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Letter

# Pretreatment Methods for Human Nasopharyngeal Swabs to Increase the Signal to Noise Ratio of High Sensitivity Immunoassays

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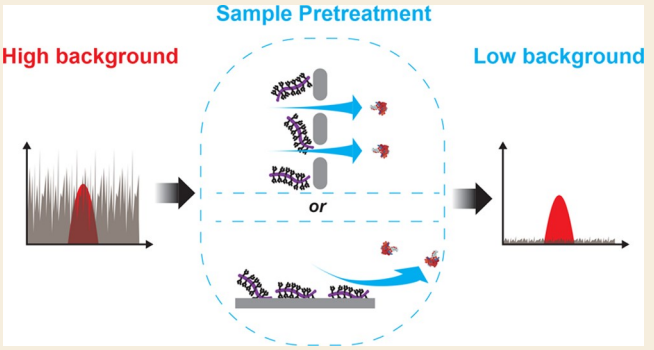
**ABSTRACT:** Mucous samples collected through nasopharyngeal (NP) swabs are considered gold standard specimens for the detection of respiratory pathogens. Matrices of these highly viscous samples often cause significant background noises in immunoassays, especially immunoassays with high sensitivity. We demonstrated such nonspecific background signals in both a chemiluminescence enzyme-linked immunosorbent assay (ELISA) and a novel highly sensitive immunoassay called Microbubbling SARS-CoV-2 Antigen Assay (MSAA). We developed and demonstrated the effectiveness of two quick sample pretreatment methods, filtration and preadsorption, to decrease nonspecific signals and increase the signal-to-noise ratio (SNR). Using these pretreatment methods, the SNR (at $3.6 \times 10^4$ copies/mL of inactivated SARS-CoV-2) was increased by 42.4-fold (95% CI 41.0−43.8) and 67.1-fold (95% CI 57.9−76.3) in the MSAA, and 1.3-fold (95% CI 0.9−1.7) and 1.8-fold (95% CI 1.6−2.0) in the chemiluminescence ELISA assay. Sample pretreatment methods developed in this study are broadly adaptable for the development of immunoassays for highly viscous samples.




**KEYWORDS:** *immunoassay, signal-to-noise ratio, nasopharyngeal swabs, pretreatment methods, microbubbling assay, digital assay, infectious diseases, mucin*

## INTRODUCTION

Acute respiratory infections, such as severe acute respiratory syndrome (SARS), severe acute respiratory syndrome-2 (SARS-2), Middle East respiratory syndrome (MERS), and Influenza are the leading causes of infectious diseases worldwide[1] and the most common causes of both illness and mortality in children under 5.[2] The key to infection control and prompt treatment of these diseases is sensitive and accurate diagnostic methods, which often require mucous samples collected from the respiratory tract. Samples collected using nasopharyngeal (NP) swabs are considered gold standard diagnostic specimens. Studies comparing specimen types including saliva, oropharyngeal (OP), nasal (NS), and NP swabs for SARS-CoV-2 nucleic acid amplification testing (NAAT) demonstrated that NP swab testing resulted in the highest positive rates or lowest cycle threshold (Ct) values.[3−5]

The current gold standard testing method for the detection of respiratory pathogens in NP swabs is reverse transcription polymerase chain reaction (RT-PCR), which requires highly trained medical personnel, specialized instrumentation, and supply limited reagents.[6,7] Moreover, positive nucleic acid results do not always predict infectivity. Studies showed that infectious SARS-CoV-2 viruses were absent from most specimens taken 8 days after symptom onset, despite recurrent positive RT-PCR results.[8−11] On the other hand, antigen testing results of SARS-CoV-2 were shown to correlate better with viral culture results than with nucleic acids, which may provide good risk prediction of transmissibility.[12,13] One major disadvantage of most commercially available rapid antigen immunoassays is low analytical sensitivity compared with RT-PCR. For example, some antigen immunoasssays for SARS-CoV-2 detection are 100 000-fold less sensitive than RT-PCR in patients suspected of SARS-CoV-2 infection, thereby in clinical practice more likely producing false negative results, especially for asymptomatic infections with a low viral load.[14] Our laboratory has developed several immunoassays with high analytical sensitivity, including the microbubbling SARS-CoV-2 antigen assay (MSAA).[13,15]

However, the viscous and complicated matrices in respiratory samples pose challenges for the detection of desired analytes using immunoassays, especially highly sensitive immunoassays. Immunoassays rely on high affinity and high specificity binding between antibodies with target

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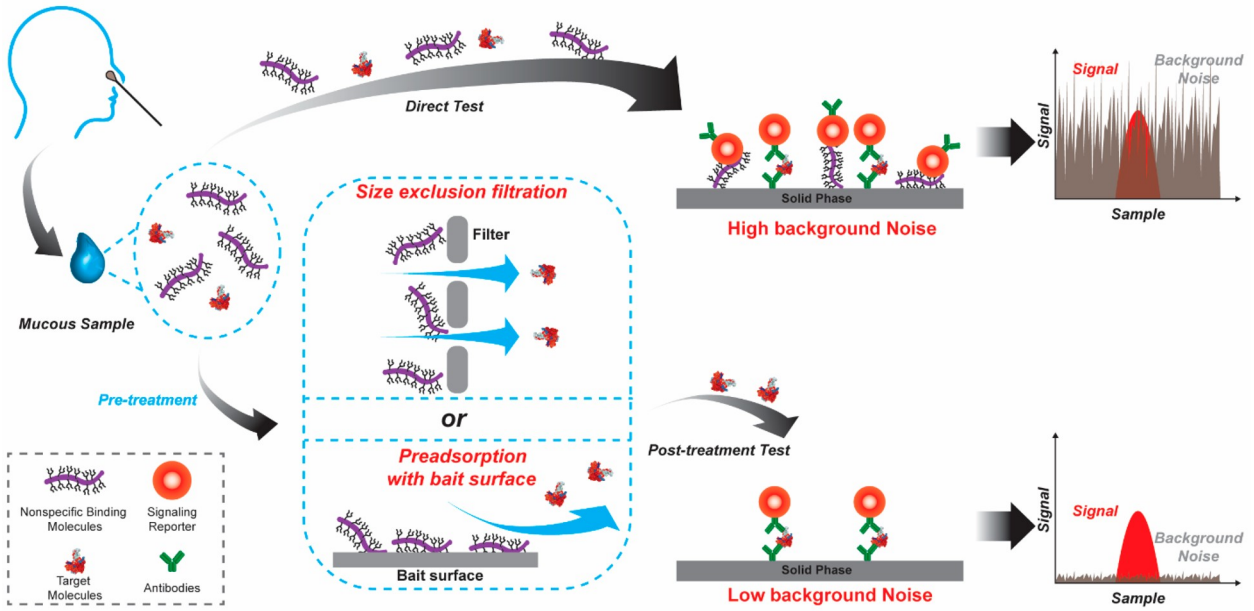


**Figure 1.** Illustration of the background noises caused by mucins in NP swabs in immunoassays and identified pretreatment methods to remove background and increase signal-to-noise ratio.

molecules. In highly sensitive immunoassays, low background signals and high signal-to-noise ratios are required to achieve high analytical sensitivity, which may be hindered by nonspecific background noises from respiratory samples. Mucin, one of the abundant components in the respiratory tract fluid network,[16] has been suggested as the culprit that causes high background noises in immunoassays. Mucins are large bottlebrush-shaped extensive glycosylated proteins (200 kDa to 200 MDa) that are viscous and can bind to a variety of surfaces through electrostatic or hydrophobic interactions.[16] To protect the respiratory tract from the attack of airborne droplets and particles containing viruses and irritants, secreted mucins connect with each other via end-to-end disulfide bonds to form heterogeneous networks with porous structures in nano/microsizes to capture inhaled particles on the surfaces of the respiratory tract.[16] The reproducible and selective removal or blocking of viscous porous mucins is important for sensitive and precise analysis using immunoassays. Previous studies have shown it is critical to remove or block mucins in saliva samples for immunoassays.[17−22] The current gold standard approach for saliva processing for immunoassay is the freeze−thaw−centrifuge (FTC) approach, in which saliva samples are treated with a freeze−thaw cycle followed by high-speed centrifugation (~14 000g) to precipitate the mucin aggregates.[19] However, the FTC approach requires freezers, high-speed centrifuges, and long processing times that are not amenable to field testing. Other approaches, including using blocking agents[20,21] and chemical treatments,[22] have also been investigated to decrease the background signals from saliva samples in different immunoassays. *N*-Acetyl-L-cysteine (NAC) as a mucolytic agent has also been used to reduce the background signal in immunofluorescence staining by breaking the disulfide bonds in the mucin network in throat wash samples.[23] While many pretreatment approaches for saliva and other respiratory tract fluid samples have been reported, the extent of background noises caused by NP swab samples and pretreatment measures to decrease NP swab background in high-sensitivity immunoassays are part of an understudied area.

In this report, we demonstrate the highly variable levels of background noises caused by individual NP swab samples in immunoassays as illustrated in Figure 1 top row. A chemiluminescence enzyme-linked immunosorbent assay (ELISA) and the Microbubbling SARS-CoV-2 Antigen Assay (MSAA) for the detection of SARS-CoV-2 nucleocapsid protein (N-Protein) are used as two example immunoassays with high sensitivity. The MSAA was previously developed by our group for the detection of SARS-CoV-2 N-Protein with a limit of detection (LOD) of 0.5 pg/mL. In the MSAA, individual sandwich complexes formed between magnetic bead/N-Protein/platinum nanoparticle (PtNP) are distributed in microwells in a microchip. Bright-field images of oxygen microbubbles generated through PtNP catalysis of $H_2O_2$ decomposition are captured using a smartphone camera, enabling a facile and highly sensitive signal readout.[13] The number and size of microbubbles correlate linearly with the target molecule concentration.[13,15]

We hypothesize that mucins increase background signals in immunoassays through nonspecific binding between signaling reporters and the surfaces of the capture solid phase, such as horseradish peroxidase (HRP) and the surfaces of 96-well plate wells in the chemiluminescence ELISA and the magnetic microbeads (2.8 $\mu$m) and PtNPs (140 nm) in the MSAA. Based on this hypothesis, we explored different pretreatment approaches to reduce the background signals from NP swab samples in both assays. Both size exclusion filtration and preadsorption with bait surfaces can effectively reduce the background signals and increase the signal-to-noise-ratio (SNR) in the two model immunoassays (Figure 1, lower row).

## MATERIALS AND METHODS

### Clinical Swab Samples

The study was approved by the Institutional Review Board (IBR) of the University of Pennsylvania. The NP swab samples were collected from patients presented to the Hospital of University of Pennsylvania as part of the routine clinical care for the qualitative detection of SARS-CoV-2 using RT-PCR and transported to the clinical laboratory


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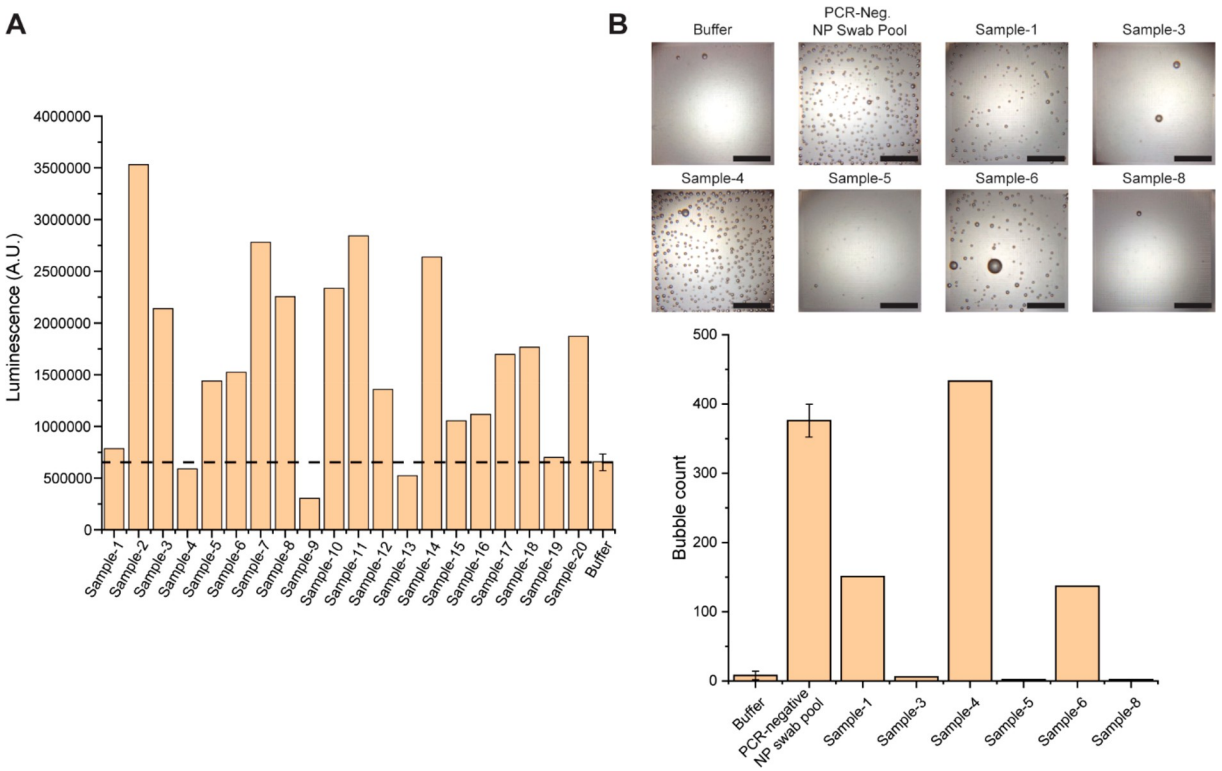


**Figure 2.** Highly variable background signals from individual RT-PCR-negative NP swab samples in the (A) chemiluminescence ELISA and (B) MSAA for SARS-CoV-2 N-protein; the raw microbubble images from MSAA are shown in the top panel, and the bubble count bar graph is shown in the lower panel. All scale bars represent 1 mm. Mean ± standard deviation; $n$ = 3.

in a 3 mL saline tube (Becton Dickinson cat#15439) according to standard clinical operating procedure. Residual swab samples were transferred to the research lab within 48 h of sample collection. Once the samples were in the research lab, Halt Protease inhibitor cocktail was immediately added at a 1:100 v/v ratio following the manufacturer's instruction and the samples were then stored at −80 °C. Before the immunoassay testing, samples were thawed at room temperature and then tested. For the PCR-negative pool, the same protocol was used after 100 PCR-negative samples were mixed in the research lab.

## Filtration Pretreatment of NP Swab

NP swab samples of 150 $\mu$L were processed using the centrifugal filter (Pierce protein concentrators PES, 100 K MWCO) by centrifuging at 12 000$g$ for 10 min at room temperature (22 °C), and 100 $\mu$L of the filtrate was collected and used for each assay subsequently.

## Preadsorption Pretreatment of NP Swab

NP swab samples of 150 $\mu$L were preincubated with 2 × $10^6$ carboxyl magnetic beads as the bait surface for 10 min at room temperature (22 °C). Beads were then pulled down using a magnet, followed by the collection of 100 $\mu$L of the supernatant for each assay subsequently.

## Microbubbling SARS-CoV-2 Antigen Assay (MSAA)

NP swab samples (200 $\mu$L) were first mixed with a virus lysis buffer of 10% TWEEN 20 (100x, 2 $\mu$L) and incubated at room temperature for 30 min. The protocol for the MSAA was then followed as previously published. Briefly, sample solutions (100 $\mu$L) were incubated with suspensions of 500 000 capture antibody functionalized magnetic beads on a roller (12 rpm) at room temperature for 30 min. The beads were then separated using magnets, washed three times with PBS buffer pH 7.4 containing 0.01% TWEEN 20, and then resuspended in 100 $\mu$L of 250 ng/mL biotinylated detection antibody in PBS containing 1% bovine serum albumin (BSA) on a roller (12 rpm) at room temperature for 30 min. The beads were then separated using magnets, washed three times with PBS buffer pH 7.4 containing 0.01% TWEEN 20, and then resuspended in 100 $\mu$L of 1 $\mu$g/mL NeutrAvidin functionalized PtNP in PBS containing 1% BSA on a roller (12 rpm) at room temperature for 30 min. The beads were then separated using magnets, washed three times with PBS buffer pH 7.4 containing 0.01% TWEEN 20, and resuspended in 100 $\mu$L of 30% $H_2O_2$. The magnetic beads slurries were then added into the chambers of the microbubbling microchips. The microbubbling microchips were placed on neodymium disc magnets for 1 min to pull down the beads to the bottom of the microchips. Images of microbubbles in the microwell arrays were captured via a smartphone camera attached to a mobile microscope. The same protocol was used for the counting of bubble numbers as previously published.[13] Briefly, a computer vision algorithm was designed to process these images to detect microbubbles and count the number of detected bubbles, whose radius was above and below a set threshold (50 $\mu$m, corresponding to 8 pixels in the images).

## Chemiluminescence ELISA for SARS-CoV-2 Antigen Detection

A volume of 50 $\mu$L of 10 $\mu$g/mL capture antibody (N-1) was added in a well of 96-well half area microplate and incubated at 4 °C overnight to coat the surface of the microplate well. Then the well was washed three times with PBS buffer containing 0.05% TWEEN 20. A volume of 150 $\mu$L of 5% BSA was added into the well and incubated for 2 h at room temperature (22 °C) to block the wells. Then the wells were washed three times with PBS buffer containing 0.05% TWEEN 20. For the filtration pretreatment, lysed NP swab samples were processed using the centrifugal filter (Pierce protein concentrators PES, 100 K MWCO) by centrifuging at 12 000$g$ for 10 min, collecting the filtrate for following assays. For the preadsorption pretreatment, samples were preincubated with 2 × $10^6$ carboxyl magnetic beads as the bait surface for 10 min at room temperature (22 °C). Beads were then pulled down with a magnet to collect the supernatant for the following assays. Then 50 $\mu$L of treated or untreated samples was added into each well and incubated for 30 min at room temperature (22 °C). The wells were washed 3 times with PBS with 0.05%


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TWEEN 20. A volume of 50 μL of 250 ng/mL biotinylated detection antibody in PBS containing 1% BSA was added into the wells and incubated for 30 min at room temperature (22 °C). The wells were washed three times with PBS with 0.05% TWEEN 20. Next, 50 μL of Pierce High Sensitivity Streptavidin-HRP (prediluted, catalog number: 21134) was added into the wells and incubated for 30 min at room temperature (22 °C). The wells were again washed three times with PBS with 0.05% TWEEN 20. After that, 50 μL of FemtoGlow Horseradish Peroxidase CL (HRP-CL) substrate was added into the well. The chemiluminescence signal was subsequently read with a plate reader (Tecan, Infinite 200 PRO).

## RESULTS AND DISCUSSION

### Background Signal from NP Swab Samples in High-Sensitivity Immunoassays

To investigate the extent of background signals from the NP swab samples, we first used the widely used chemiluminescence ELISA as a model high sensitivity immunoassay (LOD for SARS-CoV-2 N-Protein at 13.1 pg/mL, as shown in Figure S1) to measure luminescence signals of 20 NP swap samples with negative SARS-CoV-2 RT-PCR results. Among these samples, 85% (17/20) of the samples generated nonspecific signals higher than that of the blank buffer control (Figure 2A) at variable levels in the chemiluminescence ELISA. Six individual NP swab samples with negative SARS-CoV-2 RT-PCR results and a negative NP swab pool (100 NP swab samples with negative RT-PCR results for SARS-CoV-2 pooled together) were tested using the MSAA with the same pair of capturing and detecting antibodies (dose response curve and LOD previously reported[13]). Strong nonspecific background signals were observed in the negative NP swab pool and 50% (3/6) of individual negative samples (Figure 2B). As the volumes of these clinical NP swab samples were limited, only five individual samples were tested using both the chemiluminescence ELISA and the MSAA. In these five samples, no strong correlations of background signals between chemiluminescence ELISA and MSAA were observed. For example, although no strong background signal from sample 4 was observed in Figure 2A, the MSAA result of sample 4 showed a much higher background reading than that of the buffer control in Figure 2B. Although different assay reagents and signal reading methods were used in these two assays, which might cause differences in absolute signal levels, these factors alone cannot fully explain the lack of correlation in relative background levels. The significantly different background patterns might be due to the heterogeneity of mucins in the NP swab samples and their different interactions with the different solid surfaces involved in these two assays (polystyrene (PS) microplate wells in chemiluminescence ELISA, and magnetic iron oxide microbeads and PtNP in MSAA). The porous and viscous mucin networks are known to bind and trap micro/nanoparticles, which may explain the increased background signals in MSAA. The variations of background signals among individual NP swabs were high in both assays, likely due to the high variability in the matrix compositions of individual samples. Similar findings have been reported with saliva samples.[18] Therefore, in order to obtain sensitive and specific results for the target analyte (in this case SARS-CoV-2 antigen), it is critical to minimize or eliminate the highly variable background signals from each sample for each assay of interest. Due to the limited volume of individual NP swab samples and the large variations between individual sample matrices, we used the negative NP pool in the following part of this report to identify effective methods to remove the collective background signals.

### Methods to Decrease Nonspecific Background Signals

**Blocking Nonspecific Binding.** Previous studies reported that polyethylene glycol (PEG) or BSA modification can block nonspecific binding in immunoassays.[21,24] To investigate if these measures lead to decreased background signal in the MSAA, we coated magnetic beads with BSA or PEG without the capturing antibody and used these beads in the MSAA to test the negative NP swab pool. Strong background signals were still observed under both conditions (Figure S1). The background signals persisted without the detecting antibody in the assay (Figure S2), indicating the background signals were entirely nonspecific and unrelated to the antibodies used.

Several studies have investigated using blocking reagents to lower background signals in immunoassays.[20,21,25] We tested a variety of blocking reagents (sodium dodecyl sulfate (SDS), dextran, polyethylene glycol (PEG), poly(vinyl alcohol) (PVA), polyvinylpyrrolidone (PVP)PVX (1:1 mixture of PVA and PVP), cellulose, Triton X100, ChemiBlock, polyethylenimine (PEI), polystyrenesulfonates (PSS), sucrose) by adding them into the negative NP swab pool with MSAA. None of those blocking reagents successfully eliminated the nonspecific background signal (Figure S3).

**Pretreatment of NP Swab Samples.** *Mucolytic Agent Treatment.* After secretion, individual mucin molecules can connect with each other via end-to-end disulfide bonds to form heterogeneous networks with porous structures in nano/microsizes.[16] Therefore, mucolytic agents that can break disulfide bonds, such as NAC, are commonly used in treating samples from respiratory tract to minimize the interferences of mucins.[26] For example, NAC has been used to reduce the background signal in immunofluorescence staining for the detection of SARS virus in throat wash samples.[23] We hereby tried to pretreat NP swab samples with NAC at different concentrations for 30 min at room temperature and then tested them with MSAA. As shown in Figure S4, pretreatment with NAC decreased the background signal in MSAA compared with the untreated sample. But a significant background signal still existed even after increasing the concentration of NAC to 10%, which is 20-fold higher than the concentration previously used in immunofluorescence staining.[23] This finding was consistent with the previously reported treatment with NAC with throat wash samples, in which small amounts of mucin were still seen after the treatment of NAC.[23] But this small amount of mucin might still interfere with assay performance especially for immunoassays with high analytical sensitivity such as MSAA.

*Dilution.* We then explored diluting swab samples to minimize the background signals. The negative NP swab pool was serially diluted up to 30 times and then tested using the MSAA. The majority of the strong background signals remained (Figure S5). Extremely high dilutions might eventually remove the background signals but we suspect analytical sensitivity will suffer greatly. Therefore, dilution alone is not a feasible approach to remove the background signals in NP swabs for immunoassays. This finding is consistent with those found with saliva samples.[22]

*Molecular Filtration.* Filtration can be used to remove interfering components in clinical samples for immunoassays; for example, filters made of glass fiber[27] and polypropylene (PP)[28] have been used to pretreat saliva samples to lower


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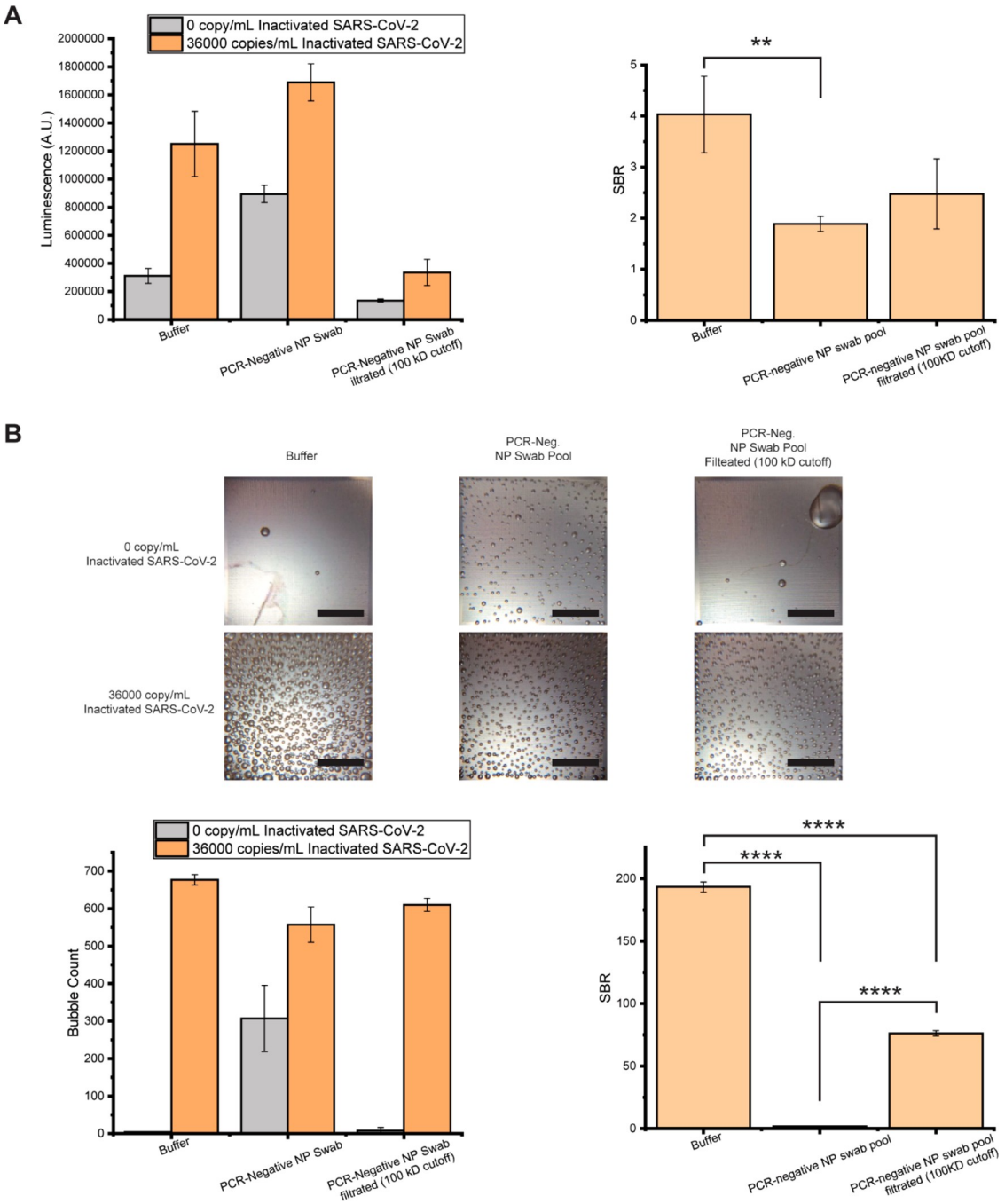


**Figure 3.** Reduction of nonspecific signals and improvement of signal-to-noise ratio (SNR) using filtration in (A) chemiluminescence ELISA and (B) MSAA for the detection of N-Protein of SARS-CoV-2. Scale bar: 1 mm. *$P \leq 0.05$; **$P \leq 0.01$, ***$P \leq 0.001$; ****$P \leq 0.0001$. Mean ± standard deviation; $n = 3$.

background signals in immunoassays. However, to our best knowledge, filtration has not been used in the pretreatment of NP swab samples to lower the background signals in immunoassays. A molecular filter can be used to eliminate undesirable molecules with a certain molecular weight cutoff. Since the molecular weight of N-Protein is 45.6 kDa and the molecular weight of mucins is above 200 kDa,[16,29] we pretreated NP swab samples with a centrifugal filter (100 kDa cutoff) and then collected filtrate to be used for both the chemiluminescence ELISA and the MSAA. RT-PCR negative NP swab pool and negative NP swab pool spiked with 36 000 copies/mL inactivated SARS-CoV-2 viruses were treated using this method and tested using the chemiluminescence ELISA. As shown in Figure 3A, the filtration pretreatment decreased both specific and nonspecific signals for SARS-CoV-2 in the swab pool. However, the SNR increased 1.3- fold (95% CI 0.9−1.7), although still lower than the SNR of the buffer. More pronounced improvement in SNR was observed for the MSAA (Figure 3B, 42.4-fold, 95% CI 41.0−43.8), with the majority of the SARS-CoV-2 specific signals retained while nonspecific signals greatly reduced. Two RT-PCR-positive NP swab clinical samples (Sample 1 and Sample 2) were pretreated using the same filtration method and tested using MSAA (Figure S6A). Filtration pretreatment reduced the absolute


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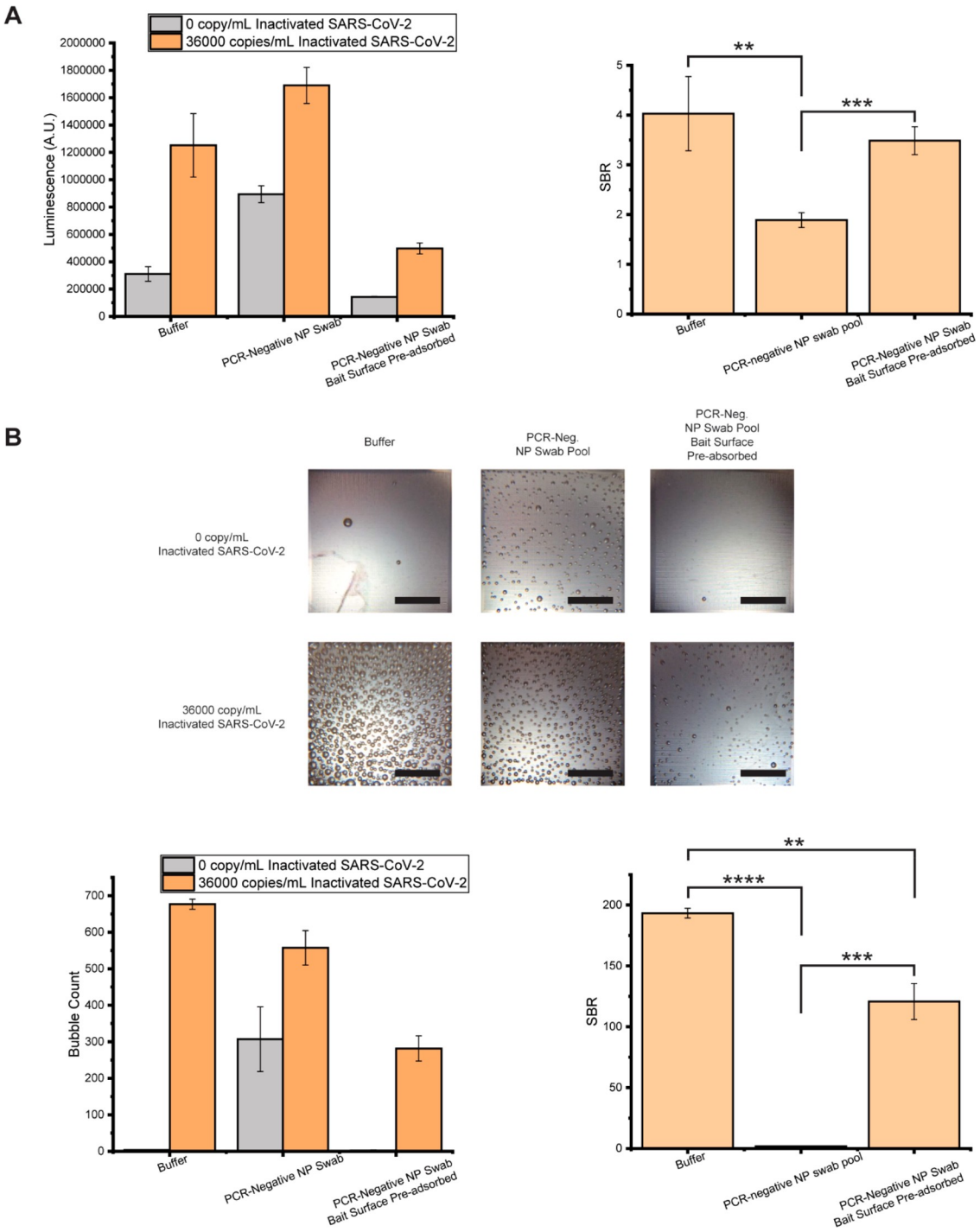


**Figure 4.** Reduce nonspecific signals and improve SNR using preadsorption in (A) chemiluminescence ELISA and (B) MSAA for the detection of N-Protein of SARS-CoV-2. All scale bars represent 1 mm. *$P \leq 0.05$; **$P \leq 0.01$, ***$P \leq 0.001$; ****$P \leq 0.0001$. Mean ± standard deviation; $n$ = 3.

level of assay signals, likely due to the combination of nonspecific signal removal and some N-Protein loss during the filtration. However, assay readout was still robust, indicating the presence of SARS-CoV-2 antigens, with about 25-fold improvement of the LOD in MSAA, as shown in Figure S7 and Table S1. The better improvement for MSAA is likely due to the lower analytical sensitivity and narrower dynamic range of MSAA. These results suggest that centrifugal filtration pretreatment is an effective method to remove nonspecific background signals and enhance SNR for NP swabs in highly sensitive immunoassays.

*Preadsorption Using Bait Surfaces.* Although the centrifugal filtration pretreatment was effective, it increases assay costs and requires a high-speed centrifuge. To identify another pretreatment solution that is more friendly for in-field use, we experimented with preabsorption using bait surfaces. Preadsorption has been widely used in ligand screening, such as phage display,[30] and in lowering antibody cross talks in Western blot and ELISA when serum samples are used.[31,32]


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But to our best knowledge, preadsorption has not been used before to lower the background signals in NP swab samples for immunoassays. Unconjugated magnetic beads were used as bait surfaces to adsorb the nonspecific binding moieties in NP swab samples before they interact with assay components. The NP swab pool with or without spiked SARS-CoV-2 was first incubated with bait magnetic beads. The bait beads were then pulled down using a magnet, and the supernatant was collected for immunoassays. As shown in Figure 4A, the SNR of the SARS-CoV-2 spiked NP swab pool increased by 1.8-fold (95% CI 1.6−2.0) in the chemiluminescence ELISA, though it is still lower than the SNR of the SARS-CoV-2 spiked buffer. A more pronounced SNR improvement was observed in the MSAA (Figure 4B, 67.1-fold, 95% CI 57.9−76.3), with some SARS-CoV-2 specific signal showing as decreased while nonspecific signals greatly reduced. Two RT-PCR-positive clinical NP swab samples (Sample 3 and Sample 4) were pretreated with bait preadsorption and tested using the MSAA (Figure S4B). Like the filtration method, while preadsorption decreased overall absolute signals, robust readout was retained, with about 64-fold improvement of the LOD in MSSA, as shown in Figure S7 and Table S1, indicating the presence of SARS-CoV-2 antigens. Similar to the filtration method, the better improvement on MSAA is likely due to the lower analytical sensitivity and narrower dynamic range of MSAA. This suggests that the preadsorption pretreatment is also an effective method to remove nonspecific background signals and enhance SNR from NP swabs in highly sensitive immunoassays. The more pronounced effect for MSAA is likely due to the nano/micro particles used in MSAA. Mucous components in the NP swab sample, such as mucins, usually form heterogeneous networks with nano/micro porous structures to capture and trap foreign particles. Therefore, they are more likely to cause high background signals in immunoassay using nano/micro particles and are more effectively removed using porous microparticles.

## CONCLUSIONS AND OUTLOOK

In conclusion, we have demonstrated the highly variable nonspecific background signals from individual NP swab respiratory samples in both a chemiluminescence ELISA assay and the MSAA with femtomolar analytical sensitivity. These nonspecific signals likely resulted from the nonspecific binding of mucins to assay surfaces and were unrelated to antibody specificity. We further demonstrated the effectiveness of two pretreatment methods, filtration and preadsorption, to remove nonspecific background signals and increase the SNR for both assays. Although this study only used two assays detecting SARS-CoV-2 N-protein as model immunoassays, our proof of concept study indicates that the identified sample pretreatment methods can be potentially applied to the detection of other targets in NP swab samples in other immunoasssays, especially highly sensitive immunoassays. Furthermore, the pretreatment methods can also be potentially applied to and modified for other respiratory samples, though this study only focused on NP swab samples. The effectiveness of both pretreatment methods depends on the abundance of mucins in the respiratory sample relative to the capacity of the filter or bait surfaces. In future studies, the type of filter and bait surface may be further optimized to further enhance the SNR and result in more streamlined workflows.

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsmeasuresciau.2c00024.

Experimental materials; cell culture and virus titration; fabrication of microbubbling microchips; functionalization of superparamagnetic microbeads and platinum nanoparticles; dose−response curve of chemiluminescence immunoassay; BSA and PEG were unable to block background signals from NP swabs in the MSAA; screening of blocking reagents to remove the background signals from NP swabs in the MSAA; NAC pretreatment was unable to completely remove background signal from NP swabs in the MSAA; serial dilution was unable to decrease background signal from NP swabs in the MSAA; compare the signals of RT-PCR-positive NP swab samples in MSAA with/without pretreatment; comparison of dose−response curves with different sample types; comparison of LODs with different sample types (PDF)

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### Notes

The authors declare no competing financial interest.

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