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# Comparison of anti-peptide and anti-protein antibody-based purification techniques for detection of SARS-CoV-2 by targeted LC-MS/MS

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ABSTRACT

The COVID-19 pandemic has necessitated exploration of alternative testing methods for detection of Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2) to ensure clinical laboratories can continue to provide critical testing results. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) is established in many clinical laboratories due its high specificity and sensitivity, making it a logical alternative methodology. However, matching the sensitivity of quantitative reverse transcription-polymerase chain reaction (qRT-PCR) remains challenging, which forced utilization of antibody-based enrichment prior to targeted LC-MS/MS analysis. When utilizing antibody purification techniques, investigators must decide whether to enrich the target protein or peptides, but there are few studies comparing the two approaches to assist in this decision-making process. In this work, we present a comparison of intact protein and peptide antibody-based purification for LC-MS/MS based detection of SARS-CoV-2. We have found that protein purification yields more intense LC-MS/MS signals, but is also less specific, yielding higher noise and more background when compared to peptide purification techniques. Therefore, when using traditional data analysis techniques, the enrichment technique that provides superior sensitivity varies for individual peptides and no definitive overall conclusion can be made. These observations are corroborated when using a novel machine learning approach to determine positive/negative test results, which yielded superior sensitivity when using protein purification, but better specificity and area under the ROC curve when performing peptide purification.

## 1. Introduction

The ongoing COVID-19 pandemic caused by Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2) continues to put a strain on global health care resources, including clinical laboratories. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) remains the "gold-standard" for detecting SARS-CoV-2 [1], but alternative testing methodologies are being explored to ensure clinical laboratories are able to meet the demand. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) detection of viral proteins has been proven to be a highly specific and accurate technique, playing an integral role in the clinical laboratory [2–11]. However, matching the sensitivity of qRT-PCR is challenging on even the most advanced instrumentation, and is imperative for establishing LC-MS/MS testing for SARS-CoV-2 detection.

Maximizing the detection sensitivity of LC-MS/MS instrumentation requires extensive purification of target proteins or peptides to minimize suppression caused by matrix components [12–14]. Antibody-based purification or immunopurification (IP) has been established as one of the most specific and selective purification techniques [15–18]. When utilizing this approach, an antibody against the target is immobilized and the sample is then incubated with the antibody allowing for capture of the

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target. Following several washing steps, the target is eluted off the antibody and is ready for LC-MS/MS analysis. In the case of proteomic analyses, these purifications typically use antibodies against the target protein, which can be digested after elution if necessary. However, an alternative approach developed by Anderson and coworkers [15,18] termed Stable Isotope Standards and Capture by Anti-Peptide Antibodies (SISCAPA), utilizes antibodies against target peptides to purify after trypsin digestion. This approach minimizes the impact of protein interactions, allows for simultaneous isolation of the target peptides and isotopically labeled peptides improving the precision of the resulting measurement, and theoretically reduces background by isolating only select peptides as opposed to all peptides derived from a target protein.

Despite the widespread use of antibody-based purification in research and clinical proteomics, few studies have directly compared optimized anti-protein and anti-peptide based approaches for detection of proteotypic peptides. A notable example of such a comparison was published by Levernæs and coworkers in 2019 [19]. In this work, the investigators utilize an anti-protein antibody with an established linear epitope to isolate the tryptic peptide that corresponds to the epitope amino acids in the protein progastrin releasing peptide. This unique approach allowed for the assessment of peptide purification without requiring production of anti-peptide antibodies. In our investigation, we compare methods using both anti-peptide and anti-protein antibodies for LC-MS/MS detection of SARS-CoV-2 in nasopharyngeal (NP) swab samples, an application with unparalleled challenges in terms of sensitivity and throughput requirements. We have previously published our extensive discovery and method development work, which serves as a foundation for this investigation [9]. Here, we present a comparison of peptide and protein purification approaches in terms of workflow, traditional sensitivity metrics, and testing results from a previously described machine leaning model [9].

## 2. Experimental methods

### 2.1. Chemicals and reagents

We obtained phosphate buffered saline (PBS) from Bio-Rad (Hercules, CA, USA), deoxycholate (DOC), iodoacetamide (IAA), and Trifluoroacetic acid (TFA) from ThermoFisher Scientific (Waltham, MA), Zwittergent Z3-16 from CalBiochem (MilliporeSigma, Billerica, MA, USA), dithiothreitol (DTT), Tosyl-L-lysyl-chloromethane hydrochloride (TLCK), and Tris Base from MilliporeSigma, isotopically labeled peptides as internal standards (IS) from New England Peptide (Gardner, MA, USA), anti-peptide monoclonal antibodies from SISCAPA Assay Technologies, and anti-nucleocapsid monoclonal antibody from Sino Biological (Wayne, PA, USA, Cat# 40143-R001). All antibodies were coupled to custom MSIA D.A.R.T.'S (ThermoFisher Scientific, Tempe, AZ).

### 2.2. Calibration and quality control

Recombinant SARS-CoV-2 nucleocapsid protein (97-077) purchased from ProSci (Fort Collins, CO, USA) was used to make calibrators. Calibrators were made by spiking the recombinant protein into pooled qRT-PCR negative NP swabs in PBS at concentrations of 1 pM, 2.5 pM, 5 pM, 25 pM, and 100 pM. Given the sensitivity challenges of this type of testing, the calibration curve was designed to focus on low concentration samples instead of attempting to interpolate concentrations of the entire patient population. Quality control samples were prepared by pooling qRT-PCR negative NP swabs (negative QC), qRT-PCR samples with a cycle threshold (Ct) of 31 (Low QC), 27 (Medium QC), and 25 (High QC). Calibrators and QC were purified, digested, and analyzed as described above.

### 2.3. NP swab samples

NP swab samples collected in PBS were initially analyzed by qRT-PCR at Mayo Clinic using the Roche cobas 6800/8800 test which has been approved for use under the Emergency Use Authorization (EUA) by the United States Food and Drug Administration. Based on the qRT-PCR results 120 positive samples and 120 negative samples with adequate volume (over 1.5 mL) for analysis by both purification techniques were selected. To enhance our ability to compare the sensitivity of the techniques, the Ct distribution of the selected samples was intentionally skewed to higher Cts (lower viral loads). A histogram of the Ct distributions of positive samples analyzed in this study is shown in Figure S1 (generated using analyze-it, analyze-it Software, Ltd., Leeds, UK).

### 2.4. Preparation of NP swab samples when using anti-protein antibody-based purification

All clinical samples were de-identified prior to analysis. Nasopharyngeal (NP) swab samples were collected in PBS and 750 μL of the sample was transferred to a 96 well plate. The virus was inactivated by adding 15 μL of Z3-16 and incubating at 70 °C for 30 min. Following a 10 min cooling period at 4 °C, antibody-based purification was performed using the anti-nucleocapsid protein monoclonal antibody coupled to MSIA D.A.R.T.'S. When using MSIA D.A.R.T.'S, antibodies were shipped to ThermoFisher Scientific for immobilization using carbodiimide crosslinking chemistry. The purification procedure was conducted using the automated Versette liquid handling system. The tips were first washed with PBS and then nucleocapsid protein was captured over a period of 1.75 h. Following capture, the tips were washed twice with 300 μL of PBS and then twice with 300 μL of water. The nucleocapsid protein was eluted with 100 μL of 0.2% TFA and 0.002% Z3-16 in water. The purified sample was immediately trypsin digested (rapid digest kit, Catalog#VA1060, Promega, Madison, WI). Sample eluent was mixed with 300 μL of digest buffer followed by the addition of 1 μg of trypsin and incubated at 70 °C for 1 h. The digestion was stopped by adding TFA to a final concentration of 1% and isotopically labeled internal standards were added.

### 2.5. Preparation of NP swab samples when using anti-peptide antibody-based purification

750 μl of each nasopharyngeal swab sample was transferred to a 96-well plate. Viral inactivation and reduction of proteins was done by addition of 15 μL of 1 M DTT and 15 μL of 13% DOC and incubating at 70 °C for 30 min. Alkylation was done using 45 μL of 1 M IAA, vortexed, and incubated in dark for 30 min. Finally, 250 μL of 1 M Tris-HCl pH 8.0 buffer was added and sample was digested using 6.25 μg of Worthington TPCK treated trypsin (ThermoFisher) by incubating at 37 °C for 1 h. The reaction was stopped by adding 5 μg of TLCK to each sample. Target peptides were then purified using anti-peptide monoclonal antibodies coupled to MSIA D.A.R.T.'S. When using MSIA D.A.R.T.'S, antibodies were shipped to ThermoFisher Scientific for immobilization using carbodiimide crosslinking chemistry. The capturing, washing and elution was done as described in the previous section.

### 2.6. Targeted parallel reaction monitoring (PRM) analysis

The peptides were first loaded onto EvoTips (EvoSep Inc., Odense, Denmark) as per manufacturer's instructions. Briefly, the $C_{18}$ EvoTips were activated using 20 μL of 0.1% formic acid in 100% acetonitrile followed by equilibration with 20 μL of 0.1% formic acid in water. Activation and equilibration were carried out at 700 x g for 1 min using a benchtop centrifuge. The sample was loaded at 500 x g for 5 min followed by washing using 0.1% formic acid once. Last, the tips were loaded with 100 μl of 0.1% formic acid and processed for targeted mass spectrometry analysis.

Parallel reaction monitoring (PRM) analysis was performed on an Exploris 480 mass spectrometer (ThermoFisher Scientific, San Jose, CA) and interfaced with a preformed gradient LC system (EvoSep One, EvoSep Inc.). Peptides were eluted at a flow rate of 1.5 μL/minute and peptide separation was carried out using an 8 cm analytical column (Dr. Maisch $C_{18}$AQ, 1.5 μm, 150 μm x 8 cm) with an 11.5 min gradient. Data acquisition parameters included MS1 scan from *m/z* 500–1200 at a resolution of 60,000 (*m/z* 200) followed by retention time scheduled PRM analysis of target and corresponding IS peptides as shown in Table S1. The PRM parameters included: Orbitrap resolution of 30,000 (*m/z* 200), AGC target value of $1 \times 10^5$, injection time of 50 ms, isolation window of *m/z* 2 and HCD normalized collision energy of 30.

### 2.7. Targeted LC-MS/MS data processing

The PRM spectra were used for subsequent traditional and machine learning based data analysis. Traditional data analysis was done in TraceFinder (version 5.1, ThermoFisher) by summing selected fragment ions (±10 ppm) shown in Table S2 to produce extracted ion chromatograms that were integrated to produce total signal areas. We elected to use area to align with common practice in clinical laboratories, which are largely predicated on integration of chromatographic peaks to produce peak areas that can be quantitated based on external calibration curves. All signals at the retention time of the isotopically labeled internal standards were integrated and the areas of all signals were exported to Excel to define positive/negative results. These results were determined by establishing $a + 3$ standard deviation confidence interval (CI=mean+3*SD) based on the signal area in the negatives. This CI was then applied to all the signals to determine positive/negative results for each peptide.

When performing machine learning based analysis data were imported into Skyline [20] and fragment ion chromatograms were manually integrated. Then, the fragment ion intensities were exported from skyline and (natural) log transformed. A supervised machine learning method was used to select the optimal fragments and determine their weights to maximize the detection performance of the targeted mass spectrometry assay. All computations were performed in R (version 4.0.1). For this, we utilized an ensemble-based machine learning approach encoded in the Super Learner as described previously [21]. This method was configured to use a generalized linear model via penalized maximum likelihood (glmNET), generalized linear model (glm) and random forest model; all configured to use binomial distributions. This machine learning method was used to determine whether samples were positive or negative by mass spectrometry testing.

### 2.8. Untargeted LC-MS/MS analysis and data processing

Samples for untargeted experiments were prepared as described above. LC-MS/MS analysis for untargeted discovery proteomics experiments were carried out using an Ultimate 3000 RSLCnano system (ThermoFisher Scientific) connected to an Orbitrap Exploris 480 mass spectrometer. Samples were analyzed using an untargeted single-shot DDA method. The peptides were loaded onto a trap column (PepMap $C_{18}$ 2 cm × 100 μm, 100 Å) at a flow rate of 20 μl/min using 0.1% formic acid and separated on an analytical column (EasySpray 50 cm × 75 μm, $C_{18}$ 1.9 μm, 100 Å, Thermo Scientific) with a flow rate of 300 nL/min and a linear gradient of 5 to 40% solvent B (100% ACN, 0.1% formic acid) over 70 min. Both precursor and fragment ions were acquired in the Orbitrap mass analyzer. Precursor ions were acquired in *m/z* range of 350–1800 with a resolution of 120,000 (at *m/z* 200). Precursor fragmentation was carried out using higher-energy collisional dissociation (HCD) method using normalized collision energy (NCE) of 27. The fragment ions were acquired at a resolution of 30,000 (at *m/z* 200). The scans were arranged in top-speed method with 3 s cycle time between MS and MS/MS. Ion transfer capillary voltage was maintained at 1.9 kV. For internal mass calibration, lock mass option was enabled with polysiloxane ion (*m/z* 445.120025) from ambient air.

The raw mass spectrometry data were searched using Andromeda [22] in the MaxQuant software suite (version 1.6.7.0) against combined protein database containing UniProt human protein database, SARS-CoV-2 protein sequences, and common MS contaminants. The search parameters included a maximum of two missed cleavages; carbamidomethylation at cysteine as a fixed modification for samples that were reduced and alkylated; N-terminal acetylation and oxidation at methionine as variable modifications. Precursor tolerance was set to 10 ppm and MS/MS tolerance to ±0.02 Da. False discovery rate was set to 1% at the peptide-spectrum matches (PSMs), peptide and protein levels.

## 3. Results and discussion

A simplified schematic of the respective sample preparation procedures for the anti-peptide and anti-protein antibody-based purification techniques are shown in Fig. 1. Although the processes are similar, the most important implication of the inherent differences in methodologies is the benefits of reducing the total protein concentration prior to trypsin digestion. Digesting samples with less protein content allowed us to use less enzyme (1 μg vs. 6.25 μg) and made using the Promega Rapid Digest kit viable from a financial perspective. When using this product, we were able to eliminate the reduction/alkylation steps from our procedure because the digestion is performed under denaturing conditions (70 °C) and shorten the trypsin digestion incubation time by 1 h, which shortened the procedure by 1.5 h and eliminated steps. To compare the relative specificity and sensitivity of these techniques, both were utilized for the analysis of 120 positive samples with high Ct values by qRT-PCR and 120 negative samples.

LC-MS/MS analysis revealed several interesting characteristics of both techniques, which can be visualized in the representative extracted ion chromatograms for both the anti-peptide and anti-protein antibody-based purification from the most sensitive peptides AYNVTQAFGR and QQTVTLLPAADLDDFSK are shown in Fig. 2. Chromatograms from the lower performing peptides DGIIWVATEGALNTPK, NPANNAAIVLQLPQGTTLPK, and GQGVPINTNSSPDDQIGYYR are shown in Figure S2. The signal in the positive sample XICs was much higher when performing protein purification than peptide purification, although the factor by which it was higher varied considerably based on the affinity of the respective anti-peptide antibodies.

Due to the specificity of the LC-high resolution MS/MS technique used in this work, the noise in the chromatograms was generally very low, making the signal-to-noise algorithms in the software an ineffective method of assessing the results. Additionally, when attempting to differentiate positive/negative results using this methodology, the signal present at the expected retention time (based on coeluting IS) is of the utmost importance. Therefore, we elected to establish confidence intervals based on the signal present in the negatives as a threshold for positive/negative classification and a means to assess the sensitivity and specificity of a traditional XIC signal integration workflow. To do this, a CI was created based on the signal (area) in negative samples. This threshold was then applied to all samples to yield sensitivity, which was defined as the percentage of the 120 positive samples analyzed with signals above the threshold, and specificity defined as the percentage of the 120 negative samples below the threshold. A comparison of the results from these analyses can be seen in Table 1 for the peptides AYNVTQAFGR, QQTVTLLPAADLDDFSK, GQGVPINTNSSPDDQIGYYR, and NPANNAAIVLQLPQGTTLPK. This analysis was not conducted for the peptide DGIIWVATEGALNTPK due to extremely high carry-over on the LC-MS/MS system. In conjunction with the aforementioned chromatograms, these data show that the peptide purification is far more specific, as evidenced by the lower positive/negative threshold areas and higher percent specificities compared to protein purification. This is especially the case for the peptide NPANNAAIVLQLPQGTTLPK, which

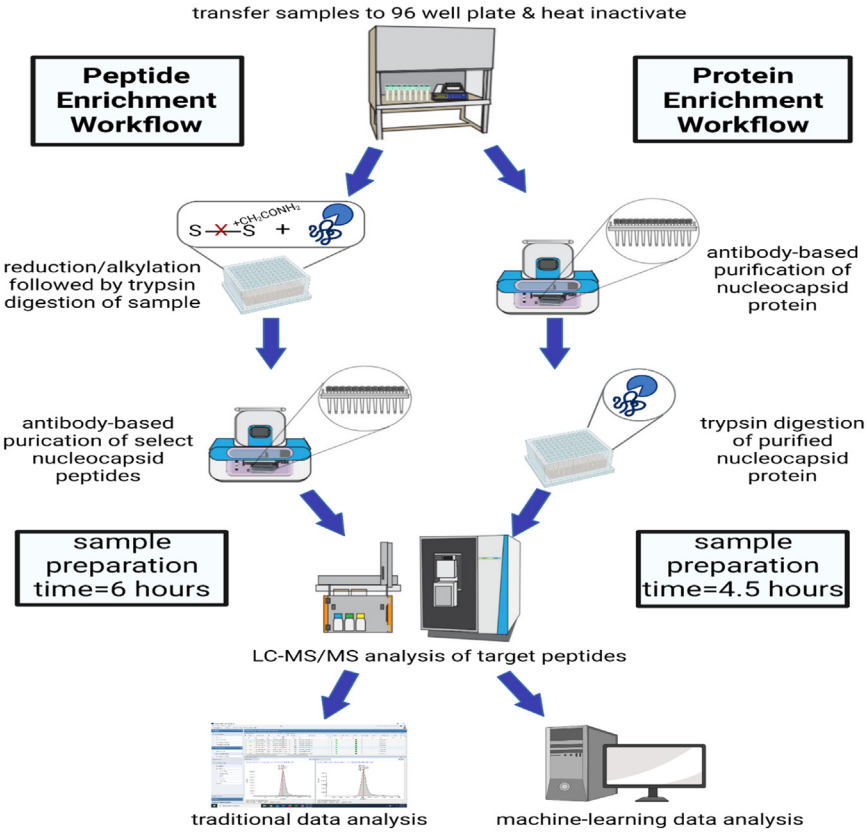


**Fig. 1.** Diagram of sample preparation procedures for the anti-peptide and anti-protein antibody-based purification techniques. When performing peptide enrichment samples are digested prior to purification and the sample preparation process takes 6 h. In contrast, protein enrichment necessitates post-purification digestion and only required 4.5 h of preparation time. Both enrichment and digestion products then underwent equivalent LC-MS/MS analysis followed by both traditional and machine learning data analysis.

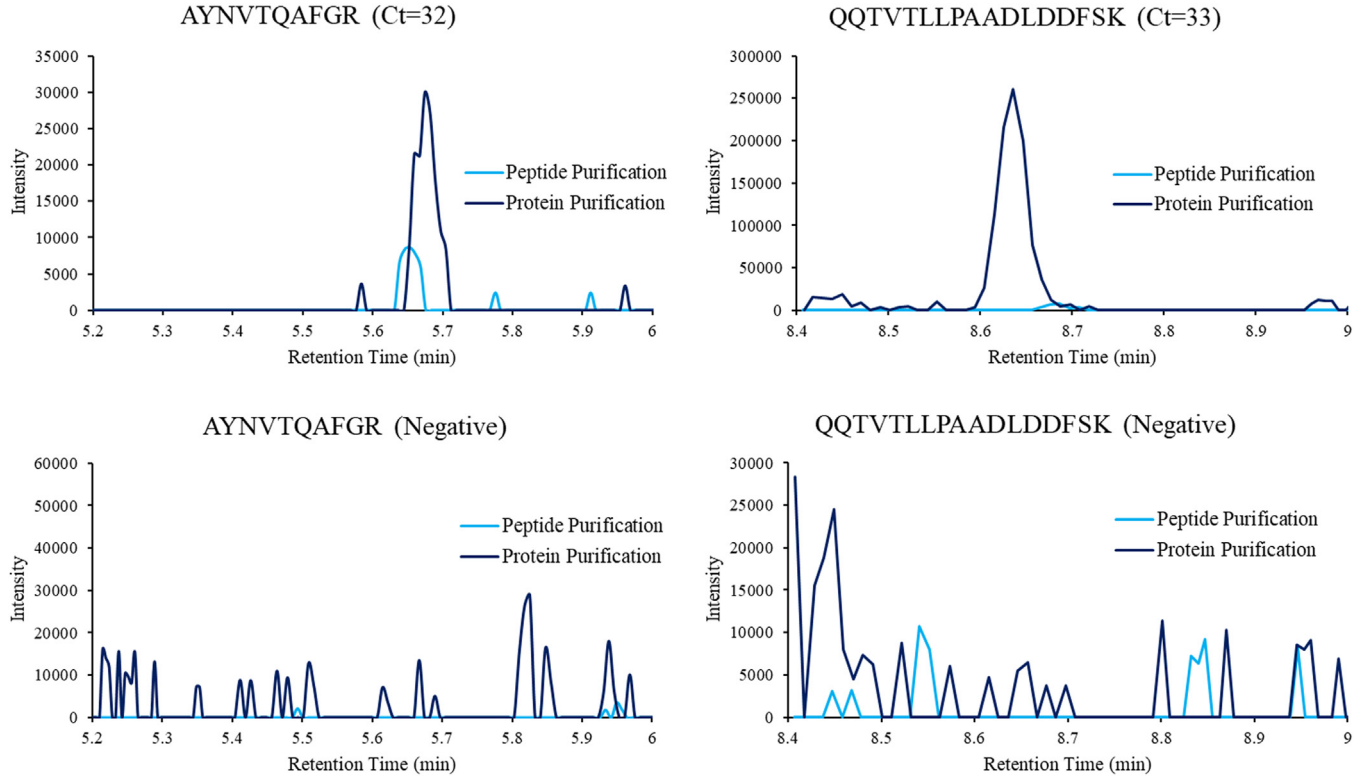


**Fig. 2.** Overlaid representative extracted ion chromatograms from targeted LC-MS/MS analysis of low viral load positive samples and negative samples (based on initial qRT-PCR results). Peptide enrichment yields lower signals in the positive samples, but lower background/noise in the negative samples. The inverse is true for protein enrichment.

**Table 1**
Comparison of positive/negative area thresholds, sensitivity, and specificity for the peptide and protein purification techniques. The positive/negative area threshold is established by calculating a confidence interval based on the signal intensities (area) in the XICs of the negative samples by qRT-PCR. The percent sensitivity is the number of positive samples (out of 120 analyzed) that were above this threshold and the specificity is the number of negative samples (out of 120 analyzed) below this threshold.

| Peptide-IP Technique | Positive/Negative Area Threshold (a.u.) | Sensitivity (%) | Specificity (%) |
|---|---|---|---|
| AYNVTQAFGR-Peptide IP | 9110 | 55.8 | 99.2 |
| AYNVTQAFGR-Protein IP | 14,391 | 53.3 | 97.5 |
| QQTVTLLPAADLDDFSK-Peptide IP | 5054 | 50 | 99.2 |
| QQTVTLLPAADLDDFSK-Protein IP | 32,359 | 58.3 | 97.5 |
| NPANNAAIVLQLPQGTTLPK-Peptide IP | 355 | 38.3 | 97.5 |
| NPANNAAIVLQLPQGTTLPK-Protein IP | N/A | N/A | N/A |
| GQGVPINTNSSPDDQIGYYR-Peptide IP | 413 | 28 | 97.5 |
| GQGVPINTNSSPDDQIGYYR-Protein IP | 10,738 | 37.5 | 95 |

**Table 2**
Test results from machine learning analysis of anti-peptide antibody purified samples.

| | Prediction | | |
|---|---|---|---|
| qRT-PCR Result | Negative | Positive | |
| Negative | 120 | 0 | Specificity=100% |
| Positive | 77 | 43 | Sensitivity=35.8% |

**Table 3**
Test results from machine learning analysis of anti-protein antibody purified samples.

| | Prediction | | |
|---|---|---|---|
| qRT-PCR Result | Negative | Positive | |
| Negative | 119 | 1 | Specificity=99.2% |
| Positive | 66 | 54 | Sensitivity=45% |

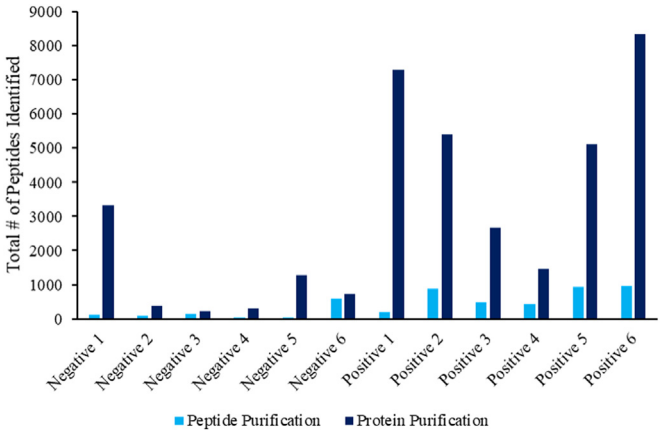


**Fig. 3.** Total peptides identified following antibody-based purification and untargeted LC-MS/MS analysis. As expected based on the background in the targeted analyses, peptide purification results in significantly fewer nonspecific peptides identified.

exhibited significant interference signal that prohibited establishing of a CI threshold. This can be visualized in Figure S2. However, despite the higher positive/negative thresholds, the protein level purification still yielded a higher percent sensitivity for three of the four peptides analyzed, indicating that the increase in signal intensity was proportionally higher than the increase in nonspecific background signals, which is corroborated by the larger signal intensities in the chromatograms, shown in Fig. 2 and Figure S2. For example, in the positive samples shown in Fig. 2 the signal intensity exceeds the described positive cut-off threshold when protein purification is utilized, but does not when peptide purification is used. This is reflective of the generally lower sensitivity when using peptide purification, which is exacerbated when using the machine learning approach described below.

These data were also analyzed via a novel machine learning approach for determining positive/negative results, which can be seen in Tables 2 and 3. Anti-peptide antibody-based purification resulted in 100% specificity and 35.8% sensitivity, whereas anti-protein antibody-based purification produced one false positive and a sensitivity of 45%. These data align with the findings from traditional analyses above; peptide purification is more specific, but protein purification is more sensitive. Receiver operating characteristic (ROC) curves for these results are shown in Figure S3. Peptide purification yielded a slightly better area under the curve (AUC) than protein purification (0.798 vs. 0.779) indicating slightly better performance of this technique overall for this challenging application.

As another means of comparing the specificity of the anti-protein and anti-peptide purification techniques we performed untargeted data-dependent LC-MS/MS analysis of 6 negative samples and 6 samples with high viral load based on qRT-PCR testing. The number of peptides identified in each sample by the MaxQuant database search against the human and SARS-CoV-2 proteome is shown in Fig. 3. Many nonspecific peptides from human proteins were identified; however, the anti-peptide antibody-based purification greatly reduced the number of nonspecific peptides identified. These data further support the conclusion that the peptide purification technique isolates the targets to a greater extent than the protein purification.

Our results largely align with the findings of Levernæs and coworkers [19], as they also noticed reduced background/noise when performing peptide purification when compared to protein purification; they also noticed the coinciding drop in signal intensity. However, they note a significant improvement in sensitivity for peptide purification compared to protein purification, whereas we have generally found protein purification to be more sensitive for our application. This discrepancy could be due to the much more proteinaceous sample (serum) they are analyzing causing increased benefits of the greater reduction in background signal. Our sample (NP swap) is much less complex by comparison. Additionally, the machine learning approach used herein is intended to minimize the impact of noise/background and would therefore disproportionally benefit from the increased signal and be more robust in the presence of noise.

We must note that the focus of this work was to compare sensitivities of two sample preparation techniques for LC-MS/MS analysis, not compare favorably to qRT-PCR. An underappreciated consideration when comparing LC-MS/MS to qRT-PCR is the Ct distribution analyzed because the viral load varies by many orders of magnitude in patient samples. In order to maximize our ability to compare sensitivities of the sample preparation techniques, we have skewed the Ct distribution to lower viral loads in this investigation. Therefore, this work is not a true reflection of the performance of the techniques relative to qRT-PCR. As

we discuss in our previous work, to effectively compare LC-MS/MS and qRT-PCR, we believe the Ct distribution should reflect the population of samples analyzed by the reference method [9]. When the distribution is not intentionally skewed, LC-MS/MS compares much more favorably to qRT-PCR (98% sensitivity, 100% specificity).

## 4. Conclusions

Decisions on whether to use anti-peptide or anti-protein antibody-based purifications must be made on a case-by-case basis. Many factors must be weighed when making this decision, such as throughput requirements, abundance of the target protein, propensity for interfering signals, and the relative affinities of the available antibodies. From an enzyme consumption perspective, it is advantageous to perform purification prior to digestion, which allows less enzyme to be used. Our results show that protein level purification resulted in higher signals when measured by LC-MS/MS, but were also less specific and yielded more noise and interfering background signals. Therefore, when using traditional chromatographic integration-based approaches for performing SARS-CoV-2 testing, the sensitivity relative to qRT-PCR was highly analyte (peptide) dependent. Machine learning analysis showed that protein purification was more sensitive, but peptide purification was more specific and resulted in a slightly better AUC overall.

## Data availability

The mass spectrometry data have been deposited to the ProteomeXchange Consortium via the MassIVE partner repository (PMID: 27924013) with the data set identifier MSV000088755.

## Author contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

## Abbreviations

LC, liquid chromatography; MS/MS, tandem mass spectrometry; MS, mass spectrometry; RT-PCR; real time-polymerase chain reaction; PBS, phosphate buffered saline; TFA, Trifluoroacetic acid; IS, internal standards; NP, nasopharyngeal; PRM, parallel reaction monitoring; Ct, cycle threshold; CV, coefficient of variation; LLOQ, lower limit of quantitation; SNP, single nucleotide polymorphisms; XIC, extracted ion chromatogram.

## Declaration of Competing Interest

All authors declared no conflict of interest.

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## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.sampre.2022.100018.

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