# Development and translation of a method of clinical utility for LC-MS/MS analysis to detect SARS-CoV-2 antigens from ONP swabs and saliva

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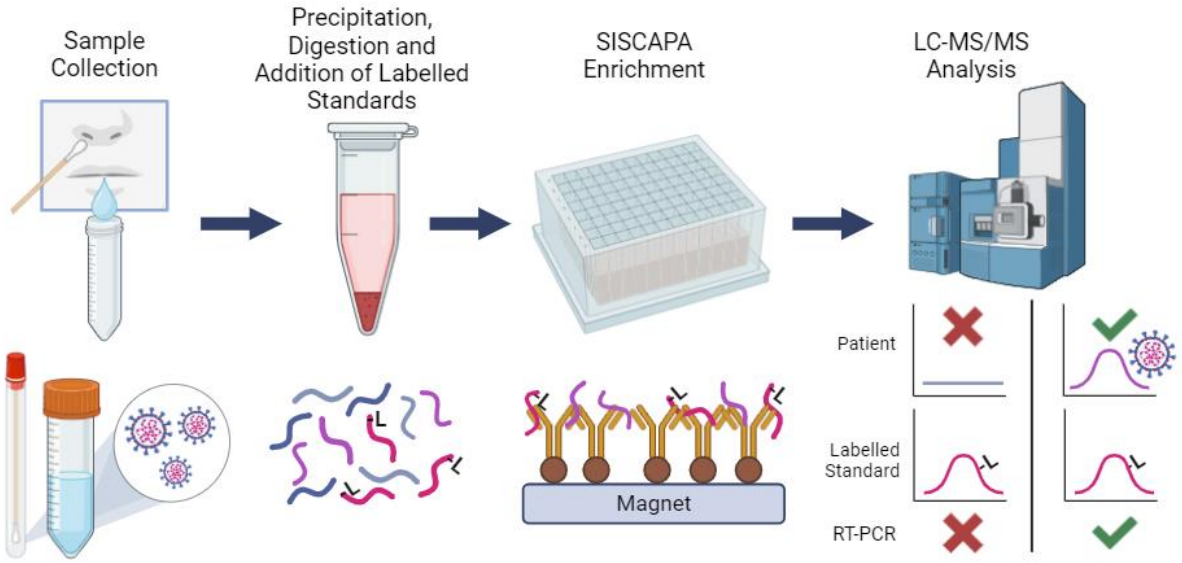
## Graphical Abstract




## Abstract

During the COVID-19 pandemic, development of diagnostic tests was vital to chart the course and to reduce the impact of the infection. Continued testing and surveillance of vaccine escape will continue for years to come which presents an opportunity to integrate such testing into clinical biochemistry laboratories that form part of integrated healthcare testing. Here we describe a protocol for a targeted mass spectrometry based proteomic assay (COVIDCAP) developed to detect SARS-CoV-2 peptides from oro-nasopharyngeal swabs (ONP) and saliva. This uses novel SISCAPA antibodies bound to magnetic beads and subsequent analysis of captured and purified SARS-CoV-2 nucleocapsid (NCAP) peptides. The method involves immediate deactivation of the sample using an ethanolic solution. This simultaneously inactivates the virus and denatures viral proteins at sampling in contrast to the approach for RT-PCR testing, with benefits for the assay as well as for downstream processing. A plate-based preparation of the samples involving acetone precipitation followed by a short tryptic digestion and subsequent immunocapture allows LC-MS detection and quantification of peptides from the NCAP protein, in a 3-minute inject-to-inject assay with an LOD of 20 attomoles from starting sample. For 576 ONP swab samples taken as exemplars here, the sensitivity and specificity of this analysis is shown to be 97.0% and 96.6% respectively.

# Contents

# 1. Introduction

On 11th March 11 2020 the WHO declared, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) a global pandemic.[1,2] As of 13th August 2024, there have been 775,830,200 confirmed cases of COVID-19, including 7,056,108 deaths reported to WHO and a total of 13.64 billion vaccine doses administered.[3] Whilst the pandemic was declared over on 5th May 2023,[2] the virus is still prevalent and continues to be a major cause of infection and death. SARS-CoV-2 gains entry into a host cell by binding to angiotensin converting enzyme II (ACE2) on the cell surface via its spike protein[4,5] which is also seen in SARS-CoV infections that led to the SARS pandemic in 2002-2003.[6,7] The sheer scale of the COVID-19 pandemic, including the number of people infected as well as mutated forms of the virus have given rise to a myriad of clinical presentations in both acute and later stages.[8] As the initial stages of infection mimic the effects of other respiratory viral infections, early precision diagnosis including in routine healthcare settings, is crucial to assist patient prognosis and public health measures.

## 1.1 Study Objectives

The COVID-19 MS Coalition was formed in 2020 during the COVID-19 pandemic to combine and coordinate UK mass spectrometry (MS) labs for the study of SARS-CoV-2, including its diagnosis and prognosis.[9] The protocol described herein is an output from that coalition which built on work described previously.[10–12] In work commissioned by the United Kingdom's Department of Health and Social Care (DHSC) in October 2020, eight academic laboratories in the UK (so called Pilot 1, or P1 labs) were appointed to determine if MS was suitable for detection and quantification of SARS-CoV-2 in oro-nasopharyngeal (ONP) or saliva samples and to develop a method of clinical utility, with applicability for high-throughput (HT) screening. The study was designed to allow rapid translation into routine clinical biochemistry laboratories, with several crucial criteria; i) the sampling should be the same or similar to that already used in ongoing national screening and testing programmes, ii) the samples should be deactivated on entry into the laboratory, iii) sample preparation should be simple and amenable to use in a routine clinical laboratory with minimal additional training to the target user, and iv) the MS platforms should be mid-range bench top systems, capable of running up to 500 tests per day at a sensitivity and specificity matching the gold-standard real time reverse transcriptase polymerase chain reaction (RT-PCR) assay. The DHSC commissioned The Francis Crick Institute (London, UK) to perform RT-PCR tests on duplicate ONP swabs collected

at the same time as those for MS analysis to provide a benchmark quantification cycle (Cq) value for comparison. Additional comparator RT-PCR tests were conducted at Falcon[13] study hospital sites for the at-hospital tests and at Milton Keynes Biocentre (MKBC) for the at-home tests.

## 1.2 Protocol Summary

The COVIDCAP method translated from preliminary research efforts,[10–12] is described herein. Firstly, ONP swab and saliva specimens are collected in a 70:30 ethanol:aqueous ammonium acetate solution, which deactivates SARS-CoV-2 at the point of sampling. Proteins are precipitated with acetone and the resulting pellet resuspended with a mild, MS-compatible protein denaturant. In 96-well plate format, known amounts of stable isotope labelled standard (SILS) heavy peptides for each of the three nucleocapsid (NCAP) peptides of interest are added for relative quantification before digesting the proteins with trypsin. The digestion is quenched after 30 minutes prior to SISCAPA (Stable Isotope Standards and Capture by Anti-Peptide Antibodies) enrichment.[14] There are three SISCAPA antibodies bound to magnetic beads that correspond to the three selected NCAP peptides. The beads are added to the samples and mixed for 1 hour. The 96-well plate is then placed on a patented magnetic array to attract the beads to the side of the well, thus enabling supernatant removal. The supernatant can be stored to allow subsequent analysis of, for example: other SARS-CoV-2 peptides not selected in this method; other viruses that may be present in the sample; quality control indicators; and other markers. The beads retained in the well are washed three times and then the peptides bound are eluted with an acidic buffer. This solution is transferred to a new 96-well plate which is seated on a SISCAPA autosampler magnetic plate to ensure no remaining beads are injected into the LC-MS system. The LC-MS method uses a reversed-phase C18 column with a 1-minute gradient, and a multiple reaction monitoring (MRM) tandem MS method that targets two transitions for each SARS-CoV-2 NCAP peptide of interest. An in-depth description of the protocol development can be found in the Supplementary Information.

## 1.3 Comparison with other methods

The most common methods to detect viruses in patient samples are nucleic acid tests and serology tests. Nucleic acid tests identify viruses by targeting their DNA or RNA, depending on the type of virus. For SARS-CoV-2, thermal inactivation of the virus at 56°C prior to nucleic

acid testing affected nucleic acid integrity of the single-stranded RNA and caused false negatives in RT-PCR tests.[15] However, comparing PCR with other routine methods of detection demonstrated that it is the more sensitive technique during acute infections, hence it is considered the gold-standard approach.[16,17] In any pandemic the supply of reagents to enable population-level testing can become limited; finding alternative testing modalities to diversify and build resilience into the diagnostic supply chain was a motivation for this study. This effort provides a framework for development of MS assays capable of deployment for population wide testing akin to that used for newborn screening in all developed nations.[18]

Serology tests encompass a wide range of assays which can be performed at the point-of-care, akin to the COVIDCAP method herein, but also include point-of-use rapid antigen detection tests.[19] For SARS-COV-2 testing in the UK, lateral flow chromatography immunoassays were widely used, due to their speed, portability, and ease of use although less sensitive than RT-PCR at low viral-loads.[20] The benefit of point-of-care versus point-of-use testing depends on many factors including prevalence of disease, relationship between infectivity and symptoms, and the concomitant public health measures that are required to contain viral spread. In some countries it was recommended that patients with viral symptoms who receive a negative rapid antigen detection result undergo further testing with more sensitive assays.[21,22] As SARS-COV2 remains endemic in the world, and predominantly dangerous to immune compromised individuals, there is a still a need for reliable point-of-use and point-of-care testing, which would also be the case for future pandemic viruses.

## 1.4 Rationale for use of mass spectrometry as an alternative modality for testing

RT-PCR is a so called 'gold-standard' assay for detecting SARS-CoV-2 in clinical specimens, however false-negative and false-positive results do occur.[23] MS instruments display high sensitivity and high specificity, are readily capable of multi-target analysis, and are routinely used for diagnostic and prognostic measurements in clinical laboratories[24–27]. Many hospitals have ready access to mass spectrometers which are routinely used for HT applications such as newborn screening and immunosuppressant drug monitoring.[28,29] In the UK in 2019-2020 over 600k babies were tested for metabolic disorders such as phenylketonuria (PKU) using MS[30] and therefore there was existing capability for MS-based assays to detect SARS-COV-2 infection. Newborn screening with MS targets endogenous disease specific metabolites in blood who abundance alters based on the genetic predisposition, whereas for the presence

of an exogenous virus, the more likely markers of infection are exogenous virus-based compounds. These include RNA from inside the viral capsid or viral proteins. Both analytes have been examined using MS in prior investigations of viral infection; multiplexing PCR with electrospray ionisation (ESI)-MS (PCR-ESI-MS) was used as the first identifier of the pandemic-causing 2009/H1N1 influenza virus.[31] Additionally, Majchrzykiewicz-Koehorst *et al.* investigated the use of MALDI-TOF and LC-MS/MS to rapidly detect multiple influenza A strains via unique peptides in isolates and in mixed samples with other respiratory viruses spiked into clinical specimens.[32]

A proteomic assay targeting the SARS-CoV-2 nucleocapsid protein (NCAP) was deemed the most appropriate, considering NCAP is the most abundant protein across the virus' proteome. In the early stages of the COVID-19 pandemic, Ihling *et al*. demonstrated that the NCAP protein could indeed be found from dilute gargle solutions of COVID-19 patients, using a relatively short LC-MS/MS method, and identified one of the peptides that are used in this study.[33] Singh *et al.* also showed that asymptomatic detection of SARS-CoV-2 is possible using MS, identifying peptides from the spike glycoprotein and replicase polyprotein 1ab.[34] A proof-of-concept proteomic study by Bezstarosti *et al.* showed that SARS-CoV-2 proteins can be detected after a short 30-minute digest, down to the mid-attomole concentration range using parallel reaction monitoring (PRM)-MS.[35] These pioneering early investigations used specialised research lab based instruments more suited to proteomic discovery workflows, and not suited to routine, HT MS. Other MS approaches to SARS-CoV-2 detection included MALDI-TOF MS which was shown to be able to detect NCAP tryptic peptides from nasopharyngeal swabs.[36,37] Cardozo *et al.* translated such proteomic methods from discovery MS platforms to a targeted triple quadrupole assay, and used magnetic bead enrichment followed by turbulent flow chromatography with fast analysis times which meant that more than 500 samples could be analysed in 24 hours.[38] Van Puyelde *et al.* also developed a targeted proteomics MS assay using a bench top triple quadrupole.[11] They demonstrated relatively high sensitivity and hypothesized that RT-PCR Cq (quantification cycle) of viral RNA could correlate to MRM detection of NCAP (assuming 300 NCAP molecules per virion). They postulated that in a volume of 10 µL, a Cq of 25 would correlate to 41 amol of NCAP.[11]

These and other studies showed that MS could be used to detect infection using instruments that are found in routine clinical biochemistry laboratories, although higher sensitivity was

required to measure the viral loads associated with asymptomatic infection (Cq >25). The most instructive for the protocol described herein, with a view to scale up, was the generic and broadly applicable MS assay developed by Dhaenens and colleagues, named CoV-MS.[39] In all cases the need for higher sensitivity was cited, which meant development of robust enrichment strategies to enable detection of peptides from proteins of SARS-CoV-2 against a background of host proteins and other molecules from the initial sampling. A solution to this which was applied to SARS-CoV-2 diagnosis was achieved with SISCAPA technology[14,40] wherein antibodies are raised to target tryptic peptides, and attached to magnetic beads for capture and subsequent removal of non-target material prior to analysis. The requirement for such an enrichment process was identified early in the development of the COVIDCAP method.

## 1.5 Preliminary work leading to the harmonised COVIDCAP method

Three reports (MS1, MS2, and MS3) were provided by each of the P1 labs across the first month of testing to ensure up-to-date and accurate monitoring of protocol development. These reports can be found in the supplementary data set. The aim of MS1 was to report on the workflow optimisation for SARS-CoV-2 antigen detection based on published literature. MS2 would report on the performance of optimised methods and MS3 would report on the hybrid harmonised method.

P1 labs initially determined the sequence coverage of recombinant NCAP donated from the University of Sheffield[41] and spike protein purchased from Peak Proteins using untargeted proteomics with identical samples over a three-week period (Supplementary Data). Subsequently, limit of detection (LOD) and limit of quantification (LOQ) using different MS platforms for recombinant NCAP and spike proteins were established (Supplementary Data). Following these discovery approaches, three peptides from NCAP were chosen for targeted analysis. Initially, the respective vendor software packages were used to analyse raw data files, many of which carry out a background subtraction and apply smoothing algorithms to the data. It was later decided to switch to Skyline[42] for analysis as it is vendor-neutral and open access so raw data files were imported into the platform post-acquisition. Skyline was used to identify peptides and generate optimal collision energy profiles and transitions for these peptides. Elution time profiles were compared for each peptide across the collaboration labs. The NCAP protein is the most abundant protein in the virus and its amino acid sequence

causes the native protein to be intrinsically disordered[43] in aqueous organic solution and thus amenable to rapid proteolysis. Additional work was conducted to test the detection of peptides from respiratory viruses such as Influenza A and B, as well as other human coronaviruses (229E, NL63, OC43, and HKU1). We ensured that there was no interference from these viruses with the peptides chosen for SARS-CoV-2 in this assay (Supplementary Data).

Once LODs and LOQs of the target peptides had been determined for different instruments, the methods were applied to clinical specimens. It was found in this initial investigation that MALDI platforms were not suited to the use case, mainly based on LOD and the unsuitable sample preparation steps involved. The method developed thereafter focussed on triple quadrupole platforms from different vendors.

## 2. Procedure

A comprehensive list of expected materials and equipment required for this method is provided in the supplementary information.

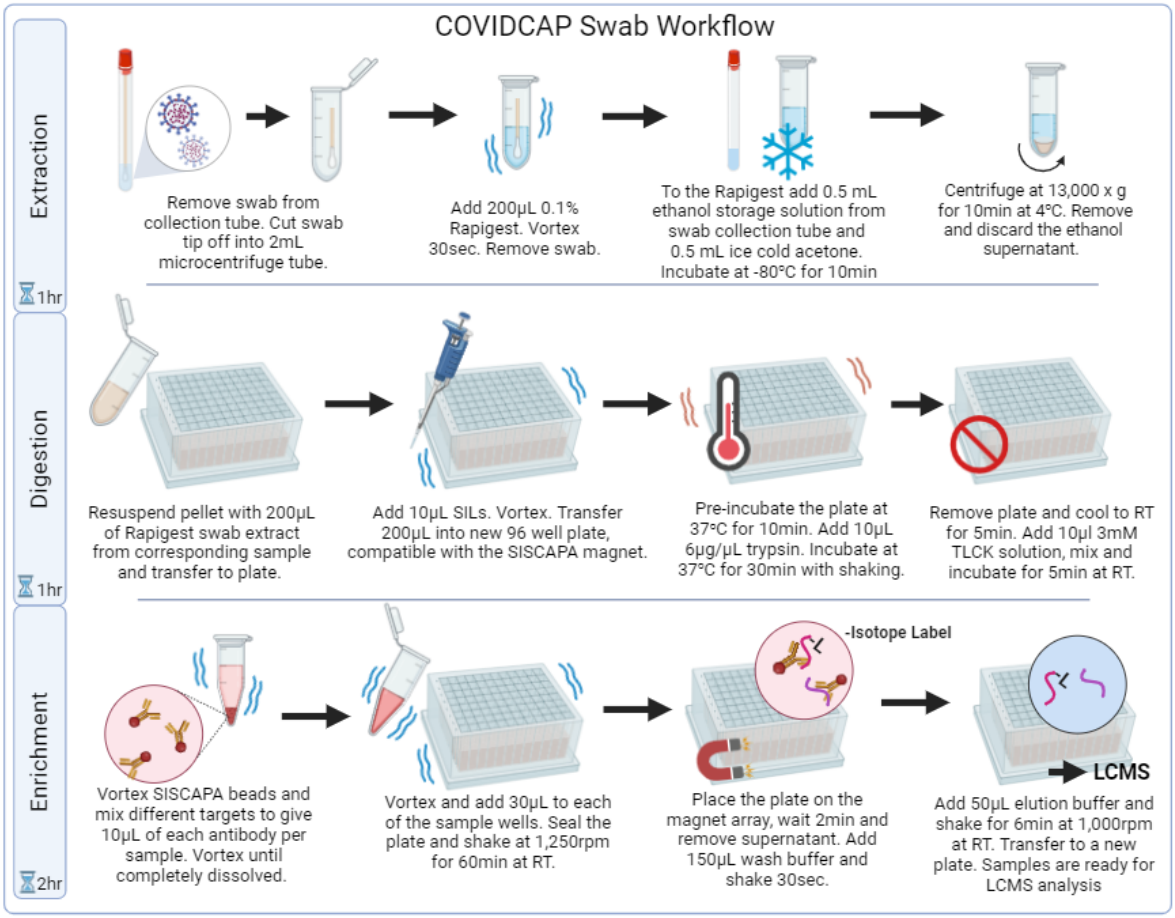


*Figure 1: Overview of the COVIDCAP swab workflow prior to LCMS analysis. The COVIDCAP method involves extraction of viral proteins from both swab and transport solution (protocol steps 1-8), followed by enzymatic digestion (protocol steps 9-11) and SISCAPA enrichment (protocol steps 12-22) of the target NCAP peptides. Proteins are precipitated from ONP swab and transport solution, digested with trypsin and stable-isotope labelled standards (SILS) are added for quantification. The SILS are as follows: using $^{13}C_6{}^{15}N_4$ Arg; ADE* modified using $^{13}C_6{}^{15}N_4$ Arg; and DGI* modified using $^{13}C_6{}^{15}N_2$ Lys. SISCAPA magnetic beads are added and bind to selected NCAP target peptides. A magnetic plate is used to enrich for these peptides and finally the peptides are eluted ready for LC-MS analysis. Figure created using BioRender.*

This method follows pre-analytical quality control and unboxing of samples (See Supplementary Information and Video 1 https://flic.kr/p/2q9Tx7t ). The procedure consists of solubilising and digesting the proteins followed by enrichment of the marker Covid-19 peptides (Figure 1)

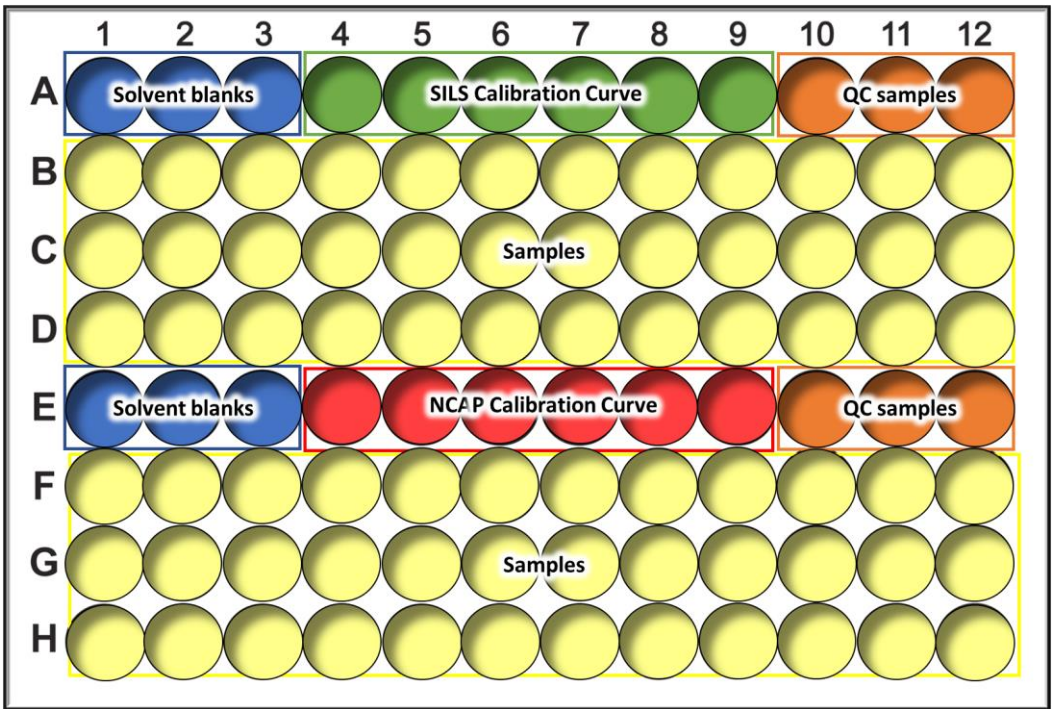



*Figure 2: Suggested plate layout for a 96 well plate includes blanks, calibration curves and QC samples.*

*This layout ensures that 72 samples can be run on one plate, with solvent blanks to wash the LC column, QC samples to assess any losses that may occur throughout the MS run, and calibration curves for both Stable Isotope Labelled Standards (SILS) and NCAP preparations. The calibration curves can be removed once LOD and LOQ have been determined for the LCMS system, leaving space for 84 samples. Sample and QC injection order can be randomised to reduce systematic bias.*

## 2.1 Swab extraction (TIMING: 1 hr)

Note: there were many types of swabs in use for SARS-CoV-2 testing, see Supplementary Information S6 Figure S1.

Note: for saliva extraction and downstream preparation method please see Supplementary Information S11 and Video 2 https://flic.kr/p/2q9RdcV

1. Add 0.5 mL acetone to the required number of 2 mL microcentrifuge tubes and place in the freezer at -80°C for 30 mins.
2. Remove the swab from the collection tube and cut the tip off into an empty 2 mL microcentrifuge tube or 96-well plate (depending on centrifuge rotor available). See Video 3, https://flic.kr/p/2q9LDLg
3. Add 200 µL of 0.1% Rapigest digestion solution to the swab and vortex for 30 sec. Pulse briefly in a microcentrifuge to bring down liquid/foam and then remove the swab from the tube and discard. The swab extract can be stored in the tube at RT until needed in step 7, i.e. all the extracts are completed.
4. Remove the ethanol storage solution from the swab collection tube (typically 0.5-0.7 ml) and transfer to the ice-cold acetone tubes in the -80°C freezer and incubate for 10 min.
5. Centrifuge at 13,000 x g for 10 min at 4°C.
6. **CRITICAL** Remove and discard the supernatant, leaving the pellet.

   **THIS MAY BE A BREAK POINT at which the pellet(s) could be stored at -80 °C**
7. Resuspend the pellets with 200 µL of Rapigest swab extract from the corresponding sample from step 3 and transfer to a LoBind Eppendorf 1 mL 96 well plate (leave empty wells for the synthetic peptide calibrators, NCAP calibrators, SIL blank and elution buffer blank).

8. Add 10 µL of the internal standard SIL solution and briefly vortex to mix, and then transfer 200 µL of the solution (avoiding generating bubbles) into a new 96 well plate, compatible with the SISCAPA magnet.

**THIS MAY BE A BREAK POINT at which the plate(s) could be stored at -80 °C**

## 2.2 Digestion (TIMING: 1 hr)

Note: for digestion method see Video 4 https://flic.kr/p/2q9RdbC

9. Pre-incubate the 96 well plate at 37°C for 10 min.
10. **CRITICAL** Add 10 µL of 6 µg/µL trypsin solution to each of the wells to start the digestion then incubate the plate at 37°C for 30 min with shaking.
11. Remove the plate and allow to cool at room temperature for 5 min. Add 10 µL of 3 mM TLCK (Tosyl-L-lysyl-chloromethane hydrochloride) solution, mix and incubate for 5 min at RT, then proceed to SISCAPA enrichment.

**THIS MAY BE A BREAK POINT at which the plate(s) could be stored at -80 °C**

## 2.3 SISCAPA Enrichment (TIMING: 3 hrs)

Note: for SISCAPA enrichment method see Video 5 https://flic.kr/p/2q9Rddm

12. **CRITICAL** Vortex SISCAPA bead solutions and then mix an equal proportion of the three SISCAPA bead solutions to give 10 µL of each antibody per sample (total volume based on the number of samples to be run) and vortex until completely dissolved.
13. **CRITICAL** Vortex and add 30 µL of this mixture to each of the wells with sample. Agitate mixture before aliquoting into each well. **TROUBLESHOOTING**
14. Seal the plate with a removable plate seal and shake the samples for 60 mins at room temperature at 1,250 rpm.

**Note:** The shaking must be vigorous enough to not let the beads settle at the bottom of the well.

15. Remove the plate seal and place the samples on the magnet array to allow the beads to migrate to the side of the well. This may take 1-2 mins and should look like the plate shown in Figure 3. If not, wait for 1 more minute.
16. **CRITICAL** Remove the supernatant, being careful to not touch the beads.

Note: This supernatant can be retained as it can be used for measuring other COVID virus peptides, 'winter viruses' proteins, and also the cellular markers for quality control of the sample (amylase and BPIFB1 protein) or other markers.

17. Remove from magnet and add 150 µL SISCAPA wash buffer to the beads and shake for 30 seconds at 1,250 rpm.
18. Place the samples on the magnetic array and allow the beads to migrate to the side of the tube/well.
19. **CRITICAL** Pipette off the SISCAPA wash buffer and discard, then repeat wash and removal for a total of three times, taking no more than 15 mins.

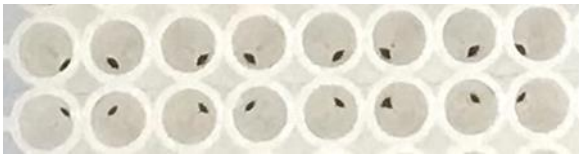


*Figure 3: 96-well plate wells with magnetic beads attracted to SISCAPA magnet underneath*

*The main utility of the SISCAPA antibody-bound beads is that target peptides can be enriched easily using the patented magnetic beads and magnetic plate. The beads, seen as dark spots in the figure, are attracted to the sides of compatible 96 well plates, leaving the rest of the well clear for washing and pipetting steps.*

20. Add 50 µL SISCAPA elution buffer to the beads and shake for 6 minutes at 1,000 rpm at room temperature.
21. Place the samples on the magnetic array and allow the beads to migrate to the side of the tube/well. This should take approximately 1 minute, if not wait for 1 more minute.
22. **CRITICAL** Take 50µL of supernatant containing the eluted peptides into a new plate compatible with the LCMS autosampler. Seal the plate with an LC-MS compatible plate seal (e.g. silicone seal with slits).

    **The sample plate is ready for tandem MS analysis.**

**THIS MAY BE A BREAK POINT at which the plate(s) could be stored at -80 °C**

## 2.4 Tandem Mass Spectrometry (TIMING: 5 hrs)

A generic method is supplied here which is optimised for separations using a Waters i-Class UPLC System and detection using a Waters Xevo TQ XS. Details of methods for triple

quadrupole MS platforms from other manufacturers utilised in the development of this protocol is provided in the Supplementary Material (S12).

23. The up-front liquid chromatography system should be equipped with a 50 mm C18 column, preceded by a frit 0.2 μm.
24. Cool the autosampler plate holder to 10°C
25. Locate the magnetic plate for the autosampler in your autosampler according to the manufacturer's instructions and place the 96-well plate onto the magnet in the autosampler
26. Currently, one injection of 20 µL is performed per sample using a 1 min gradient and 3 min inject to inject time using the method "Moonshot_SARS_CoV_2_MRM_3min" available in the Supplementary Information. A list of peptides and associated MRM method details as used on a Waters TQ-XS triple instrument is shown in Table 1. Cone voltage for each peptide was kept at 35V. MS method details for other triple quadrupole instruments can be found in the Supplementary Information.
27. As soon as possible after analysis, remove the plate from the autosampler, seal and store the remaining sample at -20 °C. This sample can be used for a duplicate run if necessary (for example if the initial run fails)

| No. | Time (min) | Peptide sequence (z state indicated) | Q1 | Q3 | Auto dwell (ms) | Collision Energy (V) | Type |
|---|---|---|---|---|---|---|---|
| 1 | 0.8-1.2 | ADETQALPQR.3 | 376.8 | 400.2 | 22 | 11 | |
| 1 | 0.8-1.2 | ADETQALPQR.3 | 380.1 | 410. 2 | 22 | 11 | |
| 2 | 1.75-2.20 | DGIIWVATEGALNTPK.3 | 562.3 | 572.3 | 19 | 15 | |
| 2 | 1.75-2.20 | DGIIWVATEGALNTPK.3 | 562.3 | 700.3 | 19 | 15 | Quant |
| 2 | 1.75-2.20 | DGIIWVATEGALNTPK.3 | 564.9 | 580.3 | 19 | 15 | |
| 2 | 1.75-2.20 | DGIIWVATEGALNTPK.3 | 564.9 | 708.4 | 19 | 15 | Sil Quant |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 2 | 1.75-2.20 | DGIIWVATEGALNTPK.2 | 842.9 | 1001.5 | 19 | 29 | Qual |
| 2 | 1.75-2.20 | DGIIWVATEGALNTPK.2 | 846.9 | 1009.5 | 19 | 29 | Sil Qual |
| 3 | 1.30-1.60 | AYNVTQAFGR.2 | 563.7 | 578.3 | 22 | 18 | |
| 3 | 1.30-1.60 | AYNVTQAFGR.2 | 563.7 | 679.3 | 22 | 18 | Quant |
| 3 | 1.30-1.60 | AYNVTQAFGR.2 | 563.7 | 778.4 | 22 | 19 | |
| 3 | 1.30-1.60 | AYNVTQAFGR.2 | 563.7 | 892.4 | 22 | 19 | Qual |
| 3 | 1.30-1.60 | AYNVTQAFGR.2 | 568.7 | 588.3 | 22 | 18 | |
| 3 | 1.30-1.60 | AYNVTQAFGR.2 | 568.7 | 689.3 | 22 | 18 | Sil Quant |
| 3 | 1.30-1.60 | AYNVTQAFGR.2 | 568.7 | 788.4 | 22 | 19 | |
| 3 | 1.30-1.60 | AYNVTQAFGR.2 | 568.7 | 902.4 | 22 | 19 | Sil Qual |
| 4 | 0.8-1.2 | ADETQALPQR.2 | 564.7 | 400.2 | 22 | 19 | Quant |
| 4 | 0.8-1.2 | ADETQALPQR.2 | 564.7 | 584.3 | 22 | 20 | Qual |
| 4 | 0.8-1.2 | ADETQALPQR.2 | 564.7 | 712.4 | 22 | 20 | |
| 4 | 0.8-1.2 | ADETQALPQR.2 | 569.7 | 410.2 | 22 | 19 | Sil Quant |
| 4 | 0.8-1.2 | ADETQALPQR.2 | 569.7 | 594.3 | 22 | 20 | |
| 4 | 0.8-1.2 | ADETQALPQR.2 | 569.7 | 722.4 | 22 | 20 | Sil Qual |
| 5 | 1.60-2.20 | CHAPS | 615.9 | 561.7 | 19 | 20 | |

*Table 1: MRM method details for target peptides. The charge state selected for each peptide is indicated with the number after the sequence tag. Sil Quant/Qual refers to the data from the stable isotopically labelled (SIL) peptide. With a dwell time of 22 msec for each of 26 transitions there are at least 15 data points across the peak.*

## 2.5 Data reporting process

In the development of the method the most intense product consistently observed for each peptide was taken as the quantifier ion from the Skyline generated extracted ion chromatograms (XIC) (Table 2). These allow the quantity of the target viral peptide to be compared to the abundance found from the corresponding SILS for each parent and product ion pair to develop the acceptance criteria for a positive test. Calibration curves should be constructed for each analytical platform (SI) and the relative signal intensity, number of points across a peak, acceptable LOD/linearity in the calibration curve together with the intensity of the peaks for high and low QC values, were used to assess batch to batch success of analytical runs. CHAPS is included in the SISCAPA wash and elution buffers as it reduces bead adherence and including a CHAPS transition in the MRM scans acts as a system suitability test for monitoring the mass spectrometer's performance. Using freshly prepared standards the LOD was found to be as a few attomols per 10 µl injection. The LOQ from routine calibration standards was found to be 1.28 pM on Waters TQS instruments.

Applying this method to real world samples allowed the presence of SARS-CoV-2 to be discerned for samples with a RT-PCR Cq < 28 robustly. Calling a transition as positive was performed by inspection and automated in subsequent adaptation of the method.[44] For Cq > 28 samples (corresponding to weaker positive signal[45]), a blinded manual interrogation of the acquired spectra was required to confirm positive and negative status. This was not optimal for routine use, and can be improved[46]. The results obtained were compared to the RT-PCR TaqPath™ results acquired at The Francis Crick Institute (Instructions and validation specifications available from the Thermo Fisher website[47,48]).

## 2.6 Timing

A summary of the timings for this method is shown in Table 2 and is based on the example 96-well plate layout (Figure 2) for 72 samples with blanks, quality control (QC) samples and calibration curves included (see Supplementary Information S5). QC samples for monitoring a sample run are created in two levels of NCAP, one high QC and one low QC, and one blank,

with an internal standard of SIL peptides, in pooled human saliva. These may be used in place of the NCAP calibration curve when there is sufficient evidence for the sensitivity of the MS workflow. The whole protocol takes approximately 12 hours from the initial receipt of samples up to data calling of infection status with ~3 mins inject to inject per sample on machine. The time on machine is 4.8 hours per plate including QC samples, in a 24 hour period we envisage that about 5 plates could be run and after the first plate (Figure 3) subsequently plates would be more sample heavy with up to 90 samples per plate and 3 high and low QCS each respectively. This provides a per machine throughput of approximately 430 samples per day of use.

| **Protocol Step** | **Approx. timings for a 96-well plate** | **Automation?** |
|---|---|---|
| QC/calibration and solutions sample prep | 1 hr | No |
| Swab/Saliva extraction | 2 hrs | No |
| Digestion | 1 hr | Yes |
| SISCAPA Enrichment | 3 hrs | Yes |
| LC-MS | 4.8 hrs | Yes |
| **TOTAL** | **~12 hrs** | |

*Table 2: Approximate timings for protocol steps and automation capacity based on 96 injections comprising 72 swab samples and 24 QCs per plate.*

## 2.7 Troubleshooting

The study design involved the harmonised method being performed in different laboratories, on different instruments by different users, and as a consequence we were readily able to identify common challenges. The below troubleshooting guide is based on the combined observations from the laboratories involved.

| **Step #** | **Problem** | **Possible Reason** | **Solution** |
|---|---|---|---|
| 6 | Pellet not solid | Samples from different individuals can cause varied pellets to form | Can repeat the ice-cold acetone incubation to try to improve the pellet. Be cautious when removing supernatant to leave as much pellet as possible. |
| 12 | QC sample detection is lower than expected | Reduced LC-MS system sensitivity | Assess the MS system using an injection of a SIL-only sample |

| | | | |
|---|---|---|---|
| | | Not enough SISCAPA beads were added to the samples | Ensure SISCAPA beads are fully in solution before mixing and adding to samples<br><br>Clean the MS source, and any ion optics that may need cleaning |
| 27 | No or misidentification of peptide | Shifts in retention time with the LC gradient | Assess the LC system using the CHAPS fragments. Additionally, can perform an injection of a SIL-only sample<br><br>Adjust retention times for peptides to match the shift seen with the CHAPS fragments |
| 27 | Low MS response | MS contamination | Assess the MS system using the CHAPS fragments. Additionally, you can perform an injection of a SIL-only sample<br><br>Clean the MS source, and any ion optics that may need cleaning |
| 27 | Insufficient fragmentation of parent ion | Different individual instruments may need different settings | Assess the MS system using the CHAPS fragments. Additionally, you can perform an injection of a SIL-only sample<br><br>Optimise collision energies and/or source pressures and voltages for each peptide sequence |

*Table 3: Common troubleshooting problems and solutions*

## 3. Anticipated Results

Using this protocol for both swab and saliva clinical samples, mass spectrometry is able to assign an individual as being positive or negative for SARS-CoV-2 infection. A summary of the results for 551 swabs run in in two laboratories and 690 saliva samples run in two laboratories using the optimised protocol is detailed in Table 3 and exemplar data is provided in the

supplementary datasets. The three values assessed are sensitivity, specificity and accuracy. Sensitivity is defined as the extent to which actual positives are detected, e.g. the higher the sensitivity means the fewer false negatives. Specificity is a measure of how well a test can identify true negatives. Finally, accuracy pertains to the closeness of the measured value to the actual value and here we are assessing diagnostic accuracy: correct attribution of positives and negatives. On average, swab samples performed better, with higher percentages in the three values compared to those from saliva samples. Isolating 'strong' positive samples (RT-PCR Cq < 20) improved the saliva results however this is not indicative of the true sample population where Cq < 20 samples only represent a fraction of all RT-PCR positive samples. Issues from saliva samples come from matrix effects, contamination from food, drink or medicines ingested by the individual,[49] as well as there being much less control of concentration compared to swab sampling due to varying volumes of sample produced by the individual.

| | Cq Level | Sensitivity | Specificity | Accuracy |
|---|---|---|---|---|
| **Swab Set 1** | All (269) | 76.2% | 96.4% | 86.6% |
| | Cq < 28 | 97.3% | 96.3% | 96.9% |
| **Swab Set 2** | All (282) | 77.4% | 96.4% | 89.6% |
| | Cq < 28 | 98.1% | 96.4% | 96.8% |
| **Saliva Set 1** | All (319) | 36.0% | 92.9% | 65.2% |
| | Cq < 28 | 45.1% | 95.0% | 74.8% |
| | Cq < 20 | 78.9% | 95.0% | 92.8% |
| **Saliva Set 2** | All (371) | 57.1% | 89.9% | 75.5% |
| | Cq < 28 | 58.7% | 89.9% | 80.3% |
| | Cq < 20 | 67.7% | 89.9% | 87.4% |

*Table 3: Summary of saliva and swab results according to sensitivity, specificity and accuracy based on three NCAP peptides*

Figure 4 shows typical Skyline outputs for each peptide for both positive and negative swab samples that has gone through the above protocol. In the positive sample there are three transitions identified for each peptide. However, no transitions can be seen from viral peptides in the negative sample. Confirming negative diagnoses is arguably more vital in a

global pandemic as it is crucial to limit the number of false-negatives i.e. individuals infected with SARS-CoV-2 that present as negative in a diagnostic assay. Reducing the number of false-negatives would help to restrict the spread of the virus, especially when considering isolation and quarantine rules.

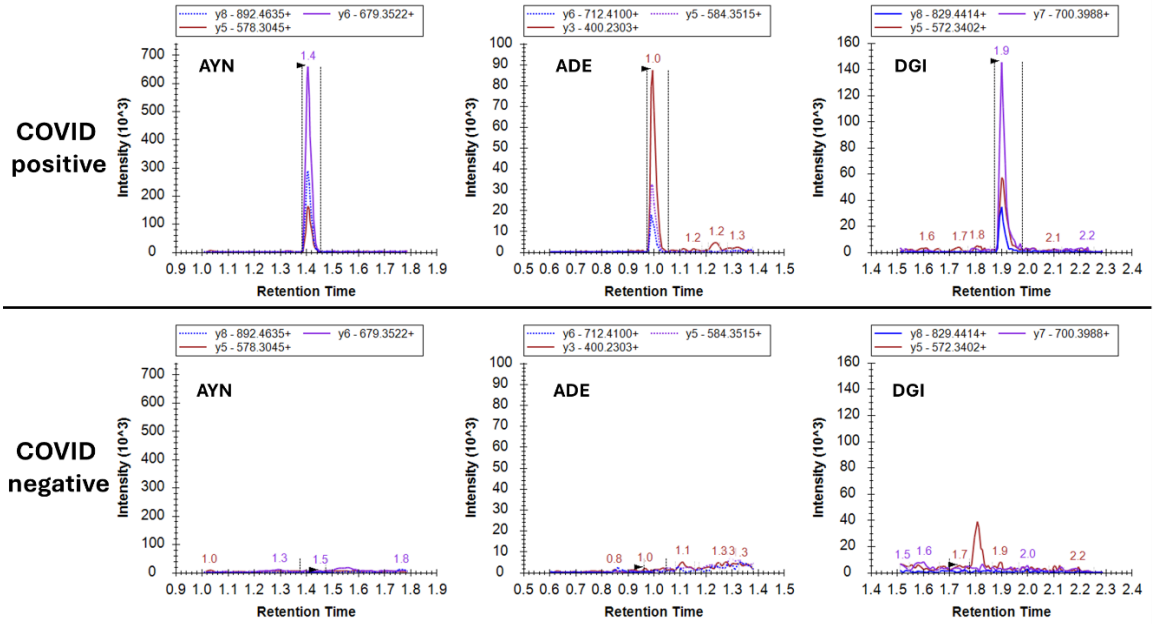



*Figure 4: Typical COVID-19 Skyline output swab samples*

*The three different peptides from COVID-19 NCAP are shown here: AYN, ADE, and DGI for a COVID positive sample (top panel) and a negative sample (bottom panel). The DGI peptide was the least reliable for data calling.*

## 4. Conclusions

The development of this protocol was a collaborative effort involving academic and clinical laboratories, the NHS, industry, international external advisors, government assistance and specimens from hundreds of willing volunteers. We have shown that the development of a clinically relevant diagnostic assay can be completed in a short amount of time and can have comparable sensitivity and selectivity to other diagnostic assays such as PCR. This protocol could be adapted to detect other viruses, following the optimisation of the relevant mAb production for the SISCAPA enrichment.

## 5. Outlook

This protocol was adapted and used in clinical labs by Hällqvist *et al*.[44] The method developed here targeted three NCAP peptides, however this was reduced to two (ADE and AYN) in the clinic due to carry-over issues with the DGI peptide.

Harnessing the MS infrastructure of hundreds of instruments and skilled technical staff for other national testing programmes such as pandemic screening would provide long-term utility. We believe that the optimisation and development process described here could be used to fulfil future needs for reliable diagnostics for any potential pandemic-causing pathogens that we may encounter in the coming decades. Using the COVIDCAP method, a single mass spectrometer has the capacity to process ~ 430 diagnostic samples in 24 hours, with sample preparation as the rate-limiting step that could be improved with innovative automation e.g. sample handling robots. Additionally, this assay is amendable to pooling prior to enrichment, where groups of patient samples could be combined and if a positive result is attained then further testing could be completed. A caveat of this pooling method however is that it is most effective with a population of majority negative samples, where the need for further testing is low.

This targeted MS method could also be applied to simultaneously detect proteins from SARS-CoV-2, Flu A/B, RSV A/B, and other coronaviruses (collectively known as the 'winter respiratory panel') facilitating differential diagnosis. SARS-CoV-2 mutations occur rapidly, as is common for RNA viruses.[50] Most mutations in the COVID-19 pandemic occurred in the spike protein, altering receptor binding and immune response to the virus, increasing infectiousness and infectivity.[51] NCAP mutations enhanced replication and transmissibility[52] however only a single mutation in one of our target peptides was identified. This mutated peptide was still captured by the SISCAPA antibodies and just required minor alteration to its MRM transitions.

Finally, this work has proven that considered collaboration between academia, industry and the NHS is of benefit to both science and medicine with other collaborative outputs[41,53]. The responsiveness of the COVID-19 MS Coalition in the UK laid the groundwork in preparedness

for future methods required to move rapidly from concept to clinic for mass population screening.

## Supplementary Information

Supplementary information is referred to in the text and contains details on the materials and reagents used, sampling logistics including the sample pack details. An overview of the protocol development including the LOD and LOQ determined, details on saliva extraction, and the detailed mass spectrometry methods used. Supplementary datasets contain the reports from all of the P1 laboratories.

## Author contributions

The protocol herein was developed by all authors, many of whom worked for long hours and attended many Zoom calls during the pandemic collectively harmonising this method to its final form. The first draft of the protocol was produced collectively by P1 laboratories as a requirement of the MS Moonshot project for the P2 laboratories to develop further. ENL reviewed these collective inputs and rationalised them into this final form with major inputs from all of the P1 labs. Special thanks given to DJLJ, RU, AT, MT, KM, PB, JH, DL and RC.

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## Competing Interests

NLA, MR, TWP, MEP and RY and are employees of SISCAPA and own or have the option to own stocks in this company.

## Bibliography

(1) Sohrabi, C.; Alsafi, Z.; O'Neill, N.; Khan, M.; Kerwan, A.; Al-Jabir, A.; Iosifidis, C.; Agha, R. World Health Organization Declares Global Emergency: A Review of the 2019 Novel Coronavirus (COVID-19). *Int J Surg* **2020**, *76*, 71–76. https://doi.org/10.1016/j.ijsu.2020.02.034.

(2) WHO. *Coronavirus disease (COVID-19) pandemic*. https://www.who.int/europe/emergencies/situations/covid-19 (accessed 2024-08-13).

(3) WHO. *COVID-19 cases | WHO COVID-19 dashboard*. https://data.who.int/dashboards/covid19/cases (accessed 2024-01-11).

(4) Hoffmann, M.; Kleine-Weber, H.; Schroeder, S.; Krüger, N.; Herrler, T.; Erichsen, S.; Schiergens, T. S.; Herrler, G.; Wu, N.-H.; Nitsche, A.; Müller, M. A.; Drosten, C.; Pöhlmann, S. SARS-CoV-2 Cell Entry Depends on ACE2 and TMPRSS2 and Is Blocked by a Clinically Proven Protease Inhibitor. *Cell* **2020**, *181* (2), 271-280.e8. https://doi.org/10.1016/j.cell.2020.02.052.

(5) Zhou, P.; Yang, X.-L.; Wang, X.-G.; Hu, B.; Zhang, L.; Zhang, W.; Si, H.-R.; Zhu, Y.; Li, B.; Huang, C.-L.; Chen, H.-D.; Chen, J.; Luo, Y.; Guo, H.; Jiang, R.-D.; Liu, M.-Q.; Chen, Y.; Shen, X.-R.; Wang, X.; Zheng, X.-S.; Zhao, K.; Chen, Q.-J.; Deng, F.; Liu, L.-L.; Yan, B.; Zhan, F.-X.; Wang, Y.-Y.; Xiao, G.-F.; Shi, Z.-L. A Pneumonia Outbreak Associated with a New Coronavirus of Probable Bat Origin. *Nature* **2020**, *579* (7798), 270–273. https://doi.org/10.1038/s41586-020-2012-7.

(6) Li, W.; Moore, M. J.; Vasilieva, N.; Sui, J.; Wong, S. K.; Berne, M. A.; Somasundaran, M.; Sullivan, J. L.; Luzuriaga, K.; Greenough, T. C.; Choe, H.; Farzan, M. Angiotensin-Converting Enzyme 2 Is a Functional Receptor for the SARS Coronavirus. *Nature* **2003**, *426* (6965), 450–454. https://doi.org/10.1038/nature02145.

(7) Zhong, N.; Zheng, B.; Li, Y.; Poon, L.; Xie, Z.; Chan, K.; Li, P.; Tan, S.; Chang, Q.; Xie, J.; Liu, X.; Xu, J.; Li, D.; Yuen, K.; Peiris, J.; Guan, Y. Epidemiology and Cause of Severe Acute Respiratory Syndrome (SARS) in Guangdong, People's Republic of China, in February, 2003. *Lancet* **2003**, *362* (9393), 1353–1358. https://doi.org/10.1016/S0140-6736(03)14630-2.

(8) Al-Aly, Z.; Davis, H.; McCorkell, L.; Soares, L.; Wulf-Hanson, S.; Iwasaki, A.; Topol, E. J. Long COVID Science, Research and Policy. *Nat Med* **2024**. https://doi.org/10.1038/s41591-024-03173-6.
(9) Struwe, W.; Emmott, E.; Bailey, M.; Sharon, M.; Sinz, A.; Corrales, F. J.; Thalassinos, K.; Braybrook, J.; Mills, C.; Barran, P. The COVID-19 MS Coalition—Accelerating Diagnostics, Prognostics, and Treatment. *The Lancet*. Lancet Publishing Group June 6, 2020, pp 1761–1762. https://doi.org/10.1016/S0140-6736(20)31211-3.
(10) Ihling, C.; Tänzler, D.; Hagemann, S.; Kehlen, A.; Hüttelmaier, S.; Arlt, C.; Sinz, A. Mass Spectrometric Identification of SARS-CoV-2 Proteins from Gargle Solution Samples of COVID-19 Patients. *J. Proteome Res.* **2020**, *19* (11), 4389–4392. https://doi.org/10.1021/acs.jproteome.0c00280.
(11) Van Puyvelde, B.; Van Uytfanghe, K.; Tytgat, O.; Van Oudenhove, L.; Gabriels, R.; Bouwmeester, R.; Daled, S.; Van Den Bossche, T.; Ramasamy, P.; Verhelst, S.; De Clerck, L.; Corveleyn, L.; Willems, S.; Debunne, N.; Wynendaele, E.; De Spiegeleer, B.; Judak, P.; Roels, K.; De Wilde, L.; Van Eenoo, P.; Reyns, T.; Cherlet, M.; Dumont, E.; Debyser, G.; t'Kindt, R.; Sandra, K.; Gupta, S.; Drouin, N.; Harms, A.; Hankemeier, T.; Jones, D. J. L.; Gupta, P.; Lane, D.; Lane, C. S.; El Ouadi, S.; Vincendet, J.-B.; Morrice, N.; Oehrle, S.; Tanna, N.; Silvester, S.; Hannam, S.; Sigloch, F. C.; Bhangu-Uhlmann, A.; Claereboudt, J.; Anderson, N. L.; Razavi, M.; Degroeve, S.; Cuypers, L.; Stove, C.; Lagrou, K.; Martens, G. A.; Deforce, D.; Martens, L.; Vissers, J. P. C.; Dhaenens, M. Cov-MS: A Community-Based Template Assay for Mass-Spectrometry-Based Protein Detection in SARS-CoV-2 Patients. *JACS Au* **2021**, *1* (6), 750–765. https://doi.org/10.1021/jacsau.1c00048.
(12) Cardozo, K. H. M.; Lebkuchen, A.; Okai, G. G.; Schuch, R. A.; Viana, L. G.; Olive, A. N.; Lazari, C. dos S.; Fraga, A. M.; Granato, C. F. H.; Pintão, M. C. T.; Carvalho, V. M. Establishing a Mass Spectrometry-Based System for Rapid Detection of SARS-CoV-2 in Large Clinical Sample Cohorts. *Nat Commun* **2020**, *11*, 6201. https://doi.org/10.1038/s41467-020-19925-0.
(13) CONDOR. *FALCON - Facilitating AcceLerated Clinical validation Of novel diagnostics for COVID-19*. https://www.condor-platform.org/condor_workstreams/falcon.
(14) Anderson, N. L.; Anderson, N. G.; Haines, L. R.; Hardie, D. B.; Olafson, R. W.; Pearson, T. W. Mass Spectrometric Quantitation of Peptides and Proteins Using Stable Isotope Standards and Capture by Anti-Peptide Antibodies (SISCAPA). *J. Proteome Res.* **2004**, *3* (2), 235–244. https://doi.org/10.1021/pr034086h.
(15) Pan, Y.; Long, L.; Zhang, D.; Yuan, T.; Cui, S.; Yang, P.; Wang, Q.; Ren, S. Potential False-Negative Nucleic Acid Testing Results for Severe Acute Respiratory Syndrome Coronavirus 2 from Thermal Inactivation of Samples with Low Viral Loads. *Clinical Chemistry* **2020**, *66* (6), 794–801. https://doi.org/10.1093/clinchem/hvaa091.
(16) Holborow, A.; Asad, H.; Porter, L.; Tidswell, P.; Johnston, C.; Blyth, I.; Bone, A.; Healy, B. The Clinical Sensitivity of a Single SARS-CoV-2 Upper Respiratory Tract RT-PCR Test for Diagnosing COVID-19 Using Convalescent Antibody as a Comparator. *Clin Med (Lond)* **2020**, *20* (6), e209–e211. https://doi.org/10.7861/clinmed.2020-0555.
(17) Miller, T. E.; Beltran, W. F. G.; Bard, A. Z.; Gogakos, T.; Anahtar, M. N.; Astudillo, M. G.; Yang, D.; Thierauf, J.; Fisch, A. S.; Mahowald, G. K.; Fitzpatrick, M. J.; Nardi, V.; Feldman, J.; Hauser, B. M.; Caradonna, T. M.; Marble, H. D.; Ritterhouse, L. L.; Turbett, S. E.; Batten, J.; Georgantas, N. Z.; Alter, G.; Schmidt, A. G.; Harris, J. B.; Gelfand, J. A.; Poznansky, M. C.; Bernstein, B. E.; Louis, D. N.; Dighe, A.; Charles, R. C.; Ryan, E. T.; Branda, J. A.; Pierce, V. M.; Murali, M. R.; Iafrate, A. J.; Rosenberg, E. S.; Lennerz, J. K. Clinical Sensitivity and

Interpretation of PCR and Serological COVID-19 Diagnostics for Patients Presenting to the Hospital. *The FASEB Journal* **2020**, *34* (10), 13877. https://doi.org/10.1096/fj.202001700RR.
(18) Millington, D. S. How Mass Spectrometry Revolutionized Newborn Screening. *Journal of Mass Spectrometry and Advances in the Clinical Lab* **2024**, *32*, 1–10. https://doi.org/10.1016/j.jmsacl.2024.01.006.
(19) Mohit, E.; Rostami, Z.; Vahidi, H. A Comparative Review of Immunoassays for COVID-19 Detection. *Expert Rev Clin Immunol* **2021**, *17* (6), 573–599. https://doi.org/10.1080/1744666X.2021.1908886.
(20) Deeks, J. J.; Singanayagam, A.; Houston, H.; Sitch, A. J.; Hakki, S.; Dunning, J.; Lalvani, A. SARS-CoV-2 Antigen Lateral Flow Tests for Detecting Infectious People: Linked Data Analysis. *BMJ* **2022**, *376*, e066871. https://doi.org/10.1136/bmj-2021-066871.
(21) Eshghifar, N.; Busheri, A.; Shrestha, R.; Beqaj, S. Evaluation of Analytical Performance of Seven Rapid Antigen Detection Kits for Detection of SARS-CoV-2 Virus. *Int J Gen Med* **2021**, *14*, 435–440. https://doi.org/10.2147/IJGM.S297762.
(22) Fernandez-Montero, A.; Argemi, J.; Rodríguez, J. A.; Ariño, A. H.; Moreno-Galarraga, L. Validation of a Rapid Antigen Test as a Screening Tool for SARS-CoV-2 Infection in Asymptomatic Populations. Sensitivity, Specificity and Predictive Values. *eClinicalMedicine* **2021**, *37*. https://doi.org/10.1016/j.eclinm.2021.100954.
(23) Tahamtan, A.; Ardebili, A. Real-Time RT-PCR in COVID-19 Detection: Issues Affecting the Results. *Expert Review of Molecular Diagnostics* **2020**, *20* (5), 453–454. https://doi.org/10.1080/14737159.2020.1757437.
(24) Ng, L. L.; Sandhu, J. K.; Squire, I. B.; Davies, J. E.; Jones, D. J. L. Vitamin D and Prognosis in Acute Myocardial Infarction. *Int J Cardiol* **2013**, *168* (3), 2341–2346. https://doi.org/10.1016/j.ijcard.2013.01.030.
(25) Lane, D.; Lawson, A.; Burns, A.; Azizi, M.; Burnier, M.; Jones, D. J. L.; Kably, B.; Khunti, K.; Kreutz, R.; Patel, P.; Persu, A.; Spiering, W.; Toennes, S. W.; Tomaszewski, M.; Williams, B.; Gupta, P.; Dasgupta, I.; Endorsed by the European Society of Hypertension (ESH) Working Group on Cardiovascular Pharmacotherapy and Adherence. Nonadherence in Hypertension: How to Develop and Implement Chemical Adherence Testing. *Hypertension* **2022**, *79* (1), 12–23. https://doi.org/10.1161/HYPERTENSIONAHA.121.17596.
(26) Patel, P.; Gupta, P. K. C.; White, C. M. J.; Stanley, A. G.; Williams, B.; Tomaszewski, M. Screening for Non-Adherence to Antihypertensive Treatment as a Part of the Diagnostic Pathway to Renal Denervation. *J Hum Hypertens* **2016**, *30* (6), 368–373. https://doi.org/10.1038/jhh.2015.103.
(27) Farré-Segura, J.; Le Goff, C.; Lukas, P.; Cobraiville, G.; Fillet, M.; Servais, A.-C.; Delanaye, P.; Cavalier, E. Validation of an LC-MS/MS Method Using Solid-Phase Extraction for the Quantification of 1-84 Parathyroid Hormone: Toward a Candidate Reference Measurement Procedure. *Clin Chem* **2022**, *68* (11), 1399–1409. https://doi.org/10.1093/clinchem/hvac135.
(28) McShane, A. J.; Bunch, D. R.; Wang, S. Therapeutic Drug Monitoring of Immunosuppressants by Liquid Chromatography-Mass Spectrometry. *Clin Chim Acta* **2016**, *454*, 1–5. https://doi.org/10.1016/j.cca.2015.12.027.
(29) Carling, R. S.; Whyte, E.; John, C.; Garstone, R.; Goddard, P.; Greenfield, T.; Hogg, S. L.; Le Masurier, C.; Cowen, S.; Moat, S. J.; Hopley, C. Improving Harmonization and Standardization of Expanded Newborn Screening Results by Optimization of the Legacy

Flow Injection Analysis Tandem Mass Spectrometry Methods and Application of a Standardized Calibration Approach. *Clinical Chemistry* **2022**, *68* (8), 1075–1083. https://doi.org/10.1093/clinchem/hvac070.

(30) NHS. *NHS screening programmes in England: 2019 to 2020*. GOV.UK. https://www.gov.uk/government/publications/nhs-screening-programmes-annual-report/nhs-screening-programmes-in-england-2019-to-2020 (accessed 2024-07-31).

(31) Metzgar, D.; Baynes, D.; Myers, C. A.; Kammerer, P.; Unabia, M.; Faix, D. J.; Blair, P. J. Initial Identification and Characterization of an Emerging Zoonotic Influenza Virus Prior to Pandemic Spread. *Journal of Clinical Microbiology* **2010**, *48* (11), 4228–4234. https://doi.org/10.1128/jcm.01336-10.

(32) Majchrzykiewicz-Koehorst, J. A.; Heikens, E.; Trip, H.; Hulst, A. G.; de Jong, A. L.; Viveen, M. C.; Sedee, N. J. A.; van der Plas, J.; Coenjaerts, F. E. J.; Paauw, A. Rapid and Generic Identification of Influenza A and Other Respiratory Viruses with Mass Spectrometry. *J Virol Methods* **2015**, *213*, 75–83. https://doi.org/10.1016/j.jviromet.2014.11.014.

(33) Ihling, C.; Tänzler, D.; Hagemann, S.; Kehlen, A.; Hüttelmaier, S.; Arlt, C.; Sinz, A. Mass Spectrometric Identification of SARS-CoV-2 Proteins from Gargle Solution Samples of COVID-19 Patients. *J. Proteome Res.* **2020**, *19* (11), 4389–4392. https://doi.org/10.1021/acs.jproteome.0c00280.

(34) Singh, P.; Chakraborty, R.; Marwal, R.; Radhakrishan, V. S.; Bhaskar, A. K.; Vashisht, H.; Dhar, M. S.; Pradhan, S.; Ranjan, G.; Imran, M.; Raj, A.; Sharma, U.; Singh, P.; Lall, H.; Dutta, M.; Garg, P.; Ray, A.; Dash, D.; Sivasubbu, S.; Gogia, H.; Madan, P.; Kabra, S.; Singh, S. K.; Agrawal, A.; Rakshit, P.; Kumar, P.; Sengupta, S. A Rapid and Sensitive Method to Detect SARS-CoV-2 Virus Using Targeted-Mass Spectrometry. *Journal of Proteins and Proteomics* **2020**, *11* (3), 159–165. https://doi.org/10.1007/s42485-020-00044-9.

(35) Bezstarosti, K.; Lamers, M.; van Kampen, J.; Haagmans, B.; Demmers, J. Targeted Proteomics as a Tool to Detect SARS-CoV-2 Proteins in Clinical Specimens. *bioRxiv* **2020**, 2020.04.23.057810. https://doi.org/10.1101/2020.04.23.057810.

(36) Yoshinari, T.; Hayashi, K.; Hirose, S.; Ohya, K.; Ohnishi, T.; Watanabe, M.; Taharaguchi, S.; Mekata, H.; Taniguchi, T.; Maeda, T.; Orihara, Y.; Kawamura, R.; Arai, S.; Saito, Y.; Goda, Y.; Hara-Kudo, Y. Matrix-Assisted Laser Desorption and Ionization Time-of-Flight Mass Spectrometry Analysis for the Direct Detection of SARS-CoV-2 in Nasopharyngeal Swabs. *Anal. Chem.* **2022**, *94* (10), 4218–4226. https://doi.org/10.1021/acs.analchem.1c04328.

(37) Kollhoff, L.; Kipping, M.; Rauh, M.; Ceglarek, U.; Barka, G.; Barka, F.; Sinz, A. Development of a Rapid and Specific MALDI-TOF Mass Spectrometric Assay for SARS-CoV-2 Detection. *Clinical Proteomics* **2023**, *20* (1), 26. https://doi.org/10.1186/s12014-023-09415-y.

(38) Cardozo, K. H. M.; Lebkuchen, A.; Okai, G. G.; Schuch, R. A.; Viana, L. G.; Olive, A. N.; Lazari, C. dos S.; Fraga, A. M.; Granato, C. F. H.; Pintão, M. C. T.; Carvalho, V. M. Establishing a Mass Spectrometry-Based System for Rapid Detection of SARS-CoV-2 in Large Clinical Sample Cohorts. *Nat Commun* **2020**, *11*, 6201. https://doi.org/10.1038/s41467-020-19925-0.

(39) Van Puyvelde, B.; Van Uytfanghe, K.; Tytgat, O.; Van Oudenhove, L.; Gabriels, R.; Bouwmeester, R.; Daled, S.; Van Den Bossche, T.; Ramasamy, P.; Verhelst, S.; De Clerck, L.; Corveleyn, L.; Debunne, N.; Wynendaele, E.; De Spiegeleer, B.; Judak, P.; Roels, K.; De Wilde, L.; Van Eenoo, P.; Reyns, T.; Cherlet, M.; Dumont, E.; Debyser, G.; T'Kindt, R.; Sandra, K.; Gupta, S.; Drouin, N.; Harms, A.; Hankemeier, T.; Jones, D.; Gupta, P.; Lane, D.; Lane, C.; El Ouadi, S.; Vincendet, J.; Morrice, N.; Oehrle, S.; Tanna, N.; Silvester, S.;

Hannam, S.; Sigloch, F.; Bhangu-Uhlmann, A.; Claereboudt, J.; Anderson, L.; Razavi, M.; Degroeve, S.; Cuypers, L.; Stove, C.; Lagrou, K.; Martens, G.; Deforce, D.; Martens, L.; Vissers, J.; Dhaenens, M. Cov-MS: A Community-Based Template Assay for Clinical MS-Based Protein Detection in SARS-CoV-2 Patients. *medRxiv* **2020**, *19* (11), 2020.11.18.20231688. https://doi.org/10.1101/2020.11.18.20231688.

(40) Hober, A.; Tran-Minh, K. H.; Foley, D.; McDonald, T.; Vissers, J. P. C.; Pattison, R.; Ferries, S.; Hermansson, S.; Betner, I.; Uhlén, M.; Razavi, M.; Yip, R.; Pope, M. E.; Pearson, T. W.; Andersson, L. N.; Bartlett, A.; Calton, L.; Alm, J. J.; Engstrand, L.; Edfors, F. Rapid and Sensitive Detection of Sars-Cov-2 Infection Using Quantitative Peptide Enrichment Lc-Ms Analysis. *eLife* **2021**, *10*. https://doi.org/10.7554/eLife.70843.

(41) Brudenell, E. L.; Pohare, M. B.; Zafred, D.; Phipps, J.; Hornsby, H. R.; Darby, J.; Dai, J.; Liggett, E.; Cain, K.; Barran, P. E.; Silva, T. I. de; Sayers, J. R. Efficient Overexpression and Purification of SARS-CoV-2 Nucleocapsid Proteins in Escherichia Coli. bioRxiv January 9, 2024, p 2024.01.08.574531. https://doi.org/10.1101/2024.01.08.574531.

(42) MacLean, B.; Tomazela, D. M.; Shulman, N.; Chambers, M.; Finney, G. L.; Frewen, B.; Kern, R.; Tabb, D. L.; Liebler, D. C.; MacCoss, M. J. Skyline: An Open Source Document Editor for Creating and Analyzing Targeted Proteomics Experiments. *Bioinformatics* **2010**, *26* (7), 966–968. https://doi.org/10.1093/bioinformatics/btq054.

(43) Cubuk, J.; Alston, J. J.; Incicco, J. J.; Singh, S.; Stuchell-Brereton, M. D.; Ward, M. D.; Zimmerman, M. I.; Vithani, N.; Griffith, D.; Wagoner, J. A.; Bowman, G. R.; Hall, K. B.; Soranno, A.; Holehouse, A. S. The SARS-CoV-2 Nucleocapsid Protein Is Dynamic, Disordered, and Phase Separates with RNA. *Nat Commun* **2021**, *12* (1), 1936. https://doi.org/10.1038/s41467-021-21953-3.

(44) Hällqvist, J.; Lane, D.; Shapanis, A.; Davis, K.; Heywood, W. E.; Doykov, I.; Śpiewak, J.; Ghansah, N.; Keevil, B.; Gupta, P.; Jukes-Jones, R.; Singh, R.; Foley, D.; Vissers, J. P. C.; Pattison, R.; Ferries, S.; Wardle, R.; Bartlett, A.; Calton, L. J.; Anderson, L.; Razavi, M.; Pearson, T.; Pope, M.; Yip, R.; Ng, L. L.; Nicholas, B. I.; Bailey, A.; Noel, D.; Dalton, R. N.; Heales, S.; Hopley, C.; Pitt, A. R.; Barran, P.; Jones, D. J. L.; Mills, K.; Skipp, P.; Carling, R. S. Operation Moonshot: Rapid Translation of a SARS-CoV-2 Targeted Peptide Immunoaffinity Liquid Chromatography-Tandem Mass Spectrometry Test from Research into Routine Clinical Use. *Clin Chem Lab Med* **2022**, *61* (2), 302–310. https://doi.org/10.1515/cclm-2022-1000.

(45) Singanayagam, A.; Patel, M.; Charlett, A.; Bernal, J. L.; Saliba, V.; Ellis, J.; Ladhani, S.; Zambon, M.; Gopal, R. Duration of Infectiousness and Correlation with RT-PCR Cycle Threshold Values in Cases of COVID-19, England, January to May 2020. *Eurosurveillance* **2020**, *25* (32), 2001483. https://doi.org/10.2807/1560-7917.ES.2020.25.32.2001483.

(46) Hällqvist, J.; Lane, D.; Shapanis, A.; Davis, K.; Heywood, W. E.; Doykov, I.; Śpiewak, J.; Ghansah, N.; Keevil, B.; Gupta, P.; Jukes-Jones, R.; Singh, R.; Foley, D.; Vissers, J. P. C.; Pattison, R.; Ferries, S.; Wardle, R.; Bartlett, A.; Calton, L. J.; Anderson, L.; Razavi, M.; Pearson, T.; Pope, M.; Yip, R.; Ng, L. L.; Nicholas, B. I.; Bailey, A.; Noel, D.; Dalton, R. N.; Heales, S.; Hopley, C.; Pitt, A. R.; Barran, P.; Jones, D. J. L.; Mills, K.; Skipp, P.; Carling, R. S. Operation Moonshot: Rapid Translation of a SARS-CoV-2 Targeted Peptide Immunoaffinity Liquid Chromatography-Tandem Mass Spectrometry Test from Research into Routine Clinical Use. *Clinical Chemistry and Laboratory Medicine (CCLM)* **2023**, *61* (2), 302–310. https://doi.org/10.1515/cclm-2022-1000.

(47) Thermo Fisher. *TaqPath™ COVID-19 CE-IVD RT-PCR Kit*. https://www.thermofisher.com/order/catalog/product/A48067 (accessed 2024-07-31).

(48) Thermo Fisher. *Instructions for Use Finder for COVID-19 Molecular Diagnostics - UK*. https://www.thermofisher.com/uk/en/home/clinical/clinical-genomics/pathogen-detection-solutions/covid-19-sars-cov-2/instructions-for-use.html (accessed 2024-07-31).
(49) Spick, M.; Lewis, H.-M.; Frampas, C. F.; Longman, K.; Costa, C.; Stewart, A.; Dunn-Walters, D.; Greener, D.; Evetts, G.; Wilde, M. J.; Sinclair, E.; Barran, P. E.; Skene, D. J.; Bailey, M. J. An Integrated Analysis and Comparison of Serum, Saliva and Sebum for COVID-19 Metabolomics. *Sci Rep* **2022**, *12* (1), 11867. https://doi.org/10.1038/s41598-022-16123-4.
(50) Sanjuán, R.; Nebot, M. R.; Chirico, N.; Mansky, L. M.; Belshaw, R. Viral Mutation Rates. *Journal of Virology* **2010**, *84* (19), 9733–9748. https://doi.org/10.1128/jvi.00694-10.
(51) Markov, P. V.; Ghafari, M.; Beer, M.; Lythgoe, K.; Simmonds, P.; Stilianakis, N. I.; Katzourakis, A. The Evolution of SARS-CoV-2. *Nat Rev Microbiol* **2023**, *21* (6), 361–379. https://doi.org/10.1038/s41579-023-00878-2.
(52) Johnson, B. A.; Zhou, Y.; Lokugamage, K. G.; Vu, M. N.; Bopp, N.; Crocquet-Valdes, P. A.; Kalveram, B.; Schindewolf, C.; Liu, Y.; Scharton, D.; Plante, J. A.; Xie, X.; Aguilar, P.; Weaver, S. C.; Shi, P.-Y.; Walker, D. H.; Routh, A. L.; Plante, K. S.; Menachery, V. D. Nucleocapsid Mutations in SARS-CoV-2 Augment Replication and Pathogenesis. *PLOS Pathogens* **2022**, *18* (6), e1010627. https://doi.org/10.1371/journal.ppat.1010627.
(53) Waters. *SARS-CoV-2 | LC-MS Analysis Kit for SARS-CoV-2 Nucleocapsid Peptides | Waters*. https://www.waters.com/nextgen/gb/en/products/standards-and-reagents/sars-cov-2-lc-ms-kit-ruo.html (accessed 2024-04-24).