Mass Spectrometry Immunoassay Coupled with Peptide Enrichment to Detect Thyroglobulin by Capillary Flow LC/MS/MS in Clinical Research

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ABSTRACT

Purpose: To develop an assay to capture Thyroglobulin in biological matrix and quantitate.

Methods: Using immuno-capture sample preparation and capillary chromatography detect Thyroglobulin with a highly sensitive mass spectrometer.

Results: Thyroglobulin was quantitated down to 0.1 ng/mL in plasma with analytical reproducibility, accuracy and precision.

INTRODUCTION

As one of the most common endocrine cancers, thyroid cancers the measurement of serum thyroglobulin (Tg) is important for diagnostics and follow-up treatment. Immunoassays have been the standard technique for quantitation, however a high amount of false negative results occur. Anti-Tg auto antibodies found endogenously block the binding epitope leading to these results. A user friendly, automated technique with low dead volume and improved performance for washes over beads was utilized to determine if this method would have utility for clinical research. Sample preparation techniques of using mass spectrometric immunoassay (MSIA) with stable isotope standards and capture by anti-peptide antibodies (SISCAPA) in conjugation with capillary flow LC/MS on a triple quadrupole provides high analytical selectivity and specificity at low levels of Tg found in serum.

MATERIALS AND METHODS

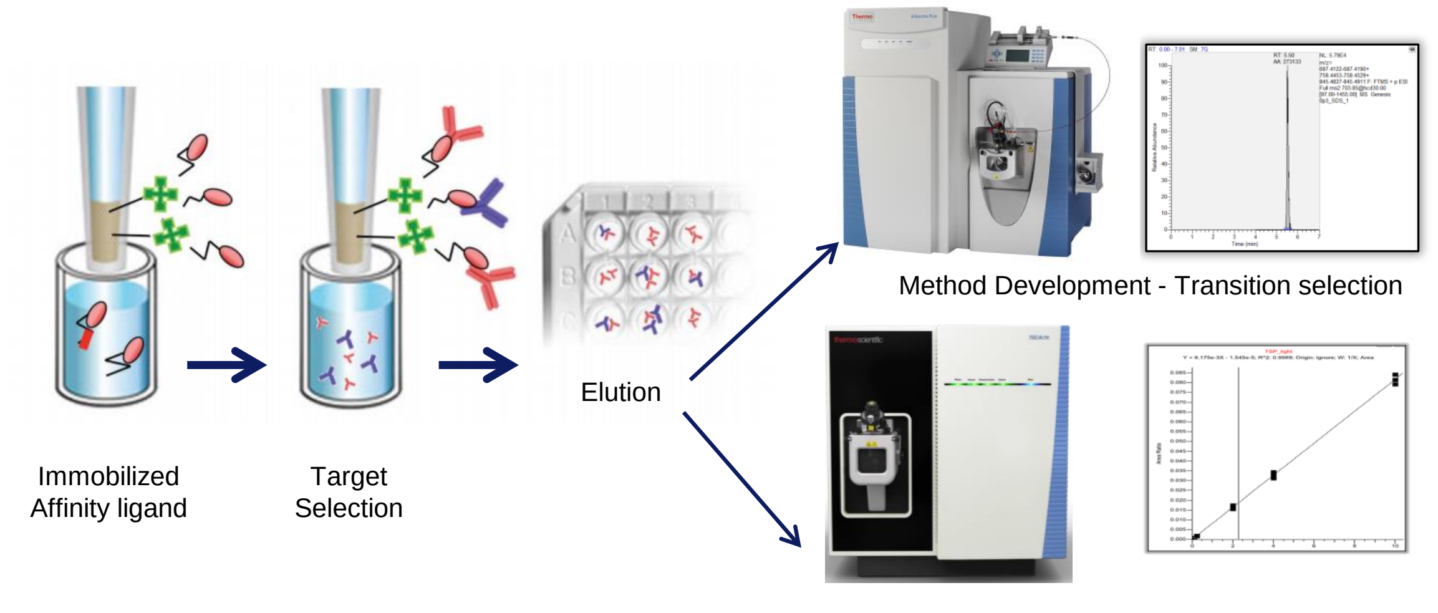
Sample Preparation

Human thyroglobulin was spiked into human heparin plasma and reduced and alkylated, followed by digestion with trypsin. Immuno-capture was than executed with a Thermo Scientific™ Finnpipette™ Novus i pipetter with Thermo Scientific™ MSIA™ D.A.R.T.'S™ derivitized with anti-TG FSP antibody (SISCAPA). Elution of the peptide of interest was placed into a 96 well plate and injected into the LC/MS. Workflow of this process is showed in Figure 1.

LC/MS Parameters

Method development was performed on a Thermo Scientific™ Dionex™ UltiMate™ 3000 LC and Thermo Scientific™ Q Exactive™ mass spectrometer. The chromatography was performed with a Thermo Scientific™ ProSwift™ RP4H, 500um x 10cm column and run with a 20 minute long method. The quantitation experiments will be verified with a Thermo Scientific™ UltiMate™ 3000 RSLCnano™ LC system equipped with a capillary flow selector. Chromatographic separation was performed using a 150 um x 15 cm Thermo Scientific™ Acclaim™ PepMap™ column packed with 2.2 um C18 using a 30 minute gradient. LC/MS parameters are found in Figure 2.

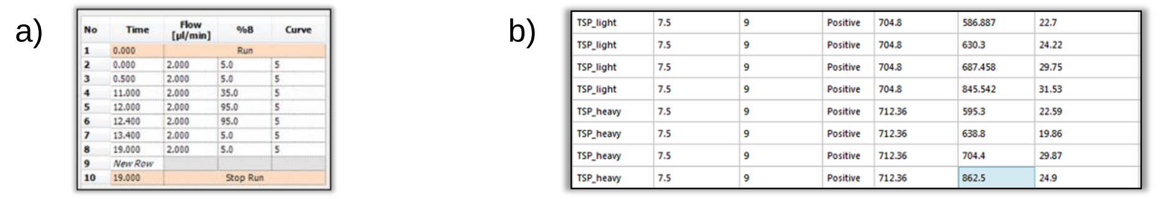
Figure 1. Workflow of MSIA immuno-capture of Tg peptide and detection through LC/MS.



Data Analysis

Quantitation data was processed in Thermo Scientific™ TraceFinder™ software and retention times were scheduled using Skyline software.

Figure 2. LC/MS parameters for quantitation on the Thermo Scientific™ TSQ Altis™ triple quadrupole MS, a) gradient from liquid chromatography and b) SRM parameters for FSP peptide.



a)

No	Time	Flow [µl/min]	%B	Curve
1	0.000	Run		
2	0.000	2.000	5.0	5
3	0.500	2.000	5.0	5
4	11.000	2.000	35.0	5
5	12.000	2.000	95.0	5
6	12.400	2.000	95.0	5
7	13.400	2.000	5.0	5
8	19.000	2.000	5.0	5
9	*New Row*			
10	19.000	Stop Run		

b)

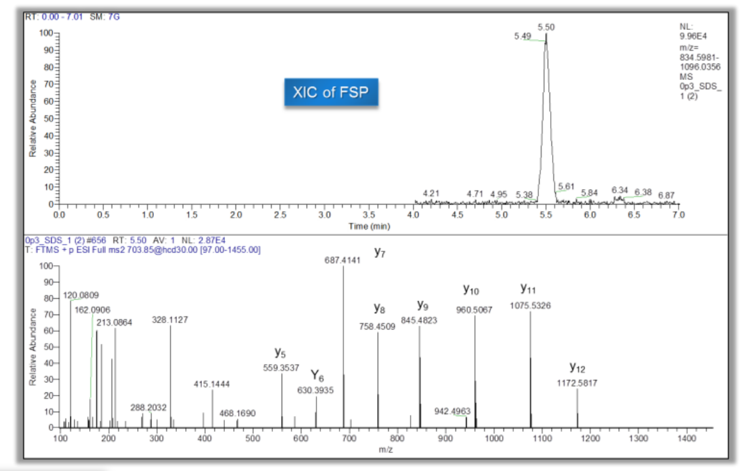
TSP_light	7.5	9	Positive	704.8	586.887	22.7
TSP_light	7.5	9	Positive	704.8	630.3	24.22
TSP_light	7.5	9	Positive	704.8	687.458	29.75
TSP_light	7.5	9	Positive	704.8	845.542	31.53
TSP_heavy	7.5	9	Positive	712.36	595.3	22.59
TSP_heavy	7.5	9	Positive	712.36	638.8	19.86
TSP_heavy	7.5	9	Positive	712.36	704.4	29.87
TSP_heavy	7.5	9	Positive	712.36	862.5	24.9

RESULTS

Method Development

Sample conditions and liquid chromatography parameters were developed on the Q Exactive MS platform and UltiMate 3000 RLSC nano system. Transitions of FSP peptide are shown in the high resolution data in Figure 3 In order to get the peptide to stick to the column, the gradient had to begin at 5% mobile phase B. Chromatography of the peptide of interest can be seen comparing to retention time standard PRTC in Figure 4. In addition, in order for the peptide to elute off the MSIA tips higher percent organic was needed, but good peak shape and retention was achieved with 0.4% TFA and 20% ACN as a reconstitution solvent. In this solution conditions a higher injection can be used without fronting of the chromatography, demonstration of this is shown in Figure 5.

Figure 3. XIC of FSP peptide on the Q Exactive MS and the bottom panel is the MS/MS spectrum.



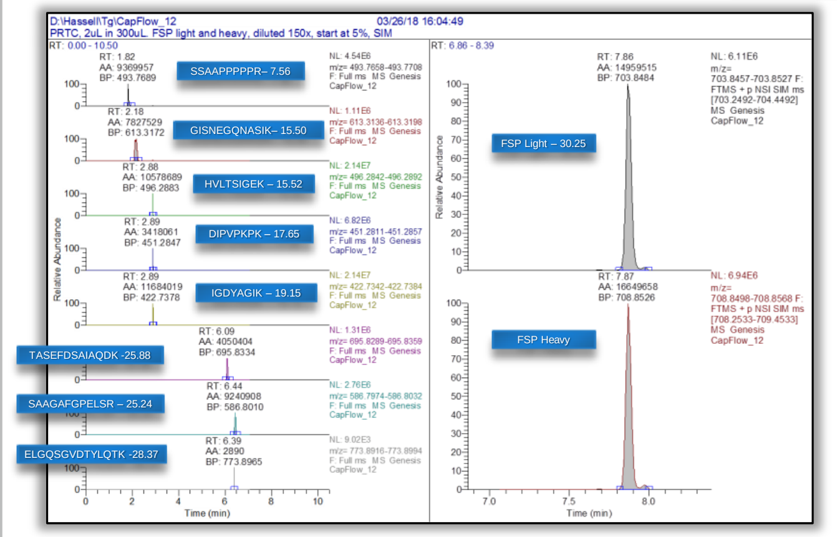
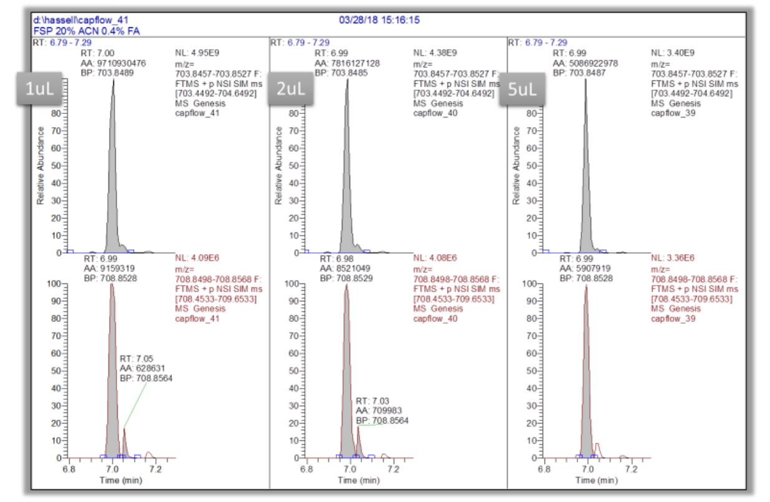


Figure 4. SRM chromatograms of PRTC peptides in the right panel and FSP peptides in the left with the peptides corresponding isoelectric points (pIs.)

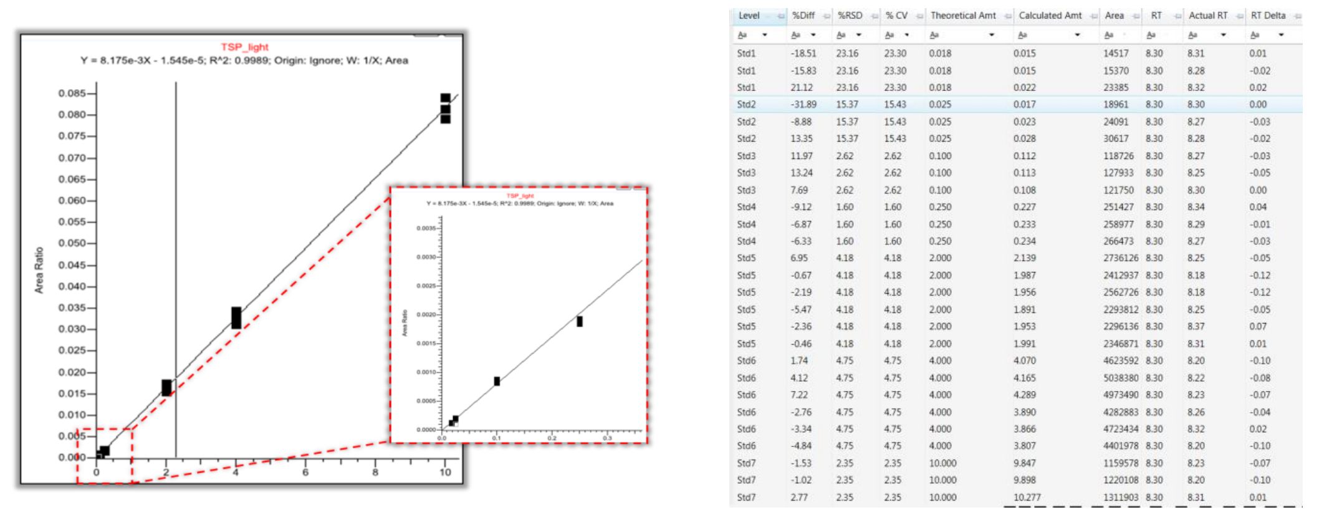
Figure 5. SRM chromatograms of different injections volumes of FSP diluted in 0.4% TFA and 20% ACN.



Neat Calibration Curve

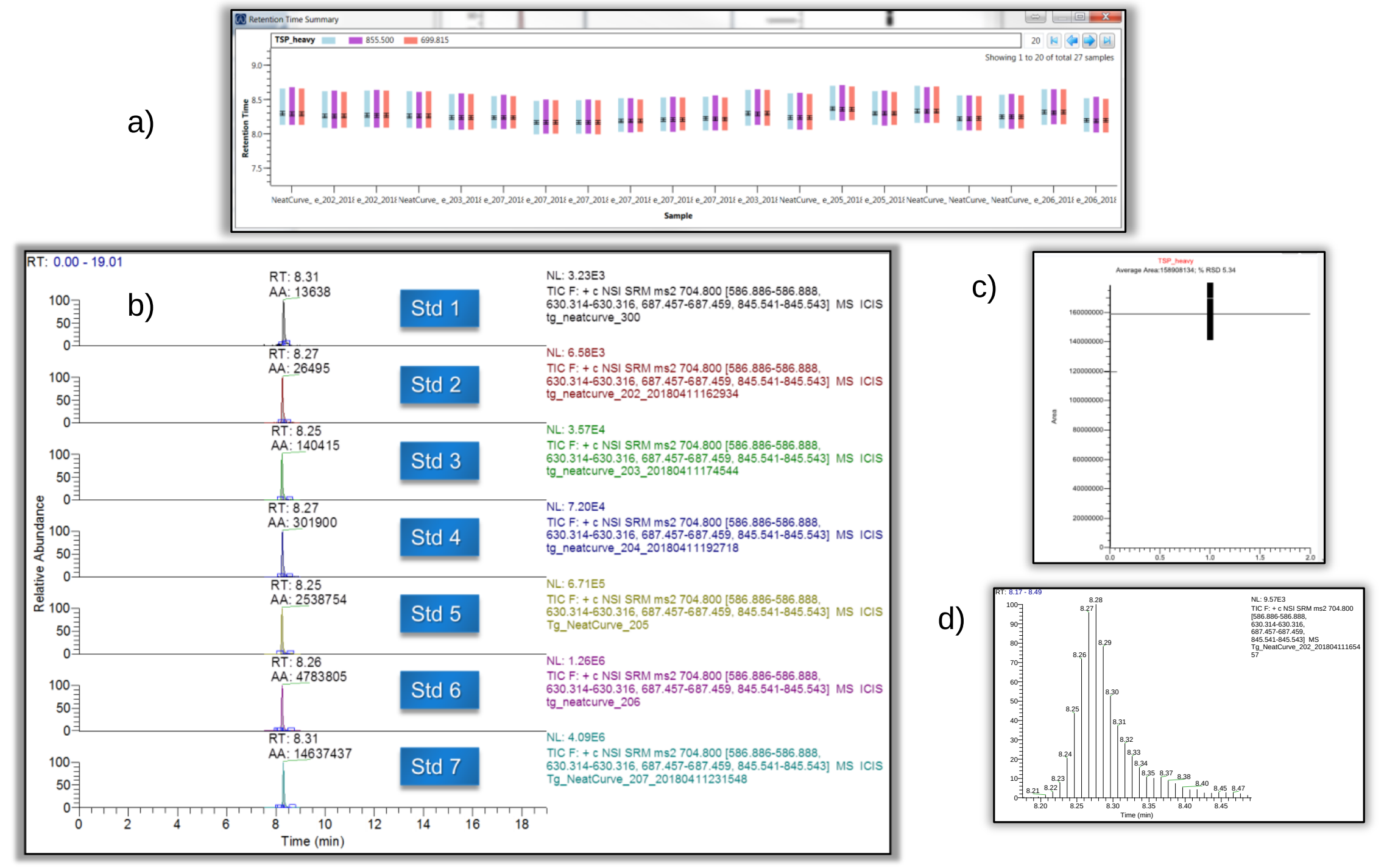
A neat calibration curve was prepared in 0.4% TFA and 20% ACN and injected in triplicate. A heavy labeled was used an the internal standard. As shown in Figure 6, the linearity of the assay is demonstrated, from 15 pg/mL to 10 ng/mL. The CVs and % difference were all below 25%.

Figure 6. Neat calibration curve is shown to the left and the analytical results are presented on the right. Inset of the low end of the curve is shown as well.



Level	%Diff	%RSD	% CV	Theoretical Amt	Calculated Amt	Area	RT	Actual RT	RT Delta
Std1	-18.51	23.16	23.30	0.018	0.015	14517	8.30	8.31	0.01
Std1	-15.83	23.16	23.30	0.018	0.015	15370	8.30	8.28	-0.02
Std1	21.12	23.16	23.30	0.018	0.022	23385	8.30	8.32	0.02
Std2	-31.89	15.37	15.43	0.025	0.017	18961	8.30	8.30	0.00
Std2	-8.88	15.37	15.43	0.025	0.023	24091	8.30	8.27	-0.03
Std2	13.35	15.37	15.43	0.025	0.028	30617	8.30	8.28	-0.02
Std3	11.97	2.62	2.62	0.100	0.112	118726	8.30	8.27	-0.03
Std3	13.24	2.62	2.62	0.100	0.113	127933	8.30	8.25	-0.05
Std3	7.69	2.62	2.62	0.100	0.108	121750	8.30	8.30	0.00
Std4	-9.12	1.60	1.60	0.250	0.227	251427	8.30	8.34	0.04
Std4	-6.87	1.60	1.60	0.250	0.233	258977	8.30	8.29	-0.01
Std4	-6.33	1.60	1.60	0.250	0.234	266473	8.30	8.27	-0.03
Std5	6.95	4.18	4.18	2.000	2.139	2736126	8.30	8.25	-0.05
Std5	-0.67	4.18	4.18	2.000	1.987	2412937	8.30	8.18	-0.12
Std5	-2.19	4.18	4.18	2.000	1.956	2562726	8.30	8.18	-0.12
Std5	-5.47	4.18	4.18	2.000	1.891	2293812	8.30	8.25	-0.05
Std5	-2.36	4.18	4.18	2.000	1.953	2296136	8.30	8.37	0.07
Std5	-0.46	4.18	4.18	2.000	1.991	2346871	8.30	8.31	0.01
Std6	1.74	4.75	4.75	4.000	4.070	4623592	8.30	8.20	-0.10
Std6	4.12	4.75	4.75	4.000	4.165	5038380	8.30	8.22	-0.08
Std6	7.22	4.75	4.75	4.000	4.289	4973490	8.30	8.23	-0.07
Std6	-2.76	4.75	4.75	4.000	3.890	4282883	8.30	8.26	-0.04
Std6	-3.34	4.75	4.75	4.000	3.866	4723434	8.30	8.32	0.02
Std6	-4.84	4.75	4.75	4.000	3.807	4401978	8.30	8.20	-0.10
Std7	-1.53	2.35	2.35	10.000	9.847	1159578	8.30	8.23	-0.07
Std7	-1.02	2.35	2.35	10.000	9.898	1220108	8.30	8.20	-0.10
Std7	2.77	2.35	2.35	10.000	10.277	1311903	8.30	8.31	0.01

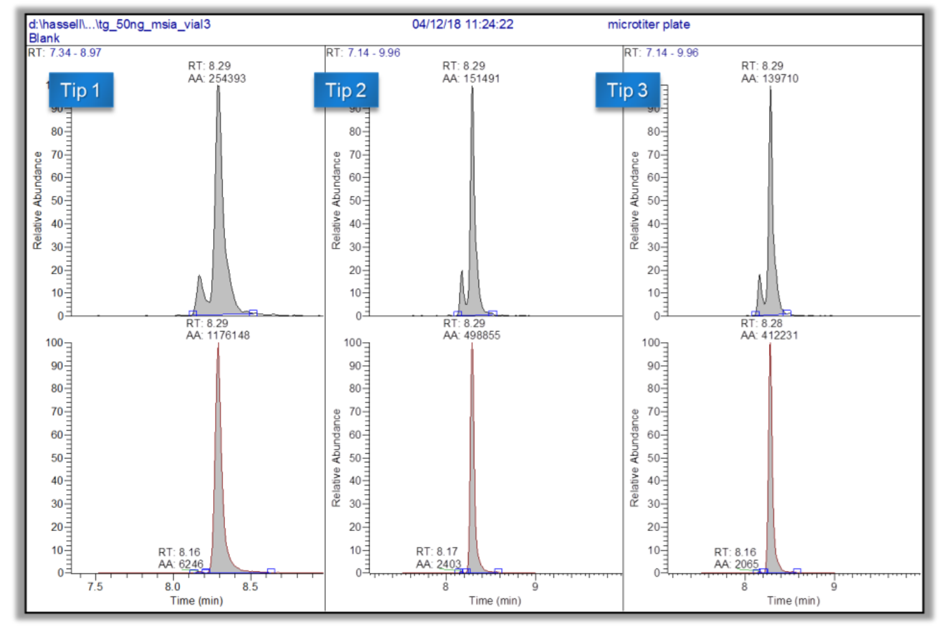
Figure 7. From neat samples that were diluted in 20% ACN and 0.4% TFA, a) is the retention times of the heavy peptide b) is the SRM chromatograms of the calibration curve of the neat samples and c) is the heavy labeled peak area showing RSDs and d) is the LLOQ of the curve demonstrating the points on the peak.



Plasma Calibration Curve

Reproducibility of elution off different MSIA tips were examined and shown in Figure 8, the ratios between heavy to light is consistent but further investigation is underway to improve reproducibility off the tips. The plasma calibration curve was prepared used MSIA tips and spiking in and co-captured FSPDDSAGASA(Heavy L)L(Heavy R). The calibration curve is from 0.1 to 200 ng/mL and is found in Figure 9. The analytical results demonstrated all percent difference and CVs that were under 15% and are shown in Figure 10.

Figure 8. SRM chromatograms of elution off of three different MSIA tips. The interference in the light peptide was not quantitated and is an interference in the plasma samples.



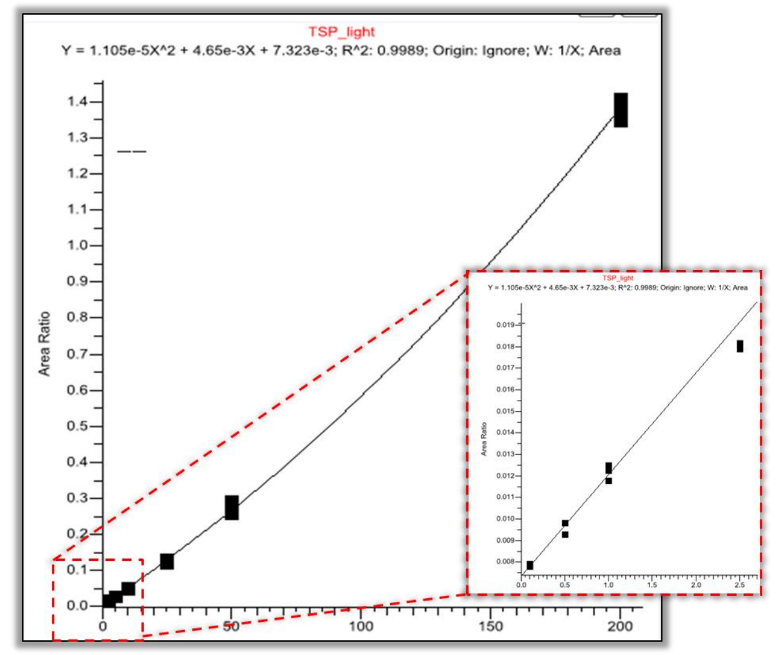
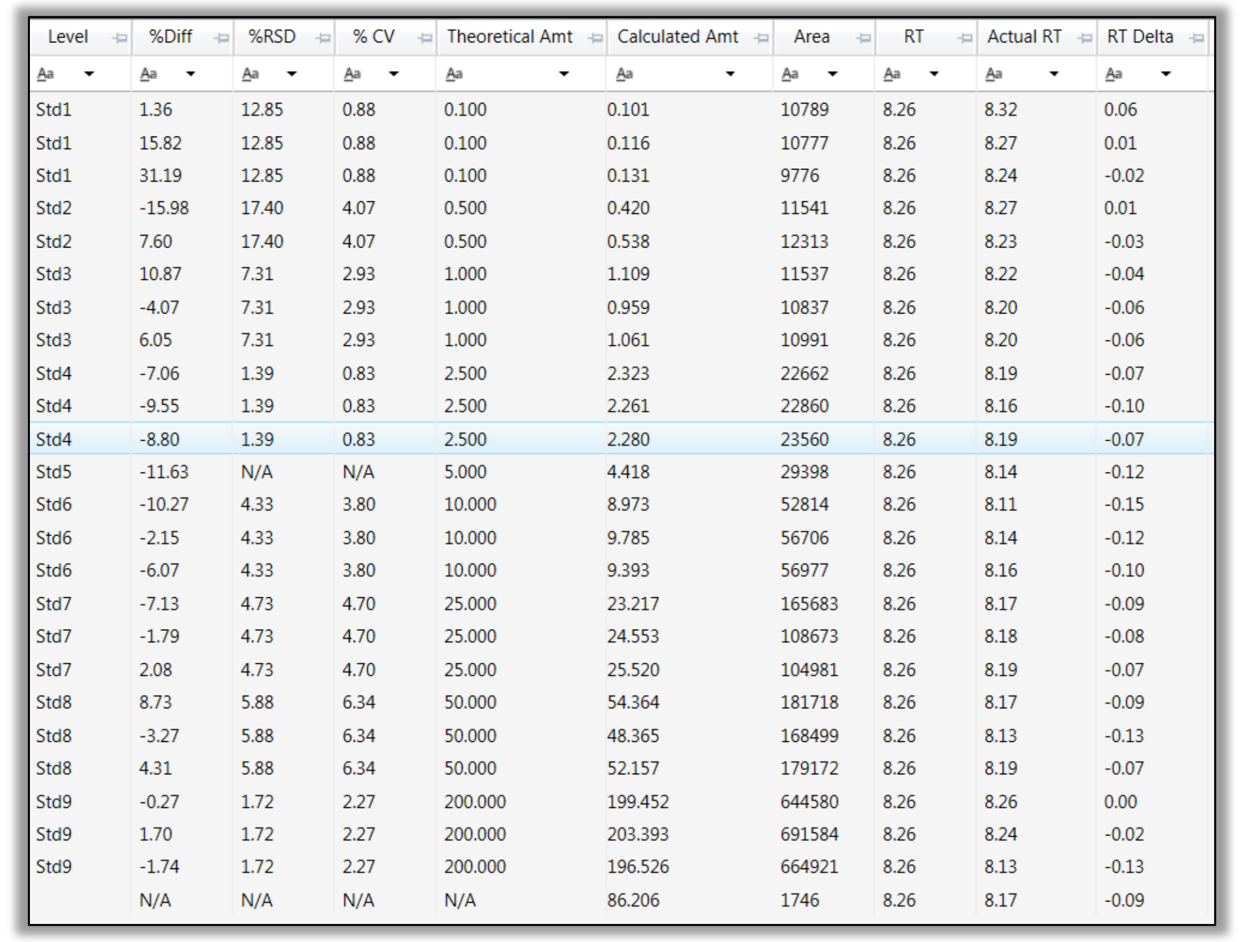


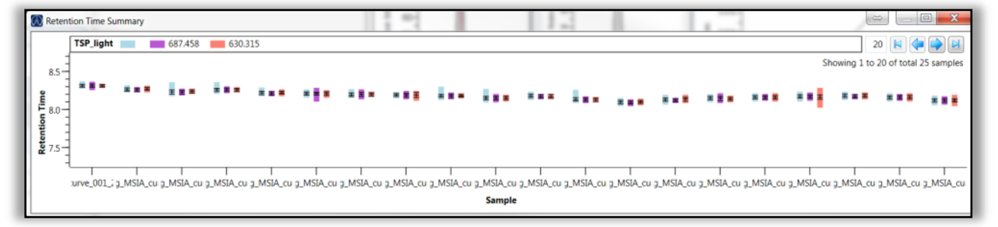
Figure 9. Calibration curve of plasma samples and the inset displays the bottom points of the calibration curve.

Figure 10. Analytical results calculated from peak area in TraceFinder software. Values are based on calibration curve of plasma samples extracted using MSIA.



Level	%Diff	%RSD	% CV	Theoretical Amt	Calculated Amt	Area	RT	Actual RT	RT Delta
Std1	1.36	12.85	0.88	0.100	0.101	10789	8.26	8.32	0.06
Std1	15.82	12.85	0.88	0.100	0.116	10777	8.26	8.27	0.01
Std1	31.19	12.85	0.88	0.100	0.131	9776	8.26	8.24	-0.02
Std2	-15.98	17.40	4.07	0.500	0.420	11541	8.26	8.27	0.01
Std2	7.60	17.40	4.07	0.500	0.538	12313	8.26	8.23	-0.03
Std3	10.87	7.31	2.93	1.000	1.109	11537	8.26	8.22	-0.04
Std3	-4.07	7.31	2.93	1.000	0.959	10837	8.26	8.20	-0.06
Std3	6.05	7.31	2.93	1.000	1.061	10991	8.26	8.20	-0.06
Std4	-7.06	1.39	0.83	2.500	2.323	22662	8.26	8.19	-0.07
Std4	-9.55	1.39	0.83	2.500	2.261	22860	8.26	8.16	-0.10
Std4	-8.80	1.39	0.83	2.500	2.280	23560	8.26	8.19	-0.07
Std5	-11.63	N/A	N/A	5.000	4.418	29398	8.26	8.14	-0.12
Std6	-10.27	4.33	3.80	10.000	8.973	52814	8.26	8.11	-0.15
Std6	-2.15	4.33	3.80	10.000	9.785	56706	8.26	8.14	-0.12
Std6	-6.07	4.33	3.80	10.000	9.393	56977	8.26	8.16	-0.10
Std7	-7.13	4.73	4.70	25.000	23.217	165683	8.26	8.17	-0.09
Std7	-1.79	4.73	4.70	25.000	24.553	108673	8.26	8.18	-0.08
Std7	2.08	4.73	4.70	25.000	25.520	104981	8.26	8.19	-0.07
Std8	8.73	5.88	6.34	50.000	54.364	181718	8.26	8.17	-0.09
Std8	-3.27	5.88	6.34	50.000	48.365	168499	8.26	8.13	-0.13
Std8	4.31	5.88	6.34	50.000	52.157	179172	8.26	8.19	-0.07
Std9	-0.27	1.72	2.27	200.000	199.452	644580	8.26	8.26	0.00
Std9	1.70	1.72	2.27	200.000	203.393	691584	8.26	8.24	-0.02
Std9	-1.74	1.72	2.27	200.000	196.526	664921	8.26	8.13	-0.13
	N/A	N/A	N/A	N/A	86.206	1746	8.26	8.17	-0.09

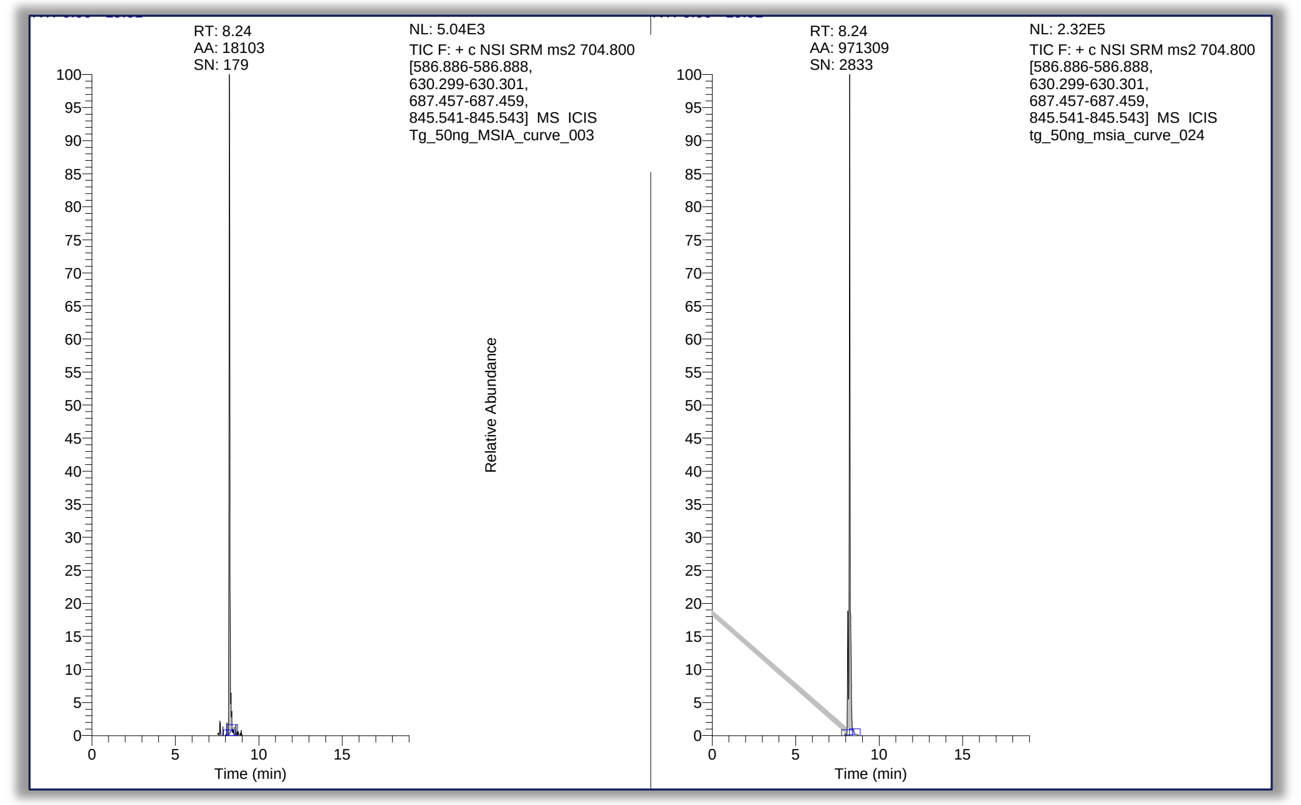
Figure 11. Retention time reproducibility of plasma samples running 2uL/min. The different colors represent different fragment ions for the light FSP peptide.



Low flow chromatography retention time reproducibility is an important matter especially in complex matrices, presented in Figure 11. Each color representation a different transition for the targeted peptide. This chart was made in the TraceFinder software.

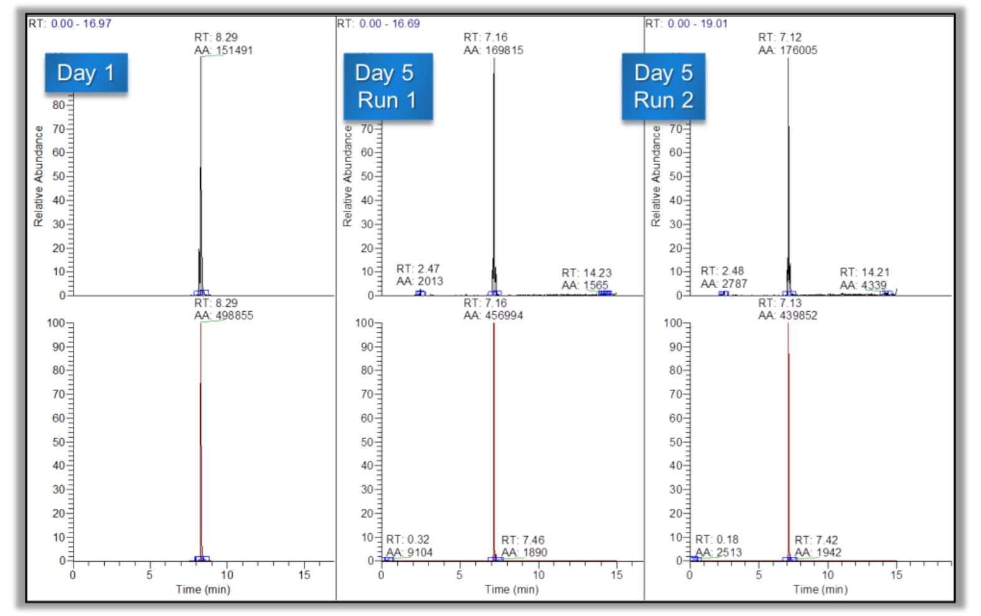
The SRM chromatograms of the lowest and highest point of the curve are shown in Figure 12. Based on the peak area and signal to noise the lowest point could be diluted further than 0.1 ng/mL.

Figure 12. SRM chromatograms of the light peptide LLOQ and ULOQ.



Finally, the stability of the MSIA eluted samples were examined over a five day period, the stability of the SRM chromatograms are found in Figure 13. The samples were kept in the auto sampler at 4ºC.

Figure 13. SRM chromatograms of stability across five days of the vials sitting in the autosampler.



CONCLUSIONS

- This affinity based sample prep allows for no interference from anti-TG autoantibodies that can be found in plasma endogenously.
- This tip based immuno-capture is time saving and has the capability of running in high throughput laboratories.
- Capillary flow LC/MS gives the sensitivity needed to reach below ng/mL and also provides the robust needed for running large batches of samples.

Future Work

- Experiments will be performed to see if better recovery and reproducibility from the MSIA could be possible.
- Obtain clinical samples and do correlation studies to assays being run in these laboratories.

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TRADEMARKS/LICENSING

ThermoFisher
SCIENTIFIC