

A quick and robust mass spectrometry-based method for the detection of SARS-CoV-2

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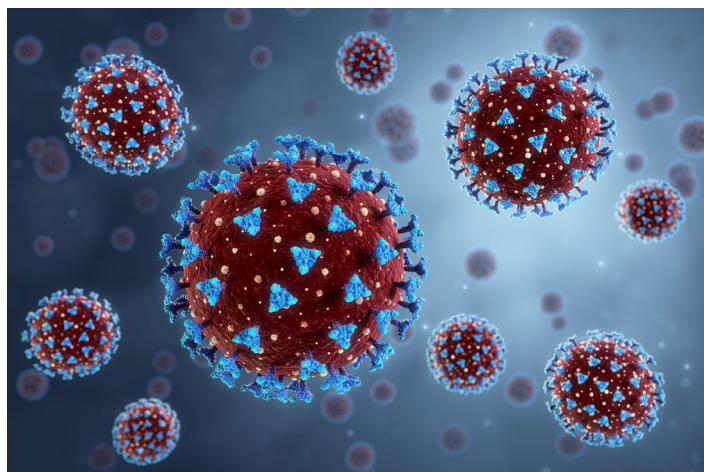
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Goal

To develop a robust, quick, and reliable method using the Thermo Scientific™ TSQ Altis™ MD mass spectrometer to allow the absolute quantitation of peptides from SARS-CoV-2 enzymatic digestions.

Application benefits

- Simple offline sample preparation, with high recovery
- Reliable detection and absolute quantification of peptides from digested SARS-CoV-2 spike and nucleocapsid proteins down to low/sub-femtomole on-column concentrations
- Highlights the viability of two different sample matrices



Abstract

The development of a bottom-up proteolytic workflow has been demonstrated as a reliable means of detection for SARS-CoV-2 peptides that are biomarkers of an active COVID-19 infection.¹ In this technical note, a quick and robust mass spectrometry-based method for the detection of such biomarkers is reported. Six peptides were observed from recombinant protein samples that were spiked either onto nasopharyngeal swabs or into saliva, placed in viral transport media, and enzymatically digested. Sub/low-femtomole on-column detection and quantification limits were observed for each peptide, demonstrating the feasibility of using a TSQ Altis MD mass spectrometer for COVID-19 testing.

Introduction

SARS-CoV-2 is a highly infectious virus that has created a global COVID-19 pandemic, resulting in over 4.5 million reported deaths (as of August 2021).² Attempts to contain the virus have only had limited success, partly due to its spread by asymptomatic carriers.³ This emphasizes the need for widespread and regular testing, not just of the symptomatic, but also of the general population.

Real-time reverse transcription-polymerase chain reaction (RT-PCR) has proven to be the gold standard method in the detection of COVID-19 infections. It relies on the amplification and visualization of the viral RNA that encodes SARS-CoV-2. Although PCR has demonstrated high sensitivity (80%) and specificity (>98%),⁴ a shortage of reagents and trained scientists resulted in a backlog of tests and inconsistent processing times during the height of the SARS-CoV-2 pandemic. This highlights the need to develop orthogonal methods, not just for the current pandemic, but to create a robust and economical system capable of sufficient testing in future pandemics or infectious disease outbreaks.

Testing for COVID-19 infection is not limited to the viral RNA detected by PCR. The SARS-CoV-2 viral particle also contains numerous copies of spike glyco-(P0DTC2), envelope (P0DTC4), membrane (P0DTC5), and nucleocapsid (P0DTC9) proteins, all of which are putative biomarkers of an active COVID-19 infection (Figure 1). Therefore, any technique that can reliably detect the presence of these components could be used to test for infection.

The recent appearance of new variants⁵ has emphasized the importance of COVID-19 tests being able to detect biomarkers that are unlikely to be affected by mutations. As most mutations appear to be in the externally located spike

protein,⁶ targeting the centrally located nucleocapsid protein seems to be a logical route. However, targeting the spike protein could provide a viable way to detect and adjust for variants, with the added advantage of the second biomarker to decrease the possibility of a false positive result.

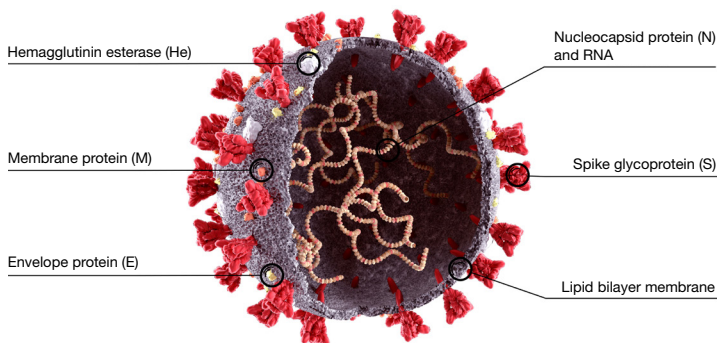


Figure 1. Structure of the SARS-CoV-2 virus. Each SARS-CoV-2 particle consists of four different proteins (S, N, M, E) and the RNA viral genome.

One approach capable of detecting COVID-19 proteins, including variants, is bottom-up mass spectrometry (MS), as shown in Figure 2. Enzymatic digestion of proteins results in the creation of peptides, which may be separated by liquid chromatography (LC). These peptides are easier to identify than intact proteins (due to their size), and numerous peptides can be targeted from each protein, thereby decreasing the chances of a false positive. Consequently, the best course of action was to create a mass spectrometry-based peptide quantification method as an analogous technique to complement existing RT-PCR methods. This not only allows the expansion of COVID-19 testing, but also is capable of accessing information not discernible by PCR, such as the viral load of each positive case.⁷⁻⁸ Moreover, additional experimental parameters could be added to detect specific known variants. This highlights the importance of being able to search for numerous peptides within each experiment.⁹⁻¹¹

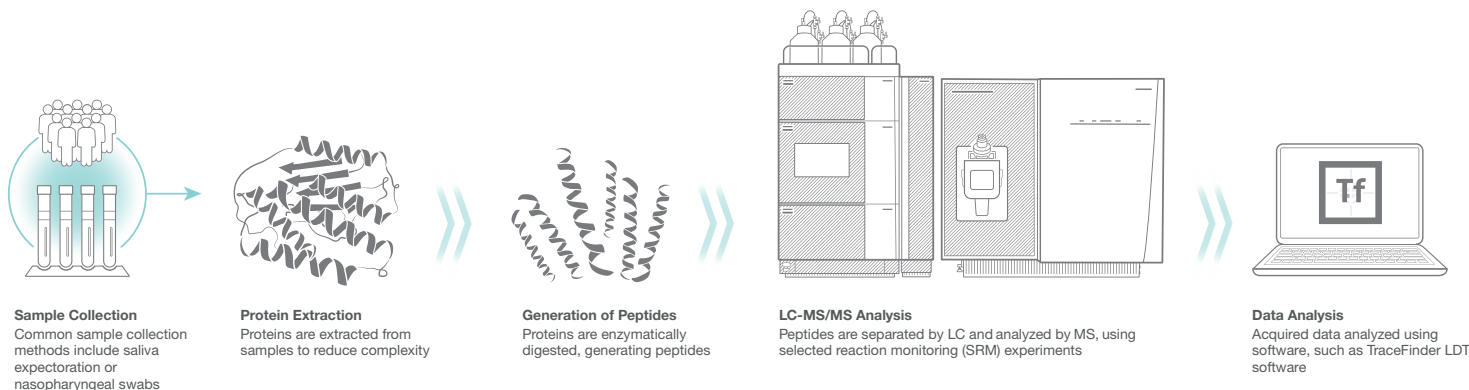


Figure 2. Bottom-up LC/MS workflows allow the detection of numerous peptides from various target proteins

Another key consideration in COVID-19 testing is sample collection. Two of the most common sample collection methods are via nasopharyngeal swabs (NPS) and by expectorating saliva. Although collection by NPS is an invasive approach, it is one of the most well-established sample collection methods and therefore will be considered in this work. In comparison, the collection of saliva is simple and unobtrusive, and the viral load in saliva has been shown to be a better indicator of the severity of each COVID-19 case.¹²⁻¹³ Such an indicator could prove to be vital information in the early allocation of healthcare resources to the required individuals. Many other viable sample collections exist, not limited to nasopharyngeal wash solutions,¹⁴ oropharyngeal swabs,¹⁵ salt-water gargle solutions,¹⁶ and sponges.¹⁷

Methods

Viral Transport Media (VTM) preparation: Preparation followed Centers for Disease Control and Prevention (CDC) guidelines.¹⁸ Ten milliliters of inactivated fetal bovine serum (Gibco) was added to 500 mL of Hanks' Balanced Salt Solution (MP Biomedicals). Gentamicin and Amphotericin B (Gibco) were added for final concentrations of 100 and 0.5 µg/mL.

Sample preparation (nasal fluids): Stable isotope-labeled standards (SIS, Biosyn, Table 1) were added to varying concentrations of equimolar quantities (C1–C10, Table 2) of recombinant spike glycoprotein (P0DTC2, Invitrogen) and nucleocapsid protein (P0DTC9, Invitrogen). The mixes were each spiked into 50 µL of pooled nasal fluids from healthy donors (Innovative Research). Samples were vortexed (30 seconds) and pipetted onto NPSs. The NPSs were then stored in 3 mL of VTM (25 °C, 10 minutes) and vortexed (30 seconds). Protein precipitation was conducted by the addition of 12 mL of ice-cold acetone, incubation (-80 °C, 10 minutes), and centrifugation (4 °C, 4,100 RCF, 10 minutes). The supernatants were removed, and the pellets were dried (room temperature, 30 minutes), before being resuspended in Thermo Scientific™ SMART Digest™ buffer to a final volume of 200 µL. Trypsin digestions were carried out as per the Thermo Scientific™ SMART Digest™ Trypsin Kit's protocol (70 °C, 800 RPM, 90 minutes). Samples were diluted 10-fold in distilled water (dH₂O) with 0.2% formic acid to varying concentrations (0.01–10.0 fmol/µL), with SIS peptides present at a final concentration of 1.0 fmol/µL. Samples were then briefly centrifuged, and the supernatant was transferred to a clean Eppendorf tube.

Table 1. Isotope-labeled peptides used as internal standards. C-terminal residues were R (¹³C₆, ¹⁵N₂) or K (¹³C₆, ¹⁵N₂) modified.

Isotope-labeled peptide sequence	Unlabeled peptide mass (Da)	Isotope-labeled peptide mass (Da)	Protein source—accession number	Residues
GWIFGTTLD <u>S</u> K	1224.6259	1232.6400	P0DTC2	103–113
AYNVTQAF <u>G</u> R	1126.5640	1136.5723	P0DTC9	267–276
ADETQALP <u>Q</u> R	1128.5643	1138.5726	P0DTC9	376–385
KADETQALP <u>Q</u> R	1256.6593	1266.6676	P0DTC9	375–385
DGIIVVATEGALNTP <u>K</u>	1684.8904	1692.9045	P0DTC9	128–143
NPANNAIVLQLPQGGTTL <u>P</u> K	2060.1498	2068.1639	P0DTC9	150–169

Table 2. Preparation of varying concentrations of equimolar P0DTC2 (137 kDa) / P0DTC9 (49.4 kDa) with internal standards. Standard mix consists of 0.2 pmol/µL of each isotope-labeled standard (Table 1).

	P0DTC2 (0.5 µg/µL) P0DTC9 (0.55 µg/µL) Standard Mix (0.2 pmol/µL)		Concentrations of peptides (fmol/µL) after SMART Digest and dilution		
			P0DTC2	P0DTC9	Standards
C1	P0DTC2: 11 µL 10 × Standard Mix: 2 µL	P0DTC9: 3.6 µL dH ₂ O: 3.4 µL	10	10	1.0
C2	C1: 10 µL	Standard Mix: 10 µL	5.0	5.0	1.0
C3	C2: 10 µL	Standard Mix: 10 µL	2.5	2.5	1.0
C4	C3: 8 µL	Standard Mix: 12 µL	1.0	1.0	1.0
C5	C4: 10 µL	Standard Mix: 10 µL	0.50	0.50	1.0
C6	C5: 10 µL	Standard Mix: 10 µL	0.25	0.25	1.0
C7	C6: 8 µL	Standard Mix: 12 µL	0.10	0.10	1.0
C8	C7: 10 µL	Standard Mix: 10 µL	0.050	0.050	1.0
C9	C8: 10 µL	Standard Mix: 10 µL	0.025	0.025	1.0
C10	C9: 8 µL	Standard Mix: 12 µL	0.010	0.010	1.0

Sample preparation (saliva): SIS were added to varying concentrations of equimolar quantities (C1–C10) of recombinant spike glycoprotein (P0DTC2) and nucleocapsid protein (P0DTC9). The mixes were each spiked into 200 μ L pooled saliva samples from healthy donors, which were added to 3 mL of VTM and vortexed (30 seconds). Protein precipitation was conducted by the addition of 12 mL of ice-cold acetone, incubation (-80 $^{\circ}$ C, 10 minutes), and centrifugation (4 $^{\circ}$ C, 4,100 RCF, 10 minutes). The supernatants were removed, and the pellets were dried (room temperature, 30 minutes), before being resuspended in SMART Digest buffer to a final volume of 200 μ L. Trypsin digestions were carried out as per the SMART Digest Trypsin Kit's protocol (70 $^{\circ}$ C, 800 RPM, 90 minutes). Samples were diluted 10-fold in dH₂O with 0.2% formic

acid to varying concentrations (0.01–10.0 fmol/ μ L), with SIS peptides present at a final concentration of 1.0 fmol/ μ L. Samples were then briefly centrifuged, and the supernatant was transferred to a clean Eppendorf tube.

Liquid chromatography: Peptide separation was performed using a Thermo Scientific™ Vanquish™ MD UHPLC system (Figure 3) and a Thermo Scientific™ Hypersil GOLD™ C18 column (2.1 \times 50 mm, 1.9 μ m, P/N 25002-052130). Column temperature was set to 40 $^{\circ}$ C and flow rate to 0.5 mL/min. Mobile phase A: 0.2% formic acid in dH₂O (Thermo Scientific™ Optima™, P/N W8). Mobile phase B: 0.2% formic acid in 80% acetonitrile, 10% isopropanol, and 10% dH₂O. 10 μ L of each sample was injected and chromatographic separation was carried out with a gradient as described in Table 3.



Figure 3. The developed method used a Vanquish MD UHPLC system and TSQ Altis MD mass spectrometer.

Table 3. Liquid chromatography pump flow gradient

Time (min)	% A	% B	Gradient type	Curve
0.0–0.5	99	1	Step	5
0.5–3.0	30	70	Ramp	5
3.0–3.5	1	99	Step	5
3.5–4.0	99	1	Step	5

Mass spectrometry: Analysis was performed using a TSQ Altis MD mass spectrometer, operating in positive ion mode (3.5 kV) with further source conditions shown in Table 4. Final selected reaction monitoring (SRM) transitions of the six peptides chosen to be targeted are shown in Table 5. Two product mass transitions (ADETQALPQR and

AYNVTQAFGR) differed by only one Dalton, which is within the applied Q1 resolution window. However, the clear discrepancy in the mass of each product ion allows clear Q3 differentiation between the two peptides and ensures the selectivity of the method. A steep linear gradient was used to improve sample throughput, while still providing good separation of targeted peptides (Figure 4).

Table 4. Mass spectrometer source settings

Parameter	Setting	Parameter	Setting
Polarity	Positive	Cycle time	0.4 s
Sheath gas	55 Arb	Q1 resolution	1.2 FWHM
Aux gas	15 Arb	Q3 resolution	1.2 FWHM
Sweep gas	3 Arb	Source fragmentation	0
Ion transfer tube temperature	325 °C	Chromatographic peak width	6 s
Vaporizer temperature	350 °C	CID gas	2 mTorr

Table 5. Optimized SRM transitions and collision energies (CEs) for peptides from SARS-CoV-2 protein digests and corresponding SISs

Peptide sequence	Retention time (min)	Peptides			SISs		
		Q1 (Da)	Q3 (Da)	CE (eV)	Q1 (Da)	Q3 (Da)	CE (eV)
KADETQALPQR	1.52	419.558	400.230	12	422.888	410.220	11
			673.315			673.315	
			744.352			744.352	
ADETQALPQR	1.59	564.786	400.230	20	569.781	410.220	20
			513.314			523.304	
			584.352			594.342	
AYNVTQAFGR	1.95	563.786	679.352	20	568.781	689.342	20
			778.421			788.411	
			892.464			902.454	
NPANNAIVLQLPQGTTLPK	2.22	1030.579	841.478	33	1034.581	849.483	33
			1082.620			1090.626	
			1195.705			1203.710	
GWIFGTTLDSK	2.30	612.817	868.441	22	616.819	876.446	21
			664.351			672.356	
			721.373			729.378	
DGIIVWATEGALNTPK	2.32	842.949	1001.526	24	846.952	1009.531	22
			1100.595			1108.600	
			1286.674			1294.679	

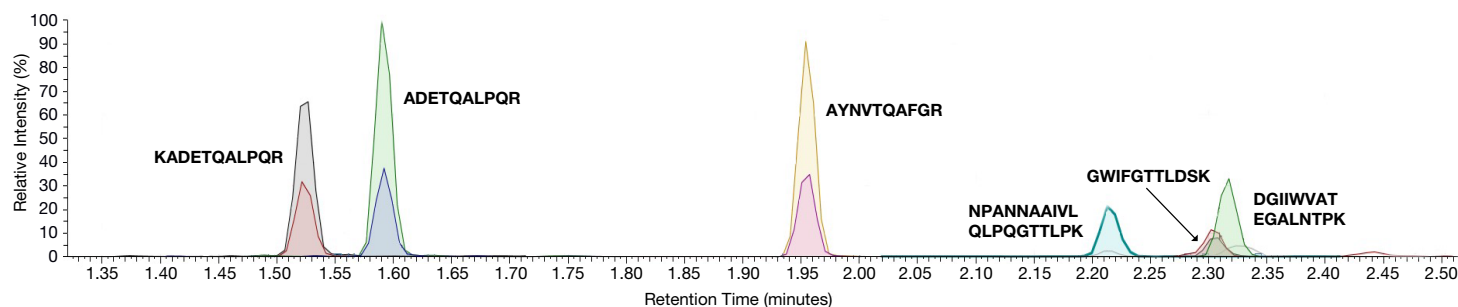


Figure 4. A chromatographic trace demonstrating the separation of the targeted peptides. Each peptide has a less abundant isotope-labeled standard with near identical retention times (± 0.01 minutes).

Data processing: Post-acquisition data analysis was carried out using Thermo Scientific™ TraceFinder™ LDT 1.0 software.

Analysis of spiked nasal fluid and saliva samples in VTM: Samples were analyzed in triplicate using optimized SRM conditions. Limits of detection (LODs) were determined using TraceFinder LDT 1.0 software as the lowest

concentration at which the peptide was detected in triplicate injections. Calibration curves were then fitted with % RSD < 15%, % CV < 15%, and R² > 0.99 to determine the limits of quantitation (LOQs) for each peptide (Figure 5). Details of calibration, retention time, LOD, LOQ, and linearity range are shown for samples in nasal fluids and saliva (Table 6).

Table 6. Determined LODs and LOQs for nasal fluid and saliva samples. Each calibrator was acquired in triplicate, with LODs determined to be the lowest concentration at which triplicates resulted in peptide detection and LOQs to be the lowest concentration where all RSD and CV values remain under 15%.

Peptide sequence	SIS—C-terminal residue modification	Retention time (min)	LOD (fmol on column)	LOQ (fmol on column)	Linearity range (fmol on column)	Weighting	R ²
Samples in nasal fluid							
KADETQALPQR	¹³ C ₆ , ¹⁵ N ₄	1.52	0.25	1.0	1.0–100.0	1 / x	0.9952
ADETQALPQR	¹³ C ₆ , ¹⁵ N ₄	1.59	0.25	0.5	0.5–100.0	1 / x	0.9970
AYNVTQAFGR	¹³ C ₆ , ¹⁵ N ₄	1.96	0.25	0.5	0.5–50.0	1 / x	0.9955
NPANNAIVLQLPQGGTTLPK	¹³ C ₆ , ¹⁵ N ₂	2.22	2.5	5.0	5.0–50.0	Equal	0.9958
GWIFGTTLDISK	¹³ C ₆ , ¹⁵ N ₂	2.31	5.0	10.0	10.0–100.0	Equal	0.9908
DGIWVATEGALNTPK	¹³ C ₆ , ¹⁵ N ₂	2.32	2.5	2.5	2.5–50.0	1 / x	0.9872
Samples in saliva							
KADETQALPQR	¹³ C ₆ , ¹⁵ N ₄	1.52	0.25	1.0	1.0–50.0	1 / x	0.9964
ADETQALPQR	¹³ C ₆ , ¹⁵ N ₄	1.59	0.25	0.5	0.5–100.0	1 / x	0.9975
AYNVTQAFGR	¹³ C ₆ , ¹⁵ N ₄	1.95	0.25	0.5	0.5–25.0	1 / x	0.9905
NPANNAIVLQLPQGGTTLPK	¹³ C ₆ , ¹⁵ N ₂	2.22	2.5	2.5	2.5–50.0	1 / x	0.9939
GWIFGTTLDISK	¹³ C ₆ , ¹⁵ N ₂	2.30	5.0	10.0	10.0–100.0	Equal	0.9885
DGIWVATEGALNTPK	¹³ C ₆ , ¹⁵ N ₂	2.32	2.5	5.0	5.0–50.0	1 / x	0.9951

Discussion

Absolute quantitation of the six targeted peptides was performed by including the corresponding isotope-labeled standard for each peptide to mitigate measurement uncertainty, to confirm the retention times and to correct for any possible matrix effects. LODs and LOQs were determined (Figure 5A for nasal fluid and Figure 5B for saliva) for the six most promising peptides, with all % RSD and % CV below 15%, and R² values greater than 0.99. Five of the chosen peptides were from the nucleocapsid, and one was from the spike protein. This allowed the detection of biomarkers from two different proteins in the COVID-19 testing workflow developed from this method, thereby decreasing the chances of a false positive. Clear chromatographic separation was observed for each of the five non-isomeric nucleocapsid peptides. Minimal variance in retention time was observed for all peptides in each

acquired sample (± 0.01 minutes, Figure 6) and every peptide showed an almost identical retention time (± 0.01 minutes) to the corresponding SIS.

The LOD and LOQ of each targeted peptide was lower than reported by comparable methods using longer run-times, demonstrating the ability of the TSQ Altis MD mass spectrometer to provide a quick and reliable way to test for COVID-19 infections.

The reproducibility, robustness, and reliability of this method provides the basis for the automation of data processing for a faster turnaround time. Demonstrating accurate automated peak picking and integration, as well as TraceFinder software flagging routines, are also essential to instill confidence in analysis without manual interpretation.

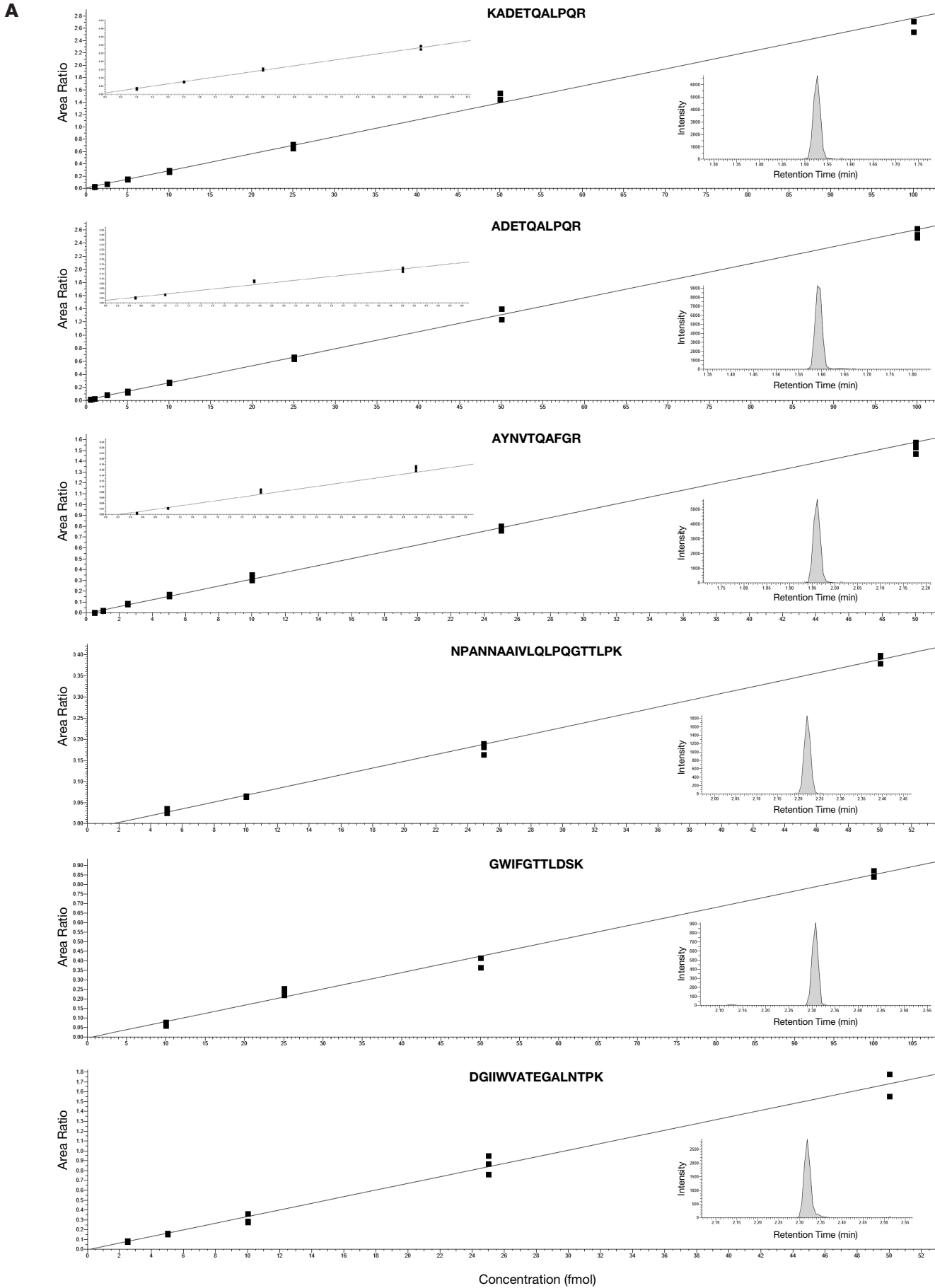


Figure 5A. Sample spectra and calibration curves for each targeted peptide from proteins spiked into nasal fluids

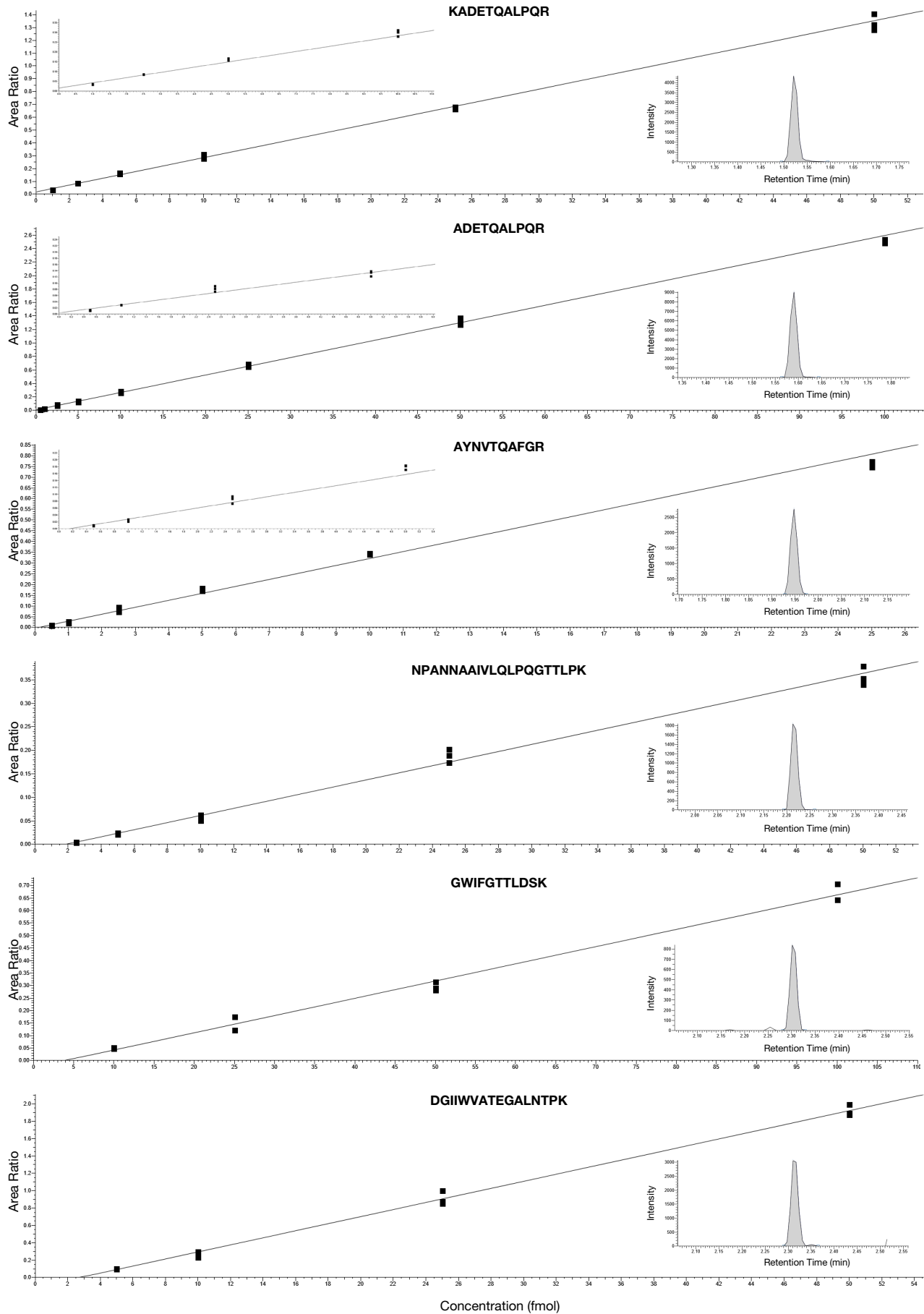
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Figure 5B. Sample spectra and calibration curves for each targeted peptide from proteins spiked into saliva

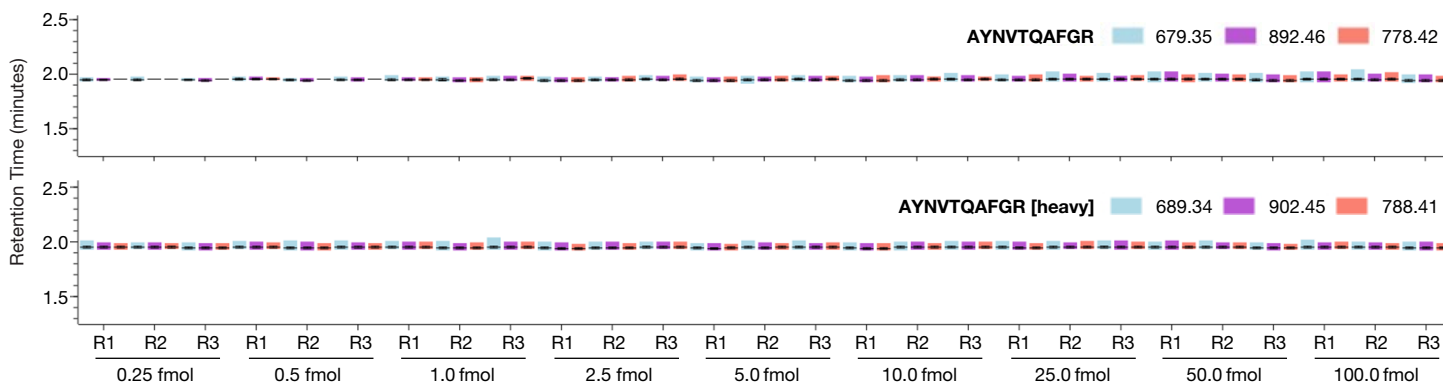


Figure 6. Retention time summary for each product ion from AYNVTQAFGR

Conclusion

A robust, quick (4-minute), and reliable targeted peptide absolute quantification assay has been developed for the detection of SARS-CoV-2 proteins from nasal fluid and saliva. Optimal conditions were determined, and the five best performing nucleocapsid peptides were chosen, along with the best performing spike peptide. Spiked nasal fluid on NPS and saliva samples were stored in VTM to closely mimic a COVID-19 test, and detection limits were determined to be between 0.25 and 5.0 fmol on column, with quantitation limits of between 0.5 and 10.0 fmol on column.

The method presented in this technical note must also be shown to demonstrate close agreement with PCR results acquired from the corresponding samples. This

would validate the effectiveness of the test and also allow conditions to be set for what constitutes a positive and negative result. Various sample preparations should also be evaluated with the goal of improving result accuracy, detection limits, and processing times, while also maintaining a low cost.

Using a mass spectrometry-based bottom-up proteolytic approach also allows for biomarkers from other infections to be detected within the same assay. This could initially involve the extension of the method to a respiratory infection testing panel, with potential targets being influenza, respiratory syncytial virus, or tuberculosis, as such infections may carry symptoms that could be mistaken for COVID-19. Such a method could be rapidly deployed to combat future testing shortages.

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