A Distributable LC-MS/MS Method for the Measurement of Serum Thyroglobulin

Supplemental Material

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STANDARD OPERATING PROCEDURE

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Unofficial copy, Do Not Use For Patient Care

Specimen Collection and Handling

One (1) mL of serum (serum separator tube) is preferred, for a 400 µL minimum volume. Serum from red top tubes is acceptable if tube is processed without delay (< 2 hours).

Equipment

Antistatic Device (ZEROSTAT, Fisher NC9597214)
Centrifuge, mini microcentrifuge
Centrifuge, benchtop microcentrifuge (accuSpin™ Micro 17, Fisher Scientific)
Centrifuge, benchtop (Beckman Allegra X-22)
Microcentrifuge tube racks
Chemical Fume Hood
Graduated cylinder, glass, 1L
Graduated cylinder, glass 10-25 mL
Graduated cylinder, glass 100 mL
Graduated cylinder, glass 500 mL
Magnet, for 1.5 mL tubes (Dynamag™-2, Invitrogen™ 12321D)
Mass spectrometer, triple quadrupole (Xevo TQ-S, Waters)
pH meter
Pipet, serological
Pipette, single-channel, 1-20 µL (Rainin LTS™)
Pipette, single-channel, 20-200 µL (Rainin LTS™)
Pipette, single-channel, 100-1000 µL (Rainin LTS™)
Pipette, repeater, 100-1200 µL (Rainin)
Vortex mixer (Vortex Genie 2, Scientific Industries SI-0236)
Vortex mixer, multitube (VWR)
Rotator, end-over-end (Labquake™, Thermo Scientific™ 415110Q)
Scale
Stir plate
Syringe, glass, 1 mL
Thermomixer, with 1.5mL Thermoblock (Eppendorf 5382000023, 5360000038)
Timer
UHPLC System (ACQUITY, Waters)

Supplies

Autosampler vials (Total Recovery, Waters 186000384C)
Beaker, glass, 500 mL
Chromatography column (ACQUITY UPLC HSS T3, Waters 186003538)
Chromatography pre-column (ACQUITY UPLC HSS T3 VanGuard, Waters 186003976)
Conical tubes, screw cap, 15mL
Conical tubes, screw cap, 50mL
Glass reagent bottles, 100 mL
Glass reagent bottles, 250 mL
Glass reagent bottles, 1 L
Magnetic stir-bars
Microcentrifuge tubes, Low-Retention, 0.6 mL (Fisher 02-681-311)
Microcentrifuge tubes, Low-Retention, 1.5 mL (Fisher 02-681-320)
Microcentrifuge tubes, LoBind Protein, 1.5 mL (Eppendorf ® 022431081)
Microtubes, 5.0 mL (Eppendorf ® 0030119401)
Parafilm
pH test strips 5-10 (EMD 9588-3)
Pipette tips
Sample Storage Assembled Screw Vial Kits, 20mL (Thermo Scientific™ 03-375-25)
Serological pipet tips, 10 mL and 50 mL, disposable
Microplate sealer, aluminum sealer (Silverseal, Greiner Bio-One 676090),

Chemicals

Acetic acid, glacial (Fisher Scientific BP2401).
Acetonitrile, LC-MS Grade (Optima™, Fisher Scientific A955)
Antibody, rabbit monoclonal, 0.5 mg/mL, CPTAC-39c peptide (SISCAPA Assay Technologies, Tg-FSP-31)
Bovine serum albumin (BSA), ≥98%, (Sigma-Aldrich A7030)
CHAPS hydrate, >=98% (Millipore Sigma C3023)
Deoxycholic Acid Sodium Salt (Fisher Scientific BP349)
Dimethylsulfoxide, LC-MS (Thermo Scientific™/Pierce™ 85190)
Formic acid, 98-100% (Suprapur®, Millipore Sigma 1116701000)
Hydrochloric acid, 1N (Fisher Scientific SA48-4)
Iodoacetamide, 98% (Thermo Scientific AC12227-0050)
Magnetic Beads (Dynabeads™ M-280 Tosyl activated, Invitrogen 14204)
Methanol, LC-MS Grade (Optima™, Fisher Scientific A456)
Peptide, stable-isotope labeled, sequence FSPDDSAGASALL(C13(6))R (Anaspec)
    MH+1 = 1412.7 (monoisotopic)
    Stock solution: 2 mg/mL in DMSO
Potassium chloride, ACS reagent, 99.0-100.5%, (Sigma-Aldrich P-3911)
Serum, chicken (Equitech-Bio, Inc. SC30)
Sodium azide, Solution 5% (w/v) (VWR BDH7465-2)
Sodium chloride (Sigma-Aldrich S3014)
Sodium hydroxide, 1N (Fisher Scientific 02-004-129)

Sodium phosphate, dibasic anhydrous (Fisher Scientific S374),
Sodium phosphate, monobasic monohydrate (Fisher Scientific BP330)
Sodium tetraborate decahydrate, ≥99.5% (ReagentPlus ®, Sigma-Aldrich B9876)
TCEP solution, 0.5 M (Bond-Breaker™, Thermo Scientific™ 77720)
TLCK Trypsin Inhibitor (Sigma-Aldrich T7254)
Tris base, ≥99.9% (Trizma®, Sigma-Aldrich T4661)
Trypsin, TPCK-treated (Worthington LS003741)
Water, deionized (18Ω)
Water, LC-MS Grade

Calibration & Internal Standard Normalization

Five-point external calibrators are prepared by diluting an assayed patient population pool (n>=400 discrete samples) that has a thyroglobulin value >10ng/mL with chicken serum which is deficient for the peptides monitored. Aliquots of 450 µL are prepared and stored at -20°C in low retention 0.6 mL tubes, suggested expiration at 2 years.

Target Calibrator Setpoints:

Calibrator Level	Concentration
Standard A	0.1 ng/mL
Standard B	0.3 ng/mL
Standard C	3.5 ng/mL
Standard D	10 ng/mL
Standard E	15 ng/mL

Quality Control

Four process-level controls (negative, ultra-low, low, and high) are prepared as follows:

- *Negative:* Aliquot 450 µL chicken serum and freeze in 0.6 mL low-retention microcentrifuge tubes at -20°C.
- *Ultra-low:* Pool population sera with a mean target of 0.15 ng/mL. Aliquots of 450uL are prepared and frozen in 0.6 mL low retention tubes and stored at -20°C.
- *Low:* Dilute population sera pool with chicken serum to a mean target of 0.5 to 1.0 ng/mL final concentration. Aliquots of 450uL are prepared and frozen in 0.6 mL low retention tubes and stored at -20°C.
- *High:* Dilute population sera pool with chicken serum to a mean target of 6.0 to 8.0 ng/mL final concentration. Aliquots of 450 uL are prepared and frozen in 0.6 mL low retention tubes and stored at -20°C.

A mean and standard deviation will be established for the control material on the basis of a minimum 10-20 measurements. Confirm that the mean, SD and CV are consistent with previous lots of comparable control materials.

Quality Assurance

Carryover of 0.14% was observed at Tg concentration of 3,204 ng/mL. Any sample > 0.10 ng/mL that immediately follows a sample with an estimated concentration ≥ 50 ng/mL are repeated.

Working Reagent Preparation

10X Phosphate Buffered Saline (10X PBS)

1. Add 750mL deionized water to a 1 L bottle containing a stir-bar.
2. Weigh and add:
    a. 80.65g Sodium chloride
    b. 5.24g Sodium phosphate, monobasic, monohydrate
    c. 10.77g Sodium phosphate, dibasic, anhydrous
    d. 2.01g Potassium chloride
3. Stir until chemicals have dissolved.
4. Adjust to pH 7.4 using HCl and NaOH (Sigma)
5. Adjust to 1L in graduated cylinder with deionized water.
6. Store at room temperature for up to 6 months.

2% CHAPS

1. In a 50 mL conical tube, combine:
    a. 1 g CHAPS
    b. 50mL deionized water
2. Invert to mix.
3. Store at room temperature for up to 1 year.

PBS - 0.1% CHAPS

1. In a 100 mL glass bottle, combine:
    a. 85mL deionized water
    b. 10 mL 10X PBS
    c. 5 mL 2% CHAPS
2. Store at 4-8°C for up to 1 month.

PBS - 0.1% CHAPS & 0.02% sodium azide

1. In a 50mL conical tube, combine:
    a. 5 mL 10X PBS
    b. 2.5 mL 2% CHAPS
    c. 0.2 mL 5% sodium azide
2. Adjust final volume up to 50mL with deionized water, invert to mix.
3. Store at 4-8°C for up to 1 year.

100 mM Sodium Borate, pH 9.5

1. In a 1 L bottle containing stir-bar, combine:
    a. 250 mL deionized water
    b. 11.44g sodium tetraborate decahydrate
2. Mix until dissolved.
3. Adjust to pH 9.5 with 1M HCl and 1N NaOH, as needed.
4. Adjust to 300mL with deionized water in a 500 mL graduated cylinder.

5. Store at room temperature for up to 2 years.

0.2 M Tris pH~10.8

1. In a 250 mL bottle, combine:
    a. 6.057 g Trizma base and add to a clean 250mL bottle
    b. 250 mL deionized water
2. Invert to mix until Tris is dissolved.
3. Check pH is ~10.8.
4. Store at room temperature for up to 6 months.

10 mM HCl

1. In a 100 mL bottle, combine:
    a. 99 mL deionized water
    b. 1 mL 1M HCl (add using 1 mL glass syringe)
2. Store at room temperature for up to 6 months.

Elution Buffer: 2.5% Acetic acid - 0.1% CHAPS

1. In a 20mL glass vial, combine:
    a. 18.5 mL of deionized water
    b. 1 mL of 2% CHAPS
    c. 0.5 mL of glacial acetic acid
2. Invert to mix.
3. Store at room temperature for up to 1 month.

10% BSA Stock

1. Remove BSA solid from 2-8°C and warm to room temperature prior to opening the bottle.
2. In a 15 mL conical tube, combine:
    a. 1.45 g BSA
    b. 14.5 mL PBS
3. Invert repeatedly and vortex occasionally until BSA is in solution.
4. Make 200 µL aliquots in 0.6mL tubes.
5. Store at -20°C for up to in 1 year.

PBS - 0.1% BSA Preparation

1. In a 15 mL conical tube, combine:
    a. 1 mL of 10X PBS
    b. 9 mL of deionized water
2. Remove 100 µL of the total volume
3. Add 100 µL 10% BSA
4. Invert to mix.
5. Expires at 24 hours.

0.2 M Tris - 0.1% BSA

1. In a 1.5mL tube, combine:
    a. 990 µL 0.2M Tris
    b. 10 µL 10% BSA
2. Invert to mix.
3. Expires at 24 hours.

20% Deoxycholate (DOC) - 0.19 M TRIS - 0.03 M TCEP

1. In a 20 mL vial, combine:
    a. 2g deoxycholate (DOC)
    b. 9.4 mL 0.2 M TRIS
    c. 0.6 mL TCEP
2. Invert to mix.
3. Store at room temperature for up to 1 month.

0.06 M Iodoacetamide (IAA)

1. In a 5mL microtube, weigh 0.040 - 0.045 g of IAA.
2. Multiply weight in grams by 90.1095 to calculate volume of deionized water in mL to add.
    a. Example: 0.04 g x 90.1095 = 3.60 mL of water.
3. Vortex until dissolved.
4. Use immediately.

10 mg/mL TPCK* treated Trypsin in 10 mM

1. In a 5 mL microtube, weigh 40 - 45 mg of Trypsin.
2. Divide weight in mg by 10 to calculate volume of 10 mM HCl in mL to add.
    a. For example: 40 mg ÷ 10 = 4.0 mL of 10 mM HCl.
3. Vortex until dissolved.
4. Use immediately.

*TPCK, Tosyl phenylalanyl chloromethyl ketone

3 mg/mL TLCK trypsin inhibitor in 10 mM HCl

1. In a 1.5mL tube, weigh out 3 - 3.5 mg of TLCK Trypsin inhibitor.
2. Divide weight by 3 to calculate the amount of 10 mM HCl to add to tube.
    a. For example: 3 mg ÷ 3 = 1.0 mL of 10 mM HCl.
3. Vortex until dissolved
4. Use within 24 hours.

5% ACN - 0.1% formic acid

1. In a 100 mL bottle, combine:
    a. 94.9 mL LC-MS grade water
    b. 5mL of Optima LC/MS grade acetonitrile
    c. 0.1mL formic acid >98%.
2. Vortex/invert to mix
3. Store at room temperature for up to 1 year.

Primary Intermediate (FSP Peptide) IS Solution, **50 µM** in 5% ACN - 0.1% formic acid

1. In a low retention 1.6 mL tube, combine:
    a. 64.7µL 5% ACN - 0.1% formic acid
    b. 35.3 µL isotopically-labeled FSP peptide stock solution (2 mg/mL)
2. Vortex to mix.
3. Store at -20°C.

Secondary Intermediate (FSP Peptide) IS Solution, **0.5 µM** in 5% ACN - 0.1% formic acid

1. Into a low retention 1.6 mL tube, combine:

a. 990 µL 5% ACN - 0.1% formic acid
b. 10µL *primary* intermediate FSP IS solution (50 µM)
2. Vortex to mix.
3. Store at -20°C.

Working Intermediate (FSP Peptide) IS Solution, **0.5 nM** in 5% ACN - 0.1% formic acid

1. Into a 15 mL conical tube, combine:
    a. 9.99mL 5% ACN - 0.1% formic acid
    b. 10 µL *secondary* intermediate FSP IS (0.5µM)
2. Vortex to mix.
3. Aliquot 1 mL volumes into 1.6 mL low retention tubes.
4. Store at -20°C.

Mobile Phase A: 2% DMSO - 0.1% Formic Acid in Water

1. 1L bottle, combine:
    a. 979 mL Optima LC-MS grade water
    b. 20 mL of LC-MS grade DMSO
    c. 1mL formic acid (>98%).
2. Mix by inversion.
3. Stable at room temperature for up to 6 months.

Mobile Phase B: 2% DMSO - 0.1% Formic Acid in Methanol

1. In a 1 L HPLC bottle, combine:
    a. 979 mL Optima LC-MS grade methanol
    b. 20 mL of LC-MS grade DMSO
    c. 1 mL formic acid (>98%).
2. Mix by inversion.
3. Stable at room temperature for up to 6 months.

Needle Wash: 0.1% Formic Acid in Acetonitrile

1. In a 0.5 L HPLC bottle, combine:
    a. 499.5 mL Optima LC-MS grade acetonitrile
    b. 0.5 mL formic acid (>98%)
2. Mix by inversion.
3. Stable at room temperature for up to 6 months.

Purge Solvent: 0.1% Formic Acid in 10% Methanol

1. In a 0.5 L HPLC bottle, combine:
    a. 449.5 mL acetonitrile (LC-MS grade)
    b. 50 mL methanol (LC-MS grade)
    c. 0.5 mL formic acid (>98%)
2. Mix by inversion.
3. Stable at room temperature for up to 6 months.

Seal Wash: 10% Methanol in water

1. In a 0.5 L HPLC bottle, combine:
    a. 450 mL acetonitrile (LC-MS grade)
    b. 50 mL methanol (LC-MS grade)
2. Mix by inversion.
3. Stable at room temperature for up to 6 months.

Beads for Immunoaffinity Purification

1. Turn on thermomixer and preheat to 37°C.
2. For bead preparation, label a 1.5mL low retention tube as follows:
    a. *Date of preparation:*
    b. *Uses:*
    c. *Reactions:*
3. Calculate the required volume of tosyl activated beads (7.5 µL of beads per reaction).
    a. Example: For 24 reactions, 24 x 7.5 = 180 µL of tosyl activated beads are required.
4. Re-suspend commercial bead slurry (30 mg/mL) by gentle mixing and inversion over several minutes. Beads are well re-suspended when there are no clumps of beads remain.
5. Pipet the calculated bead volume into the bottom of the labeled 1.5 tube.
6. Place the tube on the magnet.
7. Remove the storage buffer using a pipet.
8. Perform *pre-coupling* bead wash (repeatedly, x3):
    a. Add 200µL of sodium borate (100 mM)
    b. Vortex gently (speed 4-6) for 15 seconds, avoiding contact between beads and cap.
    c. Centrifuge briefly, and apply the tube to magnet.
    d. Remove wash buffer using a pipet.
9. Calculate and add the required volume of 100mM sodium borate (8µL per reaction).
    a. Example: For 24 reactions, 24 x 8 = 192 µL sodium borate are required.
10. Calculate and add the required volume of antibody (4µL per reaction)
    a. For each reaction we need 2 µg (+/- 10%) of antibody in 12 µL total volume and antibody concentration is about 0.5 µg/µL.
    b. Example: For 24 reactions, 24 x 4 = 96 µL antibody are required.
11. Vortex gently for 15 seconds, avoiding contact of bead mixture with tube cap.
12. Incubate bead-antibody mixture in the thermomixer (preheated to 37°C) shaking at 1400rpm for 1 hour. During the incubation:
    a. Thaw 10% BSA.
    b. Prepare both PBS - 0.1% BSA and 0.2M Tris - 0.1% BSA.
13. Remove the bead-antibody mixture from the thermomixer and allow cooling to room temp.
14. Invert the tube and briefly centrifuge.
15. Place the tube in the magnet.
16. Remove supernatant.
17. Perform *post-coupling* bead wash (repeatedly, x4):
    a. Re-suspend the beads in 200µL PBS-0.1% BSA
    b. Vortex gently for 15 seconds, avoiding contact between beads and cap.
    c. Centrifuge briefly, and apply the tube to magnet.
    d. Remove wash buffer using a pipet.
    e. Repeat steps a-d twice, for a total of 3 washes.
18. Remove supernatant.
19. Re-suspend beads in 400µL 0.2M Tris - 0.1% BSA
20. Incubate bead-antibody mixture at 37°C at 1400rpm for 1-3 hours (block unbound bead sites).
21. Remove the bead-antibody mixture from the thermomixer and allow cooling to room temp.
22. Briefly centrifuge.

23. Place the tube on the magnet, allow the beads to migrate to the side of the tube and then remove the supernatant.
24. Perform *final* bead wash (x3):
    a. Re-suspend the beads in 200µL PBS - 0.1% CHAPS
    b. Vortex gently for 15 seconds, avoiding contact between beads and cap.
    c. Touch down and place the beads on the magnet.
    d. Remove wash buffer using a pipet.
    e. Repeat steps a-d twice, for a total of 3 washes.
25. Calculate and add final storage buffer volume:
    a. 10 µL of PBS - 0.1% CHAPS - 0.02% NaN3 per reaction
    b. Example: 240 µL per 24 reactions
26. Vortex gently for 15 seconds then briefly centrifuge.
27. Store beads at 4°C.

Sample Preparation

1. In a 1.5 mL tube (Protein LoBind), add 400 µL serum.
2. Add 100 µL of 20% DOC/0.19M Tris/0.03M TCEP (pH 10.8). Vortex briefly.
3. Incubate in thermomixer at 40°C, shaking at 1400 rpm for 1 hour.
4. Remove tube from thermomixer, invert, and briefly centrifuge.
5. Add 100 µL of 0.06 M IAA. Vortex briefly. Incubate in the dark for 30 minutes.
6. Invert tubes and briefly centrifuge.
7. Add 100 µL of 0.2 M Tris and 20 µL of working IS solution (0.5 nM). Vortex and briefly centrifuge.
8. Add 100 µL trypsin (10 mg/mL, in 10 mM HCl). Vortex gently.
9. Incubate in thermomixer at 37°C, shaking at 1400 rpm for 30 minutes.
10. Invert tubes and briefly centrifuge.
11. Add 20 µL TLCK (3 mg/mL in 10 mM HCl). Vortex gently.
12. Incubate in thermomixer at RT, shaking at 1400 rpm for 5 minutes.
13. Invert tubes and briefly centrifuge.
14. Add 10 µL of magnetic bead preparation.
    a. Mix beads well prior to addition to samples, but avoid contact with tube lid.
    b. When adding across multiple tubes, vortex bead preparation after every two tubes to prevent bead settling
15. Rotate tubes at room temp. for 1 hour.
16. Briefly centrifuge, put tubes on magnet. Remove digest supernatant with a pipette.
17. Add 200µL of PBS/0.1% CHAPS. Vortex gently.
18. Centrifuge briefly, place tubes on magnet and remove wash buffer. Repeat step 9-10 one more time for a total of 2 washes.
19. Add 50 µL of 2.5% acetic acid/0.1% CHAPS (elution buffer). Vortex at setting of 5-7 then place on RT thermomixer for 5 minutes. Do not let IA bead slurry come into contact with lid.
20. Briefly centrifuge, and place tubes on magnet.
21. Transfer 50 µL eluent to new 1.5 mL tubes and centrifuge for 5 minutes at 13,000 rpm.
22. Save IA bead tubes after elution, beads will be collected, cleaned and re-used. See Bead Cleaning Protocol.
23. Put tubes on magnet and transfer eluents to total recovery vials.

Bead Regeneration Protocol

1. Post-elution, re-suspend beads in 100 µL of PBS - 0.1% CHAPS and transfer volume back to labeled tube.
2. Once all beads have been collected remove the PBS - CHAPS buffer.
3. Add 200 µL of elution buffer, gently vortex and briefly centrifuge.
4. Place tube on magnet and remove elution buffer
5. Repeat steps 3 - 4 two more times.
6. Add 200 µL of PBS – 0.1% CHAPS, gently vortex and briefly centrifuge.
7. Place tube on magnet and remove buffer.
8. Repeat steps 6 -7 two more times.
9. Add 10µL of PBS – 0.1% CHAPS – 0.02% NaN3 per reaction, gently vortex for 15 seconds.
10. Store beads at 4°C. Beads can be cleaned and re-used 5 times.

Liquid Chromatography Settings

Sample Manager

Parameter	*Value*
Column Temperature (celsius)	45
Sample Temperature (celsius)	5
Pre-Inject Wash	0 sec
Post-Inject Wash	6 sec
Injection type	N/A
Extension Loop Size	50 µL
Needle Size	15 µL
Syringe Size	100 µL
Injection volume	40 µL

Binary Solvent Manager Settings

Event	*Time (min)*	*Flow (mL/min)*	*%A*	*%B*	*Curve*
1	Initial	0.3	80.0	20.0	Initial
2	0.50	0.3	80.0	20.0	11
3	3.50	0.3	33.0	67.0	6
4	3.60	0.6	5.0	95.0	11
5	4.90	0.6	80.0	20.0	11
6	6.90	0.3	80.0	20.0	11

Run Time: 7.0 min

Mass Spectrometry Settings

MS Tune Settings

Parameter	*Value*
Capillary	1.4 kV

Cone	26 V
Desolvation Temp	500 °C
Desolvation Gas	1000 L/Hr
Cone Gas	150 L/Hr
Resolutions	Mass Resolution/Calibration Dependent
Collision Gas Flow	External control gauge 7-9 psi
Collision Energy	Variable See Transitions

MS Method Events

Time (mins)	*Event*	*Action*
0.00	Solvent Delay	Begin
2.30	Flow State	LC
2.40	Solvent Delay	End
3.40	Flow State	Waste
3.50	Solvent Delay	Begin

Transitions

Compound	*Transition*	*Cone (V)*	*Collision(V)*
FSP	704.0404>586.90	26	20
FSP	704.0404>586.91	26	20
FSP	704.0404>586.92	26	20
FSP IS	707.0300>589.89	26	20
FSP IS	707.0300>589.90	26	20
FSP IS	707.0300>589.91	26	20

ASSAY VALIDATION

Linearity. A commercially available pooled human serum (Golden West MSG4000, lot C10003) was diluted to 4 additional levels in Tg-negative chicken serum (Equitech-Bio, Inc), and evaluated in duplicate over 3 days (6 total measurements at each level).

Lower limit of quantification. Three samples, made by diluting a pool of 4 low positive patient samples (~1 ng/mL) with chicken serum to target concentrations of ~0.6, 0.3 and 0.15 ng/mL, were processed in quintuplicate on 1-2 days. The %CV was used to estimate the LLOQ (target < 20 %CV).

Imprecision. Residual sera, pooled to achieve low (<1 ng/mL), mid (7-8 ng/mL), and high concentrations (>10 ng/mL), were evaluated in a 5x5 study design (5 measurements performed on each of five separate days, for a total 25 measurements). Longitudinal precision using an “ultra-low” control sample (estimated concentration 0.15 ng/mL) was evaluated over a longer period.

Interference. A pool of low positive samples was mixed at different proportions with three different samples that had known potential interferences. Samples were run in triplicate on the same day.

Carryover. Three samples with extreme Tg elevation were pooled (High Pool). A low concentration pool also processed in 10 replicates and pooled. Then the samples were injected from the same vials in the order high, high, high, low, low, low, etc.

Method comparison: LC-MS/MS vs. immunoassay. A comparison of the Beckman UniCel DxI 800 Tg immunoassay to the new LC-MS/MS assay was performed by analyzing residual patient samples with negative (N =120) and positive (N = 45) anti-Tg antibody.

Method comparison: 96-well plate versus microcentrifuge tube. Sample denaturation, reduction, alkylation, and digestion were performed as described for microcentrifuge tube assay, with substitution of tubes with a 96-well plate (Eppendorf™ Deepwell™, Fisher Scientific 951033308, Protein LoBind) and tube magnet with a 96-well magnet (V&P scientific VP 771LWAZS). The speed of thermomixer was adjusted to 1200 rpm during each incubation. Eluates were transferred to 96-well 700 µl collection plate. The plate was placed on the 96-well magnet to prevent clogging of HPLC tubing by residual beads during injection. Bias and imprecision were assessed.

SUPPLEMENTAL TABLES

Supplemental Table 1. Impact of DMSO on peak area of the FSP-IS peptide

%DMSO (v/v) [a]	Peak Area [b]		Average [c]
	Injection 1	Injection 2	
0	1864.06	2352.02	2108.04
0.5	7601.37	7809.61	7705.49
1.0	7315.99	7776.65	7546.32
1.5	7697.59	8069.25	7883.42
2.0	8372.64	8807.83	8590.24
2.5	9033.48	8962.75	8998.12
3.0	8286.19	8539.04	8412.61

[a] The percentage of DMSO in the mobile phase was increased between duplicate injections at each DMSO concentration, with a system purge between exchange of the mobile phases.
[b] Represents peak area of internal standard, resulting from injection of the working IS (0.5 nM FSP) diluted to a target concentration of 0.2 nM in 2.5% acetic acid (via mixing 4 mL of working IS and 6 mL of 2.5% acetic acid) and further diluted 1:1 with 2.5% Acetic acid - 0.1% CHAPS (equates to 4 femtomoles of peptide in a 40 microliter injection volume). Peak area reflects average of triplicate transitions (707.0300>589.89, 707.0300>589.90, 707.0300>589.91)
[c] Average peak area of Injection 1 and Injection 2

Supplemental Table 2. Impact of DMSO on charge state

Molecular Ion [a]	XIC Range [b]	XIC Intensity		% of Total [c]	
		no DMSO	2% DMSO	No DMSO	2% DMSO
MH+	1406-1408	1.36E+06	1.42E+06	0.7	0.4
MH2+	703-705	1.78E+08	3.43E+08	97.8	96.8
MH3+	469-471	3.93E+06	9.94E+06	2.2	2.8
	Sum of Total:	*1.82E+08*	*3.54E+08*		

Abbreviation: XIC, extracted ion chromatogram
[a] 5 pmol of synthetic FSP peptide (unlabeled) was injected for LC-MS/MS analysis. The peak height of the parent ions was used to evaluate consolidation across charge states (MH+, MH2+, and MH3+) in the presence of DMSO (post-column infusion of 2% in water). The MS was set to full scan mode and mass spectrum peak height was evaluated.
[b] Molecular ion charge states: MH+ (calculated m/z 1406.7), MH2+ (703.8), MH3+ (469.6).
[c] e.g., In the absence of DMSO, (MH+ XIC Intensity)/(Sum of Total) = 1.36E+06 / 1.82E+08 = 0.7%.

Supplemental Table 3. Chicken serum evaluation (dilution study)

Level [a]	Chicken Serum Diluent		Human Serum Diluent		%Difference [d]
	Expected [b]	Result	Expected [b]	Result	
1	0.00	0.01	0.00	0.02	-
2	1.56	1.62	1.56	1.60	1%
3	3.64	3.79	3.64	3.46	10%
4	5.20	5.03	5.20	4.95	2%
5	6.75	6.46	6.75	6.50	-1%
6	8.83	8.72	8.83	9.19	-5%
7	10.39	10.39	10.39	10.56 [c]	1%

[a] A pool of antibody-positive, Tg-positive human sera was diluted either in chicken serum or Tg-negative, Ab-negative human serum and analyzed on the same day (single replicate per level).
[b] Based on volumetric dilution. Values in ng/mL.
[c] The expected concentration for Level 7 was set based on the LC-MS/MS evaluation with chicken serum diluent set. The sample was re-evaluated by LC-MS/MS with the set of samples diluted in human serum.
[d] 100%*([Chicken Serum Result] - [Human Serum Result])/[Human Serum Result]

Supplemental Table 4. Linearity

Level [a]	% Human Serum [b]	Result (ng/mL) [c]	SD	%CV	Set Point [d]
1	2%	0.24	0.027	11.3	0.23
2	8%	1.19	0.042	3.5	1.15
3	39%	4.83	0.256	5.3	5.14
4	70%	8.39	0.686	8.2	8.83
5	100%	11.32	1.202	10.6	12.26

[a] Samples were prepared at 5 levels via dilution of pooled human serum (Golden West MSG4000) in Tg-null chicken serum. These 5 sample pools subsequently became the Husky Ref levels 1-5.
[b] Based on volumetric dilution of human serum with chicken serum.
[c] Mean result based on 6 total measurements
[d] Established through analysis by four different laboratories, over 3 days, in duplicate.

Supplemental Table 5. Imprecision (Near LLOQ, Short Term)

Day/Replicate [a]	1A	1B	1C	1D	1E	2A	2B	2C	2D	2E
Level A	0.57	0.60	0.65	0.62	0.63	0.58	0.57	0.61	0.55	0.62
Level B	0.35	0.34	0.30	0.29	0.31	N/A	N/A	N/A	N/A	N/A
Level C	0.12	0.21	0.17	0.17	0.12	0.15	0.16	0.14	0.16	0.19

Summary	Mean (ng/mL)	SD (ng/mL)	%CV [b]
Level A	0.60	0.032	5%
Level B	0.32	0.026	8%
Level C	0.16	0.028	18%

Abbreviation: N/A, not applicable

[a] Three samples were processed in quintuplicate on 1 or 2 days. The samples were made by diluting a pool of 4 low positive patients (~1 ng/mL) with chicken serum to achieve the desired concentration. Results are shown in ng/mL. Day 2 was not performed for Level B (results marked as "N/A").

[b] The %CV was used to estimate the LLOQ (minimum concentration with <20 %CV).

Supplemental Table 6. Imprecision (Near LLOQ, Longitudinal [a])

Replicate	Day	Tg (ng/mL)	Replicate	Day	Tg (ng/mL)	Replicate	Day	Tg (ng/mL)
1	0	0.12	**15**	105	0.13	**29**	175	0.13
2	12	0.18 [b]	**16**	106	0.13	**30**	182	0.12
3	15	0.14	**17**	111	0.14	**31**	189	0.16
4	21	0.11	**18**	112	0.13	**32**	196	0.15
5	28	0.14	**19**	119	0.13	**33**	197	0.14
6	35	0.12	**20**	121	0.12	**34**	203	0.08
7	42	0.14	**21**	125	0.13	**35**	210	0.16
8	49	0.16	**22**	126	0.15	**36**	221	1.14 [b]
9	56	0.12	**23**	133	0.12	**37**	225	0.16
10	63	0.12	**24**	140	0.13	**38**	232	0.12
11	77	0.13	**25**	147	0.12	**39**	238	0.13
12	77	0.15	**26**	155	0.14	**40**	245	0.17
13	84	0.14	**27**	161	0.15	**41**	252	0.13
14	98	0.13	**28**	168	0.17			

Summary	Mean	Std. Dev.	%CV
All Replicates	0.14	0.0189	14%

[a] Very low (<0.15 ng/mL) or negative patient samples were pooled to a target value of ~0.15 ng/mL. Aliquots (stored at -20°C) were evaluated in separate clinical runs spread over 252 days.

[b] Run was repeated due to overall QC failure. Value was excluded from calculation of summary statistics.

Supplemental Table 7. Imprecision (5x5 Experiment [a])

Level [b]	Day	Replicate A (ng/mL)	Replicate B (ng/mL)	Replicate C (ng/mL)	Replicate D (ng/mL)	Replicate E (ng/mL)	Intra-day (% CV)
Low	1	0.57	0.6	0.65	0.62	0.63	5%
	2	0.58	0.57	0.61	0.55	0.62	5%
	3	0.65	0.56	0.51	0.72	0.52	15%
	4	0.54	0.68	0.58	0.61	0.58	9%
	5	0.64	0.57	0.6	0.7	0.7	9%
	Inter-day (% CV)	8%	8%	9%	11%	11%	
Medium	1	7.56	7.67	7.54	8.01	8	3%
	2	7.58	7.75	7.25	7.66	7.7	3%
	3	7.8	7.79	7.21	7.12	7.93	5%
	4	7.12	7.38	7.2	7.73	7.41	3%
	5	7.75	7.52	7.66	8.24	7.77	3%
	Inter-day (% CV)	4%	2%	3%	5%	3%	
High	1	11.73	12.05	12.4	12.18	12.41	2%
	2	12.68	12.73	12.45	12.47	*	1%
	3	12.46	11.71	12.45	12.31	12.62	3%
	4	12.57	12.03	12.98	12.65	12.95	3%
	5	10.02	9.85	11.69	10.87	11.82	8%
	Inter-day (% CV)	9%	9%	4%	6%	4%	

Summary [c]	Mean	Std. Dev.	Mean inter-day	Mean Intra-day	TE
Low	0.61	0.056	9%	9%	13%
Medium	7.61	0.290	3%	4%	5%
High	12.09	0.813	6%	4%	7%

[a] 5 measurements performed on each of 5 separate days, for a total 25 measurements

[b] Residual sera, pooled to achieve low (<1 ng/mL), medium (7-8 ng/mL), and high concentrations (>10 ng/mL). One sample (*) in the "High" set had to be excluded to an error in sample preparation.

[c] Summary statistics across all 25 replicates for each level (24 replicates for "High" concentration)

Supplemental Table 8. Interference (Mixing Study)

Interferent [a]	Sample [b]	Mixing Ratio [c]	Replicate [d] A	B	C	Avg.	Expected [e]	%Recovery
Total Protein (TP)	TP-1	1:0	1.5	1.5	0.98	1.33	1.33	N/A
	TP-2	0.75:0.25	2.72	2.04	2.51	2.42	2.16	112%
	TP-3	0.5:0.5	3.55	4.11	2.55	3.40	2.99	114%
	TP-4	0.25:0.75	2.6	5.39	4.98	4.32	3.82	113%
	TP-5	0:1	5.58	3.71	N/A	4.65	4.65	N/A
Lipids (L)	L-1	1:0	1.22	1.34	1.23	1.26	1.26	N/A
	L-2	0.75:0.25	3.26	3.53	3.59	3.46	3.13	110%
	L-3	0.5:0.5	5.28	5.24	5.4	5.31	5.00	106%
	L-4	0.25:0.75	7.15	7.09	N/A	7.12	6.87	104%
	L-5	0:1	8.85	8.64	N/A	8.75	8.75	N/A
Hemoglobin (Hb)	Hb-1	1:0	1.09	1.39	1.11	1.20	1.20	N/A
	Hb-2	0.75:0.25	6.25	4.31	4.61	5.06	5.05	100%
	Hb-3	0.5:0.5	9.14	9.3	9.76	9.40	8.91	106%
	Hb-4	0.25:0.75	13.93	13.33	13.35	13.54	12.76	106%
	Hb-5	0:1	16.79	16.99	16.08	16.62	16.62	N/A

Abbreviations: TP, total protein; L, lipids; Hb, hemoglobin; N/A, not applicable

[a] Three sample sets were prepared containing increasing levels of interferents – total protein (TP), lipids (L), or hemoglobin (Hb).

[b] Five levels were created for each interferent by mixing pools of low Tg-positive sera (Tg 1 to 2 ng/mL) with sera from individuals with high levels of total protein (protein in TP-5 = 9.9 g/dL), lipids (triglycerides in L-5 = 412 mg/dL), or hemoglobin (0.54 g/dL in Hb-5). Samples TP-1, TG-1, and Hb-1 did not contain interferents and are representative of the sera pools used to prepare each set.

[c] Ratio of volume of the "-1" sample to the "-5" sample in each group.

[d] Samples were run in triplicate on the same day. All results are in ng/mL Tg. In three instances, testing was not performed for a third replicate (appear as "N/A" under column C).

[e] The concentrations for Levels 1 and 5 were defined based on triplicate analysis by LC-MS/MS. The expected concentrations (in ng/mL) for levels 2-4 were then calculated based on ratio of volumes mixed to prepare the samples.

Supplemental Table 9. Carryover

Injection	Sample [a]	Result (Tg ng/mL)
1	High Pool	3045.80
2	High Pool	3222.07
3	High Pool	3208.79
4	Low Pool	4.74
5	Low Pool	0.85
6	Low Pool	0.28
7	High Pool	3251.56
8	High Pool	3146.45
9	High Pool	3285.82
10	Low Pool	4.65
11	Low Pool	0.93
12	Low Pool	0.37
13	High Pool	3235.77
14	High Pool	3205.31
15	High Pool	3235.2
16	Low Pool	4.77
17	Low Pool	0.93
18	Low Pool	0.46
Summary	**Mean of High (1-3, 7-9, 13-15)**	3204.09
	Mean of Low w/ carryover (4, 10, 16)	4.72
	Mean of Low w/o carryover (6, 12, 18)	0.37
	%Carryover	0.14%
	Following a concentration of	3204.09

[a] Three samples with extreme Tg elevation were pooled (High Pool). A low concentration pool was also prepared. The high and low pools were injected in the order high, high, high, low, low, low, etc., using a single vial for each pool.

Supplemental Table 10. Method Comparison – (Vs. Immunoassay [a], TgAb Negative [b])

#	IA	MS	#	IA	MS	#	IA	MS	#	IA	MS
1	7.77	8.7	**31**	0.71	0.73	**61**	0.2	0.23	**91**	0.06	0.1
2	6.04	8.12	**32**	0.81	0.59	**62**	0.21	0.22	**92**	0.15	0
3	5.97	6.95	**33**	0.47	0.65	**63**	0.22	0.18	**93**	0.03	0.11
4	5.73	3.97	**34**	0.51	0.57	**64**	0.26	0.13	**94**	0.1	0.03
5	4.17	5.04	**35**	0.56	0.51	**65**	0.2	0.17	**95**	0.03	0.1
6	3.73	4.36	**36**	0.5	0.57	**66**	0.16	0.21	**96**	0.03	0.1
7	3.81	3.25	**37**	0.43	0.52	**67**	0.16	0.2	**97**	0.06	0.06
8	1.69	1.51	**38**	0.43	0.5	**68**	0.23	0.12	**98**	0.04	0.07
9	1.35	1.61	**39**	0.42	0.51	**69**	0.17	0.18	**99**	0.08	0.03
10	1.66	1.18	**40**	0.55	0.35	**70**	0.17	0.18	**100**	0.03	0.08
11	1.49	1.34	**41**	0.46	0.44	**71**	0.21	0.13	**101**	0.04	0.06
12	1.45	1.38	**42**	0.43	0.43	**72**	0.2	0.13	**102**	0.04	0.06
13	1.26	1.06	**43**	0.46	0.35	**73**	0.25	0.05	**103**	0.03	0.07
14	1.13	1.16	**44**	0.21	0.59	**74**	0.1	0.19	**104**	0.06	0.03
15	1.04	1.2	**45**	0.37	0.41	**75**	0.08	0.2	**105**	0.04	0.04
16	0.86	1.36	**46**	0.31	0.47	**76**	0.11	0.16	**106**	0.03	0.05
17	1.23	0.89	**47**	0.35	0.38	**77**	0.08	0.18	**107**	0.05	0.02
18	1.11	0.91	**48**	0.37	0.31	**78**	0.13	0.12	**108**	0.04	0.03
19	1.18	0.8	**49**	0.25	0.42	**79**	0.1	0.14	**109**	0.03	0.04
20	0.87	1.07	**50**	0.35	0.31	**80**	0.12	0.12	**110**	0.04	0.02
21	0.5	1.43	**51**	0.24	0.4	**81**	0.08	0.15	**111**	0.03	0.03
22	0.93	0.93	**52**	0.32	0.3	**82**	0.12	0.1	**112**	0.03	0.02
23	0.88	0.98	**53**	0.22	0.37	**83**	0.12	0.09	**113**	0.03	0.02
24	0.82	0.99	**54**	0.27	0.31	**84**	0.1	0.1	**114**	0.02	0.03
25	0.84	0.77	**55**	0.23	0.33	**85**	0.1	0.09	**115**	0.02	0.02
26	0.67	0.92	**56**	0.23	0.3	**86**	0.08	0.11	**116**	0.03	0
27	0.86	0.73	**57**	0.28	0.23	**87**	0.1	0.08	**117**	0.03	0
28	0.96	0.55	**58**	0.19	0.29	**88**	0.1	0.07	**118**	0.01	0.01
29	0.8	0.71	**59**	0.24	0.23	**89**	0.09	0.07	**119**	0	0.02
30	0.83	0.64	**60**	0.23	0.2	**90**	0.08	0.08	**120**	0.01	0

Result values in ng/mL

[a] Beckman UniCel DxI 800 Tg immunoassay

[b] Thyroglobulin Antibody < 1.0 IU/mL, analyzed using the Access Thyroglobulin Antibody II assay performed on the Beckman UniCel DxI 800 analyzer

Supplemental Table 11. Method Comparison – (Vs. Immunoassay, TgAb Positive [a])

#	TgAB	IA	MS	#	TgAB	IA	MS	#	TgAB	IA	MS
121	>2000	0.03	2.44	**136**	27.6	0	0.1	**151**	4.3	0	0.05
122	1.2	1.02	1.28	**137**	21.7	0.01	0.09	**152**	12.9	0.04	0.01
123	1.6	0.63	0.73	**138**	1.9	0.02	0.07	**153**	23.7	0.01	0.03
124	1.1	0.5	0.49	**139**	13.3	0.03	0.06	**154**	21.9	0.02	0.02
125	127.7	0.06	0.85	**140**	3.9	0.01	0.07	**155**	3.3	0	0.04
126	1.1	0.23	0.44	**141**	168.2	0.03	0.04	**156**	34.1	0.04	0
127	1	0.38	0.18	**142**	3.6	0.02	0.05	**157**	2.8	0.02	0.01
128	1.7	0.22	0.26	**143**	101.6	0.03	0.04	**158**	1.2	0	0.03
129	102.8	0.14	0.17	**144**	3.1	0.07	0	**159**	2.4	0	0.03
130	2.5	0.01	0.19	**145**	259.3	0.01	0.06	**160**	17.7	0.01	0.02
131	11.1	0.09	0.1	**146**	25.4	0.01	0.05	**161**	734.1	0.02	0
132	9.4	0.08	0.07	**147**	1647.2	0	0.06	**162**	5	0.01	0
133	20.8	0.05	0.09	**148**	5	0.06	0	**163**	2.3	0.01	0
134	235.3	0	0.13	**149**	2.9	0.03	0.03	**164**	1.5	0	0
135	33.1	0.04	0.08	**150**	11.3	0.01	0.04	**165**	37.7	3.67	3.41

Result values in ng/mL

[a] Thyroglobulin Antibody ≥ 1.0 IU/mL

Supplemental Table 12. Comparison of Tube and Plate Formats

#	Tube	Plate	#	Tube	Plate	#	Tube	Plate	#	Tube	Plate
1	1.06	1.40	**11**	0.43	0.35	**21**	3.87	3.68	**31**	5.72	6.11
2	0.09	0.14	**12**	5.22	4.95	**22**	0.64	0.81	**32**	4.68	5.60
3	0.39	0.64	**13**	0.18	0.14	**23**	0.91	1.21	**33**	3.83	3.95
4	0.43	0.52	**14**	1.67	1.37	**24**	1.02	1.18	**34**	6.54	6.73
5	1.86	1.57	**15**	0.65	0.74	**25**	0.76	0.96	**35**	3.85	3.71
6	1.18	1.48	**16**	1.00	1.00	**26**	1.20	1.16	**36**	4.06	4.29
7	1.90	2.71	**17**	4.43	4.00	**27**	0.34	0.32	**37**	3.68	4.09
8	10.03	8.33	**18**	6.92	6.26	**28**	0.55	0.46	**38**	0.78	0.83
9	7.24	6.49	**19**	0.30	0.37	**29**	1.16	1.17	**39**	4.84	5.50
10	1.08	1.25	**20**	0.93	0.84	**30**	11.35	11.17	**40**	0.26	0.40

Result values in ng/mL

Supplemental Table 13 Plate Format Imprecision Summary Statistics

Sample	n	Mean (ng/mL)	Std. Dev. (ng/mL)	%CV
TG Ultra Low Control	14	0.13	0.03	24%
Low Pool	25	1.00	0.11	11%
High Pool	25	4.00	0.43	11%

Supplemental Table 14. Husky Ref Characterization

Diluent [a]	Sample	Average Result	Standard Error of the Mean
Chicken	1	0.23	0.02
	2	1.15	0.03
	3	5.14	0.17
	4	8.83	0.28
	5	12.26	0.38
Rabbit	A	0.20	0.01
	B	0.97	0.02
	C	5.05	0.15
	D	8.81	0.26
	E	12.24	0.39

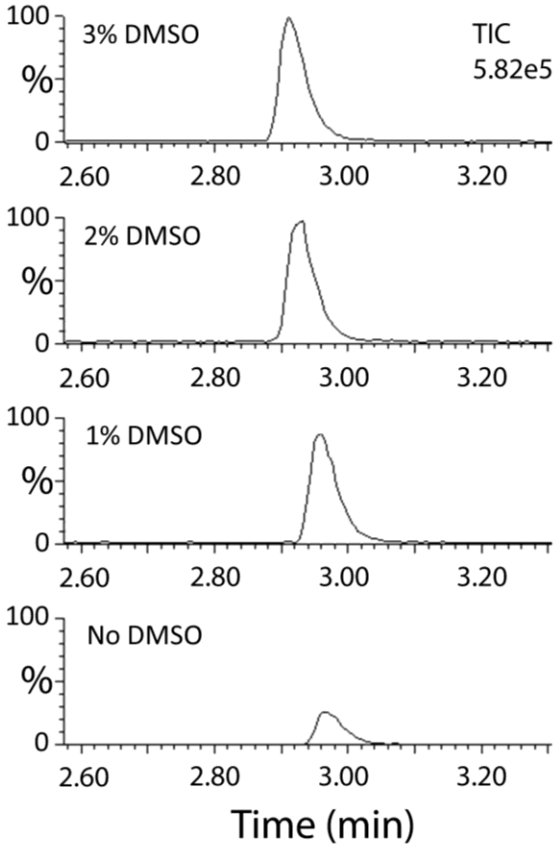
[a] The reference material was prepared at the University of Washington. One set (standards 1-5) was prepared by serial dilution of Golden West MSG4000 human serum pool with chicken serum. A second set (standards A-E) was prepared similarly, with rabbit serum as the diluent. Set points were established through analysis of the material by four different laboratories, over three days, in duplicate.

Supplemental Table 15. Interlaboratory Study (Alternative Assessment)

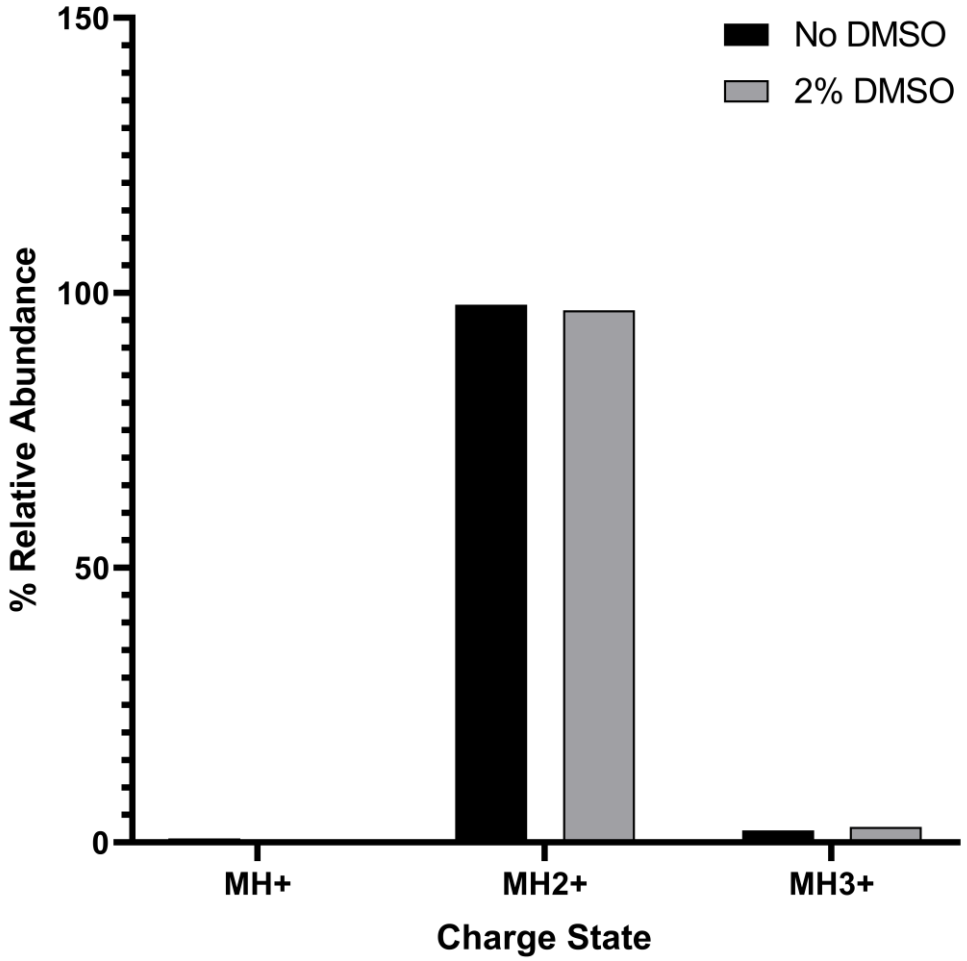
Date	Sample [a]	Results (ng/mL)				Min/Max	Mean	S.D.	%CV
		Lab A [b]	Lab B [c]	Lab C	Lab D				
4/1/2020	TG-01	2.70	2.90	2.0	2.0	2.0/2.9	2.40	0.47	19.54
	TG-02	1.80	1.60	1.2	1.5	1.2/1.8	1.53	0.25	16.39
	TG-03	16.3	16.0	12.0	12.8	12.0/16.3	14.28	2.19	15.36
7/1/2020	TG-04	8.70	10.2	7.9	10.6	7.9/10.6	9.35	1.27	13.54
	TG-05	3.20	3.30	2.7	3.7	2.7/3.7	3.23	0.41	12.75
	TG-06	4.50	4.90	3.8	5.4	3.8/5.4	4.65	0.68	14.53
10/1/2020	TG-07	0.67	0.71	0.4	0.7	0.4/0.7	0.61	0.16	26.54
	TG-08	0.45	0.43	0.3	0.3	0.3/0.45	0.36	0.09	25.76
	TG-09	3.26	3.74	2.3	3.0	2.3/3.74	3.08	0.60	19.54
3/1/2021	TG-10	1.27	1.19	0.9	1.0	0.9/1.27	1.09	0.17	15.59
	TG-11	2.93	2.53	1.9	2.4	1.9/2.93	2.44	0.42	17.41
	TG-12	6.38	6.38	5.1	6.4	5.1/6.4	6.07	0.64	10.61

[a] Samples distributed for alternative assessment evaluation constituted pooled residual samples. The participating laboratories alternate in distributing samples.
[b] University of Washington Assay
[c] The assay of Laboratory B utilized calibrators with set points also traceable to the Husky Ref.

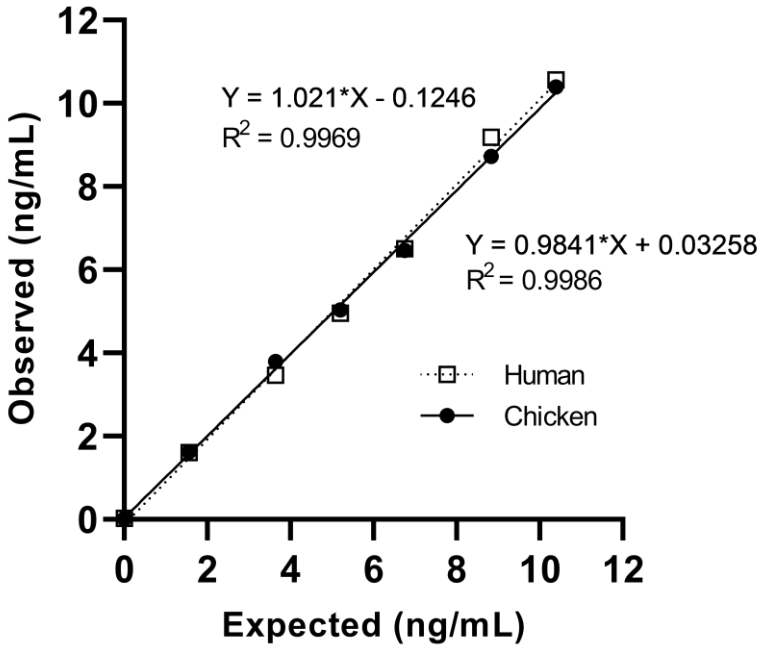
SUPPLEMENTAL FIGURES



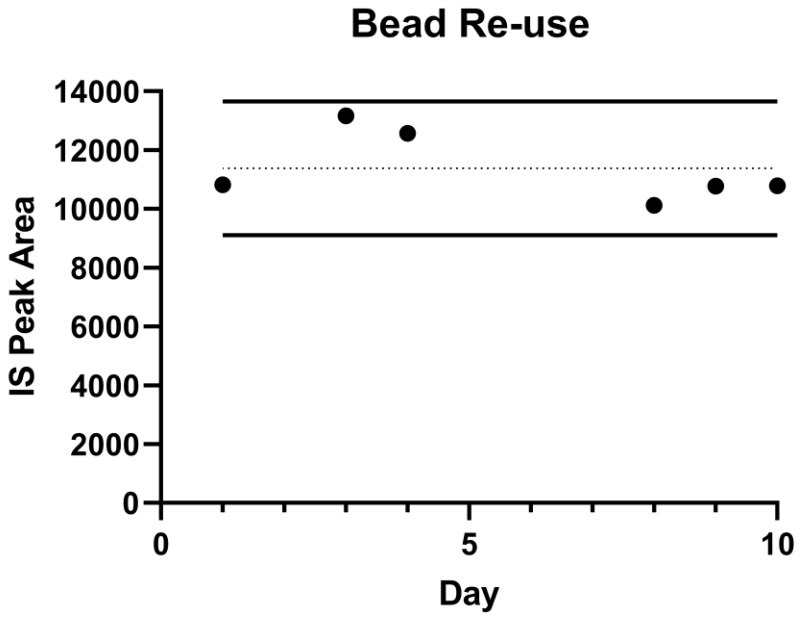
Supplemental Figure 1. Impact of DMSO on peak area of the FSP-IS peptide. Representative chromatograms are shown demonstrating an increase in peak area for the FSP stable-isotope labeled internal standard peptide (injection of 4 femtomoles peptide) with addition of DMSO to the mobile phase. The y-axis in each chromatogram is normalized to a maximal intensity of 5.82e5.



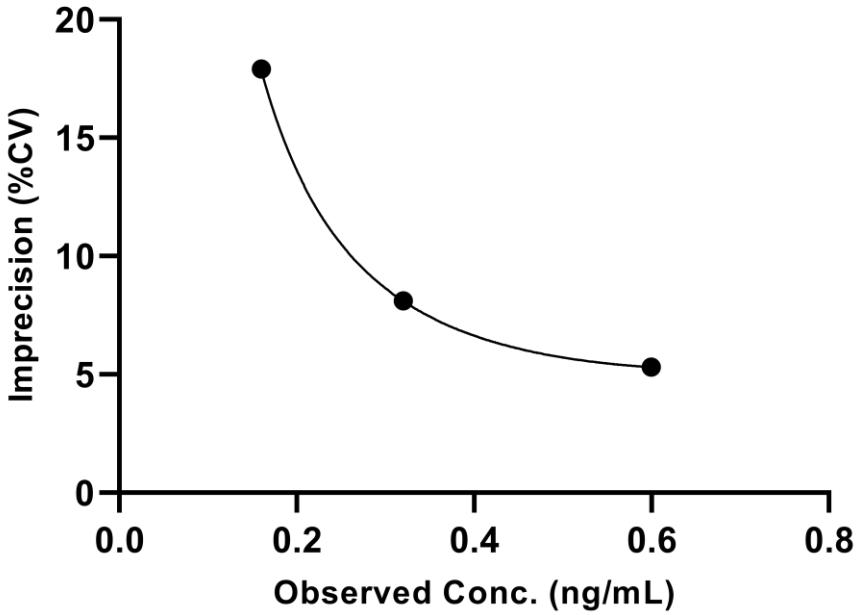
Supplemental Figure 2. Impact of DMSO on charge state. 5 pmol of purified FSP peptide (unlabeled) was injected for LC-MS analysis (full scan mode). The mass spectrum peak height of the parent ions was used to evaluate charge consolidation in the presence of DMSO (post-column infusion of 2% in water). No charge state coalescence was observed.



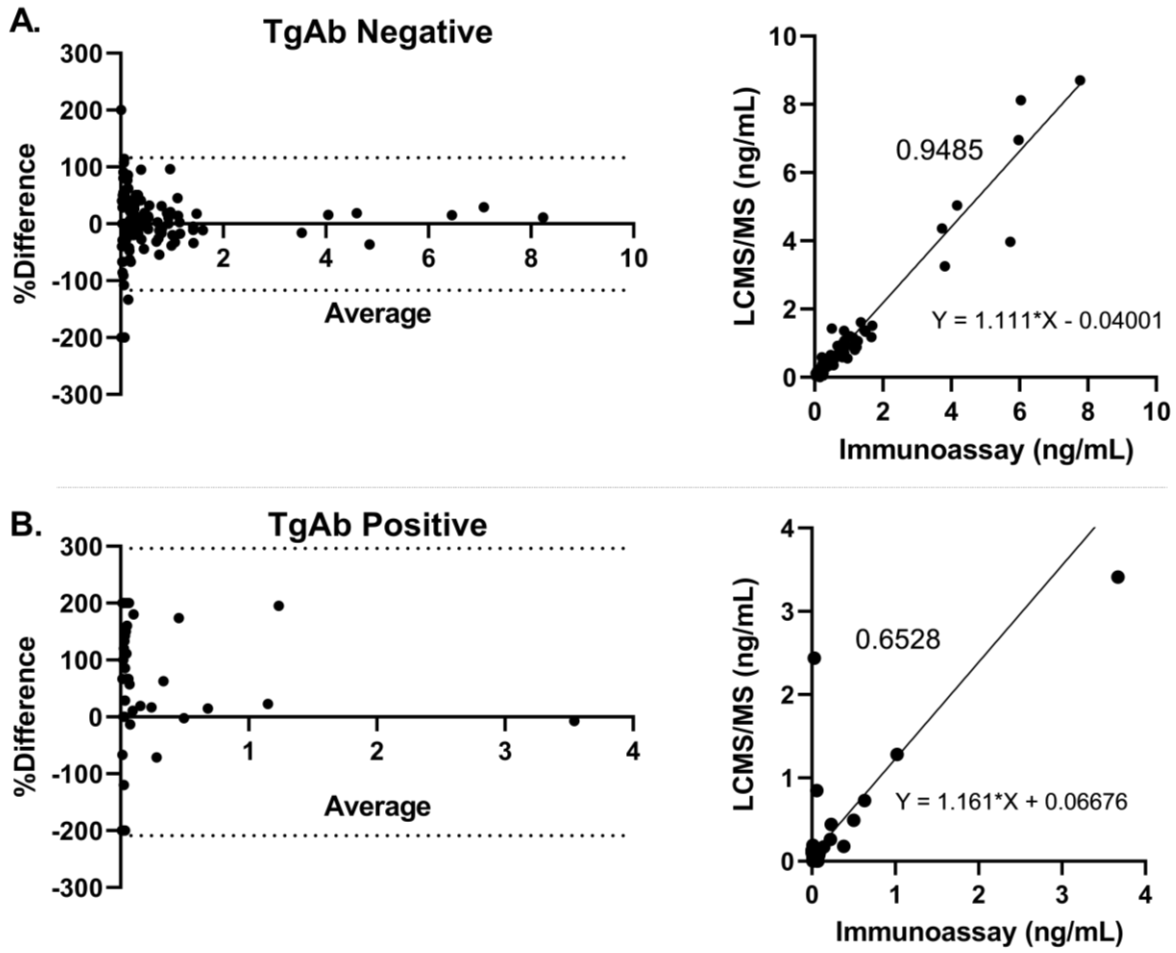
Supplemental Figure 3. Chicken serum evaluation (dilution study). Residual human sera positive for Tg Ab (≥1.0 IU/mL measured on Beckman Coulter Unicel DxI 800) were pooled and diluted in two sets, one with chicken serum and the other with Tg-negative, antibody-negative human serum and analyzed on the same day. Expected concentrations were based on volumetric dilution of the undiluted, high-level sample.



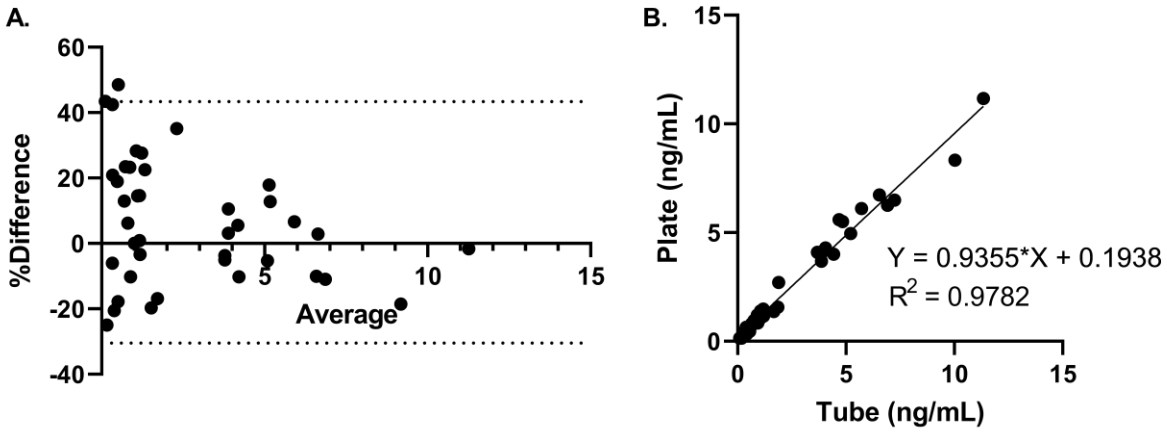
Supplemental Figure 4. Pre-validation of bead regeneration protocol. A pool of patient samples with approximately 0.15 ng/mL was digested and stored in aliquots. The peak area of IS immunoenriched with the antibody-conjugated beads is illustrated for each re-use of the beads. Average IS peak area was 11375 (dotted line), with %CV = 11%; +/-20% range = 9100 to 13650 (solid lines).



Supplementary Figure 5. Imprecision Near LLOQ (Short Term). The CV was used to estimate the LLOQ. The samples were made by diluting a pool of 4 Tg low-positive patients (~1 ng/mL) with chicken serum to achieve the desired concentrations. The points are fitted with a power series, Y=A*X^B + C*X^D, where A = 1.563, B = -1.315, C = 3.968, D = 1.119. The extrapolated imprecision at 0.15 ng/mL = 19.4%.



Supplemental Figure 6. Comparison with Immunoassay. Bland-Altman plots (left) are shown for **(A)** samples negative (n = 120) and **(B)** positive (n = 45) for thyroglobulin autoantibodies as measured by Beckman Coulter Unicel DxI 800. Pearson R^2 is indicated in each plot containing Deming regression (right). X-axis "Average" indicates average result across both platforms.



Supplemental Figure 7. Comparison of Tube and Plate Formats. (A) Bland-Altman and **(B)** Deming Regression are shown for 40 leftover clinical samples collected and processed per SOP in tubes or per an adapted procedure in a 96-well plate. X-axis “Average” in the Bland Altman plot indicates average result across both methods.

Department of Laboratory Medicine
Division of Chemistry

Certificate of Analysis

Thyroglobulin Reference Standard 'Husky Ref'

Thyroglobulin (Tg) reference standard aka 'Husk Ref' was prepared in the Hoofnagle Laboratory at the University of Washington, Department of Laboratory Medicine. Husky Ref 1-5 were prepared by serial dilution of Golden West MSG4000 human serum pool (lot#C10003) with Tg-null chicken serum (Equitech-Bio, Inc., cat # SC30, lot# 190509-0130). Husky Ref A-E were prepared similarly with Tg-null rabbit serum (Gibco, cat# 16120, lot# 2046820). Set point were established through analysis by four different laboratories, over three days, in duplicate.

Husky Ref	May2019
Storage	≤ -20°C
Standard	Tg Concentration (ng/mL)
Husky Ref 1	0.23
Husky Ref 2	1.15
Husky Ref 3	5.14
Husky Ref 4	8.83
Husky Ref 5	12.26
Husky Ref A	0.20
Husky Ref B	0.97
Husky Ref C	5.05
Husky Ref D	8.81
Husky Ref E	12.24

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Supplemental Figure 8. Husky Ref Certificate of Analysis. The reference material was prepared at the University of Washington. One set (standards 1-5) was prepared by serial dilution of Golden West MSG4000 human serum pool with chicken serum. A second set (standards A-E) was prepared similarly, with rabbit serum as the diluent. Set points were established through analysis of the material by four different laboratories, over three days, in duplicate.