SUPPORTING INFORMATION

Paper-Based Immunoassay with Signal Amplification for Sensitive Detection of Nucleocapsid Protein Toward the Diagnosis of Long COVID

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PREPARATION OF 2D-MICROFLUIDIC DEVICE

Aldehyde-functionalized paper

The Whatman-1 chromatography paper was immersed in a 0.03 M KIO_4 solution in distilled water and incubated at 80°C for 3 hours. After incubation, the paper was washed three times with distilled water to remove any unreacted KIO_4 and then air-dried at room temperature for 12 hours. The functionalized paper sheets were stored in a desiccator for at least 12 hours to ensure complete dryness before further use. The paper functionalization was performed following methods established in previous studies by our group [1,2]. Potassium periodate (KIO_4) was used to introduce aldehyde functional groups onto the paper surface, which enabled the covalent binding of capture antibodies (**Figure S1**). These aldehyde groups subsequently formed Schiff base adducts with lysine residues on the antibody surface, facilitating stable immobilization.

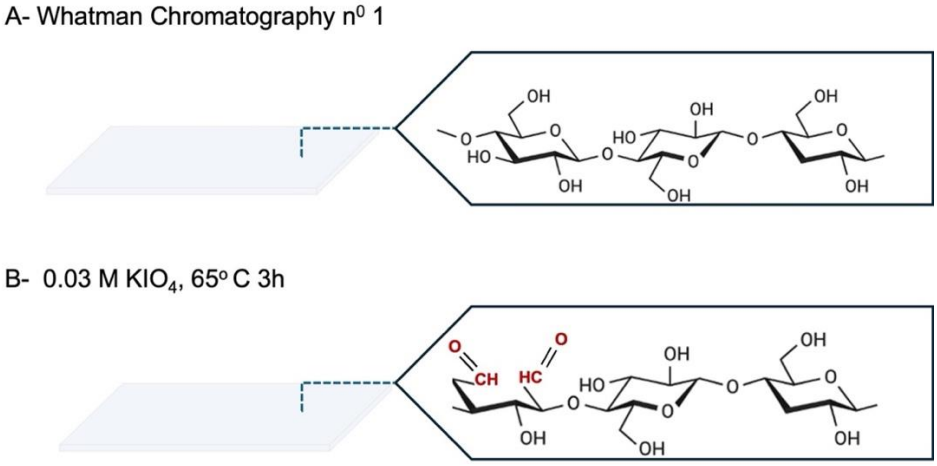

Figure S1. Preparation of aldehyde-functionalized hydrophilic test zones on paper for covalent immobilization of cAb. A- Whatman chromatography paper containing cellulose groups on its surface. B- The paper was immersed in an aqueous solution of potassium periodate at 65°C for 3 hours, resulting in the formation of aldehyde functional groups on its surface.

Wax Printing

Microfluidic channels were created on the functionalized paper substrates with aldehyde groups using wax printing, a simple and cost-effective technique that generates both hydrophobic and hydrophilic regions (**Figure S2**). This method

selectively protects certain areas of the paper, allowing hydrophilic zones to form where capture antibodies can be immobilized. A wax mask, consisting of circular, wax-free regions (3 mm in diameter), was printed on the functionalized paper using a solid ink printer set to default photo-quality parameters. The printed sheets were then heated at 150°C for 60 seconds, causing the wax to melt and spread through the paper, resulting in circular hydrophilic zones (approximately 2 mm in diameter) surrounded by hydrophobic wax barriers. The functionalized paper sheets were stored in a desiccator to preserve the reactivity of the aldehyde groups before use in the immunoassay.

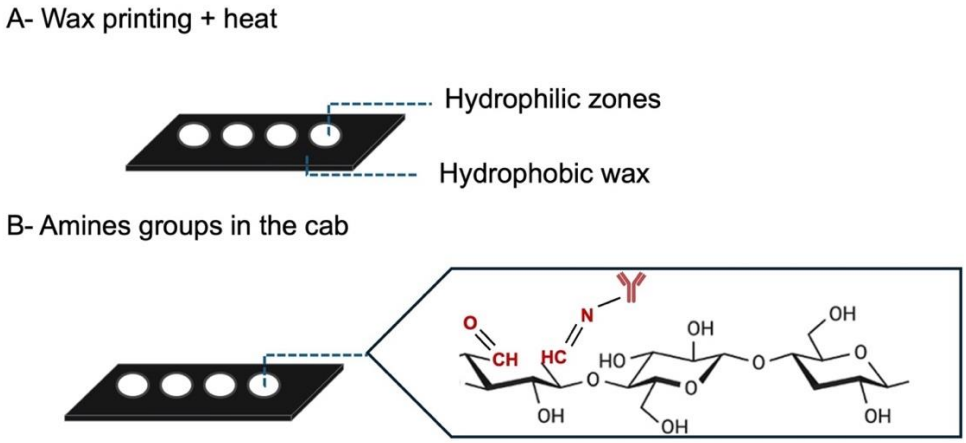

Figure S2. Paper functionalization process. A- After drying, the functionalized paper was printed with solid wax and heated to create hydrophilic test zones surrounded by hydrophobic wax barriers. B- Immobilization of the detection antibody on the functionalized paper was carried out by incubating the antibody (1 mg/mL) for 3 hours, allowing interaction between the functionalized groups on the paper and the amine groups of the antibody.

Bioactive Paper Chip

Once the paper is properly functionalized, the covalent immobilization of the cAb is performed as follows: Antibody application: A small amount (4 µL) of a 1 mg/mL capture antibody (cAb) solution is applied to the test zone on the paper. Immobilization reaction: The antibody remains in contact with the aldehyde-functionalized paper for 3 hours at room temperature. During this time, the amine groups present in the lysine residues of the antibody react with the aldehyde functional groups on the paper. The test zones were washed twice with 20 µL PBS **(phosphate-buffered saline).** This reaction forms a Schiff base, which creates a stable covalent bond, ensuring strong attachment of the antibody to the

test zone. Blocking unreacted sites: After the immobilization step, any remaining unreacted aldehyde groups must be blocked to prevent unwanted interactions in the assay. This is done by adding 20 µL of Tween-20/TBS solution and allowing it to react for another 3 hours. After the test zones were washed twice with 20 µL PBS. Formation of the bioactive paper: Once this process is complete, the paper now contains stably immobilized antibodies and is ready to be used in an immunoassay (**Figure S3**).

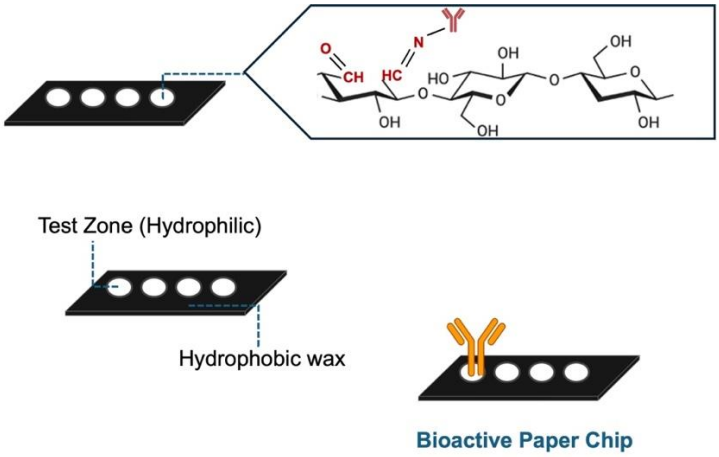

Figure S3. Bioactive paper chip. The detection antibody is immobilized in the test zone, where its amine groups bind to the hydroxyl (-OH) groups of functionalized paper. To prevent nonspecific binding, unoccupied sites are blocked, ensuring higher detection specificity. The bioactive paper chip is ready for use after the immobilization of the capture antibody and the blocking of non-specific sites.

SYNTHESIS OF THE IONIC PROBES

The ionic probe 4-(4-isothiocyanatophenetoxy)-N, N, N-trimethyl-4-oxobutan-1-aminium chloride (ITBA) [2] was synthesized in our laboratory via Steglich esterification, as previously described by Neises e Stiglich (1978) [3] (**Figure S4**). The ionic probe contains an isothiocyanate functional group at one end, which readily reacts with primary amines to form a stable thiourea linkage. This reaction enables selective modification of antibodies by targeting the lysine residues on their surface, allowing for precise functionalization.

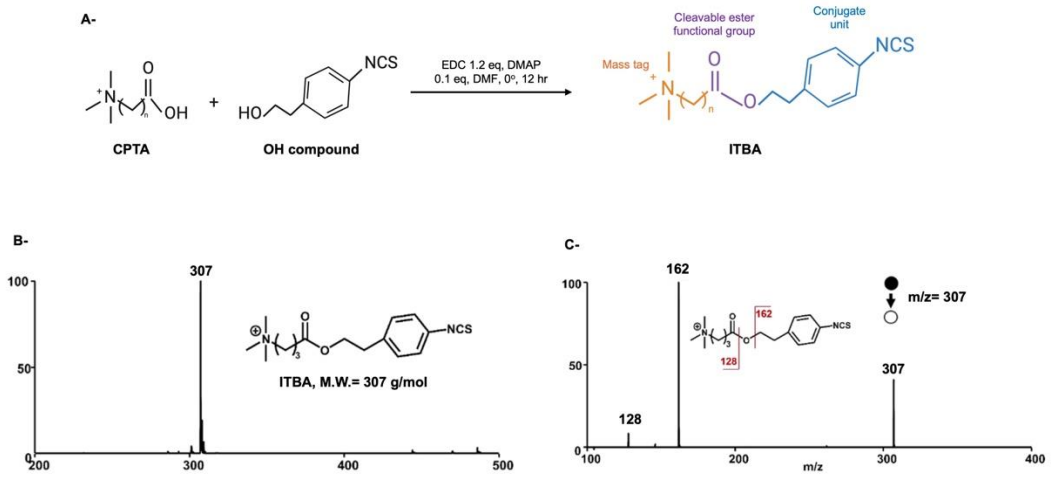

Figure S4. A- Reaction Scheme for the Synthesis of Cleavable Ionic Probes via Steglich Esterification. The final product consists of three key components: a mass tag ((3-carboxypropyl) trimethylammonium chloride, CPTA), a cleavable ester functional group, and a conjugation unit. B- MS spectrum of ITBA showing its peak at m/z 307 and C- MS/MS spectrum representing possible fragmentations for structural elucidation.

PREPARATION OF DETECTION ANTIBODY CONJUGATED WITH PROBE

The PAMAM dendrimer of the 4th generation was conjugated with the goal of amplifying the probe's signal, aiming to enhance the sensitivity of the detection device. Initially, the dendrimer was reconstituted by removing the solvent and adding a buffer solution (PBS), which ensured the stabilization of its dendritic structure and created an appropriate environment for the incorporation of the ionic probes. The ionic probes were then added to dendrimer, and the reaction was maintained for 12 hours at 4 °C, allowing the probes to bind to the available functional groups on the dendrimer. After this period, the excess probe was removed by centrifugation and washing using 3 kDa filters, ensuring that only the probes bound to the dendrimer remained in the solution (**Figure S5,i**).

Next, a glutaraldehyde solution was added. The reaction was maintained at room temperature for 10 minutes, and after this period, the excess glutaraldehyde was removed by centrifugation using 3 kDa filters (**Figure S5,ii**).

The next step involved buffer exchange for the detection antibody (dAb). The buffer exchange was performed by adding a Sodium Buffer Solution (SBS) to the antibody, followed by centrifugation and washing with 100 kDa filters. After the buffer exchange, the antibody solution was mixed with the dendrimer previously

conjugated with the ionic probe, and the reaction was maintained at 4 °C for 12 hours (**Figure S5,iii**). After this period, the solution was again centrifuged and filtered using 100 kDa filters to remove any excess unreacted reagents or impurities. The final product obtained was stored in a freezer at -20 °C until ready for use, ensuring the stability of the conjugation.

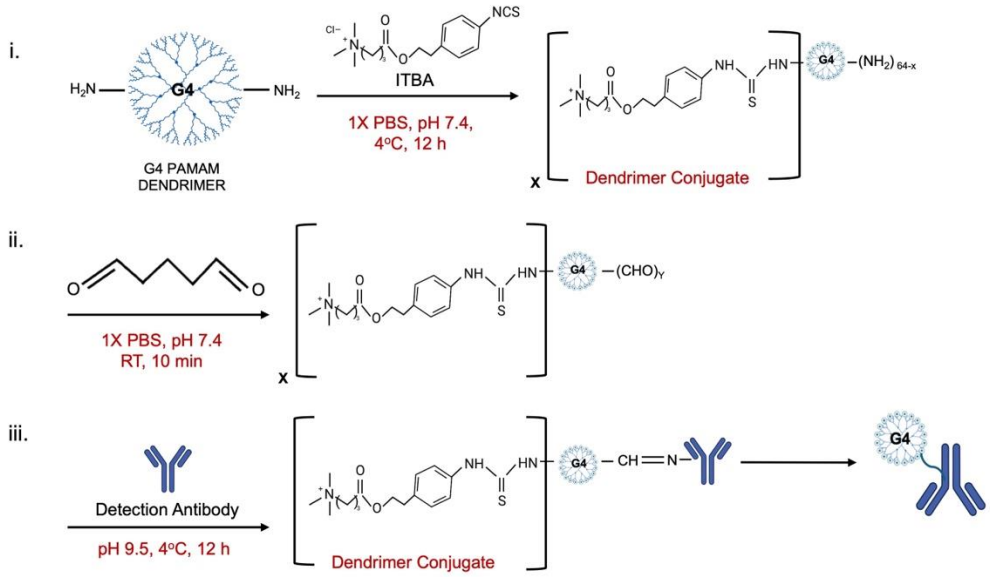

Figure S5. Conjugation process of the 4th generation PAMAM dendrimer with ionic probes and detection antibody. i. The dendrimer is reconstituted in buffer and conjugated with ITBA. ii. Glutaraldehyde is added as a crosslinking agent. iii. The antibody is pH-adjusted and conjugated with the modified dendrimer. Final Product: Detection antibody conjugated with the ionic probe and dendrimer, ready for application.

IMMUNOASSAY OPTIMIZATION

In this study, we adopted the protocol previously established by our group for malaria diagnostic methodology [2]. However, adjustments to certain steps were necessary to optimize the process's efficiency.

Capture Antibody

Modifications were made to the incubation time with the capture antibody (cAb) (**Figure S6**). We tested different incubation times, starting with 2 hours, but observed that the control presented a high signal. Subsequently, we tested overnight incubation, followed by 2 hours and 30 minutes, and finally, 3 hours.

The best performance was achieved with the 3-hour incubation, where the signal from the CPTM ionic probe (m/z 87) was more robust, and the difference between positive and negative samples was more pronounced, indicating an improvement in diagnostic accuracy.

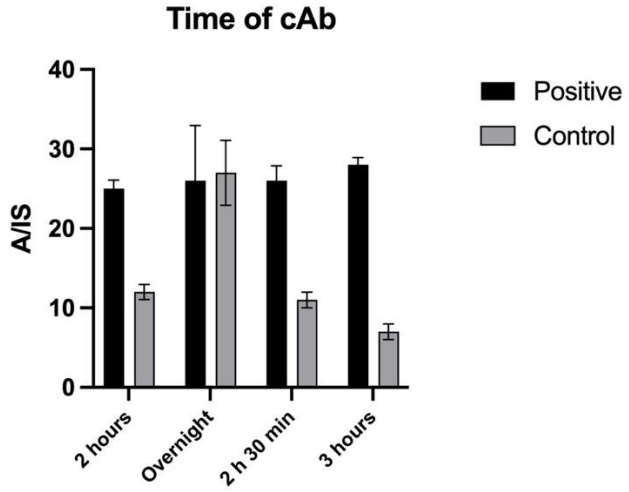

Figure S6. Optimization of incubation time for immobilization of the capture antibody. Different incubation times (2 hours, overnight, 2 hours 30 minutes, and 3 hours) were evaluated for the immobilization of the capture antibody on the test zone. The optimal time was determined based on signal intensity and the differentiation between positive and negative samples using the CPTM ionic probe (m/z 87).

Blocking Buffer Type

The choice of blocking buffer in an immunoassay is crucial for minimizing non-specific binding, which can lead to unwanted signal interference during the analysis of negative samples. Non-specific interactions with the surface of the assay paper can contribute to false positives or background noise. In our study, we tested TBS with 0.05% Tween 20, TBS, and 1% BSA in PBS (**Figure S7**). Among these, the immunoassay using TBS (0.05% Tween 20) demonstrated the lowest level of non-specific binding, providing the most accurate differentiation between positive and negative samples.

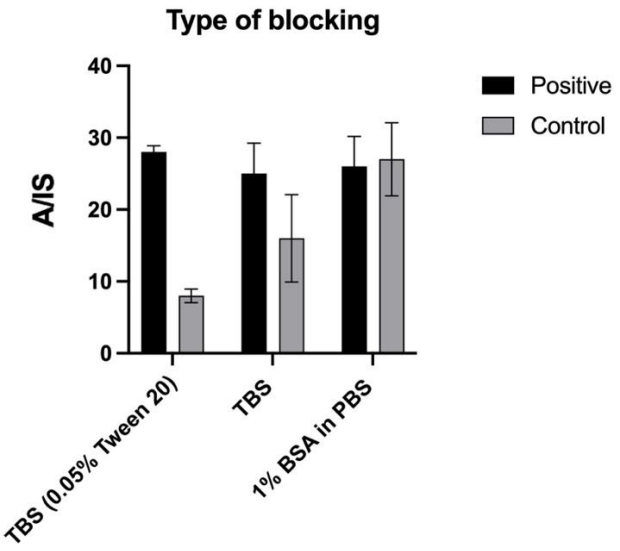

Figure S7. Optimization of blocking buffer. Three buffers were tested, including TBS with 0.05% Tween 20, TBS, and PBS supplemented with 1% BSA. The buffer containing TBS with 0.05% Tween 20 resulted in a higher signal for positive samples and a lower background signal from non-specific binding in negative samples.

Blocking Buffer Time

Additionally, we evaluated different incubation times for TBS (0.05% Tween 20) (**Figure S8**). The incubation times tested were 1 hour, 2 hours, and 3 hours. All conditions, including the positive control, showed favorable results; however, the 3-hour incubation time yielded the optimal results, demonstrating the best performance compared to the other time points.

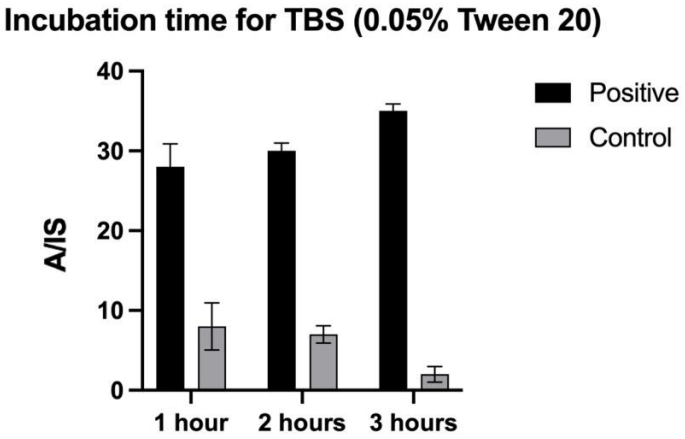

Figure S8. Optimization of incubation time for blocking using TBS (0.05% Tween 20). The highest signal for positive sample and the lowest signal for negative sample were observed with 3 hours of buffer incubation.

PS-MS ANALYSIS

To promote the cleavage of the ionic probe in the immunoassay, an 8 µL solution of **NH_4OH/H_2O** (2 M, 87:13, v/v%) was prepared and applied to the assay zone, where it was incubated for 20 minutes.

For the PSI-MS analysis, a paper strip with triangular tips was positioned beneath the reaction strip. The test zone was placed on top of a triangular paper, and both layers were securely fixed held together with a metal holder. The assembled setup was then positioned in front of the mass spectrometer, maintaining an approximately 5 mm between the paper spray tip and the MS inlet (**Figure S9**).

An 8 µL ACN:H_2O (1:1, v/v) solution was applied as a solvent directly onto the paper surface, followed by the application of a voltage of 4.5 kV. Upon solvent addition and voltage application, the analytes were extracted from the top of the paper, penetrating through the bottom layer, and reaching the MS inlet as charged electrospray microdroplets.

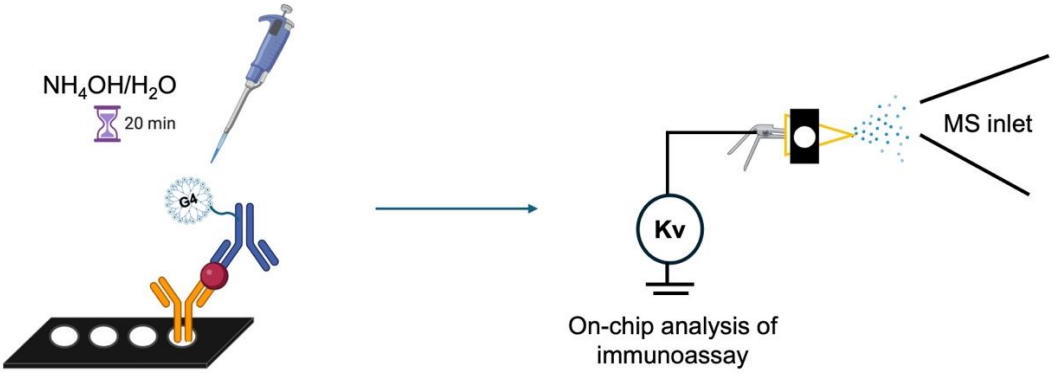

Figure S9. PS-MS analysis. The NH_4OH/H_2O solution (2 M, 87:13, v/v%) is applied to the test zone and incubated for 20 minutes. Then, the test zone is placed on top of a triangular paper and secured with a clip. After fixation, a solvent is added to the paper surface, and a voltage is applied to promote the extraction and analysis of the analytes.

References

(1) Jackson, S.; Lee, S.; Badu-Tawiah, A. K. Automated Immunoassay Performed on a 3D Microfluidic Paper-Based Device for Malaria Detection by Ambient Mass Spectrometry. *Anal Chem* **2022**, *94* (12), 5132–5139.

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(2) Lee, S.; Kulyk, D. S.; Afriyie, S. O.; Badu, K.; Badu-Tawiah, A. K. Malaria Diagnosis Using Paper-Based Immunoassay for Clinical Blood Sampling and Analysis by a Miniature Mass Spectrometer. *Anal Chem* **2022**, *94* (41), 14377–14384. https://doi.org/10.1021/ACS.ANALCHEM.2C03105/SUPPL_FILE/AC2C03105_SI_001.PDF.

(3) Neises, B.; Steglich, W. Simple Method for the Esterification of Carboxylic Acids. *Angewandte Chemie International Edition in English* **1978**, *17* (7), 522–524. https://doi.org/10.1002/ANIE.197805221.