Rapid and accurate quantitation of thyroglobulin biomarkers using the Echo® MS+ system with ZenoTOF 7600+ system

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Thyroglobulin is a protein biomarker used to monitor thyroid cancer treatment and is also responsible for synthesizing thyroid hormones. In this technical note, we describe a rapid Zeno MRM^{HR} method capable of detecting and quantifying singly and doubly charged thyroglobulin peptides using the Echo® MS+ system with ZenoTOF 7600+ system.

Serum thyroglobulin is a tumor marker used in managing patients diagnosed with differentiated thyroid carcinoma.[1] It also plays a role in synthesizing the thyroid hormones triiodothyronine (T3) and thyroxine (T4).[2] Many LC–MS/MS methods for quantifying thyroglobulin peptides have been established but runtimes take several minutes.[3]

Here, we developed a Zeno MRM^{HR} method to quantify 2 peptides rapidly and accurately without sample preparation. The 2 peptides derived from the thyroglobulin protein, VIFDANAPVAVR and FSPDDSAGASALLR, were serially diluted to create a calibration curve with values ranging from 3.91 ng/mL to 250 ng/mL in a solvent. We achieved the same sensitivity level as polyclonal antibody enrichment but at a significantly faster rate using the Echo® MS+ system with ZenoTOF 7600+ system.[4]

The Bio Tool Kit micro-application in SCIEX OS software was used to select the peptide fragments for the quantitation assay. After peptide fragment selection, the singly and doubly charged peptides were quantified using the Analytics module of SCIEX OS software. Each sample containing the 2 peptides and 2 isotopically labeled peptide analogs was analyzed at a rate of 5 seconds per sample to achieve high-quality Zeno MRM^{HR} data.

Key features of the quantitation of thyroglobulin peptides

- **Low-ng/mL level quantitation of singly and doubly charged peptides:** A lower limit of quantitation (LLOQ) of 3.91 ng/mL was achieved for thyroglobulin peptides VIFDANAPVAVR and FSPDDSAGASALLR
- **Rapid sample acquisition:** Samples were analyzed at a rate of 5 seconds per sample using the wide peak mode for Zeno MRM^{HR} quantitation

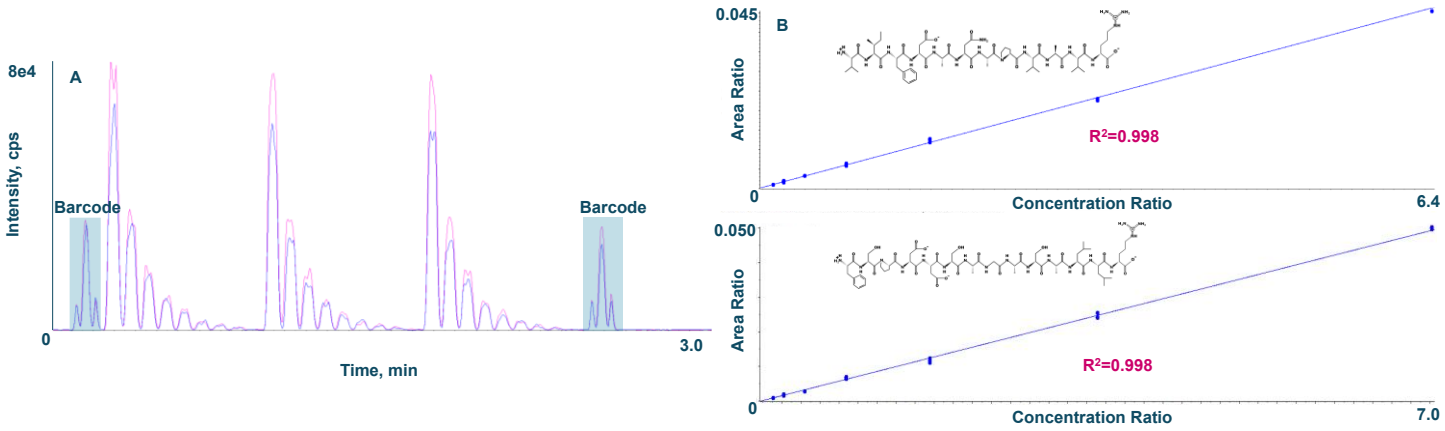


Figure 1. Representative peak ejections and calibration curves from the Zeno MRM^{HR} analysis of the thyroglobulin peptides, VIFDANAPVAVR and FSPDDSAGASALLR. A) Rapid, 5-second-wide peak ejections from the triplicate analysis of VIFDANAPVAVR (blue) and FSPDDSAGASALLR (pink). Start and finish barcodes are shown. B) Calibration curves are shown alongside the structure of the 2 peptides derived from the thyroglobulin protein. VIFDANAPVAVR and FSPDDSAGASALLR are shown at the top and bottom, respectively. Linearity was achieved between 3.91 ng/mL and 250 ng/mL, with an r^2 of 0.998. Each concentration level was run in triplicate.

- **Fast fragment selection using the Bio Tool Kit micro-application:** Fragment ions were rapidly identified, which facilitated product ion selection for the quantitation assay
- **Fast analysis with negligible carryover:** Peptide quantitation analysis was rapidly performed with negligible carryover
- **Streamlined data management:** Data acquisition and processing are integrated into SCIEX OS software

Methods

Sample preparation: Thyroglobulin peptides VIFDANAPVAVR and FSPDDSAGASALLR were diluted to 1000 ng/mL in 35:65 [v/v], water/acetonitrile. A concentration curve was prepared by serial dilution using 35:65 [v/v], water/acetonitrile covering concentrations ranging from 3.91 ng/mL to 250 ng/mL of each peptide. Isotopically labeled analogs of VIFDANAPVAVR and FSPDDSAGASALLR were analyzed as internal standards at 39.3 ng/mL and 35.6 ng/mL, respectively.

Acoustic ejection: The carrier solvent was methanol with 0.1% formic acid and its flow rate was set to 400 µL/min. A total of 70 nL of the sample was ejected over 5 seconds in wide peak mode using the SP fluid class. Each sample was ejected in triplicate.

Mass spectrometry: A Zeno MRM^{HR} method using the "peptides" workflow was developed to quantify the 2 thyroglobulin peptides and their labeled internal standards. The conditions used for this method are outlined in Tables 1–4.

Table 1. Source parameters and values.

Parameter	Value
Polarity	*Positive*
Spray voltage (V)	*5500*
Curtain gas (psi)	*25*
CAD gas (psi)	*11*
Ion source gas 1 (psi)	*90*
Ion source gas 2 (psi)	*75*
Temperature (°C)	*400*

Table 2. TOF MS parameters and values.

Parameter	Value
Scan type	*Zeno MRM^{HR}*
TOF MS start mass (m/z)	*100*
TOF MS stop mass (m/z)	*1000*
Accumulation time (s)	*0.05*
Declustering potential (V)	*40*
Time bins to sum	*8*

Table 3. TOF MS/MS parameters and values.

Parameter	Value
Q1 resolution	*Unit*
Zeno pulsing	*On*
Zeno threshold (cps)	*1000*

Data processing: Data were processed in the Analytics module and the Bio Tool Kit micro-application within SCIEX OS software. The product ions used for quantitation were m/z 213.1620 and m/z 586.8049 for VIFDANAPVAVR and FSPDDSAGASALLR, respectively.

Table 4. Zeno MRM^{HR} parameters and values.

Compound ID	Precursor ion (m/z)	TOF start (m/z)	TOF stop (m/z)	Accumulation time (s)	Declustering potential (V)	Collision energy (V)	Collision energy spread (V)	Time bins to sum
VIFDANAPVAVR	*636.36*	*100*	*1000*	*0.05*	*40*	*32*	*10*	*8*
FSPDDSAGASALLR	*703.85*	*100*	*1000*	*0.05*	*40*	*32*	*10*	*8*
VIFDANAPVAVR IS	*647.39*	*100*	*1000*	*0.05*	*40*	*32*	*10*	*8*
FSPDDSAGASALLR IS	*715.38*	*100*	*1000*	*0.05*	*40*	*32*	*10*	*8*

Rapid analysis

The analysis time for the batch of calibrators ejected in triplicate was 3.2 minutes, including the initial and final mandatory barcodes (Figure 1A).

Linearity was achieved from 3.91 ng/mL to 250 ng/mL with r^2 values of 0.9976 and 0.9978 for the VIFDANAPVAVR and FSPDDSAGASALLR peptides, respectively (Figure 1B). A summary of the quantitative performance can be found in Table 5.

Quantitation

Specific product ions were selected for the quantitation of each peptide. The Zeno MRM^{HR} scan performed a product ion scan from m/z 100–1000 (Figure 2) and the Bio Tool Kit micro-application was employed to facilitate product ion selection for the quantitation assay in SCIEX OS software (Figure 3). Together, this approach provided a fast identification of peptide fragments in the MS/MS spectrum.

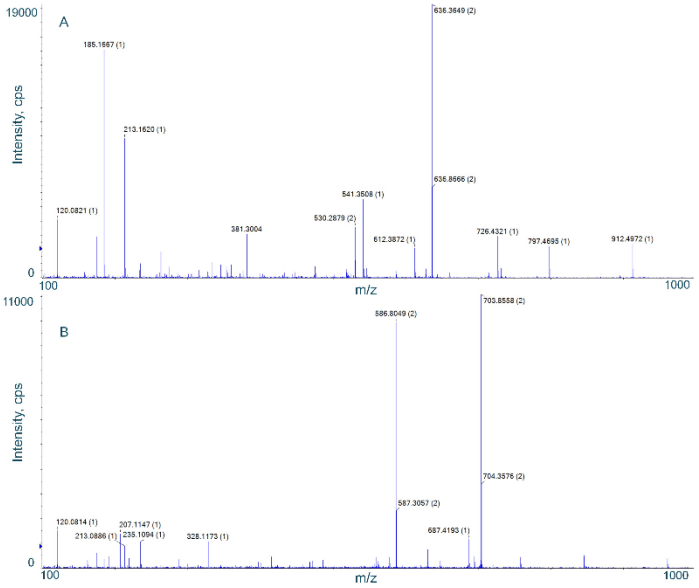
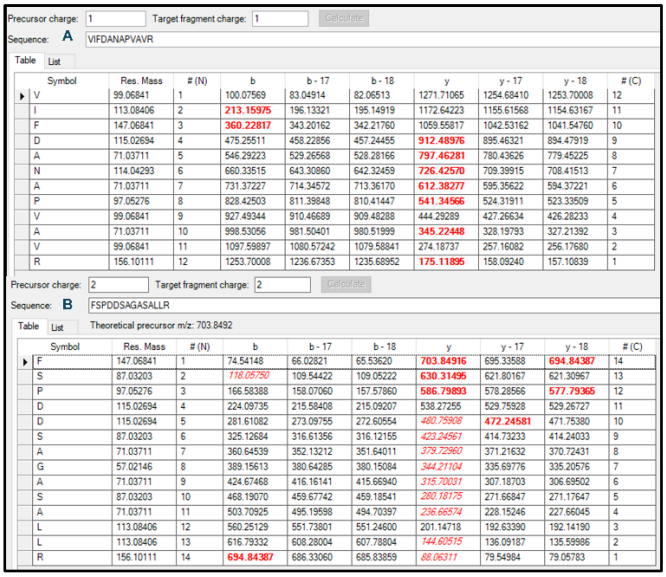


Figure 2. MS/MS spectra obtained from the Zeno MRM^{HR} scan. MS/MS spectra are shown for 250 ng/mL of VIFDANAPVAVR (A) and 250 ng/mL of FSPDDSAGASALLR (B).


Precursor charge: 1 Target fragment charge: 1 Calculate

Sequence: A VIFDANAPVAVR

Table List

Symbol	Res. Mass	# (N)	b	b - 17	b - 18	y	y - 17	y - 18	# (C)
V	99.06841	1	100.07569	83.04914	82.06513	1271.71065	1254.68410	1253.70008	12
I	113.08406	2	**213.15975**	196.13321	195.14919	1172.64223	1155.61568	1154.63167	11
F	147.06841	3	**360.22817**	343.20162	342.21760	1059.55817	1042.53162	1041.54760	10
D	115.02694	4	475.25511	458.22856	457.24455	**912.48976**	895.46321	894.47919	9
A	71.03711	5	546.29223	529.26568	528.28166	**797.46281**	780.43626	779.45225	8
N	114.04293	6	660.33515	643.30860	642.32459	**726.42570**	709.39915	708.41513	7
A	71.03711	7	731.37227	714.34572	713.36170	**612.38277**	595.35622	594.37221	6
P	97.05276	8	828.42503	811.39848	810.41447	**541.34566**	524.31911	523.33509	5
V	99.06841	9	927.49344	910.46689	909.48288	444.29289	427.26634	426.28233	4
A	71.03711	10	998.53056	981.50401	980.51999	**345.22448**	328.19793	327.21392	3
V	99.06841	11	1097.59897	1080.57242	1079.58841	274.18737	257.16082	256.17680	2
R	156.10111	12	1253.70008	1236.67353	1235.68952	**175.11895**	158.09240	157.10839	1

Precursor charge: 2 Target fragment charge: 2 Calculate

Sequence: B FSPDDSAGASALLR

Table List Theoretical precursor m/z: 703.8492

Symbol	Res. Mass	# (N)	b	b - 17	b - 18	y	y - 17	y - 18	# (C)
F	147.06841	1	74.54148	66.02821	65.53620	**703.84916**	695.33588	**694.84387**	14
S	87.03203	2	*118.05750*	109.54422	109.05222	**630.31496**	621.80167	621.30967	13
P	97.05276	3	166.58388	158.07060	157.57860	**586.79893**	578.28566	**577.79365**	12
D	115.02694	4	224.09735	215.58408	215.09207	538.27255	529.75928	529.26727	11
D	115.02694	5	281.61082	273.09755	272.60554	*480.75908*	**472.24581**	471.75380	10
S	87.03203	6	325.12684	316.61356	316.12155	*423.24561*	414.73233	414.24033	9
A	71.03711	7	360.64539	352.13212	351.64011	*379.72960*	371.21632	370.72431	8
G	57.02146	8	389.15613	380.64285	380.15084	*344.21104*	335.69776	335.20576	7
A	71.03711	9	424.67468	416.16141	415.66940	*315.70031*	307.18703	306.69502	6
S	87.03203	10	468.19070	459.67742	459.18541	*280.18175*	271.66847	271.17647	5
A	71.03711	11	503.70925	495.19598	494.70397	*236.68574*	228.15246	227.66045	4
L	113.08406	12	560.25129	551.73801	551.24600	201.14718	192.63390	192.14190	3
L	113.08406	13	616.79332	608.28004	607.78804	*144.60515*	136.09187	135.59986	2
R	156.10111	14	**694.84387**	686.33060	685.83859	*88.06311*	79.54984	79.05783	1

Figure 3. Peptide identification using the Bio Tool Kit micro-application in SCIEX OS software. Results are shown for VIFDANAPVAVR (A) and FSPDDSAGASALLR (B). Bold, red font indicates that the corresponding fragment was identified in the MS/MS spectrum as shown in Figure 2. Red italic numbers indicate a match to a fragment in a different charge state than what has been indicated.[5]

Table 5. Quantitative performance of the thyroglobulin peptide standards, VIFDANAPVAVR (A) and FSPDDSAGASALLR (B).

A

Actual concentration (ng/mL)	Mean (ng/mL)	Percent CV	Average accuracy across replicates (%)
3.91	*3.46*	*17.7*	*88.5*
7.81	*8.53*	*0.65*	*109*
15.6	*15.7*	*6.33*	*100*
31.3	*33.7*	*7.59*	*108*
62.5	*61.9*	*1.74*	*99.1*
125	*116*	*3.45*	*92.5*
250	*257*	*3.66*	*103*

B

Actual concentration (ng/mL)	Mean (ng/mL)	Percent CV	Average accuracy across replicates (%)
3.91	*3.81*	*13.5*	*97.6*
7.81	*8.41*	*10.9*	*108*
15.6	*15.0*	*11.9*	*95.9*
31.3	*33.3*	*4.55*	*107*
62.5	*56.9*	*1.75*	*91.1*
125	*124*	*3.25*	*99.5*
250	*254*	*0.770*	*102*

Conclusions

- Data acquisition at rates as fast as 5 seconds per sample was achieved using Zeno MRM^{HR} mode
- Linearity was achieved from 3.91 to 250 ng/mL for 2 different thyroglobulin peptides
- The Bio Tool Kit micro-application in SCIEX OS software facilitated fast product identification and selection
- No carryover was observed due to constant carrier solvent flow in the Acoustic Ejection Mass Spectrometry method

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