# **Supplementary Information** for

# A Combined Digital Microfluidic Test for Assessing Infection and Immunity Status for Viral Disease in Saliva

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**Note S1: Nanobody characterization in saliva**

Monovalent (biotinylated) and bivalent (fused to human IgG1 Fc region) variants of camelid nanobodies '$V_HH$ 11' and '$V_HH$ 1d' were tested to evaluate their stability and binding affinity after exposure to human saliva.

First, nanobody stability was evaluated by size exclusion chromatography (SEC). Briefly, saliva was collected from a volunteer using a Salivette® saliva collection device (Sarstedt, Germany) and clarified according to the manufacturer's instructions. One mg of each nanobody was then dissolved in either (i) saliva ("saliva" sample) or (ii) PBS ("untreated") and incubated for 1 h at 37°C. A third sample (iii) of saliva-alone was also prepared for comparison. The samples were then injected into a Superdex™ 75 10/300 GL column (for monovalent nanobodies) or Superdex™ 200 10/300 GL column (for bivalent nanobodies) in an ÄKTA FPLC protein purification system (Cytiva, BC, Canada), with detection by Absorbance at 280 nm as described previously (Rossotti et al., *Commun. Biol.* 2022, *5*, 933). The area under the curve (AUC) for the peaks corresponding to the nanobodies in the chromatograms for both untreated and saliva-treated samples (after subtracting the saliva-alone chromatogram for the saliva-treated samples) was calculated using GraphPad Prism version 10.1.2 for Windows (MA, US). As shown in Fig. S1A, the SEC profiles of the untreated nanobodies were homogenous, displaying a single peak that corresponds to nanobodies in complete integrity and stability. In contrast, the SEC profile of the saliva-alone sample displayed a heterogeneous mixture with multiple peaks. Importantly, the peaks corresponding to the nanobodies in the saliva-treated samples largely overlapped with those in the untreated samples in terms of both elution volume ($V_e$) and AUC, indicating that all four nanobodies remained stable in saliva, without signs of degradation or aggregation. Additional peaks observed in the SEC profiles of the saliva-treated samples, compared to the untreated samples, correspond to proteins or other constituents in the saliva itself.

Next, the binding activity of saliva-treated nanobodies was monitored using a sandwich ELISA, with directionally captured biotinylated $V_HHs$ on streptavidin-coated microtiter plates, as described previously (Rossotti et al., *Commun. Biol.* 2022, *5*, 933). Briefly, NUNC® Immulon 4 HBX microtiter plates (Thermo Fisher Scientific, ON, Canada) were passively coated with 2 µg/mL of streptavidin (Jackson ImmunoResearch, PA, USA) in PBS overnight at 4°C. The following day, the plates were blocked with PBSC [1% w/v casein (Sigma, ON, Canada) in PBS] for 1 h at room temperature. Solutions of (untreated) monovalent nanobodies were then added at 100 µL/well at a concentration of 5 µg/mL in PBST (PBS supplemented with 0.05% v/v Tween® 20) to saturate the streptavidin surface. Afterward, the plates were emptied and washed twice with PBST, and decreasing concentrations of recombinant spike protein in PBS were added and incubated with agitation at 300 rpm for 1 h to allow the capture of the spike protein. Saliva-treated and untreated bivalent nanobodies were added to the wells at 1 µg/mL in PBST and incubated for 1 h. The plates were then washed five times with PBST and incubated at room temperature with 100 µL/well of 1 µg/mL of HRP-conjugated goat anti-human IgG (Sigma, ON, Canada) for 1 h. Following an additional five washes with PBST, 100 µL of peroxidase substrate solution (SeraCare, MA, USA) was added to each well and incubated for 15 min at room temperature. The reaction was stopped by adding 50 µL/well of 1 N $H_2SO_4$, and the absorbance was measured at 450 nm using a Multiskan™ FC photometer (Thermo Fisher Scientific, ON, Canada).

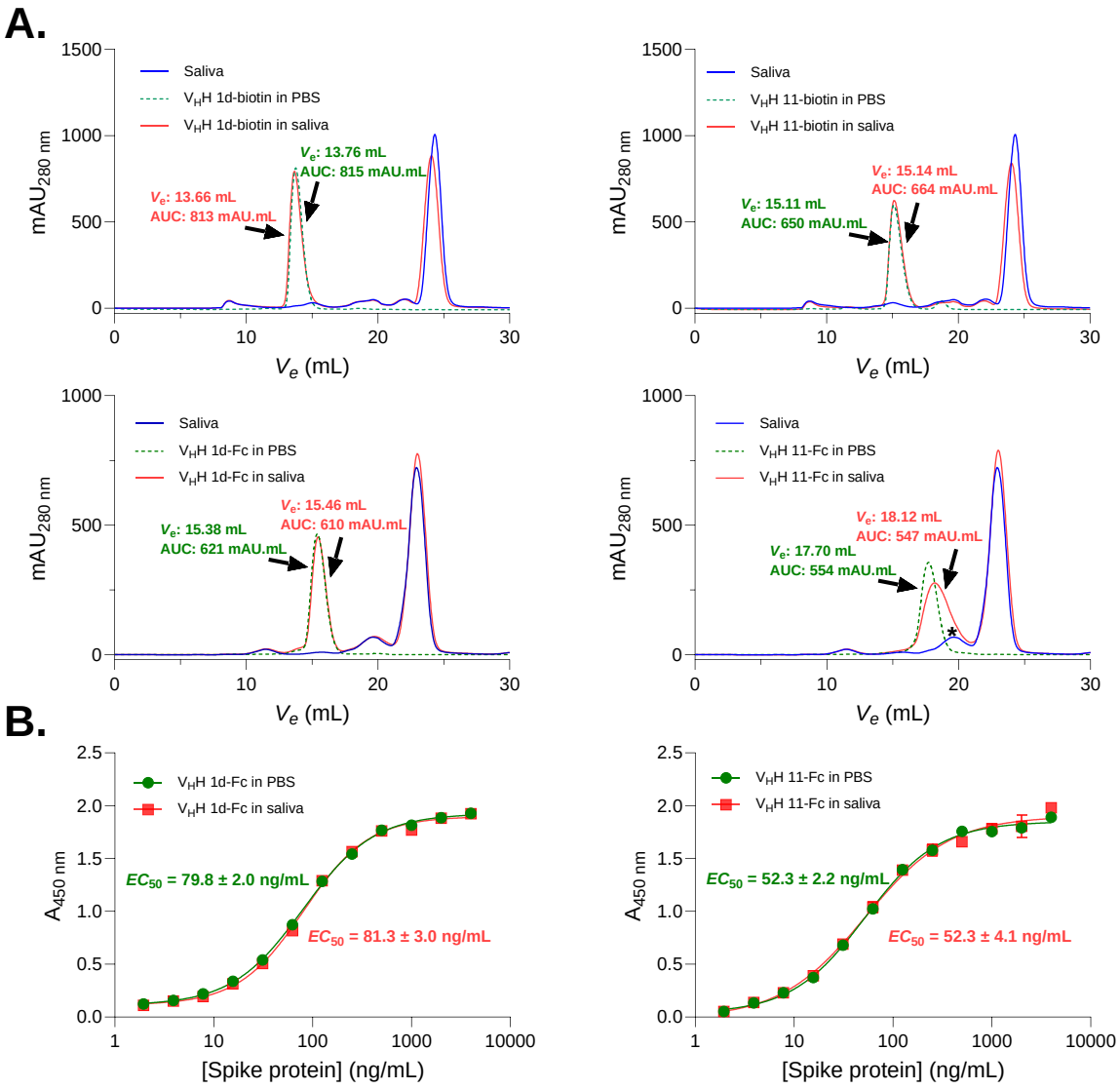


***Figure S1.*** *Nanobody characterization in saliva. (A) Size-exclusion chromatograms (plots of milliAbsorbance units at 280 nm as a function of elution volume $V_e$) for monovalent (top) and bivalent (bottom) nanobodies $V_HH$-1d (left) and $V_HH$-11 (right). Each chromatogram includes data for saliva alone (blue trace), untreated nanobody (green dashed trace), and saliva-treated nanobody (red trace). Labels indicate the elution volume and area under curve (AUC) for each nanobody peak in the untreated (green) and saliva-treated (red) samples. For $V_HH$ 11-Fc, the nanobody peak in the saliva-treated sample shifted slightly to the right compared to the untreated sample due to co-elution with a saliva component (marked by an asterisk). (B) ELISA results comparing the binding activity of untreated (green) and saliva-treated (red) bivalent nanobodies $V_HH$-1d (left) and $V_HH$-11 (right). Half-maximal effective concentration ($EC_{50}$) values are expressed as means ± SEM of two technical replicates.*

Half-maximal effective concentration ($EC_{50}$) values were determined by plotting $A_{450\ nm}$ against [spike protein] and fitting the [agonist] vs. response using the Variable Slope (four parameters) non-linear regression model to the plot using GraphPad Prism. ELISAs showed

that, for both bivalent nanobodies, $V_HH$ 1d-Fc and $V_HH$ 11-Fc, the binding curves for the saliva-treated and untreated nanobodies were nearly identical, yielding comparable $EC_{50}$ values ($EC_{50}$s of 81.3 ± 3.0 ng/mL [saliva-treated] vs. 79.8 ± 2.0 ng/mL [untreated] for $V_HH$ 1d-Fc; $EC_{50}$s of 52.3 ± 4.1 ng/mL [saliva-treated] vs. 52.3 ± 2.2 ng/mL [untreated] for $V_HH$ 11-Fc) (Fig. S1B). Collectively, these results demonstrate that all four nanobodies retain full stability and functional activity in saliva.

**Note S2: Optimization of off-chip SARS-CoV-2 infection assays**

Off-chip SARS-CoV-2 infection assays were carried out using the same 9-step procedure from the main text with the following changes. Replicates were prepared for sample (100 µL of 100 ng/mL of trimeric spike protein standard in PBS SuperBlock™) and blank (PBS SuperBlock™ alone), and in step (5), the streptavidin-HRP solutions were used at 0.5, 5.0, or 50 ng/mL. After complecting the assays, the absorbance signals generated from the sample were divided by the average signals from the bank. The results (Fig. S2) indicated that 5.0 ng/mL streptativin-HRP was optimal, which was used in all subsequent experiments.

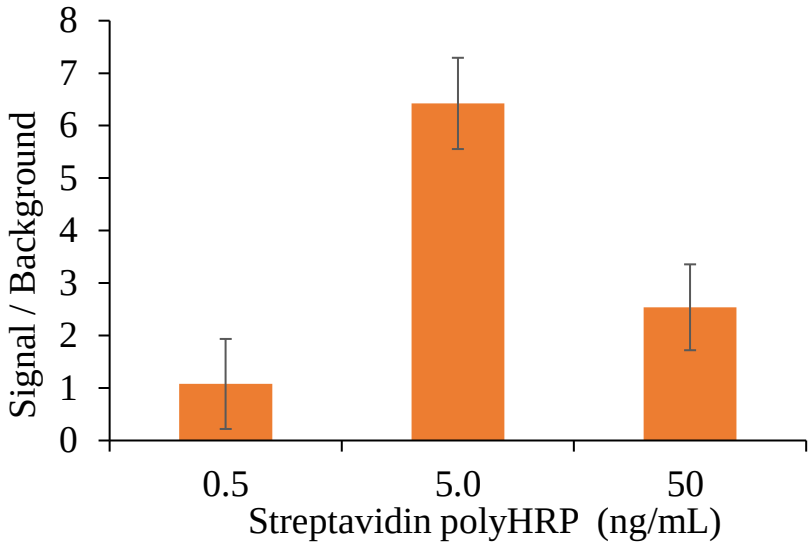


***Figure S2.*** *Optimization of streptavidin-polyHRP concentration for the off-chip infection assay. Bar plot of signal-to-background ratio for off-chip infection assay for 100 ng/mL of spike protein with 0.5, 5.0, or 50 ng/mL Streptavidin-polyHRP. Error bars represent ± one standard deviation for n = 3 replicates per condition.*

The optimized off-chip SARS-CoV-2 infection assay procedure was then applied to a dilution series of trimeric spike protein spiked in PBS SuperBlock™, with results shown in Figure S3. The LOD and LOQ for this assay were 76.6 ng/mL and 370.6 ng/mL, respectively.

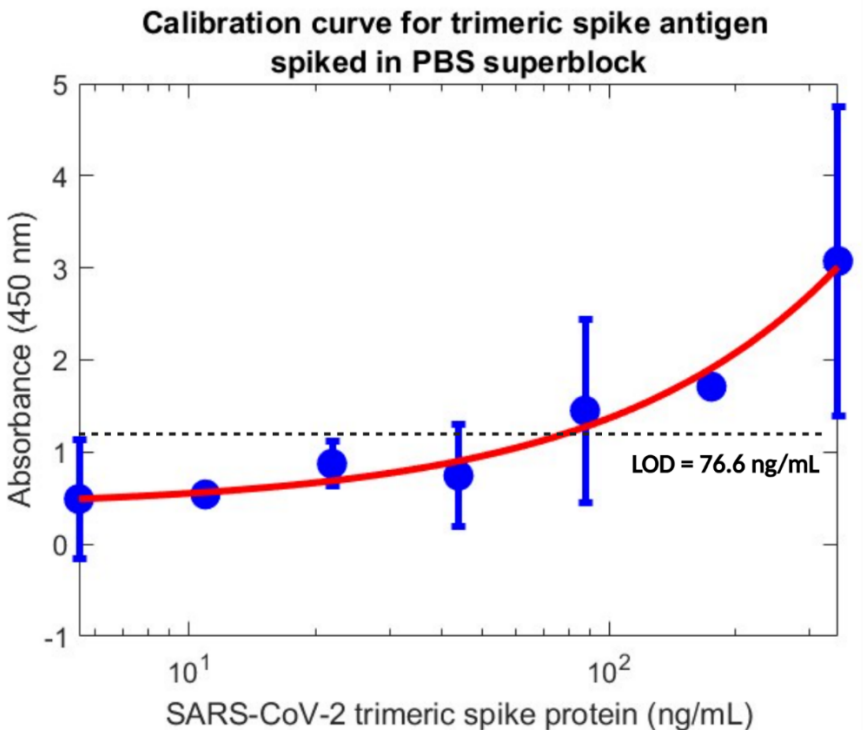


***Figure S3.*** *Semilog plot of off-chip infection assay measurements (blue markers) as a function of SARS-CoV-2 trimeric spike protein concentration spiked in PBS SuperBlock™. The curve is a 4PL fit to the data, and the dashed black line illustrates the LOD of 76.6 ng/mL. Error bars represent ± one standard deviation for n = 3 replicates per condition.*

**Note S3: Optimization of off-chip SARS-CoV-2 immunity assays**

Streptavidin-coated paramagnetic beads with two different diameters (Dynabeads® T1 = 1µm and M280 = 2.8 µm, Thermo Fisher, Rockford, IL) were evaluated in the development of off-chip assays for host IgG and IgA. The beads were modified using the same 5-step procedure described in the main text, with the following change. In step three, different amounts of purified biotinylated spike S1 protein solution were added to tubes bearing washed/resuspended beads. Specifically, 5, 10, 20, or 20 µL of S1 solution were added to the different tubes, such that the bead-coating capacity (according to the manufacturer) was 1/2, 1/4, 1/8, and maximum, respectively.

Suspensions of both bead types, modified with different loading capacities of S1, were used to run off-chip SARS-CoV-2 immunity assays using the 9-step procedure from the main text, with results shown in Fig. S4. Loading capacity of 1/2 (i.e., 20 µL of S1 solution in bead-modification step 3) was found to be sufficient for maximum performance; thus, this condition was used for all subsequent assays. Furthermore, both bead types yielded similar assay results. However, it was observed that T1 beads were more sensitive to the magnetic field during pelleting and wash steps, leading to strong aggregation that was sometimes difficult to dissociate. Thus, M280 beads were used for all subsequent assays.

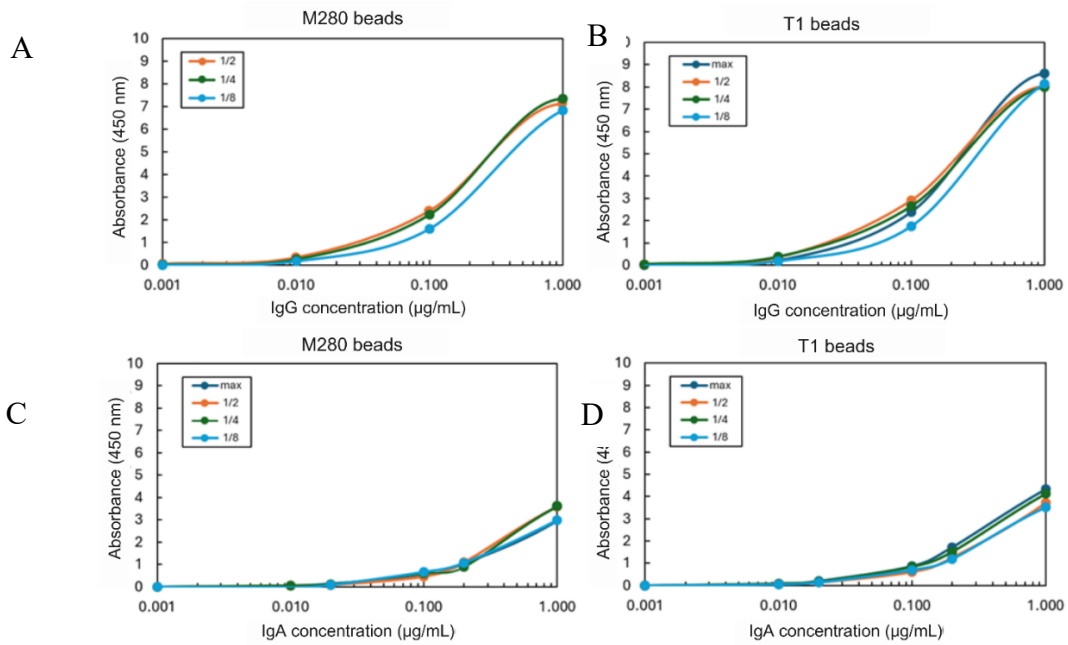


***Figure S4.*** *Off-Chip optimization of bead size and bead coating capacity for off-chip immunity assays. Semilog plots of assay signal as a function of host IgG (A, B) and host IgA (C, D) concentration, spiked in PBS SuperBlock™ for M280 (A, C) and T1 (B, D) beads modified with S1 at 1/8 (light blue), 1/4 (green), 1/2 (orange), and 'maximum' (dark blue) binding capacity.*

Next, sample incubation time was optimized for off-chip SARS-CoV-2 immunity assays. The 9-step procedure from the main text was used, except in step (3c), sample incubation times for host IgG (2.5, 5 and 10 min) and IgA (5, 10 and 20 min) were explored. After completing the assays, the absorbance signals generated from the sample were divided by the average signals from the bank. The results (Fig. S5) indicated that 5 and 10 min were optimal for host IgG and host IgA assays, respectively, which were then used in all subsequent experiments.

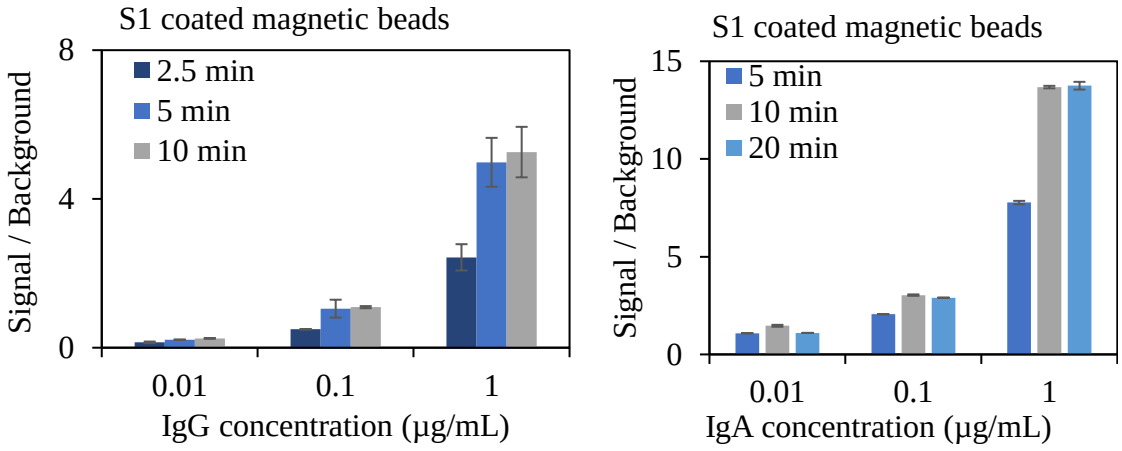


***Figure S5.*** *Optimization of sample incubation time for off-chip immunity assays. Bar plots of signal-to-background ratio as a function of host IgG concentration (left) and IgA concentration (right) spiked in PBS SuperBlock™ after incubation for 2.5 (dk. blue), 5 (med. blue), 10 (grey), or 20 (lt. blue) min. Error bars represent ± one standard deviation for n=3 replicates per condition.*

The optimized off-chip SARS-CoV-2 immunity assay procedure was then applied to a dilution series of host IgG and host IgA spiked in PBS SuperBlock™, with results shown in Figure S6. The LODs and LOQs for this assay were 4.0 and 17.4 ng/mL for host IgG and 19.1 and 59.7 ng/mL for host IgA, respectively.

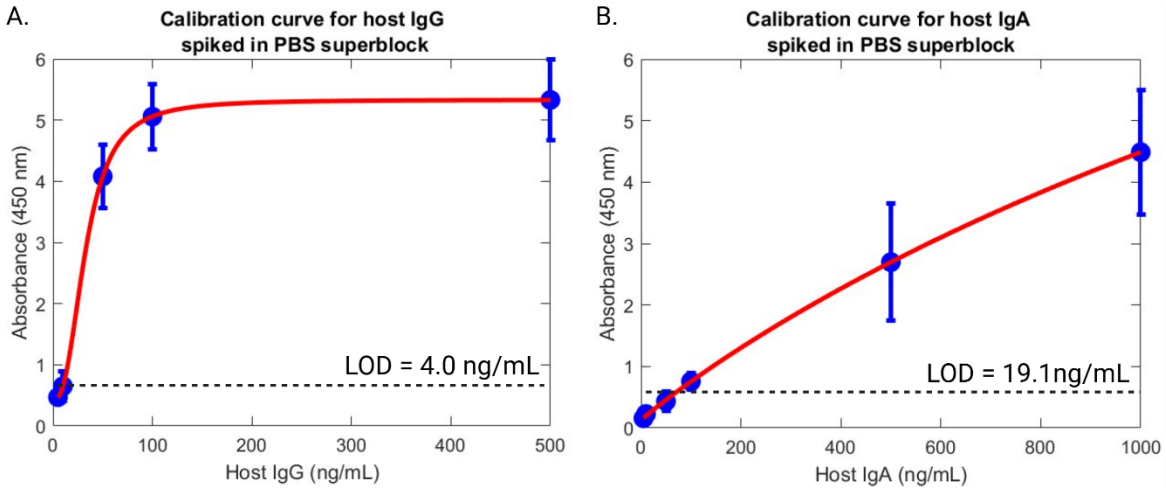


***Figure S6.*** *Plots of off-chip immunity assay measurements (blue markers) as a function of (A) host IgG and (B) host IgA spiked in PBS SuperBlock™. The curves are 4PL fits to the data, and the dashed lines illustrate the LODs of 4.0 ng/mL (IgG) and 19.1 ng/mL (IgA), respectively. Error bars represent ± one standard deviation for n=3 replicates per condition.*

Finally, when applied to saliva, we observed that cross-reactivity generated unpredictable results for the IgA assays. Thus, as described in the main text, a revised procedure was developed for IgA, in which in step (3b) a 2 µL aliquot of goat anti-human IgG was added (at excess concentration: 1 mg/mL) and allowed to complex with interfering species to prevent them from interacting with the beads. As shown in Figure S7, this measure resolved the problem and was used for all subsequent experiments.

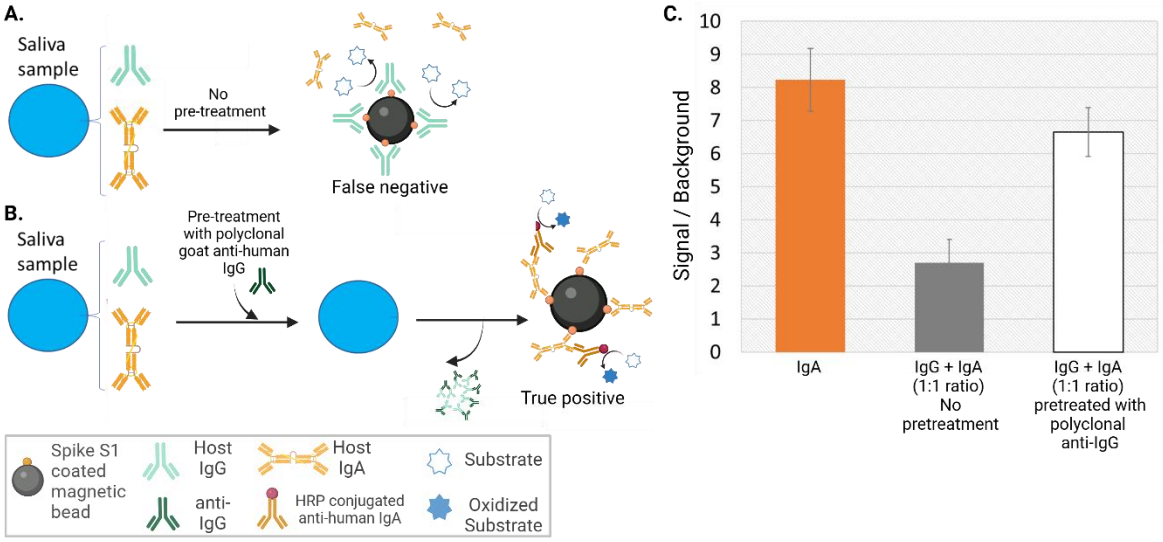


***Figure S7.*** *(A) A schematic illustrating how cross-reactivity (in this case, the presence of excess IgG, aqua markers) can prevent the binding of IgA (Orange markers) to particles, leading to false negatives. (B) Schematic illustrating how pre-treatment of the sample with polyclonal goat anti-human IgG (green markers) complexes with cross-reacting species (in this case IgG, aqua markers) allowing IgA (yellow markers) to bind and generate the measurable signal. (C) Bar plot showing the signal/background ratio for spiked samples in pooled, pre-COVID-19 saliva: recombinant human IgA (50 ng/mL, orange), a mixture of recombinant IgG and IgA (50 ng/mL each, grey) and a mixture of IgG and IgA (50 ng/mL each, white) after pre-treatment by exposure to polyclonal anti-human IgG. Error bars represent ± one standard deviation for n=3 replicates per condition.*

**Note S4: Double-assay negative control for DMF immunity assays**

Negative controls are important in portable diagnostic assays because (i) they can be used as 'background' to determine signal-to-background (S/B) ratios, and (ii) they can flag cases in which the assay results should not be trusted (if aberrant, high readings are observed). In the DMF device used here, there was no room for a separate negative control for host IgA and host IgG in the combined infection and immunity assay procedure. Thus, a new method was developed – a "double-assay negative control" for the immunity tests for host IgA and host IgG.

Briefly, in the revised procedure for DMF combined infection and immunity assays, a new version of step 8 was performed. In step eight, the five droplets dispensed from reservoirs comprised: a double-unit droplet a mixture of HRP conjugated to anti-human IgG and HRP conjugated to anti-human IgA (50 ng/mL ea.) – driven to zone A, a double-unit droplet of streptavidin-polyHRP (20 ng/mL) – driven to zone B, a double-unit droplet of HRP conjugated to anti-human IgG alone (50 ng/mL) – driven to zone C, a double-unit droplet of HRP conjugated to anti-human IgA alone (50 ng/mL) – driven to zone D, and a double-unit droplet of streptavidin-polyHRP (20 ng/mL) – driven to zone E. The remainder of the combined infection and immunity assay was unchanged.

As shown in Fig. S8, the "double assay control" (the conditions described for zone A above) gave signals very similar to the negative controls for host IgG alone or host IgA alone. Further,

all of the controls had much lower signals than those with spiked analytes. Thus, it was determined that the double-assay control could be used as the background (and for LOD/LOQ determinations) for the two assays.

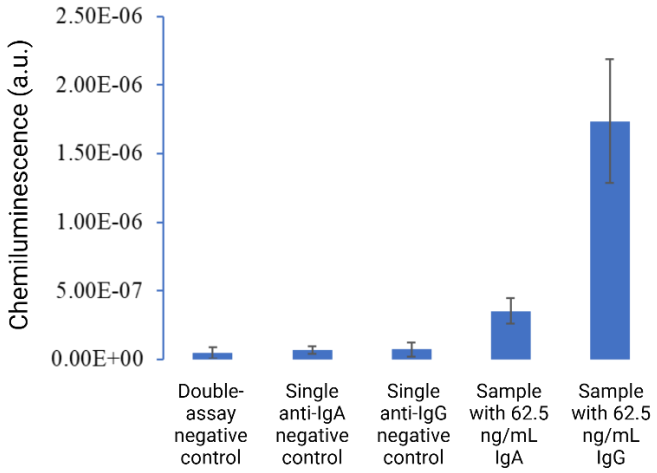


***Figure S8.*** *Bar plot of the chemiluminescent signal generated from digital microfluidic assays for the double-assay negative control (for host IgG and IgA in saliva), the conventional single-assay control for host IgA in saliva, the conventional single-assay control for host IgG in saliva, saliva spiked with 62.5 ng/mL host IgA, and saliva spiked with 62.5 ng/mL host IgG. Error bars represent* $\pm$ *one standard deviation for n=3 replicates per condition.*