[b] Each matrix was spiked nominally to an increase of 2.25 mg/dL (Level 1), 5 mg/dL (Level 2) and 8.5 mg/dL (Level 3).

[c] Increment (in **bold**) reflects the increase in measured concentration compared to the unspiked sample. The corresponding mean result for each sample/matrix appears in brackets (i.e., total concentration, rather than increment), and is the mean of 6 measurements using 5 point external calibration. The measured values are graphed in **Supplemental Figure 1**. Concentration in µmol/L can be estimated by dividing the concentration in mg/dL by 2.061807.

[d] Difference in Increment = $\frac{[\textbf{Increment in Fish Serum}]-[\textbf{Increment in Human Serum}]}{[\textbf{Increment in Human Serum}]} \times 100\%$

Supplemental Table 2. Linearity.

	Mixing Study 1: High Range				Mixing Study 2: Low Range			
Level [a]	Calculated (mg/dL) [b]	Observed (mg/dL) [c]	Recovery	%CV	Calculated (mg/dL)	Observed (mg/dL)	Recovery	%CV
1	n/a	4.5	n/a	1.8%	n/a	0.6	n/a	5.9%
2	6.1	6	98%	8.2%	1.2	1.4	117%	13.2%
3	7.7	7.9	103%	12.0%	1.9	1.7	89%	6.1%
4	9.3	9.3	100%	6.1%	2.5	2.3	92%	6.4%
5	10.9	11.6	106%	5.4%	3.1	3.1	100%	2.1%
6	12.5	11.7	94%	9.7%	3.8	3.6	95%	6.8%
7	14.1	13.6	96%	4.9%	4.4	4.1	93%	7.8%
8	15.7	15.2	97%	7.6%	5	4.7	94%	8.7%
9	17.3	17.1	99%	11.8%	5.6	5.4	96%	8.3%
10	18.9	18.7	99%	1.7%	6.3	6.5	103%	9.6%
11	n/a	20.5	n/a	9.9%	n/a	6.9	n/a	6.3%

[a] Each mixing study contained 11 levels with overlapping concentration ranges.
[b] For the two mixing studies, the expected (calculated) concentrations for levels 2 through 10 were calculated based the measured concentration by LC-MS/MS for levels 1 and 11. Concentration in µmol/L can be estimated by dividing the concentration in mg/dL by 2.061807.
[c] The observed concentration reflects mean measurement across 4 replicates.

Supplemental Table 3. Imprecision Near the Lower Limit of Measuring Interval (LLMI).

	Day 1		Day 2		Day 3		Day 4		Day 5		All Days (n = 40)	
Sample	Mean [a]	%CV	Mean	%CV	Mean	%CV	Mean	%CV	Mean	%CV	Mean	%CV
1	0.46	13%	0.41	13.1%	0.41	13.1%	0.44	11.7%	0.43	5.2%	0.43	**11.8%**
2	0.50	17%	0.46	9.5%	0.46	10.4%	0.47	8.5%	0.47	10.9%	0.47	**11.5%**
3	0.55	7%	0.49	11.7%	0.50	9.7%	0.52	8.4%	0.47	5.5%	0.50	**9.6%**
4	0.49	7%	0.44	6.8%	0.44	12.5%	0.44	7.0%	0.44	7.0%	0.46	**8.7%**
5	0.41	16%	0.41	8.8%	0.41	16.2%	0.40	12.2%	0.38	11.1%	0.40	**13.0%**

[a] Mean concentration in mg/dL of 8 replicates (performed each day). Concentration in µmol/L can be estimated by dividing the concentration in mg/dL by 2.061807.

Supplemental Table 4. Limit of Detection.

Replicate [a]	FSGTWYAMAK (2) [b]		LIVHNGYC[+57]DGR (3) [c]		
	Response	**Conc.**	**Response**	**Conc.**	**Mean (mg/dL)[d]**
Blank 1	0.00440	0.01108	0.00154	-0.00926	0.00091
Blank 2	0.00170	0.00950	0.00087	-0.00945	0.00002
Blank 3	0.00320	0.01038	0.00060	-0.00953	0.00042
Blank 4	0.00300	0.01026	0.00102	-0.00941	0.00043
Blank 5	0.00090	0.00903	0.00121	-0.00935	-0.00016
				Mean $_{blank}$	0.00032
				SD $_{blank}$	0.00042
				Limit of Blank (LOB) [e]	0.00101
				Limit of Detection (LOD) [f]	**0.08373**

[a] Blank dilution matrix (fish serum)
[b] Calibration equation (5-point external calibration): [RBP in mg/dL] = 0.587032*(Response) + 0.0085
[c] Calibration equation (5-point external calibration): [RBP in mg/dL] = 0.285804*(Response) - 0.0097
[d] Concentration in µmol/L can be estimated by dividing the concentration in mg/dL by 2.061807.
[e] LOB = Mean $_{blank}$ +1.645* SD $_{blank}$
[f] LOD = LOB + 1.645* SD $_{low}$. SD low equals 0.05, based on "Sample 5" results in Supplemental Table 3, for which SD (in mg/dL) = 0.13*0.04 = 0.05.

Supplemental Table 5. Imprecision (Low, Mid, and High Pools)

		A	B	C	D	E	Mean	SD	%CV	Total (n = 25)	
Low Pool	**Day 1**	0.65	0.51	0.50	0.57	0.71	0.59	0.09	15%	Mean	0.63
	Day 2	0.49	0.56	0.53	0.47	0.54	0.52	0.04	7%	SD	0.09
	Day 3	0.73	0.66	0.68	0.79	0.78	0.73	0.06	8%	%CV	15%
	Day 4	0.60	0.58	0.69	0.65	0.72	0.65	0.06	9%		
	Day 5	0.73	0.67	0.67	0.74	0.61	0.69	0.05	8%		
	Mean	0.64	0.60	0.62	0.65	0.67					
	SD	0.10	0.07	0.09	0.13	0.09					
	%CV	16%	11%	15%	20%	14%					
		A	**B**	**C**	**D**	**E**	**Mean**	**SD**	**%CV**	**Total (n = 25)**	
Mid Pool	**Day 1**	3.12	3.12	2.72	2.74	3.33	3.01	0.27	9%	Mean	3.01
	Day 2	2.72	2.82	2.91	2.98	3.40	3.06	0.23	8%	SD	0.26
	Day 3	2.52	3.06	3.04	2.85	2.93	2.88	0.22	7%	%CV	9%
	Day 4	3.04	3.30	3.22	3.24	3.28	3.22	0.11	3%		
	Day 5	3.21	3.35	3.14	2.78	2.48	2.99	0.36	12%		
	Mean	2.92	3.13	3.01	2.92	3.08					
	SD	0.29	0.21	0.20	0.20	0.38					
	%CV	10%	7%	7%	7%	12%					
		A	**B**	**C**	**D**	**E**	**Mean**	**SD**	**%CV**	**Total (n = 25)**	
High Pool	**Day 1**	13.24	11.87	10.71	10.17	11.28	11.46	1.18	10%	Mean	11.58
	Day 2	11.87	10.55	10.93	11.70	10.98	11.21	0.56	5%	SD	0.96
	Day 3	10.71	10.81	13.01	12.58	11.63	11.75	1.03	9%	%CV	8%
	Day 4	13.05	12.27	11.02	11.82	10.39	11.71	1.04	9%		
	Day 5	12.16	13.26	10.10	11.28	12.19	11.80	1.18	10%		
	Mean	12.21	11.75	11.15	11.51	11.29					
	SD	1.02	1.11	1.10	0.88	0.68					
	%CV	8%	9%	10%	8%	6%					

Values reflect concentrations in mg/dL. Concentration in µmol/L can be estimated by dividing the concentration in mg/dL by 2.061807.

Supplemental Table 6. Interference Summary [a]

	Hemolysate [500 mg/dL]		Protein [12 g/dL]		Triglycerides [1000 mg/dL]		uBilirubin [20 mg/dL]		cBilirubin [20 mg/dL]	
	Control	Test	Control	Test	Control	Test	Control	Test	Control	Test
n	10	10	10	10	10	10	10	10	10	10
Mean	5.03	3.91	4.21	4.04	4.84	4.89	5.67	5.83	5.63	5.66
SD	0.41	0.42	0.40	0.23	0.41	0.28	0.42	0.76	0.44	0.47
%CV	8.1%	10.7%	9.5%	5.8%	8.4%	5.8%	7.5%	13.0%	7.7%	8.4%
%Rec.		**77.6%**		**95.9%**		**101.1%**		**102.7%**		**100.5%**

[a] Assessments were made utilizing the Sun Diagnostics Assurance Interferences kits. For each, a base pool free of pathological concentrations hemoglobin, triglycerides, total protein, and bilirubin was analyzed before and after adulteration in replicates of 10. Results reflect RBP4 concentrations in mg/dL. Concentration in µmol/L can be estimated by dividing the concentration in mg/dL by 2.061807.

Supplemental Table 7. Hemolysis Interference Study A [a]

Peptide	Sample	Hgb (mg/dL)	Measured RBP Concentration (mg/dL, 6 replicates)							
			1	2	3	4	5	6	Mean	%Bias
FSG only	Low (Unspiked)	0	5.1	4.9	4.4	5.1	5.4	5.5	5.1	-
	75:25 L:H	162.50	5.1	5.6	6.2	5.8	4.2	5.8	5.5	**8.0%**
	50:50 L:H	325.00	5.6	4.9	5.6	4.6	5.7	7.1	5.6	**10.0%**
	25:75 L:H	487.50	4.4	5.1	5.0	4.8	5.5	5.2	5.0	**-0.7%**
	High Hemoglobin	650	4.9	4.7	7.0	5.3	6.2	5.4	5.6	**10.2%**
LIV3 only	Low (Unspiked)	0	5.7	5.1	6.0	6.6	5.2	5.2	5.6	-
	75:25 L:H	162.50	4.7	5.1	4.9	6.0	4.5	5.2	5.0	**-10.0%**
	50:50 L:H	325.00	4.1	4.5	4.1	3.6	4.6	4.8	4.3	**-23.5%**
	25:75 L:H	487.50	3.9	3.2	3.6	2.9	3.5	3.7	3.5	**-38.0%**
	High Hemoglobin	650	3.5	2.8	3.2	3.4	3.9	2.8	3.3	**-41.4%**
Both	Low (Unspiked)	0	5.4	5.3	6.1	6.2	4.7	5.5	5.5	-
	75:25 L:H	162.50	4.9	5.0	4.7	5.5	4.9	5.3	5.1	**-8.8%**
	50:50 L:H	325.00	4.9	4.7	4.9	4.1	5.1	5.9	4.9	**-11.0%**
	25:75 L:H	487.50	4.1	4.1	4.3	3.9	4.5	4.5	4.3	**-23.2%**
	High Hemoglobin	650	4.2	3.8	5.1	4.4	5.0	4.1	4.4	**-20.0%**

[a] A patient pool with no detectable hemoglobin (based on spectrophotometry) was spiked with Assurance Interference prepared hemoglobin or saline. These specimens were mixed from 0-100% in 25% increments. Final hemoglobin concentrations ranged from 0-650 mg/dL (n=5 hemoglobin concentrations; 6 replicates/concentration). Concentration in µmol/L can be estimated by dividing the concentration in mg/dL by 2.061807.

Supplemental Table 8. Hemolysis Interference Study B [a]

Peptide	Sample	Hgb (mg/dL)	Measured RBP Concentration (mg/dL, 6 replicates)							
			1	2	3	4	5	6	Mean	%Bias
LIV3 only	Low (Unspiked)	0	2.05	2.32	2.35	2.15	2.49		2.27	0
	50:50 L:H	370	1.56	2.09	1.69	1.74	1.89	1.78	1.79	-21%
	High Hemoglobin	740	1.69	1.61	1.58	1.37	1.61		1.57	-31%
Both	Low (Unspiked)	0	2.22	2.40	2.37	2.38	2.41		2.35	0
	50:50 L:H	370	2.03	2.33	2.22	2.12	2.03	2.01	2.12	-10%
	High Hemoglobin	740	2.13	2.24	2.14	2.07	2.23		2.16	-8%

[a] A patient pool was spiked with saline or hemolysate made by washing and freeze/thawing red blood cells. These specimens were mixed from 0-100% in 25% increments. Final hemoglobin concentrations ranged from 0-650 mg/dL (n=5 hemoglobin concentrations; 6 replicates/concentration). Concentration in µmol/L can be estimated by dividing the concentration in mg/dL by 2.061807.

Supplemental Table 9. Matrix Effect Mixing Studies Summary

Sample Pair [a]	Mix	FSG Exp. Conc. [b]	FSG Mean Conc.	FSG Bias	FSG SD	FSG %CV	LIV Exp. Conc. [b]	LIV Mean	LIV Bias	LIV SD	LIV %CV
	100:0	3.5	3.5	0%	0.17	5%	3.3	3.3	0%	0.22	7%
	75:25	4.8	4.0	-17%	0.50	13%	4.7	4.4	-7%	0.17	4%
1	**50:50**	6.1	5.2	-15%	0.64	13%	6.0	5.9	-2%	0.27	5%
	25:75	7.3	6.7	-8%	0.47	7%	7.4	7.1	-4%	0.60	8%
	0:100	8.6	8.6	0%	0.42	5%	8.7	8.7	0%	0.64	7%
	100:0	4.1	4.1	0%	0.45	11%	3.8	3.8	0%	0.76	20%
	75:25	5.2	4.9	-6%	1.60	31%	5.5	4.6	-17%	1.04	19%
2	**50:50**	5.1	5.6	10%	0.22	4%	5.0	5.3	5%	0.25	5%
	25:75	6.8	6.4	-7%	0.33	5%	6.1	6.1	-1%	0.06	1%
	0:100	7.1	7.1	0%	0.39	5%	6.9	6.9	0%	0.19	3%
	100:0	2.8	2.8	0%	0.17	6%	2.9	2.9	0%	0.36	13%
	75:25	3.3	3.7	11%	0.27	8%	3.4	3.6	7%	0.19	6%
3	**50:50**	4.0	4.5	12%	0.30	7%	4.0	4.3	7%	0.08	2%
	25:75	4.9	5.4	10%	0.27	6%	4.5	5.1	13%	0.45	10%
	0:100	6.2	6.2	0%	0.49	8%	5.8	5.8	0%	0.59	10%
	100:0	1.6	1.6	0%	0.09	6%	1.6	1.6	0%	0.20	13%
	75:25	2.5	2.8	11%	0.19	7%	2.6	2.8	9%	0.14	5%
4	**50:50**	4.0	4.1	3%	0.50	12%	3.1	4.1	31%	0.26	8%
	25:75	5.4	5.4	-1%	0.25	5%	4.9	5.3	9%	0.46	9%
	0:100	6.6	6.6	0%	0.51	8%	6.6	6.6	0%	0.50	8%
	100:0	1.3	1.3	0%	0.20	15%	1.3	1.3	0%	0.12	9%
	75:25	2.3	2.1	-10%	0.28	12%	2.1	2.1	0%	0.27	13%
5	**50:50**	3.4	2.9	-15%	0.60	18%	3.0	2.9	-3%	0.20	6%
	25:75	3.7	3.7	0%	0.27	7%	3.8	3.7	-1%	0.26	7%
	0:100	4.5	4.5	0%	0.26	6%	4.5	4.5	0%	0.19	4%

[a] Each pair was mixed in 25% increments and analyzed in triplicate per level.

[b] Concentrations are provided in mg/dL. Concentration in µmol/L can be estimated by dividing the concentration in mg/dL by 2.061807.

Supplemental Table 10. Carryover [a]

Replicate	Low – Without Alternating (mg/dL)	Alternating	
		High (mg/dL)	Low (mg/dL(
1	0.54	6.46	0.55
2	0.52	7.11	0.49
3	0.51	7.01	0.52
4	0.56	7.13	0.49
5	0.56	7.03	0.62
6	0.49	6.27	0.52
7	0.52	5.40	0.53
8	0.49	6.73	0.64
9	0.54	6.14	0.56
10	0.55	5.77	0.59
Mean	0.53	6.50	0.55
SD	0.03	0.61	0.05
%CV	**5.3%**	**9.3%**	**9.8%**

[a] A low patient near the LLMI was run 10 times sequentially (before), then run 10 more times alternating with a high patient sample (high, low, high, low...etc.). The results correspond to a carryover of 0.37% (([Alternating Low]-[Low, no alternating])/[High] = %carryover). Concentration in µmol/L can be estimated by dividing the concentration in mg/dL by 2.061807.

Supplemental Table 11. Sample Type Comparison Summary

	Red (control)		Gold SST			Lime PST		
Sample [a]	**Mean (n=6) [b]**	**%CV**	**Mean (n=6) [b]**	**%CV**	**Bias [c]**	**Mean (n=6) [b]**	**%CV**	**Bias [c]**
Sample 1	3.7	7.8%	3.7	8.1%	-0.5%	3.9	11.4%	5.2%
Sample 2	6.9	4.1%	6.5	3.9%	-5.5%	6.4	6.5%	-7.5%
Sample 3	4.4	11.6%	3.9	6.2%	-9.3%	4.0	4.2%	-7.1%

[a] From 3 healthy volunteers
[b] Concentrations in mg/dL. Concentration in µmol/L can be estimated by dividing the concentration in mg/dL by 2.061807.
[c] Compared to Red Top mean result

Supplemental Table 12. Under-recovery using EDTA-anticoagulated samples

		Control			Test (Replenished Calcium) [a]		
Sample [b]		**EDTA**	**Red Serum**	**EDTA Bias [c]**	**EDTA**	**Red Serum**	**EDTA Bias [d]**
Sample 1	Mean	1.13	1.32	-14%	1.48	1.45	13%
	%CV	7%	8%		10%	8%	
Sample 2	Mean	1.16	1.42	-18%	1.28	1.56	-10%
	%CV	9%	5%		10%	6%	
Sample 3	Mean	1.31	1.67	-21%	1.65	1.82	-1%
	%CV	10%	4%		8%	5%	

[a] The influence of EDTA on trypsin was evaluated by replenishing the calcium (final concentration 4.4 mM)
[b] n = 6 of each sample/treatment
[c] from control (red "control" serum)
[d] from control (red "test" serum)

Supplemental Table 13. Sample Stability (by Sample Type)

			Sample 1		Sample 2		Sample 3	
	Temp	**Days**	**Mean [a]**	**Bias**	**Mean [a]**	**Bias**	**Mean [a]**	**Bias**
		0	3.7		6.9		4.4	
	R.t.	1	3.7	-0.8%	6.9	0.4%	4.6	6.4%
	R.t.	2	4.0	6.3%	7.0	1.0%	4.1	-5.6%
	R.t.	7	3.7	0.4%	6.8	-0.6%	3.9	-9.8%
	R.t.	14	3.6	-3.2%	6.3	-9.0%	4.2	-2.8%
Red Top	4C	1	3.9	5.3%	7.6	10.5%	4.4	0.4%
	4C	2	4.0	6.8%	6.8	-0.6%	4.2	-2.9%
	4C	7	3.6	-2.1%	6.7	-2.8%	3.9	-9.8%
	4C	14	3.4	-7.5%	5.9	-14.3%	3.3	-24.6%
	-20C	1	4.0	7.8%	7.0	2.0%	4.6	5.0%
	-20C	2	3.7	0.6%	6.8	-1.9%	4.1	-5.3%
	-20C	7	3.3	-11.7%	5.7	-17.4%	3.8	-13.7%
	-20C	14	3.2	-13.7%	5.5	-19.7%	3.3	-25.1%

			Sample 1		Sample 2		Sample 3	
	Temp	**Days**	**Mean [a]**	**Bias**	**Mean [a]**	**Bias**	**Mean [a]**	**Bias**
		0	3.7		6.5		3.9	
	R.t.	1	3.7	-0.4%	6.9	6.3%	3.9	-0.1%
	R.t.	2	3.8	3.7%	6.5	0.5%	4.0	1.3%
	R.t.	7	3.7	0.3%	5.9	-8.6%	3.7	-5.0%
	R.t.	14	3.2	-13.2%	5.9	-8.9%	3.4	-13.9%
Gold Top	4C	1	3.2	-13.4%	7.4	14.0%	4.4	12.8%
	4C	2	3.9	4.9%	7.0	7.5%	3.2	-18.6%
	4C	7	4.6	24.7%	6.9	5.5%	4.0	0.8%
	4C	14	3.4	-7.2%	5.9	-8.9%	3.9	-1.5%
	-20C	1	3.7	-1.0%	6.1	-5.5%	4.1	4.8%
	-20C	2	3.7	0.1%	6.3	-3.8%	4.0	0.5%
	-20C	7	3.5	-4.0%	6.2	-4.2%	4.0	0.8%
	-20C	14	3.0	-18.5%	5.8	-10.3%	3.9	-1.5%

			Sample 1		Sample 2		Sample 3	
	Temp	**Days**	**Mean [a]**	**Bias**	**Mean [a]**	**Bias**	**Bias [a]**	**Mean**
		0	3.9		6.4		4.0	
	R.t.	1	3.6	-7.1%	6.5	1.6%	4.3	5.2%
	R.t.	2	3.4	-12.2%	6.6	4.3%	3.7	-7.7%
	R.t.	7	3.6	-6.9%	6.1	-3.7%	3.4	-17.1%
	R.t.	14	3.4	-14.4%	6.6	3.3%	4.4	7.7%
Lime Top	4C	1	3.7	-4.6%	6.4	0.8%	3.3	-19.1%
	4C	2	4.3	8.8%	6.0	-5.5%	3.5	-13.9%
	4C	7	3.2	-18.6%	5.3	-17.1%	3.3	-18.8%
	4C	14	3.7	-6.2%	6.6	3.7%	4.2	4.6%
	-20C	1	3.8	-3.7%	6.3	-0.9%	3.7	-8.1%
	-20C	2	3.3	-15.8%	5.8	-8.2%	3.4	-15.6%
	-20C	7	3.1	-20.7%	5.6	-12.5%	3.3	-19.0%

[a] Concentrations in mg/dL. Concentration in µmol/L can be estimated by dividing the concentration in mg/dL by 2.061807.

Supplemental Table 14. Digest Stability

	Bias (Quant by FSG 2)			Bias (Quant by LIV 3)			Bias (Quant by Avg FSG/LIV)		
Day/ Condition	**Min**	**Max**	**Mean**	**Min**	**Max**	**Mean**	**Min**	**Max**	**Mean**
Day 2 (original piercing - reinjected)	-2.2%	7.1%	2.8%	-1.2%	21.7%	10.3%	2.0%	12.3%	6.6%
Day 2 (freshly pierced)	-2.8%	7.1%	2.0%	-12.0%	15.2%	1.4%	-5.7%	8.4%	1.6%
Day 3 Diluted Digest (4C)	-4.6%	8.5%	3.0%	-34.7%	-5.1%	-18.4%	-17.4%	-2.3%	-8.2%
Day 3 Digest (4C)	-4.4%	6.2%	1.3%	-13.4%	18.7%	-0.3%	-5.2%	11.1%	0.4%
Day 8 Frozen Diluted Digest (-20C)	-5.9%	6.7%	1.2%	-11.5%	17.5%	3.1%	-5.6%	8.5%	2.1%
Day 8 Frozen Digest (-20C)	-8.0%	6.1%	0.1%	-6.2%	21.6%	9.1%	-2.7%	10.9%	4.7%

A digest with 4 distinct patients digested in 5 replicates. Plate split after stopping digestion into 6 plates:
1) Original diluted digest (injected twice - autosampler stability),
2) Another diluted digest - left on autosampler overnight and injected next day.
3) Diluted digest - stored refrigerated for 3 days
4) Undiluted digest - stored refrigerated for 3 days
5) Diluted digest - stored frozen at -20 for 8 days
6) Undiluted digest - stored frozen at -20 for 8 days

Supplemental Table 15. Reference Range Samples. [a]

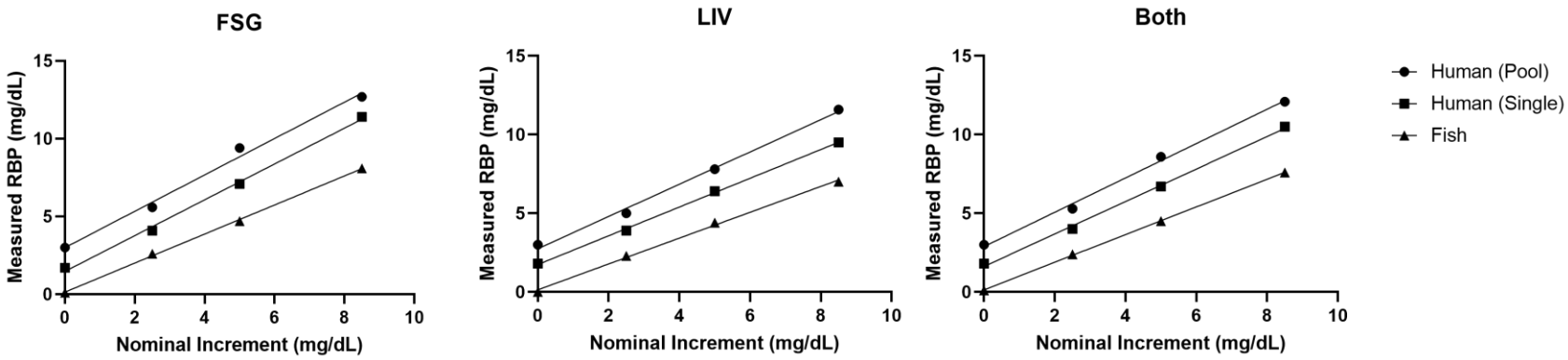
#	Sex	Age	RBP (mg/dL)	#	Sex	Age	RBP (mg/dL)	#	Sex	Age	RBP (mg/dL)	#	Sex	Age	RBP (mg/dL)
1	M	12M	3.0	*51*	M	3	2.8	*101*	M	9	1.9	*151*	F	13	3.6
2	F	13M	3.4	*52*	F	3	3.0	*102*	F	9	2.1	*152*	M	13	4.0
3	M	13M	4.4	*53*	M	3	3.1	*103*	M	9	2.4	*153*	F	13	4.1
4	F	14M	4.3	*54*	M	3	3.1	*104*	F	9	2.5	*154*	F	13	4.1
5	F	15M	2.7	*55*	F	3	3.2	*105*	F	9	2.5	*155*	M	13	4.3
6	M	15M	3.0	*56*	F	3	3.3	*106*	F	9	2.6	*156*	M	13	4.3
7	F	17M	2.5	*57*	F	3	3.3	*107*	M	9	2.6	*157*	M	13	4.4
8	M	17M	3.9	*58*	F	3	3.6	*108*	M	9	2.7	*158*	F	13	4.5
9	M	18M	2.8	*59*	M	3	3.9	*109*	M	9	2.7	*159*	F	13	4.8
10	F	19M	2.9	*60*	F	3	4.1	*110*	M	9	2.8	*160*	F	13	5.1
11	F	19M	4.2	*61*	F	5	1.9	*111*	M	9	2.9	*161*	M	15	2.7
12	M	20M	2.3	*62*	F	5	2.2	*112*	F	9	3.0	*162*	M	15	2.8
13	M	20M	3.4	*63*	M	5	2.2	*113*	F	9	3.0	*163*	M	15	2.9
14	F	21M	5.5	*64*	M	5	2.3	*114*	M	9	3.1	*164*	M	15	2.9
15	M	22M	2.3	*65*	M	5	2.5	*115*	F	9	3.1	*165*	M	15	2.9
16	M	22M	3.1	*66*	F	5	2.5	*116*	M	9	3.3	*166*	M	15	3.3
17	M	23M	2.2	*67*	M	5	2.6	*117*	M	9	3.5	*167*	M	15	3.3
18	F	23M	2.5	*68*	M	5	2.7	*118*	M	9	3.6	*168*	M	15	3.3
19	F	23M	2.6	*69*	M	5	2.8	*119*	F	9	3.8	*169*	M	15	3.4
20	F	23M	3.4	*70*	F	5	3.0	*120*	F	9	4.0	*170*	M	15	3.4
21	F	2	1.4	*71*	M	5	3.1	*121*	M	11	2.1	*171*	M	15	3.5
22	F	2	1.8	*72*	F	5	3.1	*122*	M	11	2.3	*172*	M	15	3.5
23	F	2	2.2	*73*	F	5	3.1	*123*	F	11	2.6	*173*	M	15	3.7
24	F	2	2.3	*74*	F	5	3.2	*124*	M	11	2.6	*174*	M	15	3.7
25	M	2	2.3	*75*	F	5	3.2	*125*	F	11	2.7	*175*	M	15	3.8
26	F	2	2.4	*76*	F	5	3.4	*126*	M	11	2.8	*176*	M	15	4.1
27	F	2	2.5	*77*	M	5	3.5	*127*	M	11	2.8	*177*	M	15	4.3
28	M	2	2.6	*78*	M	5	3.5	*128*	F	11	3.0	*178*	M	15	4.3
29	M	2	2.7	*79*	F	5	3.6	*129*	F	11	3.0	*179*	M	15	4.6
30	M	2	2.8	*80*	M	5	4.1	*130*	M	11	3.1	*180*	M	15	4.7
31	M	2	2.8	*81*	M	6	1.6	*131*	F	11	3.2	*181*	M	16	2.5
32	F	2	2.9	*82*	M	6	1.9	*132*	M	11	3.3	*182*	F	16	2.9
33	M	2	3.0	*83*	F	6	1.9	*133*	F	11	3.4	*183*	F	16	3.0
34	F	2	3.1	*84*	F	6	2.0	*134*	M	11	3.4	*184*	M	16	3.2
35	M	2	3.1	*85*	M	6	2.1	*135*	M	11	3.5	*185*	F	16	3.2
36	M	2	3.3	*86*	F	6	2.1	*136*	F	11	3.9	*186*	F	16	3.4
37	F	2	3.3	*87*	M	6	2.2	*137*	F	11	4.0	*187*	F	16	3.5
38	F	2	3.5	*88*	F	6	2.3	*138*	M	11	4.0	*188*	F	16	3.7
39	M	2	3.6	*89*	F	6	2.3	*139*	F	11	4.8	*189*	M	16	3.7
40	M	2	3.7	*90*	F	6	2.6	*140*	F	11	5.3	*190*	M	16	3.8
41	F	3	2.0	*91*	F	6	2.6	*141*	F	13	2.0	*191*	F	16	4.0
42	M	3	2.2	*92*	F	6	3.0	*142*	M	13	2.8	*192*	F	16	4.1
43	M	3	2.2	*93*	M	6	3.0	*143*	F	13	2.9	*193*	M	16	4.3
44	M	3	2.3	*94*	M	6	3.0	*144*	F	13	3.3	*194*	M	16	4.3
45	F	3	2.4	*95*	F	6	3.2	*145*	M	13	3.3	*195*	F	16	4.4
46	M	3	2.5	*96*	M	6	3.2	*146*	F	13	3.3	*196*	M	16	4.6
47	M	3	2.6	*97*	M	6	3.2	*147*	M	13	3.4	*197*	M	16	4.7
48	F	3	2.7	*98*	M	6	3.5	*148*	M	13	3.6	*198*	M	16	5.3
49	M	3	2.7	*99*	M	6	3.6	*149*	M	13	3.6	*199*	M	16	6.3
50	F	3	2.8	*100*	F	6	3.6	*150*	M	13	3.6	*200*	F	16	7.6

[a] Concentration in µmol/L can be estimated by dividing the concentration in mg/dL by 2.061807.

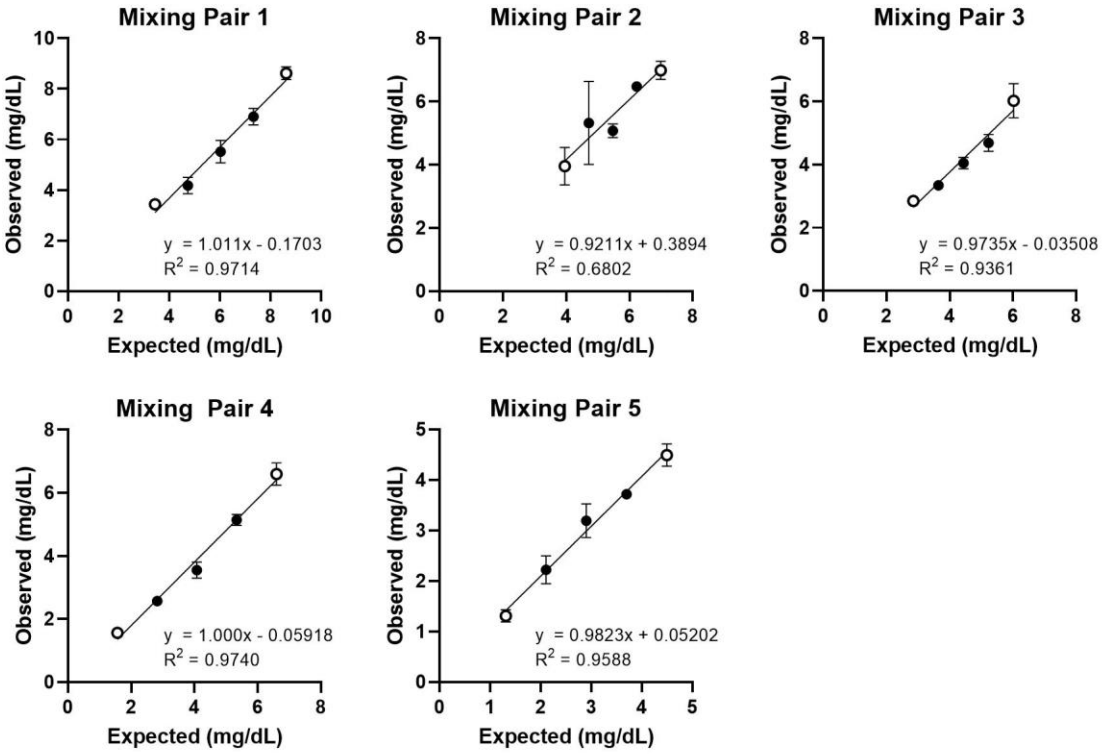
Supplemental Table 16. Method Comparison

ID	Nephelometry (mg/dL) [a]	LC-MS/MS (mg/dL)	%Bias	ID	Nephelometry (mg/dL) [a]	LC-MS/MS (mg/dL)	%Bias
01	4.3	4.5	4%	**46**	6.4	4.8	-25%
02	3.4	3.2	-7%	**47**	2	2.1	4%
03	19.6	24.4	24%	**48**	1.4	1.2	-15%
04	4.8	6.5	34%	**49**	3.1	2.7	-12%
05	2.5	3.1	23%	**50**	1.3	1.4	8%
06	1.4	0.9	-35%	**51**	2	2.3	14%
07	2.7	3.1	15%	**52**	1.4	1.3	-6%
08	2.8	2.9	2%	**53**	3.3	2.5	-23%
09	33.6 [a]	2.7	-92%	**54**	5.4	4.9	-9%
10	1.3	1.3	2%	**55**	2.3	2.8	20%
11	1.2	1.8	53%	**56**	1.5	1.3	-15%
12	1.9	2.0	4%	**57**	3.6	3.0	-17%
13	1.6	2.8	73%	**58**	1.5	1.5	-1%
14	5.1	5.4	6%	**59**	4.5	3.6	-20%
15	2.8	2.9	3%	**60**	1.6	1.6	1%
16	3.2	2.6	-18%	**61**	2.6	2.6	1%
17	1.4	1.0	-26%	**62**	1.5	1.6	7%
18	1.4	1.3	-10%	**63**	1.9	1.9	-2%
19	3.5	2.1	-39%	**64**	5.3	5.1	-4%
20	1.4	2.0	42%	**65**	1.9	2.1	12%
21	2.5	2.5	-2%	**66**	2.9	2.1	-28%
22	4.5	4.6	3%	**67**	2.9	2.3	-21%
23	<1.2	0.5		**68**	2.9	3.1	6%
24	3.8	2.4	-38%	**69**	5.9	5.5	-8%
25	2.5	3.3	32%	**70**	3.8	2.8	-26%
26	4.3	3.6	-17%	**71**	3.8	3.5	-8%
27	1.2	1.2	-2%	**72**	2	2.4	20%
28	1.7	2.4	40%	**73**	2	2.6	29%
29	3.9	2.9	-26%	**74**	2.6	2.6	-1%
30	2.3	2.4	3%	**75**	3.1	3.0	-5%
31	2.8	2.7	-2%	**76**	<1.2	1.3	
32	5.2	3.6	-32%	**77**	2.8	2.6	-9%
33	1.3	2.3	78%	**78**	3.4	2.7	-21%
34	3.4	3.9	14%	**79**	2.1	1.8	-16%
35	2.6	3.0	14%	**80**	6.1	6.1	0%
36	1.3	1.1	-15%	**81**	<1.2	0.7	
37	2.2	2.3	3%	**82**	8.6	7.7	-11%
38	1.6	1.8	14%	**83**	12.8 [a]	3.2	-75%
39	1.8	2.1	17%	**84**	4	3.6	-9%
40	2.8	2.6	-9%	**85**	1.6	1.8	15%
41	3.7	3.8	4%	**86**	2.6	2.8	9%
42	2.2	2.3	6%	**87**	2.1	2.4	14%
43	2.9	2.8	-4%	**88**	3.9	4.5	14%
44	3.2	2.9	-9%	**89**	2.1	1.8	-17%
45	3.6	2.8	-22%				

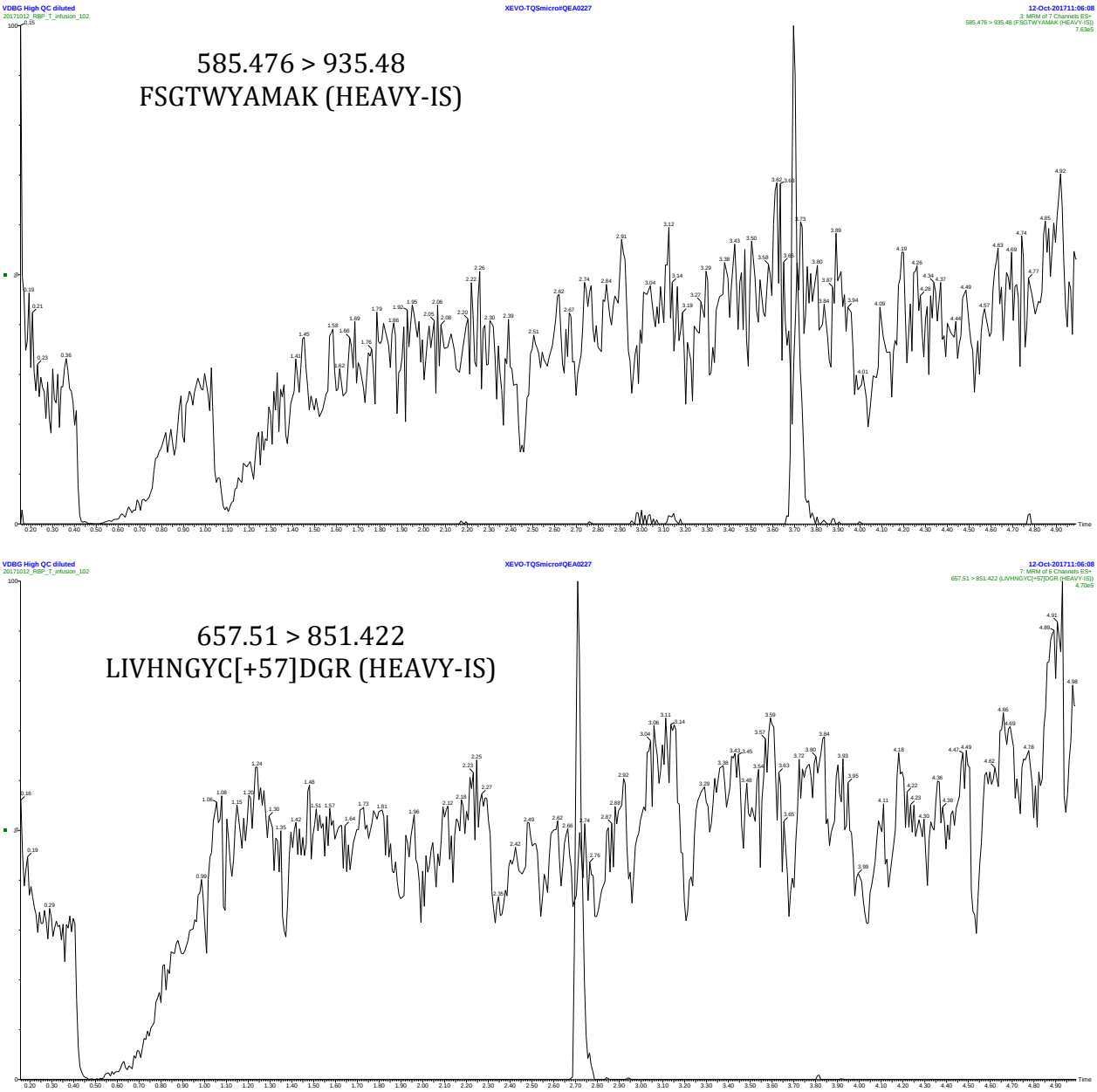
[a] For the nephelometry assay, lipemia (and other factors) can result in spuriously elevated results. Concentration in µmol/L can be estimated by dividing the concentration in mg/dL by 2.061807.



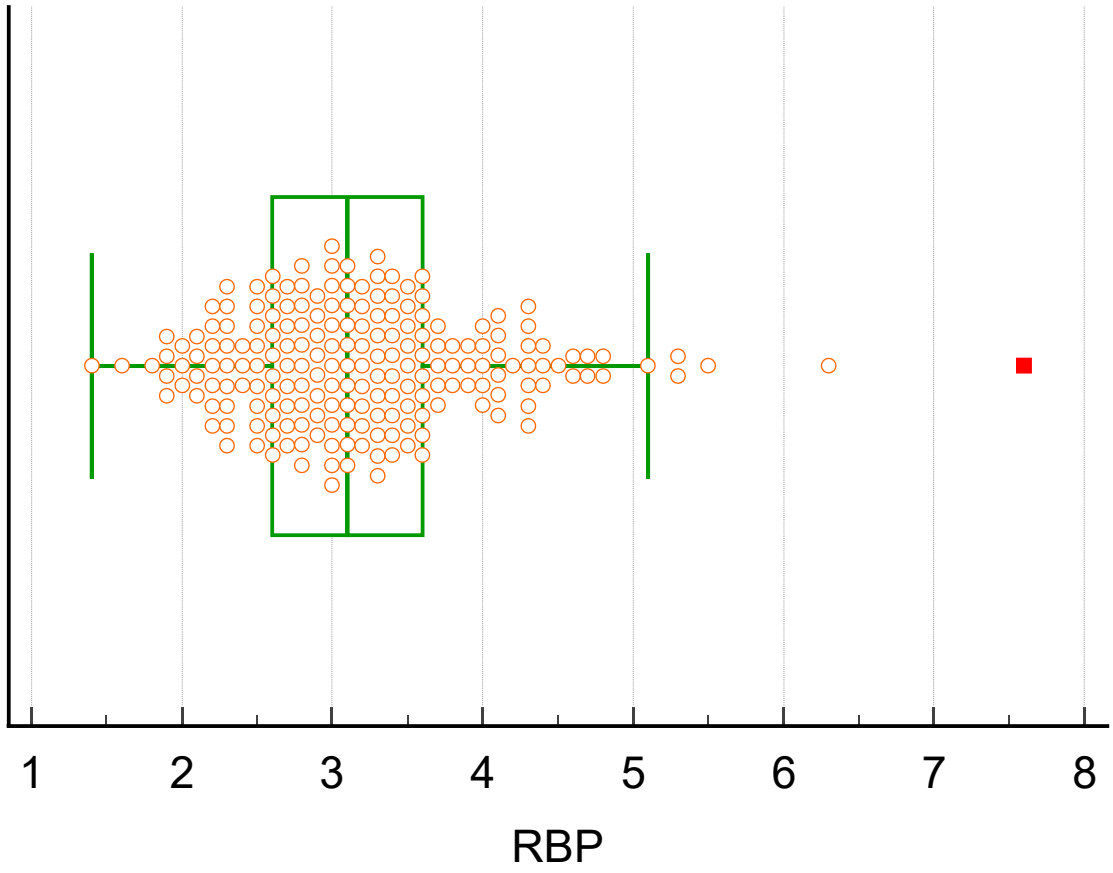
Supplemental Figure 1. Assessment of Fish Serum as Surrogate Matrix. Selected proteotypic peptides, FSGTWYAMAK (left) and LIVHNGYCDGR (middle), demonstrated similar increases in signal between human and fish sera when spiked at 3 levels. Graphed values reflect a mean results of 6 analyses per concentration per matrix. Concentration in µmol/L can be estimated by dividing the concentration in mg/dL by 2.061807.



Supplemental Figure 2 –Matrix Effects Mixing Study. Ten single-sourced human serum samples were paired together (1 low sample and 1 high sample). Each pair of samples were mixed together in the following scheme (Low (L) : High (L)): 100% L: 0% H**,** 75% L: 25% H, 50% L: 50% H, 25% L: 75% H, and 0% L: 100% H. The measured concentrations for the low and high samples (white circles) were used to calculate expected concentrations for the middle concentration samples (black circles). Concentration in µmol/L can be estimated by dividing the concentration in mg/dL by 2.061807.



Supplemental Figure 3 – T-infusion study. A solution of internal standard mixture was infused in a post-column T-infusion study. Digested human serum that did not contain the IS mixture was injected and subjected to MRM to monitor the unlabeled, endogenous peptides and the IS peptides. The chromatographic peak of the unlabeled peptide is overlaid on the signal from the constant infusion of the IS peptides.



Supplemental Figure 4. Reference Range Samples. Box and whisker plot generated in MedCalc (version 20.009, 64-bit, using Reed test for outliers). Using a non-parametric percentile method (CLSI C28-A3) the reference range lower limit was 1.9 mg/dL (90% CI 1.6 to 2.0) and reference range upper limit was 5.3 mg/dL (90% CI 4.7 to 6.3). Concentration in µmol/L can be estimated by dividing the concentration in mg/dL by 2.061807.