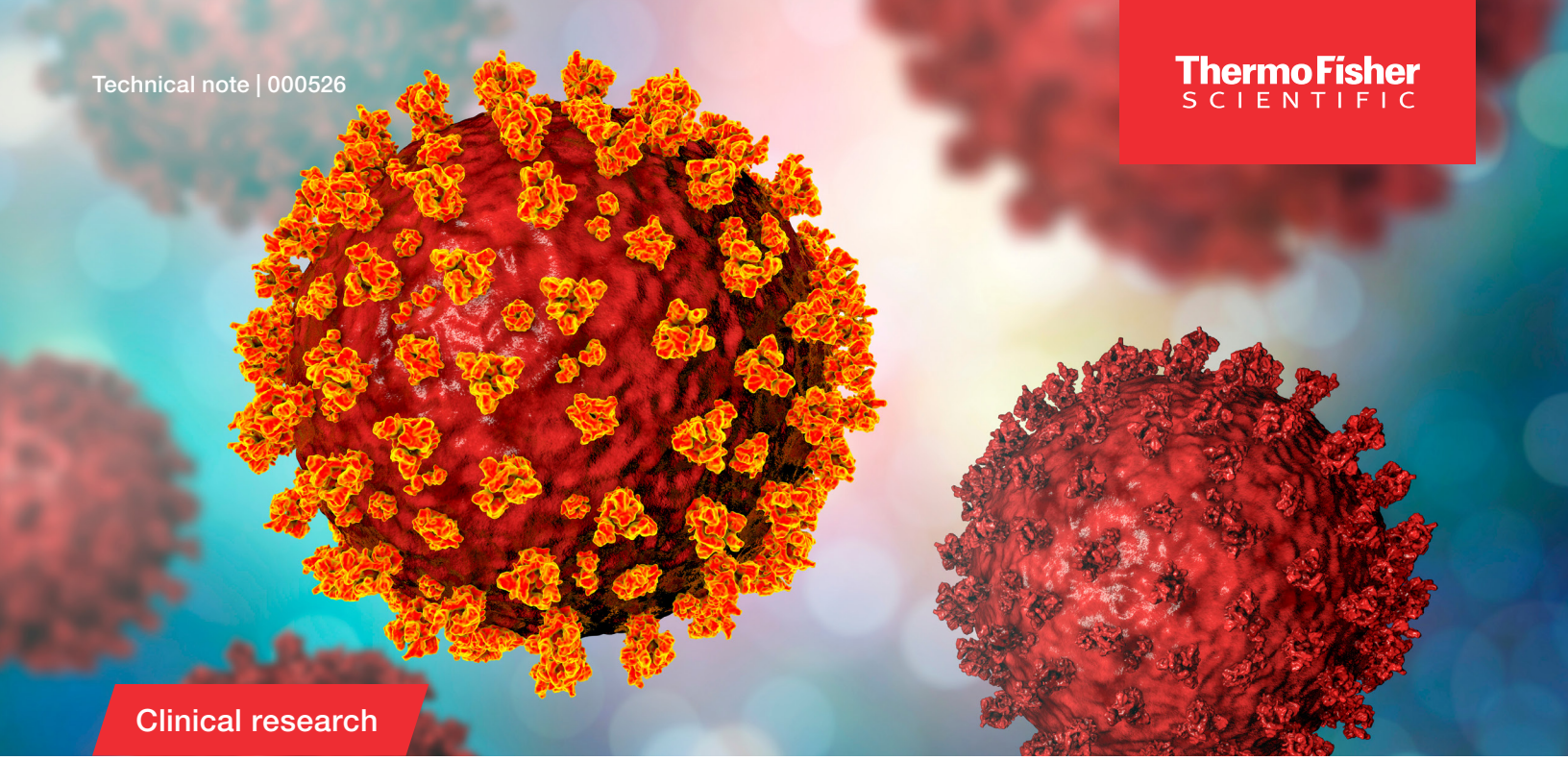
Technical note | 000526



Clinical research

Peptide enrichment of the SARS-CoV-2 nucleocapsid protein and quantitation by LC-MS

Authors

Richard J. Gibson, Stephanie N. Samra, Yvonne E. Song

Thermo Fisher Scientific, San Jose, CA

Keywords

TSQ Altis MD mass spectrometer, TraceFinder LDT, Vanquish MD HPLC system, Hypersil GOLD C18 column, SMART Digest Trypsin Kit, SISCAPA, SARS-CoV-2, COVID-19, LC-MS, reversed-phase HPLC, peptide quantitation, peptide enrichment, antibodies

Goal

To utilize a peptide enrichment method to enhance the detection of peptides from digested SARS-CoV-2-spiked nasal fluid samples

Benefits

- Enrichment of samples allows 2-minute LC-MS run times
- High sample purity without any further sample clean-up
- Reliably detects and absolutely quantifies peptides from digested SARS-CoV-2's nucleocapsid protein (down to low/sub-femtomole on column)
- Lower sample quantity/concentration required for detection and quantitation of SARS-CoV-2

Abstract

A quick and robust mass spectrometry-based method has previously been developed for the detection of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) peptides from recombinant protein samples that were spiked onto nasopharyngeal swabs, placed in viral transport media, precipitated, and enzymatically digested (TN000055). In this technical note, a peptide enrichment technique is evaluated that eliminates the need for any further sample clean-up and allows a 4-fold concentration of samples, while still obtaining sub/low-femtomole on column detection and quantification limits for peptides from the nucleocapsid protein. In addition, increased sample purity allows for shortened 2-minute LC-MS run times.

Introduction

A mass spectrometry-based workflow has previously been developed for the sub/low-femtomole on-column detection and quantitation of peptides from the nucleocapsid protein (PODTC9) of the SARS-CoV-2 virus that causes COVID-19 disease. Here, the previously developed workflow[1] is coupled with peptide enrichment, reducing the background noise by substantially removing non-targeted peptides that would normally be present in a post-trypsin digest sample.

The chosen peptide enrichment technique is known as Stable Isotope Standards and Capture by Anti-Peptide Antibodies (SISCAPA).[2-3] SISCAPA Assay Technologies (Washington, DC) have developed antibodies with specificity to certain epitopes, allowing the enrichment of targeted peptides.[4] A number of commercially available peptide-specific antibodies enrich peptides generated by an enzymatic digestion of the SARS-CoV-2 nucleocapsid protein,[5] four of which correspond to the nucleocapsid peptides detected in the aforementioned technical note. One of the commercially available SISCAPA antibodies is able to enrich both ADETQALPQR and KADETQALPQR, with the N-terminal lysine residue being the result of a missed cleavage.

The ability to enrich specific peptides could be beneficial in that it should result in samples containing the targeted peptides. Ultra-pure samples may shorten liquid chromatography (LC) run times by reducing interfering peaks and increase confidence in results. In addition, peptide enrichment techniques may be placed directly before LC injection (Figure 1), meaning that concentrated eluted samples could be injected into an LC without the need for further sample clean-up. Although this may not drastically improve on-column sensitivity, it could reduce the required viral load of patient acquired samples for COVID-19 confirmation.

Experimental

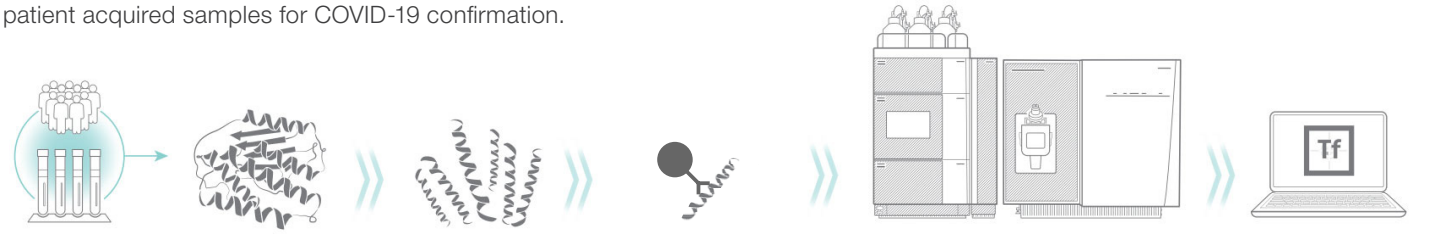
Sample preparation

Viral transport media (VTM) preparation

Preparation followed CDC guidelines.[6] Ten milliliters of inactivated fetal bovine serum (Gibco) were 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, respectively.

Preparation of digested proteins

Varying concentrations of equimolar quantities of recombinant nucleocapsid protein (P0DTC9, Invitrogen) were mixed with stable isotope-labeled standards (SISs, Table 1) and spiked into 50 µL of pooled nasal fluids from healthy donors (Innovative Research). Samples were vortexed (30 seconds) and pipetted onto nasopharyngeal swabs (NPS). 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). Pellets from the previous step were then 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 magnetic bead protocol (70 °C, 800 RPM, 90 minutes). Solutions containing the digested peptides and standards were separated from the trypsin-coated magnetic beads with a Thermo Scientific™ DynaMag™-2 magnet (1 minute). Solutions were then transferred to fresh Eppendorf tubes for SISCAPA enrichment.



Sample Collection
Common sample collection methods include saliva expectoration or nasopharyngeal swabs

Protein Extraction
Proteins are extracted from samples to reduce complexity

Generation of Peptides
Proteins are enzymatically digested, generating peptides

Sample Enrichment by SISCAPA
Peptides are enriched by peptide-specific antibodies, resulting in an ultra-pure sample

LC-MS/MS Analysis
Peptides are separated by LC and analyzed by MS, using selected reaction monitoring (SRM) experiments

Data Analysis
Acquired data analyzed using software, such as TraceFinder software

Figure 1. Coupling SISCAPA enrichment to a bottom-up LC-MS workflow allows the detection of numerous target peptides

Table 1. Stable isotope-labeled peptides used as internal standards. C-terminal residues were R ($^{13}C_6{}^{15}N_4$) or K ($^{13}C_6{}^{15}N_4$) modified.

Isotope-labeled peptide sequence	Mass (Da)	Protein	Residues
AYNVTQAFG**R**	1136.5723	P0DTC9	267–276
ADETQALPQ**R**	1138.5726	P0DTC9	376–385
KADETQALPQ**R**	1266.6676	P0DTC9	375–385
DGIIWVATEGALNTP**K**	1692.9045	P0DTC9	128–143
NPANNAAIVLQLPQGTTLP**K**	2068.1639	P0DTC9	150–169

SISCAPA enrichment

SISCAPA anti-peptide magnetic beads were premixed in equal volumes. 40 µL of the mix were then added to each digested sample and incubated (60 minutes, room temperature, 1,250 RPM). The beads were separated from the solution by a DynaMag-2 magnet (1 minute) and the supernatant was removed (stored at -20 °C). The beads were then resuspended in 150 µL of wash buffer (0.5 mM CHAPS in PBS) and shaken (30 seconds, 1,000 RPM). The peptide-SISCAPA complex was separated from solution by a magnet and the supernatant was discarded. The wash step was then repeated twice more before the beads were resuspended in 50 µL of elution buffer (0.5 mM CHAPS in PBS with 1% formic acid). The mixture was incubated (6 minutes, room temperature, 1,000 RPM) and placed on a magnet (1 minute). The supernatant was then collected, transferred to a fresh Eppendorf tube, and placed on a magnet (1 minute). The supernatant was again transferred to a fresh tube and briefly centrifuged. 25 µL of the solution were taken, with care taken not to disturb the solution at the bottom of the Eppendorf tube, and transferred to an HPLC vial with limited-volume insert.

Chromatography

Peptide separation was performed using a Thermo Scientific™ Vanquish™ MD HPLC system (Figure 2) with a Thermo Scientific™ Hypersil GOLD™ C18 column (2.1 × 50 mm, 1.9 µm, P/N 25002052130). Column temperature was set to 40 °C and flow rate to 0.5 mL/min. Mobile phase A: 0.2% formic acid in H_2O (Thermo Scientific, UHPLC Optima™ grade, P/N W8-1). Mobile phase B: 0.2% formic acid in 80% acetonitrile, 10% isopropanol and 10% H_2O. 10 µL of each sample were injected to the HPLC, and chromatographic separation was carried out with a gradient (Table 2).

Figure 2. The developed method used a Vanquish MD HPLC system and TSQ Altis MD mass spectrometer

Table 2. Liquid chromatography pump flow gradient

Time (min)	%A	%B	Gradient type	Curve
0.0–0.4	99	1	Step	5
0.4–1.2	1	99	Ramp	5
1.2–1.6	1	99	Step	5
1.6–2.0	99	1	Step	5

Mass spectrometry

Detection was performed using a Thermo Scientific™ TSQ Altis™ MD mass spectrometer, operated in positive ion mode (3,500 V, Table 3). Final selected reaction monitoring (SRM) transitions of the five peptides chosen to be targeted are shown in Table 4. A steep linear gradient was used to improve sample throughput, while still providing good separation of targeted peptides (Figure 3).

Table 3. Mass spectrometer source settings

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

Table 4. Optimized SRM transitions and collision energies (CEs) for peptides from SARS-CoV-2 proteins and corresponding SIS

Peptide sequence, origin and residues	Retention time (min)	Q1 (Da)	Q3 (Da)	CE (eV)	Q1 (Da) SIS	Q3 (Da) SIS	CE (eV) SIS
KADETQALPQR, [P0DTC9] residues 375–385	1.17	419.558	**400.230** 673.315 744.352	12	422.888	**410.220** 673.315 744.352	11
ADETQALPQR, [P0DTC9] residues 376–385	1.19	564.786	**400.230** 513.314 584.352	20	569.781	**410.220** 523.304 594.342	20
AYNVTQAFGR, [P0DTC9] residues 267–276	1.25	563.786	**679.352** 778.421 892.464	20	568.781	**689.342** 788.411 902.454	20
NPANNAAIVLQLPQGTTLPK, [P0DTC9] residues 150–169	1.30	1030.579	**841.478** 1082.620 1195.705	33	1034.581	**849.483** 1090.626 1203.710	33
DGIIWVATEGALNTPK, [P0DTC9] residues 128–143	1.33	842.949	**1001.526** 1100.595 1286.670	24	846.952	**1009.531** 1108.600 1294.679	22

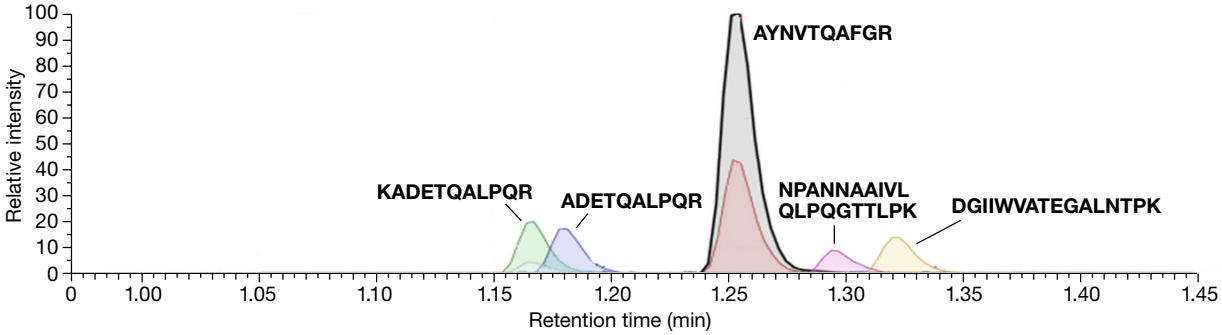


Figure 3. A chromatographic trace demonstrating the separation of the targeted peptides

Data processing

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

Analysis of spiked nasal fluid samples in VTM

Samples were analyzed in triplicate using optimized SRM conditions. Limits of detection (LODs) were determined using TraceFinder LDT 1.0 software. Calibration curves were then fitted with % RSD < 15, % CV < 15, and R^2 > 0.99 to determine the limits of quantitation (LOQs) for each peptide. Details of calibration, retention time, LOD, LOQ, and linearity range are shown in Table 5.

Table 5. Determined LODs and LOQs with each calibrator acquired in triplicate. LODs were determined to be the lowest concentration at which all three replicates resulted in peptide detection and LOQs to be the lowest concentration where all RSD and CV values remain under 15%.

Peptide	Retention time (minutes)	LOD (fmol on column)	LOQ (fmol on column)	Linearity range (fmol on column)	Type	Weighting	Origin	R^2 (% CV / % RSD)	Increase in detection limits in VTM (x-fold)	
									LOD	LOQ
KADETQALPQR	1.17	1.0	2.5	2.5–100	Linear	1 / X	Ignore	0.9925	10	8
ADETQALPQR	1.19	0.50	1.0	1.0–100	Linear	1 / X	Ignore	0.9923	20	20
AYNVTQAFGR	1.25	0.25	0.25	0.25–100	Linear	1 / X	Ignore	0.9947	40	80
NPANNAAIVL QLPQGTTLPK	1.30	10	Not quantifiable	N/A	N/A	N/A	N/A	N/A	20	N/A
DGIIWVATEGALNTPK	1.33	10	Not quantifiable	N/A	N/A	N/A	N/A	N/A	10	N/A

Discussion

Full workflow testing with four antibodies confirmed the antibodies' specificity and ability to work in conjunction. Peptide enrichment of samples not only allowed robust data acquisition but also the shortening of the LC-MS runtime from 4 to 2 minutes. Absolute quantitation of targeted peptides was then performed by including the corresponding SIS for each peptide to mitigate measurement uncertainty, confirm the retention times, and correct for any possible matrix effects. LODs and LOQs were determined for each peptide (Figure 4), with all % RSD and % CV below 15%, and R^2 values greater than 0.99. Clear chromatographic separation was observed for each non-isomeric nucleocapsid peptide with minimal variance in retention times observed (± 0.01 minutes, Figure 5). Almost identical retention times (± 0.01 minutes) were observed between each peptide and corresponding SIS.

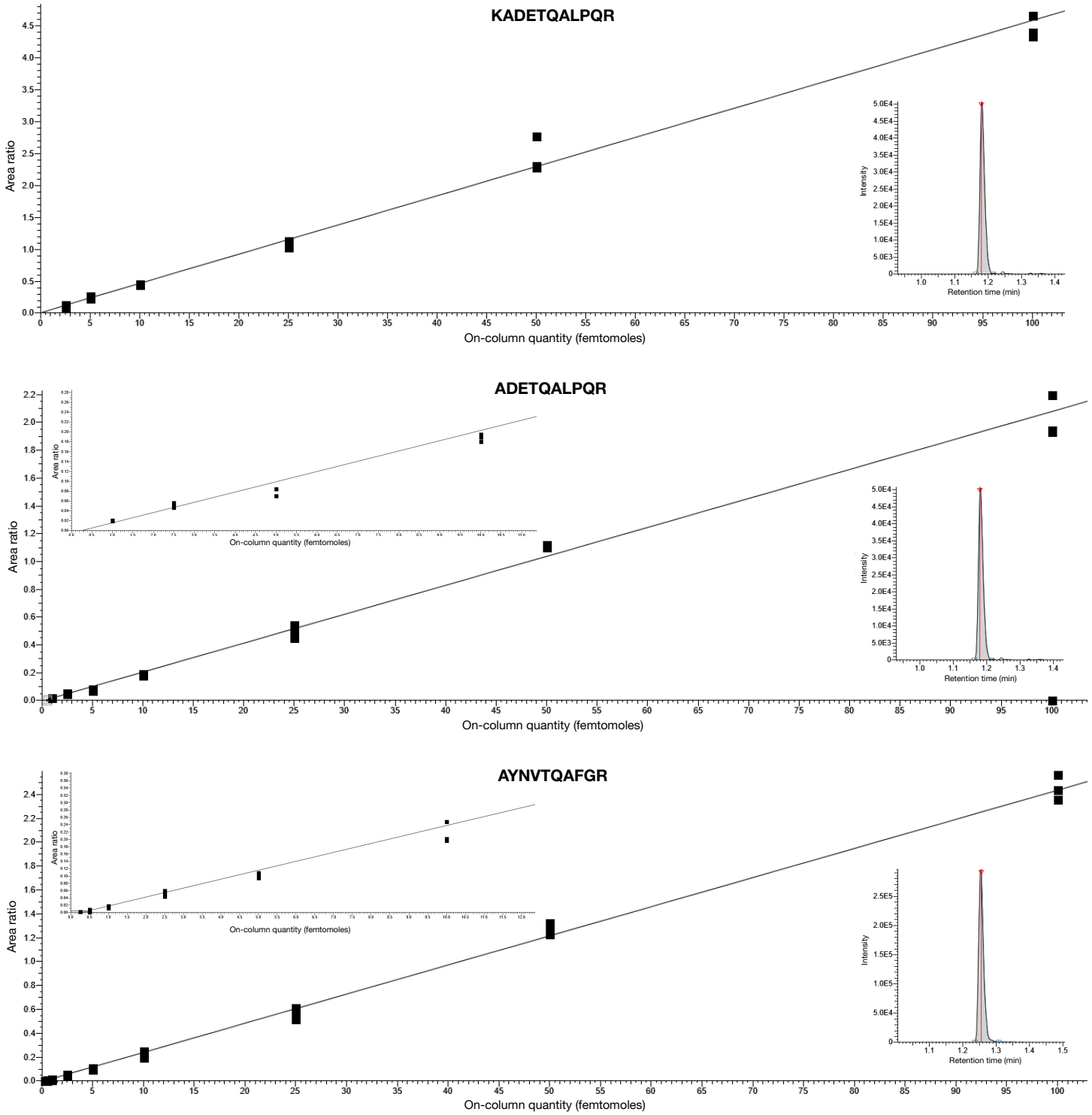


Figure 4. Sample spectra and calibration curves for each quantifiable target peptide

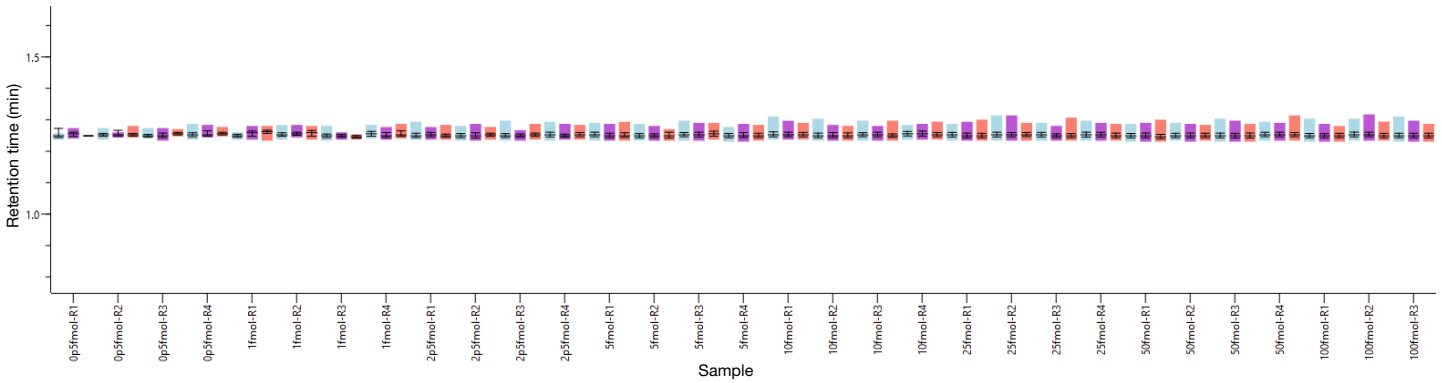


Figure 5. Retention time summary for each product ion from AYNVTQAFGR

Inclusion of the SISCAPA workflow resulted in 4-fold concentration of samples and eliminated the need for any further sample clean-up. This greatly improved the detection limits when the amount of nucleocapsid protein on each NPS is considered. The increased sample purity associated with peptide enrichment also facilitated the reduction of LC-MS run times from 4 to 2 minutes (Figure 6). Including SISCAPA in a laboratory capable of simultaneous and automated sample preparation of 96 samples per available single-channel mass spectrometer would result in approximately a 60% increase in throughput.

Although the cost of a full SISCAPA enrichment workflow may be prohibitively expensive for many labs, it could potentially be used to better examine uncertain results where targeted peptides appear to be present but cannot be completely differentiated from background noise. In such cases, the cost could be further reduced by only including the antibodies that enrich the specific peptides needed to confirm a positive test.

Another alternative workflow could include coupling a SARS-CoV-2 nucleocapsid antibody with Thermo Scientific™ Dynabeads™ (Protein A) magnetic beads, which have been shown to allow efficient protein enrichment. Each of these potential workflows has benefits and drawbacks (Table 6), and which one would be utilized is dependent on the needs of individual laboratories. Although SISCAPA is more expensive, it has the advantage of a quicker LC-MS run time, removal of all non-targeted peptides, and the ability to absolutely quantify peptides through the entire workflow. A major advantage of using Dynabeads protocols is the potential to enrich proteins directly from VTM, allowing the bypassing of precipitation steps. In addition, the removal of non-targeted proteins before trypsin digests could significantly reduce digestion times, creating quicker workflows and maximizing throughput.

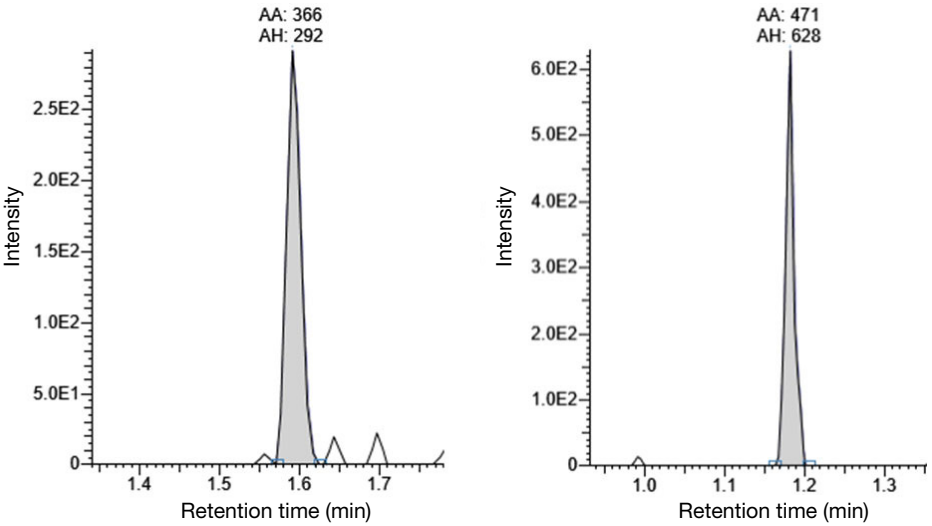


Figure 6. Chromatographic peaks for ADETQALPQR from 1.0 fmol on column samples using minimal sample preparation (left) and SISCAPA (right)

Table 6. Comparison of SARS-CoV-2 detection by LC-MS workflows

	Minimal sample preparation (TN000055)	SISCAPA peptide enrichment (TN000526)	Dynabeads (Protein A) protein enrichment with nucleocapsid antibody
Approximate cost of workflow	$10	$50	$30
Placement of enrichment steps in workflow	N/A	Post-digestion / Pre-injection	Pre-digestion
LC-MS run time	4 minutes	2 minutes	4 minutes
Sample preparation time	2.5 hours	4.25 hours	< 2.25 hours
Absolute quantitation	Yes	Yes	No

Conclusion

A robust, quick, and reliable targeted peptide absolute quantification workflow has been demonstrated to detect SARS-CoV-2 nucleocapsid protein in spiked nasal fluids. The workflow utilizes SISCAPA to enrich five peptides from the nucleocapsid protein. Detection limits were determined to be between 0.25 and 10.0 fmol on column, with quantitation limits of between 0.25 and 2.5 fmol on column for the three quantifiable peptides.

Enrichment of samples by SISCAPA has allowed significant improvement in LODs and LOQs in regards to the concentration of protein in spiked nasal fluids. In addition, the improved purity of the samples allowed quicker LC-MS run times (2 minutes), thereby increasing the throughput of the method.

References

1. Gibson, R. et al. Thermo Scientific Technical Note 000055: A quick and robust mass spectrometry-based method for the detection of SARS-CoV-2, **2021**.
2. Anderson, L. et al. Mass spectrometric quantitation of peptides and proteins using Stable Isotope Standards and Capture by Anti-Peptide Antibodies (SISCAPA). *J Proteome Res*, **2004**, *3*(2), 235–44.
3. Pope, M. et al. Anti-peptide antibody screening: selection of high affinity monoclonal reagents by a refined surface plasmon resonance technique. *J Immunol Methods*, **2009**, *341*(1–2), 86–96.
4. Anderson, L. et al. SISCAPA peptide enrichment on magnetic beads using an in-line bead trap device, *Mol Cell Proteomics*, **2009**, *8*(5), 995–1005.
5. Hober, A. et al. Rapid and sensitive detection of SARS-CoV-2 infection using quantitative peptide enrichment LC-MS/MS analysis, *Elife*, **2021**, 10.
6. Preparation of viral transport medium, Centers for Disease Control and Prevention SOP#: DSR-052-05: https://www.cdc.gov/coronavirus/2019-ncov/downloads/Viral-Transport-Medium.pdf