Targeted assay for quantification of proteins from the SARS-CoV-2 coronavirus

Using the SCIEX Triple Quad™ 5500+ System – QTRAP® Ready

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SARS-CoV-2 is the virus that causes COVID-19 infections in humans and belongs to the family of viruses known as coronaviruses. The coronavirus is an RNA-based virus, and therefore encodes its genome using RNA, similarly to influenza viruses, HIV, and rhinoviruses (common cold). Coronaviruses primarily infect human lung cells through a receptor for an enzyme called angiotensin-converting enzyme 2 (ACE2) through recognition by the spike protein.[1]

Currently, the majority of testing for active infection is carried out using a polymerase chain reaction (PCR)-based test, which can quantify the amount of viral RNA present. However, an active particle does contain a significant amount of protein encapsulating the RNA genome (Figure 1). There are a number of different viral proteins present in a varying number of copies per viral particle. The interesting question being posed by mass spectrometry researchers is whether a protein-based test could be used to detect viral particles at a level of sensitivity that would provide an alternative strategy.

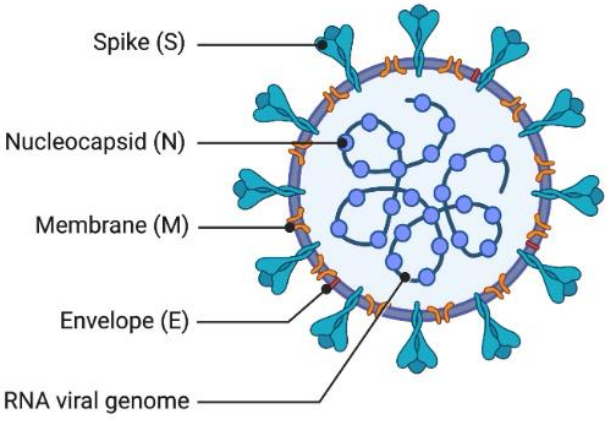

Figure 1. Structure of the SARS-CoV-2 coronavirus.[1] The coronavirus particle is a protective capsid made of nucleocapsid (N) proteins that contain the RNA genome. This is surrounded by a membrane with various viral proteins inserted, the spike (S) protein, the membrane (M) protein and the envelope (E) protein.

Here, the assay development work has been done to build a targeted peptide quantification assay for the detection of two of the viral proteins from SARS-CoV-2. Using recombinant proteins, target peptides were determined, and the MS parameters were optimized. Next, the assay was tested on recombinant proteins spiked into nasopharyngeal swabs from healthy patient samples (stored in UTM, a matrix commonly used for the collection and storage of viral samples). Detection limits were determined for the targeted peptides in this matrix. This information will be useful for researchers who want to explore the use of proteins for viral detection.

Key features of the targeted peptide quantification assay for the detection of coronavirus proteins

- A fast, robust peptide quantification assay has been developed for the detection of two of the viral proteins from the SARS-CoV-2 particle
- MRM transitions and compound dependent parameters have been optimized using the SCIEX 5500+ System
- Detection limits of 0.14–4.4 fmol protein per µL of UTM matrix
- Use of stable, isotope-labeled peptides, matched to the best performing peptides determined here, would provide enhanced quantitative robustness for the assay in the future

Methods

Sample preparation: One µg of spike glycoprotein (P0DTC2, SPIKE_SARS2) and nucleocapsid protein (P0DTC9, NCAP_SARS2) were obtained, digested with Trypsin/LysC as per standard protocols and then lyophilized. These simple digests were used for initial MRM assay development by re-suspending in 0.1% formic acid in 5% acetonitrile.

Dilution series were prepared by spiking the recombinant proteins (0, 0.05, 0.1, 0.5, 1, 5, 10, 50, 100, 500 ng) into 250 µL of two different pooled nasopharyngeal swabs in UTM from healthy donors. Samples were vortexed with ice-cold acetone (1750 µL), then centrifuged at 16,000g for 10 mins at 0 °C. Acetone was removed and pellets were left to dry.

Dried pellets were re-suspended in 250 µL of 500 mM TEAB, then 50 µL of 0.1µg/µL Trypsin/LysC was added. Digestion was done for 4 hours at 37 °C. 50 µL were removed and dried. For analysis, the samples were re-suspended in 50 µL of 0.1% formic acid in 5% acetonitrile and 5 µL was injected per analysis.

Chromatography: Peptide separation was performed using an ExionLC™ System and a Phenomenex Luna Omega Polar C18 column (2.1 x 100 mm, 3 µm). A flow rate of 0.6 mL/min was used for fast analysis times. The gradient is described in Table 1. The column temperature was 40 °C.

Table 1. Gradient for peptide separation.

Time (min)	% A	% B
0	*97*	*3*
5	*60*	*40*
5.1	*10*	*90*
5.6	*10*	*90*
5.7	*97*	*3*
7	*97*	*3*

Mobile phase A – 0.1% formic acid in water
Mobile phase B – 0.1% formic acid in acetonitrile

Mass spectrometry: MRM analysis of the selected peptides was performed using a SCIEX Triple Quad 5500+ System operating in positive ionization mode. Source conditions were an ISV of 5500 V, GS1 of 60, GS2 of 50, TEM 600 °C and CUR of 30. A Scheduled MRM™ Algorithm was used for optimum detection efficiency, using an MRM detection window of 40 sec and a target scan time of 0.5 sec. Optimized MRM transitions and compound dependent parameters are shown in Table 2.

Data processing: MRM transition optimization was performed using Skyline software. Data was processed using SCIEX OS-Q Software.

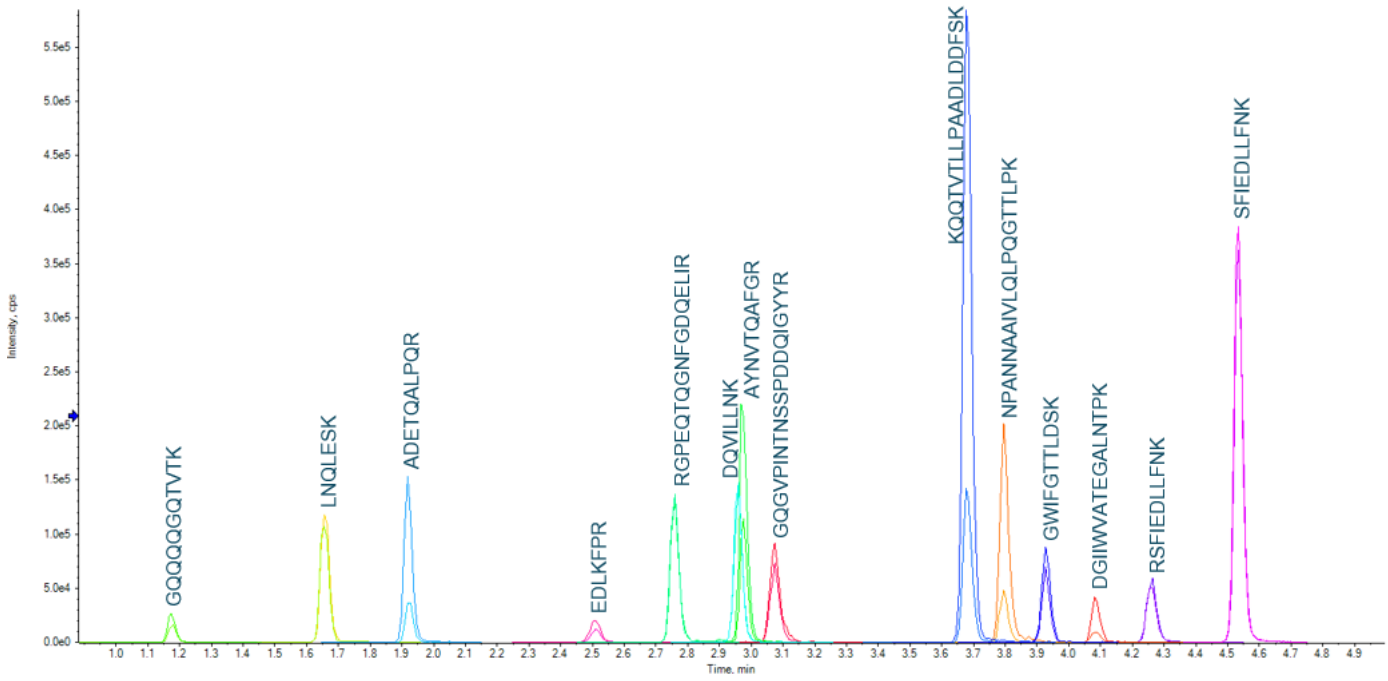

Figure 2. Chromatographic separation of the 14 peptides in the recombinant protein digests. Using high flow rates and fast run times gave sharp peaks for every peptide. Good separation was achieved for all target peptides.

Table 2. Optimized MRM transitions for quantification of key SARS-CoV-2 coronavirus proteins, SPIKE and NCAP.

Q1	Q3	RT (mins)*	ID	DP	CE
612.8	868.4	3.9	sp|P0DTC2|SPIKE_SARS2.GWIFGTTLDSK.+2y8.light	110	32.6
612.8	721.4	3.9	sp|P0DTC2|SPIKE_SARS2.GWIFGTTLDSK.+2y7.light	110	32.6
461.3	504.3	4.3	sp|P0DTC2|SPIKE_SARS2.RSFIEDLLFNK.+3b4.light	65	22.7
461.3	748.4	4.3	sp|P0DTC2|SPIKE_SARS2.RSFIEDLLFNK.+3b6.light	65	26.7
613.3	991.5	4.5	sp|P0DTC2|SPIKE_SARS2.SFIEDLLFNK.+2y8.light	110	28.7
613.3	878.5	4.5	sp|P0DTC2|SPIKE_SARS2.SFIEDLLFNK.+2y7.light	110	32.7
452.7	547.3	2.5	sp|P0DTC9|NCAP_SARS2.EDLKFPR.+2y4.light	50	28.6
452.7	419.2	2.5	sp|P0DTC9|NCAP_SARS2.EDLKFPR.+2y3.light	50	32.6
727.7	920.4	3.1	sp|P0DTC9|NCAP_SARS2.GQGVPINTNSSPDDQIGYYR.+3y16+2.light	80	27.2
727.7	563.8	3.1	sp|P0DTC9|NCAP_SARS2.GQGVPINTNSSPDDQIGYYR.+3y9+2.light	80	31.2
562.3	700.4	4.1	sp|P0DTC9|NCAP_SARS2.DGIIWVATEGALNTPK.+3y7.light	50	23.4
562.3	572.3	4.1	sp|P0DTC9|NCAP_SARS2.DGIIWVATEGALNTPK.+3y5.light	50	23.4
687.4	841.5	3.8	sp|P0DTC9|NCAP_SARS2.NPANNAAIVLQLPQGTTLPK.+3y8.light	50	29.3
687.4	865.5	3.8	sp|P0DTC9|NCAP_SARS2.NPANNAAIVLQLPQGTTLPK.+3b9.light	50	33.3
416.2	718.4	1.7	sp|P0DTC9|NCAP_SARS2.LNQLESK.+2y6.light	50	22.8
416.2	604.3	1.7	sp|P0DTC9|NCAP_SARS2.LNQLESK.+2y5.light	50	22.8
601.8	761.4	1.2	sp|P0DTC9|NCAP_SARS2.GQQQQGQTVTK.+2y7.light	80	32.1
601.8	633.4	1.2	sp|P0DTC9|NCAP_SARS2.GQQQQGQTVTK.+2y6.light	80	36.1
563.8	892.5	3.0	sp|P0DTC9|NCAP_SARS2.AYNVTQAFGR.+2y8.light	65	30.2
563.8	679.4	3.0	sp|P0DTC9|NCAP_SARS2.AYNVTQAFGR.+2y6.light	65	26.2
649.0	830.4	2.7	sp|P0DTC9|NCAP_SARS2.RGPEQTQGNFGDQELIR.+3y7.light	110	31.5
649.0	558.3	2.7	sp|P0DTC9|NCAP_SARS2.RGPEQTQGNFGDQELIR.+3b10+2.light	110	23.5
471.8	699.5	3.0	sp|P0DTC9|NCAP_SARS2.DQVILLNK.+2y6.light	50	25.6
471.8	600.4	3.0	sp|P0DTC9|NCAP_SARS2.DQVILLNK.+2y5.light	50	25.6
564.8	712.4	1.9	sp|P0DTC9|NCAP_SARS2.ADETQALPQR.+2y6.light	95	30.2
564.8	400.2	1.9	sp|P0DTC9|NCAP_SARS2.ADETQALPQR.+2y3.light	95	26.2
664.0	1078.5	3.7	sp|P0DTC9|NCAP_SARS2.KQQTVTLLPAADLDDFSK.+3y10.light	80	28.2
664.0	539.8	3.7	sp|P0DTC9|NCAP_SARS2.KQQTVTLLPAADLDDFSK.+3y10+2.light	80	28.2

* Retention times should be time scheduled based on LC and chromatography used.
EP of 10 and CXP of 20 were used for all MRM transitions.

MRM assay optimization

Using the digested recombinant peptides, MRM transitions were developed to previously selected peptides from the SPIKE and NCAP proteins. MS parameters including declustering potential (DP) and collision energy (CE) were optimized using Skyline software. Final MRM transitions (2 per peptide) were selected from a wider, optimized set of transitions on the basis of signal to noise, intensity and specificity in matrix. Final MRM transitions to the 14 best peptides are shown in Table 2.

A simple linear gradient was used for peptide chromatography, which provided good separation of all targeted peptides (Figure 2).

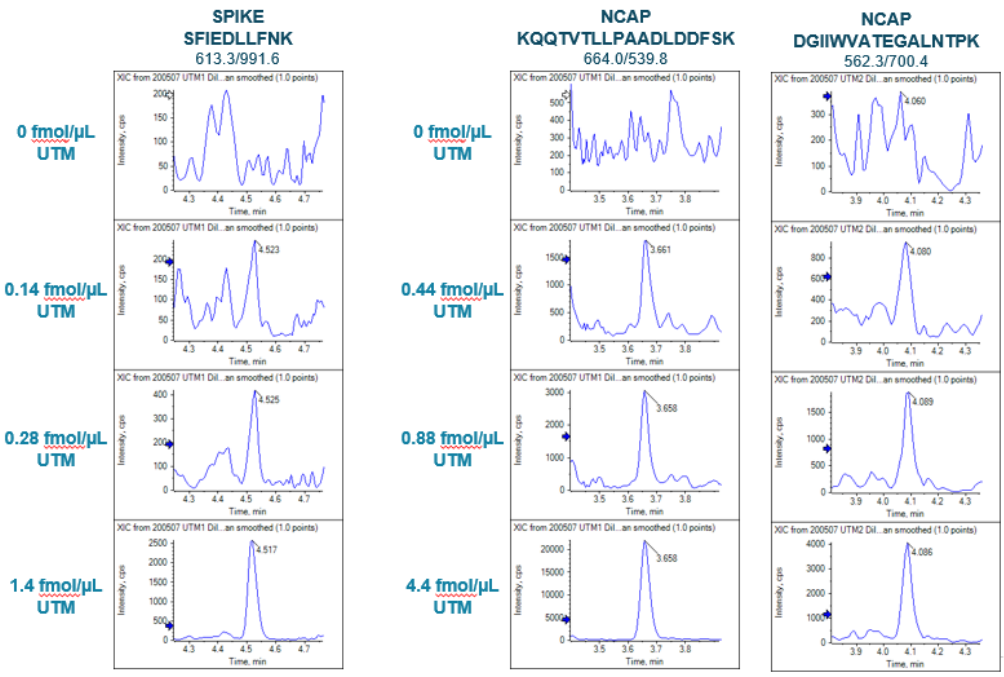

Figure 3. Example data from three peptides. Signals from three of the better performing peptides are shown here for the low end of the dilution series.

Dilution series

Dilution series were prepared by spiking the recombinant proteins into pooled nasopharyngeal swabs in UTM from healthy donors. After addition of the recombinant protein, acetone precipitation was performed, then the protein pellet was re-suspended and digested with Trypsin/LysC. The samples were then analyzed using the optimized MRM assay.

The typical bioanalytical criteria for determination of the lower limits of quantification (LLOQ) are accuracy of ±20% and precision of <20% at LLOQ, and <15% across the calibration curve. A range of LLOQs were found for the 14 peptides and are outlined in Table 3. Examples of the MRM signal observed from three of those peptides in UTM matrix are shown in Figure 3. Example calibration curves are shown in Figure 4.

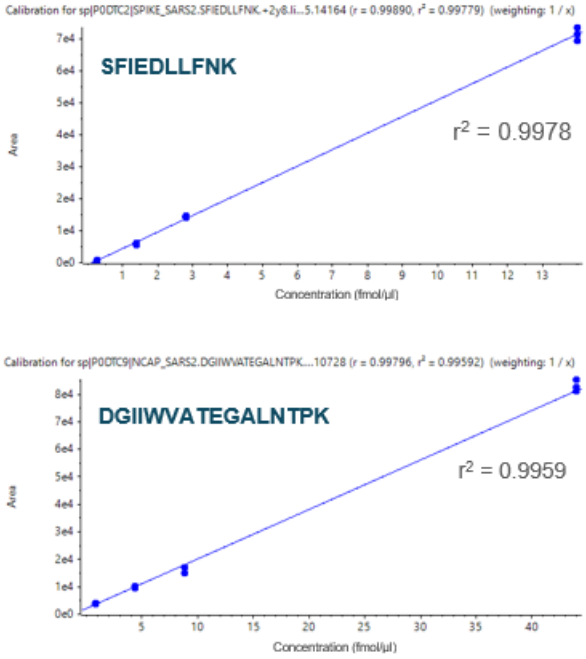

Figure 4. Calibration curve examples. Good linearity was observed across the dilution series for the majority of tested peptides with r^2 values ≥0.99.

Table 3. Lower limits of detection and quantification obtained for the 14 peptides. The LLODs ranged from 0.14–4.4 fmol/µL in pooled nasopharyngeal swabs in UTM. The best responding peptides are in bold.

Peptide	LLOQ in UTM (fmol/µL)	Area %CV for LLOQ	LLOD in UTM (fmol/µL)	LLOD on column (fmol)
SPIKE.GWIFGTTLDSK	*1.4*	*16*	*1.4*	*6*
SPIKE.RSFIEDLLFNK	*0.28*	*16*	*0.28*	*1.2*
SPIKE.SFIEDLLFNK	*0.28*	*19*	*0.14*	*0.6*
NCAP.EDLKFPR	*4.4*	*5.4*	*4.4*	*18*
NCAP.GQGVPINTNSSPDDQIGYYR	*4.4*	*1.3*	*4.4*	*18*
NCAP.DGIIWVATEGALNTPK	*0.88*	*3.9*	*0.44*	*1.8*
NCAP.NPANNAAIVLQLPQGTTLPK	*4.4*	*7.0*	*4.4*	*18*
NCAP.LNQLESK	-	-	*N/A**	-
NCAP.GQQQQGQTVTK	*4.4*	*7.9*	*4.4*	*18*
NCAP.AYNVTQAFGR	*4.4*	*14*	*4.4*	*18*
NCAP.RGPEQTQGNFGDQELIR	*0.88*	*9.8*	*0.88*	*3.7*
NCAP.DQVILLNK	*4.4*	*22*	*4.4*	*18*
NCAP.ADETQALPQR	*4.4*	*17*	*4.4*	*18*
NCAP.KQQTVTLLPAADLDDFSK	*0.44*	*4.0*	*0.44*	*1.8*

**Detection limit obscured by interfering peaks at <4.4 fmol/µL*

Conclusions

Here a targeted peptide quantification assay has been developed and tested on nasopharyngeal swabs in a typical sample collection medium for the detection and quantification of the presence of SARS-CoV-2 coronavirus proteins. The assay has been optimized for sensitivity and for fast, robust chromatography in a high-throughput environment. Next steps are to test this assay on a wider range of matrices to confirm detection limits. In addition, use of stable, isotope-labeled peptides is planned to provide further improvements to the quantitative accuracy of this method.

Note that this assay has not been validated on real test samples and should not be used for patient testing. Much more work would be required to translate this peptide quantification assay into a useful assay for determination of viral particle numbers.

References

1. King J, Kosinski-Collins M, Sundberg E. Coronavirus Structure, Vaccine and Therapy Development.

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