Supporting Information

"The immunoprobe aggregation state is central to dipstick immunoassay performance"

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Experimental Section

Gold Particle Synthesis

20 nm AuNPs (more often used)

47.2 g of MilliQ water is weighed into a glass bottle which was placed into a water batch. 1.4 mL of 10 mg/mL $HAuCl_4$ (Sigma, product code: 254169) was pipetted in. A magnetic stirrer was used to stir the solution fast enough so that there is a visible vortex which does not touch the stirrer. The water in the bath was brought to a boil and the reaction was left to fully equilibrate for at least 15 minutes. After this time the lid of the bottle was carefully removed and 0.9 mL of 10 mg/mL sodium citrate, originally tribasic sodium citrate (Sigma, product code: S4641) were added under vigorous stirring. The lid was placed back on the bottle and it was left to react for ~15 minutes. Final concentrations of $HAuCl_4$ and sodium citrate were 0.83 mM and 0.62 mM, respectively.

During this time the dispersion turned from a light yellow, to black, to purple and finally a brownish red. Typically the resulting particles had a diameter of ~50 nm by Z Average in DLS and ~20 nm by Number Mean in DLS. The dispersion was opaque and had a dark red colour.

Small AuNPs (used in divergent synthesis)

The small NPs were done much the same way except 0.5 mL of 10 mg/mL $HAuCl_4$ was added initially and 1 mL of sodium citrate was added after the 15 minute equilibration period. The final concentrations were 0.3 mM and 0.7 mM, respectively.

The solution was a translucent wine red after the reaction. Typically particles had a diameter of ~20 nm by Z average in DLS and ~8 nm by Number Mean in DLS.

Synthesis of PEG backfilled immunoprobes (PB)

mPB

The appropriate amount of as synthesised AuNPs were pipetted into a LoBind Eppendorf (Sigma, product code: Z66613). 25 µL of 2 mg/mL (13 µM) anti mouse IgG (Sigma, product code: M8642) were added per mL of AuNPs, for a final antibody concentration of 0.32 µM and the solution was left to shake gently at 18 – 22°C for an hour. At this time 5 µL of 1 mg/mL (0.2 mM) MeO-PEG_{5000}-SH (NanoCS, product code: PG1-TH-5k) was added and the dispersion for a final concentration of 0.1 µM. The dispersion was left in the same conditions for a further hour. Unless otherwise specified the Eppendorf was then centrifuged four times into PBS (8 000 rpm for 10 minutes). Resuspension volume was reduced by about 25% of the initial volume each spin (e.g. initial 1000 µL, w1 750 µL, w2 500 µL, w3 250 µL, w4 50 µL). The dispersion was finally redispersed in 1/20th of the initial volume (e.g. 50 µL for an initial volume of 1 mL). Vortexing and brief sonication (up to 3 seconds at a time) were used to disperse the particles if needed.

We observed that higher temperatures or more vigorous homogenization (e.g. shaking) led to unstable final product. Immunoprobes also tended to stuck to the walls of Eppendorfs other than the LoBind resulting in a very low final concentration.

The as made immunoprobes were stored in a 4°C fridge for up to three weeks.

zPB

Anti Zika NS1 immunoprobes were synthesised much the same way only 20 µL of 2.4 mg/mL (16 µM) anti Zika NS1 was added instead of anti mouse IgG. Final antibody concentration during the reaction was 0.37 µM.

dPB

Anti Dengue NS1 immunoprobes were done much the same way only 25 µL of 2 mg/mL (13 µM) anti Dengue NS1 was added instead of anti mouse IgG. The final antibody concentration in the reaction was 0.32 µM.

Synthesis of NHS immunoprobes (NHS)

mNHS

The appropriate amount of as synthesised AuNPs were pipetted into a LoBind Eppendorf to which 5 uL of 45 mg/mL NHS-PEG_{3400}-SH (NanoCS, product code: PG2-NSTH-3k) per mL of AuNPs were added. Final concentration was 0.63 mM. The dispersion was left shaking (700 rpm) for an hour to react at 20 – 25°C. Particles were then spun twice into the same volume of PBS (8000 rpm for 10 min).

25 µL per mL of AuNPs of 2 mg/mL (13 µM) anti mouse IgG were added and the dispersion was left to react for an hour at 20-25°C while shaking at 700 rpm. Final antibody concentration was 0.32 µM. After this time immunoprobes were purified by centrifugation the same was as PB immunoprobes.

These immunoprobes did not aggregate when different conditions were used or stick to the Eppendorf walls during centrifugation if placed in different plastic.

zNHS

Anti Zika NS1 NHS immunoprobes were done much the same way, however, 20 µL of 2.4 mg/mL anti Zika NS1 was added. Final antibody concentration during the reaction was 0.37 µM.

dNHS
Anti Dengue NS1 NHS immunoprobes were done much the same way, however, 25 µL of 2 mg/mL anti Dengue NS1 was added. The final antibody concentration in the reaction was 0.32 µM.

Synthesis of Hz immunoprobes (Hz)
mHz
The appropriate amount of as synthesised AuNPs were pipetted into a LoBind Eppendorf to which 5 µL of 50 mg/mL (15 mM) hydryzide-PEG$_{3400}$-SH per mL of AuNPs were added. Final PEG concentration was 71 µM. The dispersion was left shaking (700 rpm) for an hour to react at 20 – 25°C. Particles were then spun three times into the same volume of PBS (8 000 rpm for 10 minutes) to remove excess PEG.
50 µL per mL 1 mg/mL (6.5 µM) activated anti mouse IgG (procedure below) were added per mL of AuNPs and the dispersion was left to react for an hour at 20-25°C while shaking at 700 rpm. Final antibody concentration was 0.32 µM. Final immunoprobes were washed the same as NHS and PB ones.

zHz
Anti Zika NS1 NHS immunoprobes were done much the same way, however, 22 µL of 2.1 mg/mL anti Zika NS1 was added. Final antibody concentration was 0.30 µM.

dHz
Anti Dengue NS1 NHS immunoprobes were done much the same way, however, 30 µL of 1.7 mg/mL anti Dengue NS1 was added. Final antibody concentration was 0.33 µM.

Protein activation for Hz reaction
Anti mouse IgG
To activate, antibodies were initially dissolved from powder in 0.1 M Na$_2$HPO$_4$ (Fisher Scientific, product code: 15538454) to which 0.1 M NaIO$_4$ (Sigma, product code: 311448) was added in a volumetric ratio of 10 to 1 (i.e. 1 µL of NaIO$_4$ per 10 µL of antibodies). The solution was mixed and left to react for at least 30 mins, stationary at RT.

Anti Zika NS1
4 µL of 0.1 M NaIO$_4$ were added to 20 µL of anti Zika NS1 antibody (2.4 mg/mL) and left for at least 30 mins, stationary at RT.

Anti Dengue NS1
5 µL of 0.1 M NaIO4 were added to 25 uL of anti Dengue NS1 antibody (2 mg/mL) and left for at least 30 mins, stationary at RT.

Divergent mPB synthesis
Quadruple PEG
Procedure was the same as the regular PEG backfill, but 20 µL of 1 mg/mL PEG5000 were used per 1 mL of AuNPs.

Quadruple IgG
Procedure was the same as the regular PEG backfill, but 100 µL of 2 mg/mL anti mouse IgG were added per 1 mL of AuNPs.

Small NP synthesis
Procedure was the same as the regular PEG backfill, but 66 µL of 2 mg/mL anti mouse IgG were added per 1 mL of 8 nm AuNPs and 13 µL of 1 mg/mL PEG5000.

HEPES synthesis
Particles were washed into 40 mM HEPES pH 7.7 once prior to protein addition. After which the reaction is the same as the regular PB immunoprobes.

Small AuNP HEPES Synthesis
A solution of gold was prepared by measuring 11.6 mg of gold (III) chloride hydrate and dissolving it with 1.0 mL of MilliQ water. The prepared solution was stored in a clear glass vial and covered with foil until needed.
Sodium citrate (NaCit) solution was made fresh for every synthesis by combining 25 mg of it with 2.5 mL of Milli-Q water.
49.5 mL MilliQ water and 500 µL Au solution were added to a 250 mL Erlenmeyer flask. A stir bar was dropped inside the flask and a short-stemmed glass funnel was placed on its neck. Once setup, the flask was put on top of a cold hot

plate, with the dial of the stirrer set on slow and the heat on number 3 (Corning stirrer). This heat setting was maintained until the solution started to boil at which point, the setting was changed to 2.5. 1.0 mL NaCit was added and the flask was left for 10 minutes. After the 10-minute time period, the heat was turned off and the flask was left on the hot plate, stirring, for an additional 15 minutes. After the 15-minute duration, the solution was removed from the hot plate and allowed to cool to room temperature. The Au NP solution was stored in a 50 mL orange-coloured cap falcon tube.

Particle characterization
UV-Vis
5 µL of immunoprobes were dispersed in 195 µL of PBS in one well of a 96 well plate. The plate was placed in a SpectraMax M5 (Molecular Designs) plate reader and measured from 400 to 800 nm at a 1 nm interval.

DLS
The dispersions made for UV-Vis were taken out of the well and placed into a semi-micro (1.5 mL) plastic cuvette (Sigma, BR759115). 100 µL (total volume of 300 µL in the cuvette) of additional PBS was added and the dispersion was vortexed, then placed in the instrument.
Each DLS (Horiba SZ-100) measurement was the result of 5 runs on automatic and was done at 25°C. The running medium was always specified as water while the particle material was gold (n = 0.2 – 3.32i).

IgG binding in DLS
The same glass cuvette (l = 1 mm) was used for all binding measurements. Dilutions of the desired antigen was made in the desired medium (usually 30 mg/mL BSA or HS). In a typical experiment six total dilutions were made where each was a ¼ dilution of the previous. At this time all solutions had a volume of 60 µL. 4 µL of immunoprobes were added to the as made samples after which they were placed in the cuvette and measured.
Measurement always started from the lowest concentration. After the initial dispersion was measured 15 µL of running buffer (1 to 1 volumetric mixture of 1% Tween 80 in water and 50% sucrose in water) were added and the mixture was measured for further 10 – 20 runs.
After both measurements the dispersion was taken out of the cuvette and placed back in the initial Eppendorf. Often those dispersions were run on dipsticks (details below).
The cuvette was washed with water and acetone between uses.

Salt titration in DLS
NaCl (Fisher Scientific, product code: 10055850) was dissolved in PBS for a final concentration of 1 M. That solution was mixed 1 to 1 vol. with PBS to form a 0.55 M solution. 5 µL of immunoprobes were dispersed in 295 µL of salt solution in a semi-micro plastic cuvette and measured as described above. Each measurement consisted of 20 runs.

Running and analysis dipsticks
Nitrocellulose (UniSart CN 140, product code 1UN14ER100070) was cut into shape using a laser cutter. Strips were bound to the wick using DCN backing cards (product code MIBA-020). Dipsticks were covered and stored in a dry place.

Running dipsticks
The as made dipsticks were stained with the required reagents. Those were 0.3 uL of 2 mg/mL anti mouse IgG/Zika NS1/Dengue NS1 on the test line and rabbit or goat secondary antibody (anti rabbit or anti goat IgG) on the control line. The dipsticks were left to dry for at least 10 minutes at RT.
The antigen was added to the required volume of the running medium and diluted via serial dilution. In a typical experiment 5 µL of a 1 in 100 dilution of the antigen was added to 55 µL of the running medium and homogenized. The serial dilution was conducted as 30 µL of the as made solution were placed in a tube containing 30 µL of the running medium, etc. The final 30 µL were discarded. The negative control contained 30 µL of the running medium.
2 µL of PB and 4 µL of NHS and Hz immunoprobes were added to the as made antigen solution, homogenized and left stationary at RT for 20 – 60 minutes. After this time 15 µL of running buffer were added. The dispersion was spun in a mini centrifuge (~ 1.5 krpm), vortexed and left for further 5 – 15 minutes. The dipsticks were placed in Eppendorfs and left to run.
After the full sample volume had diffused through the paper, 1% tween 80 was ran through the dipstick to remove any non-specifically bound particles. The paper was left overnight to dry.

Dipstick analysis
Distribution analysis
The dry dipsticks were taped to a white sheet of paper and scanned. The resulting images were cropped and rearranged so that they are vertical and aligned with each other. Resulting images were analysed using the gel analysis tool in ImageJ.
The integration of the as obtained signal was copied into excel and converted to fractions. This was done to minimize any gradients in grayscale which may be present in the image.

K_D^{Eff} analysis

Images obtained from distribution analysis were cropped so that only the nitrocellulose was visible. This new image was imported into ImageJ. The measurement tool was used to analyse each area. Briefly a 36 pixel area rectangle was drawn and used to measure the mean grayscale value on an "empty" area of the nitrocellulose. Typically this was the area just below the test line, more rarely it was above the control line. After the rectangle was moved to the test and control lines measuring their mean grayscale values.

Similarly to the distribution procedure a new background was obtained for each individual nitrocellulose to reduce any gradients which may be present. Image size and size of the measurement area were kept constant.

The resulting data was transferred into excel where the difference between the background and test line was calculated. The resulting grayscale signal was fit using equation 1 in main text by an in house written python script.

LOD Analysis

LOD was obtained by averaging one or a number of points where no binding was observed to obtain the background signal. Multiplying that grayscale value by four and then fitting it in a reformatted version of equation 1 in main text.
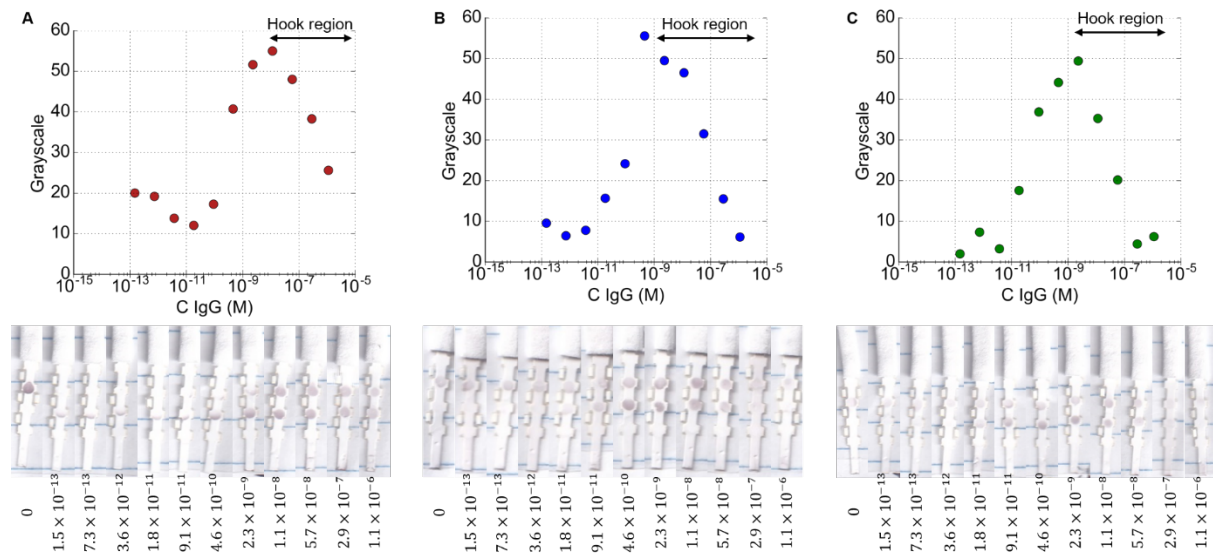
Figures

Figure S1. Antigen titration of (A) mPB, (B) mNHS and (C) mHz immunoprobes in a wide concentration region. Graphs show the hook and titration regions.
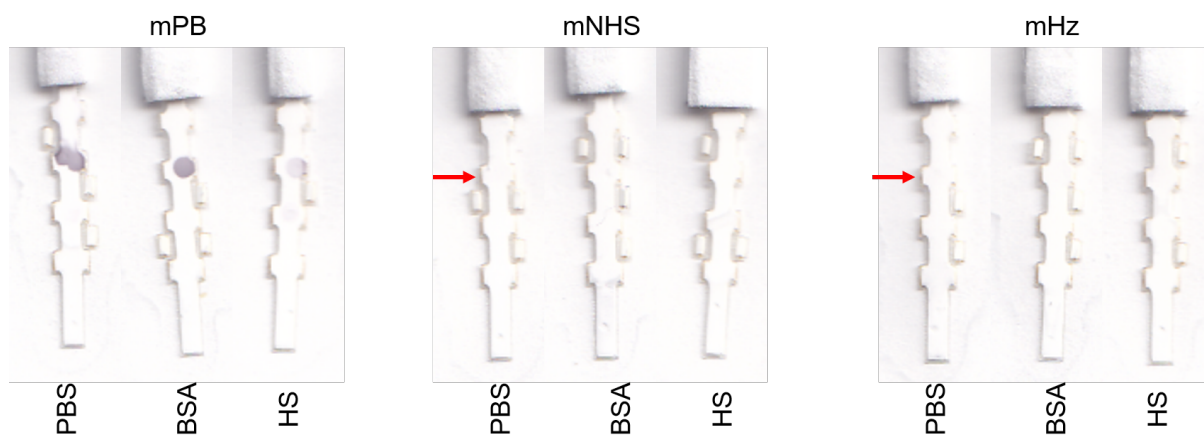
Figure S2. mPB, mNHS and mHz immunoprobes run on dipsticks in different media without antigen. Those include BSA and HS where protein corona is a factor, and PBS where protein corona can not form. Red arrows highlight the lack of signal on the control line in any media.
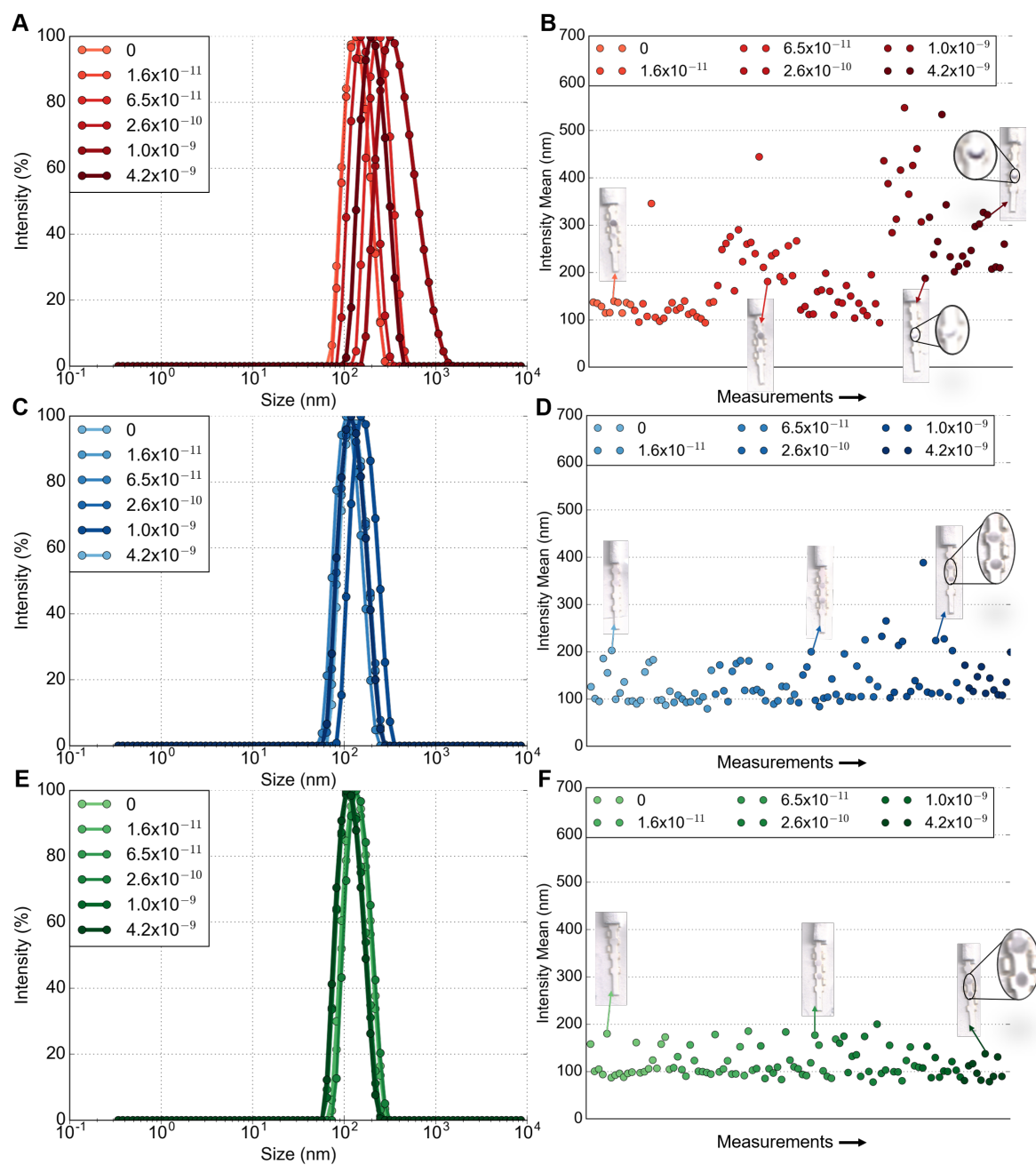
Figure S3. Immunoprobe distribution and mean intensity diameter with addition of mouse IgG and over time. Test done for (A and B) mPB, (C and D) mNHS and (E and F) mHz immunoprobes in 30 mg/mL BSA.
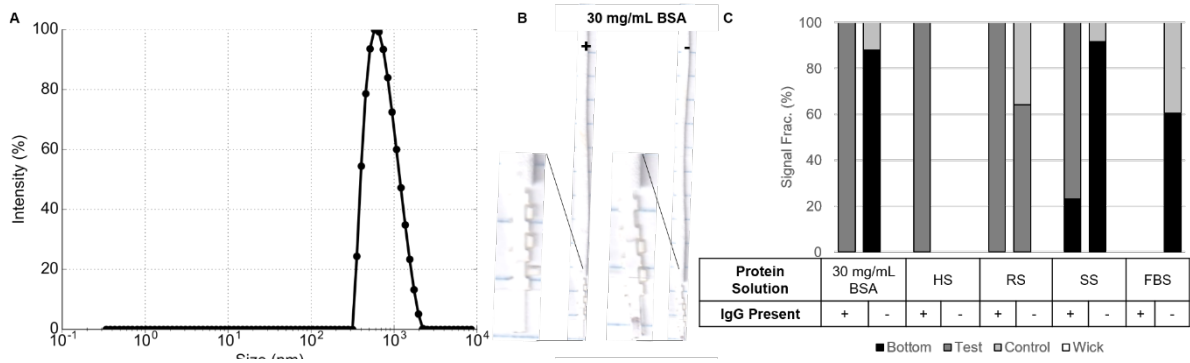
Figure S4. (A) DLS distribution of Abs immunoprobes in PBS showing aggregation. (B) Dipstick assay of mAbs immunoprobes in the presence (+) and absence (-) of antigen showing low signal mostly found at the bottom of the strip. (C) Immunoprobe distribution of Abs immunoprobes in a variety of running media 30 mg/mL BSA, human serum (HS), rabbit serum (RS), sheep serum (SS) and fetal bovine serum (FBS). Immunoprobe distributions are comparable in different running media. Immunoprobes either do not enter the dipstick or are found mostly at the bottom.
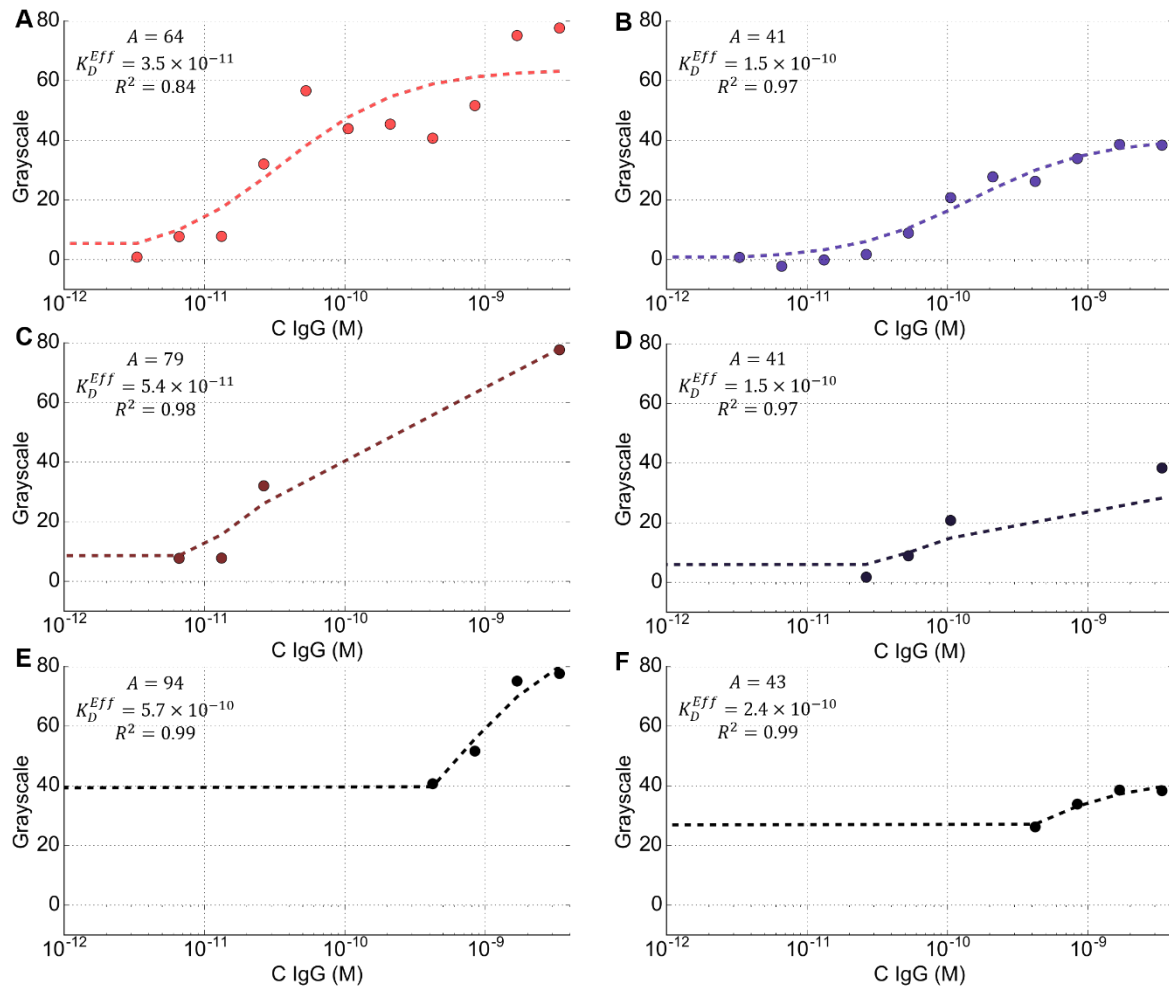
Figure S5. Langmuir fitting of the full titration curve for (A) mPB and (B) mNHS immunoprobes compared to the fitting of the beginning (C for mPB and D for mNHS) and end (E for mPB and F for

mNHS) of the titration region. $K_D{}^{Eff}$ and R^2 are provided showing a 29% RSD for mNHS and 130% RSD for mPB immunoprobes.
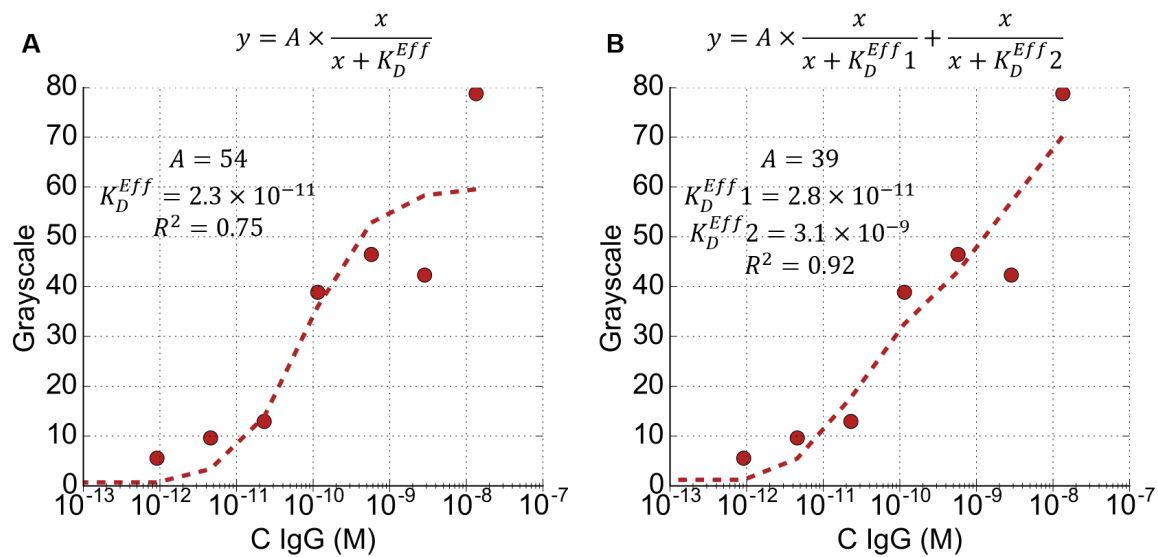
A

$$y = A \times \frac{x}{x + K_D^{Eff}}$$

$A = 54$
$K_D^{Eff} = 2.3 \times 10^{-11}$
$R^2 = 0.75$

B

$$y = A \times \frac{x}{x + K_D^{Eff}1} + \frac{x}{x + K_D^{Eff}2}$$

$A = 39$
$K_D^{Eff}1 = 2.8 \times 10^{-11}$
$K_D^{Eff}2 = 3.1 \times 10^{-9}$
$R^2 = 0.92$

Figure S6. Comparison between a single Langmuir and semi-bimodal Langmuir fitting of a PB antigen titration curve. Semi-bimodal fitting is better in terms of R^2 and shows two distinct $K_D{}^{Eff}$ values two orders of magnitude apart. Data can also be fit to a sum of two Langmuir curves, though the mathematical fitting is more difficult.

A

$A = 44 \pm 3$
$K_D^{Eff} = 2.3 \times 10^{-11} \pm 1.3 \times 10^{-11}$

B

$A = 61 \pm 2$
$K_D^{Eff} = 8.2 \times 10^{-11} \pm 7.4 \times 10^{-11}$
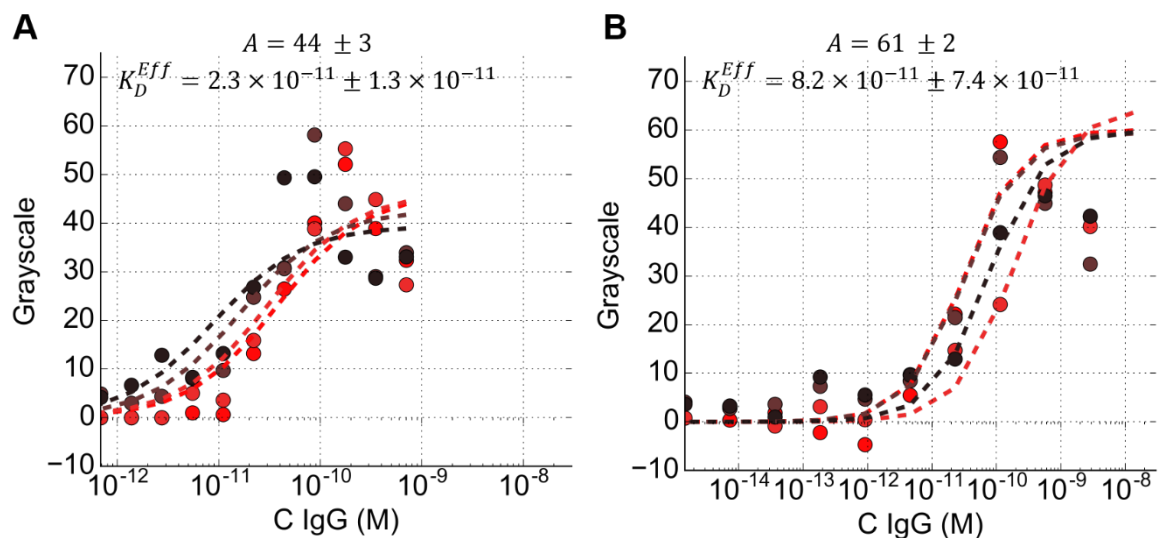
Figure S7. The effect of changing the titration range from up to (A) $7 \times 10^{-10} M$ to (B) $1.3 \times 10^{-8} M$ on the Langmuir fitting parameter (A and $K_D{}^{Eff}$) for four mPB immunoprobe batches. Same four batches used in A and B. Measurements were done two days apart in the same conditions.

Table S1. Langmuir fitting parameters for mPB, mNHS and mHz immunoprobes using different analytical procedures.

Sample	Individual Analysis			Appended Analysis			Summed Analysis			K_D^{Eff}
	A	K_D^{Eff} (M)	R^2	A	K_D^{Eff}(M)	R^2	A	K_D^{Eff}(M)	R^2	Variability*
mPB	54 ± 9	4.0×10^{-11} $\pm 4.8\times10^{-11}$	0.83 ± 0.05	54	2.7×10^{-11}	0.81	56	2.8×10^{-11}	0.84	22%
mNHS	31 ± 6	6.8×10^{-11} $\pm 4.8\times10^{-11}$	0.90 ± 0.07	31	6.3×10^{-11}	0.87	31	6.3×10^{-11}	0.99	4.5%
mHz	33 ± 11	10×10^{-11} $\pm 4.8\times10^{-11}$	0.88 ± 0.03	32	10×10^{-11}	0.77	32	10×10^{-11}	0.98	1.1%
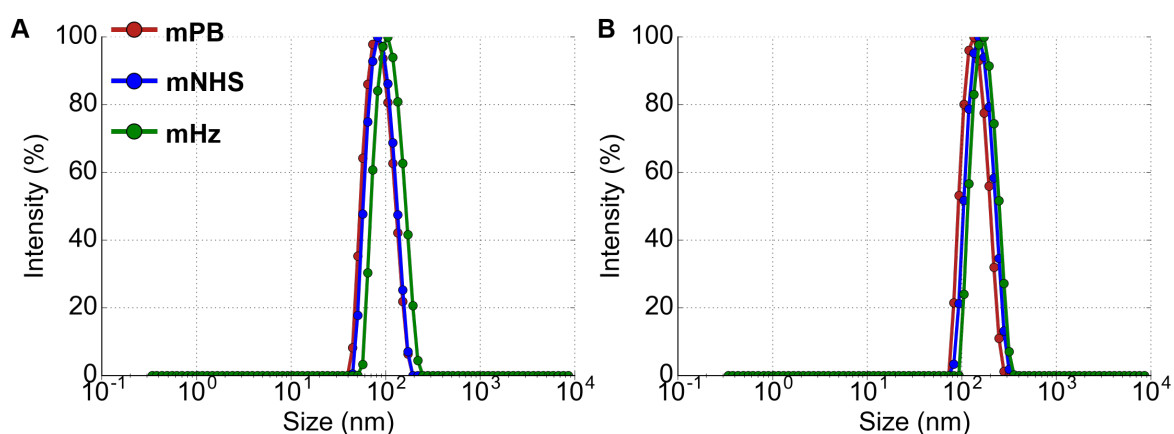
Figure S8. mPB, mNHS and mHz immunoprobe dispersion in (A) PBS compared to (B) HS.

Table S2. Z Average and intensity mean diameter, and PdI of PBS, NHS and Hz immunoprobes in PBS and HS.

Sample	PBS			HS		
	Z Average (nm)	PdI	Intensity Mean (nm)	Z Average (nm)	PdI	Intensity Mean (nm)
mPB	74	0.17	86	148	0.13	152
mNHS	75	0.16	70	167	0.22	159

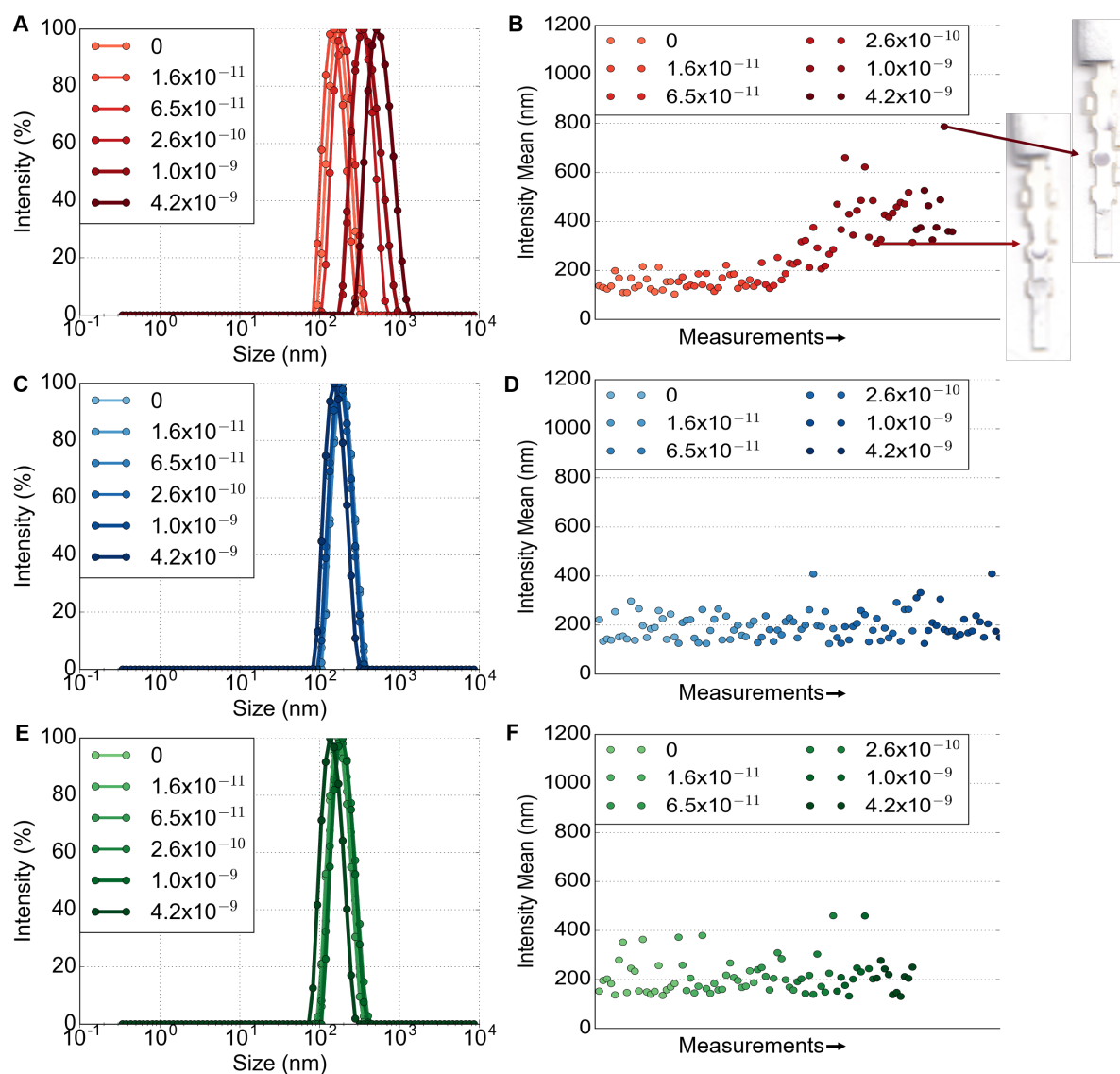| mHz	108	0.14	101	198	0.23	163

Figure S9. Immunoprobe distribution and mean intensity diameter with addition of mouse IgG and over time. Test done for (A and B) mPB, (C and D) mNHS and (E and F) mHz immunoprobes in HS.
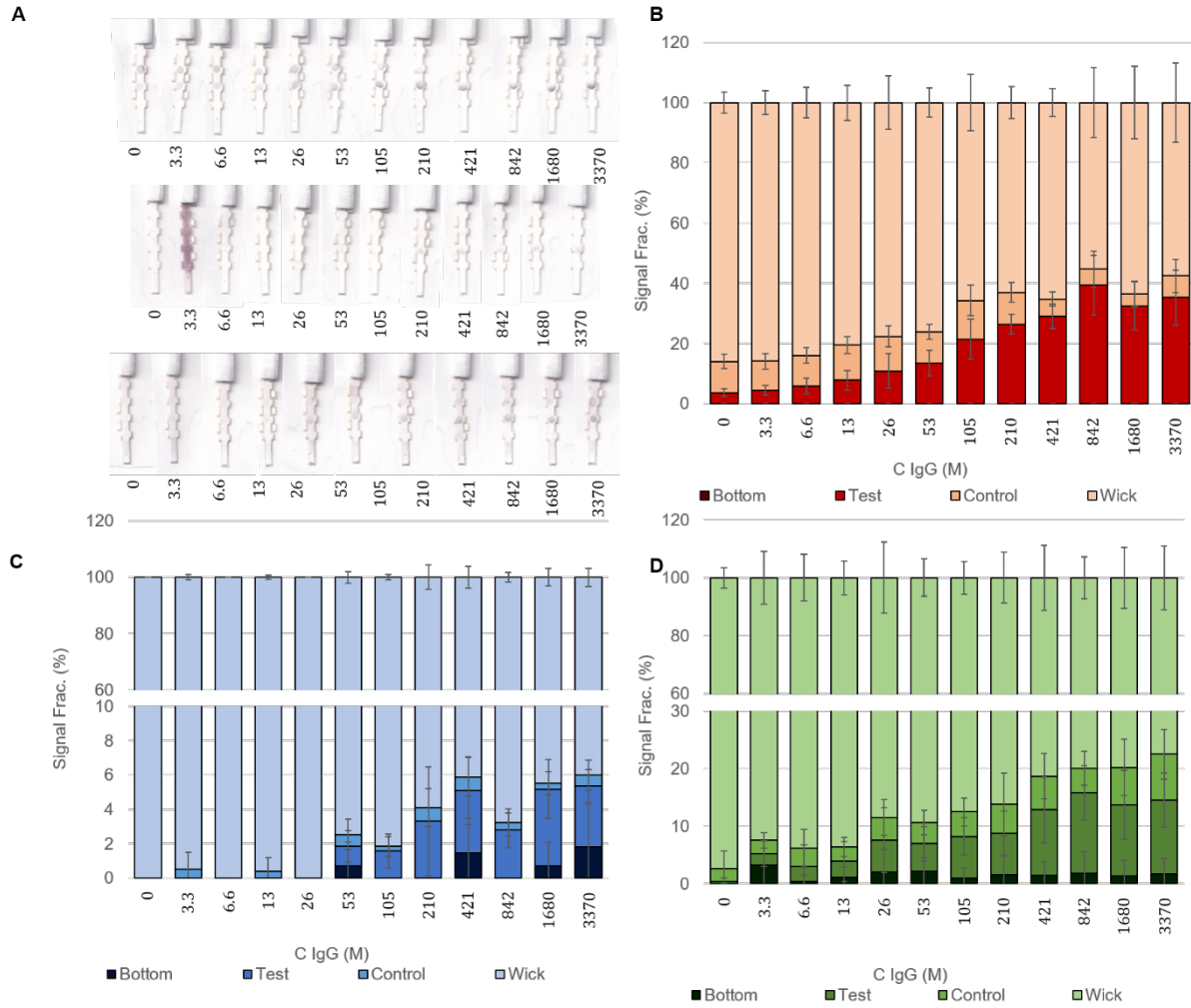
Figure S10. Distribution of immunoprobes on dipstick assays in HS shown as (A) pictures and bar charts for (B) mPB, (C) mNHS and (D) mHz immunoprobes.

Figure S11. DLS used to characterize the size distributions of anti (A) zika and (B) dengue NS1 immunoprobes in PBS and changes therein during antigen binding. This is done for (C-D) zPB immunoprobes in 30 mg/mL BSA and (E-F) HS and compared to (G-H) dPB in 30 mg/mL BSA showing similar behaviours. Conversely (I-J) zHz immunoprobes do not change their size distribution with antigen addition.

Table S3. Mean size by Z average and intensity of PB, NHS and Hz immunoprobes in PBS and HS and SPR obtained by UV-Vis.

Sample	PBS			HS			SPR in PBS
	Z Average (nm)	PdI	Intensity Mean (nm)	Z Average (nm)	PdI	Intensity Mean (nm)	
zPB	205	0.24	89	194 ± 49	0.23 ± 0.01	163 ± 25	549
zNHS	200 ± 113	0.16 ± 0.05	99 ± 18	-	-	-	-
zHz	118 ± 86	0.16 ± 0.05	158 ± 38	231	0.23	177	567
dPB	199	0.20	95	760	0.22	115	-
dNHS	131	0.22	94	-	-	-	-
dHz	303	0.15	210	-	-	-	-
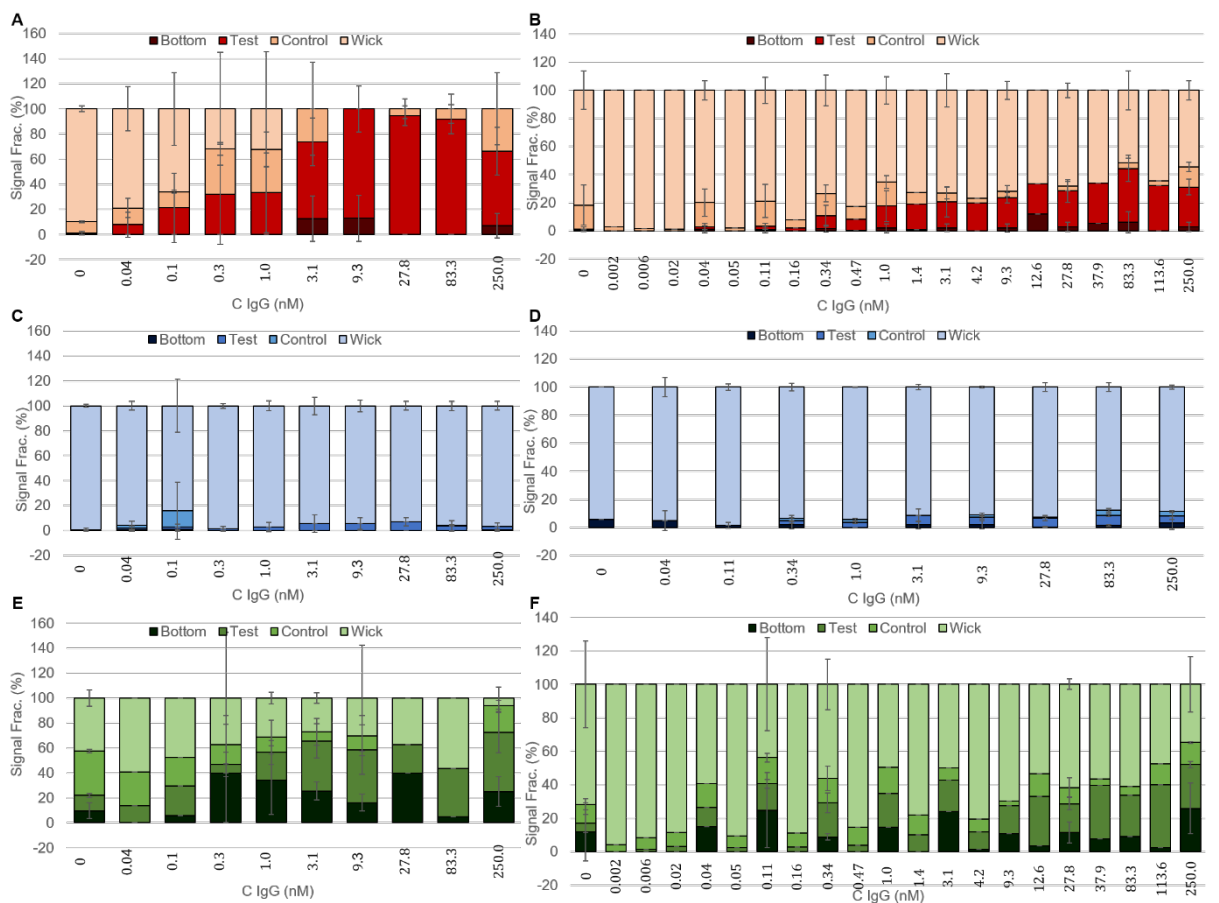
Figure S12. Dipstick distributions of zPB in (A) 30 mg/mL BSA and (B) HS with antigen titration. Comparable distributions of (C and D) zNHS and (E and F) zHz. N experiments specified in Table 2 (main paper).
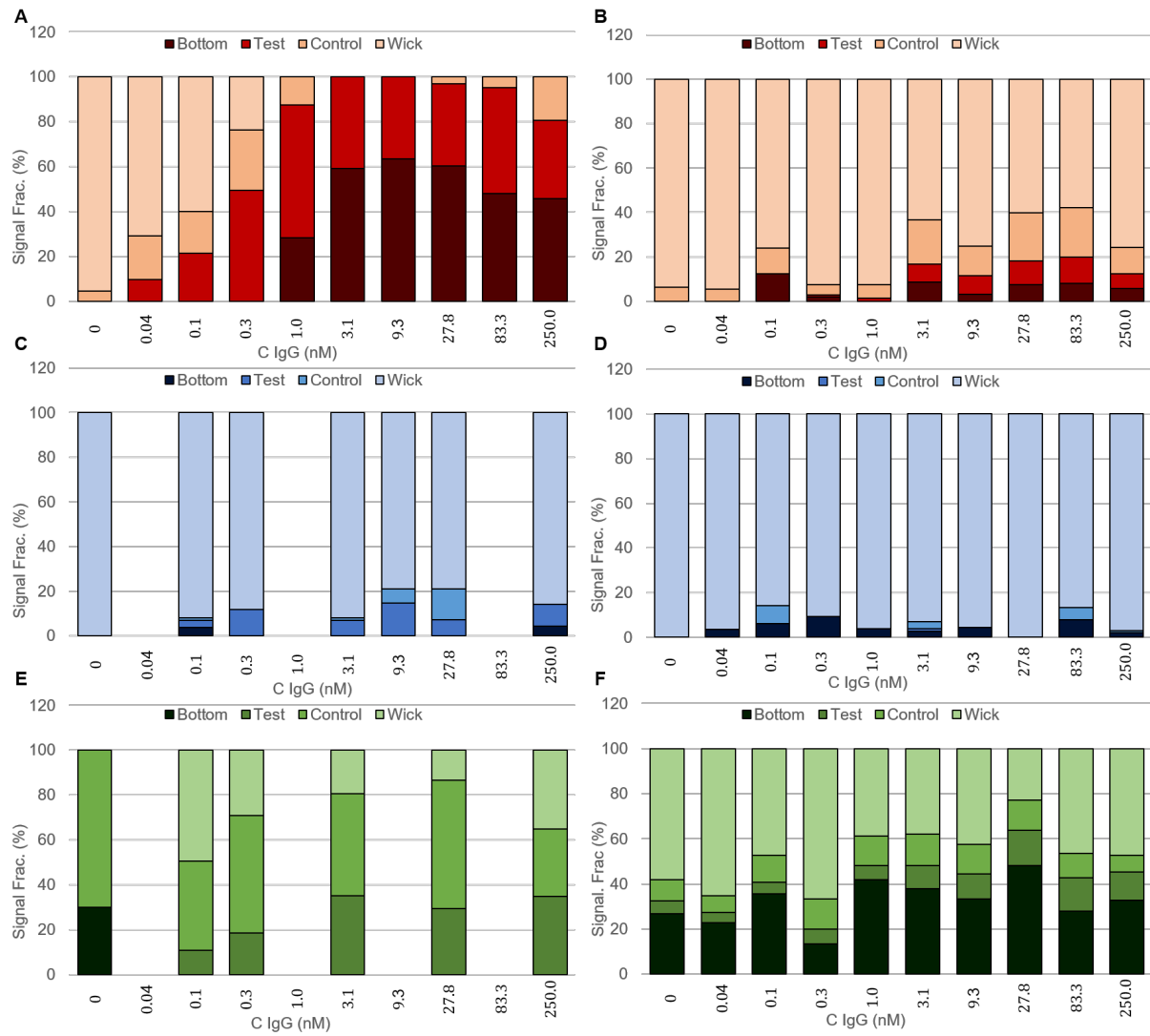
Figure S13. Dipstick distributions of dPB in (A) 30 mg/mL BSA and (B) HS with antigen titration. Comparable distributions of (C and D) dNHS and (E and F) dHz. N=1 in all cases.
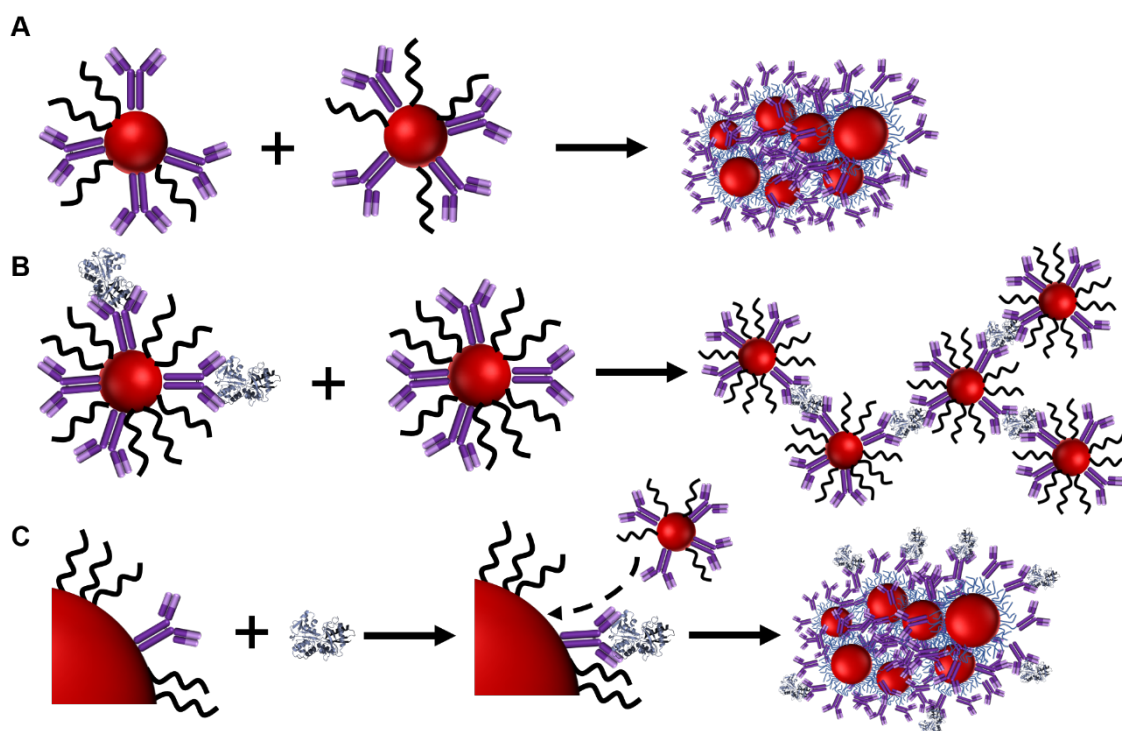
Figure S14. Schematic representation of the three proposed aggregation strategies: (A) insufficient surface stabilization, (B) aggregation through specific interactions and (C) aggregation due to changes on the NP surface with antigen binding.
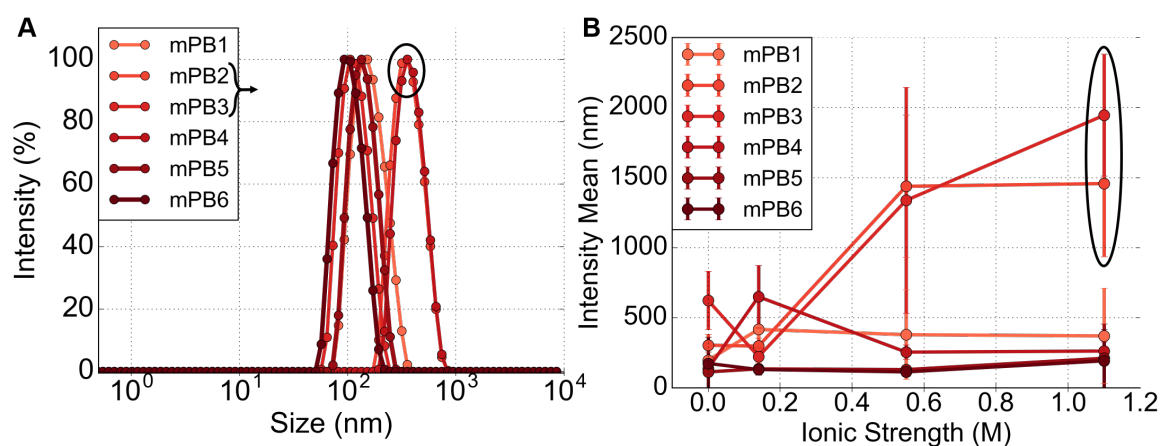

Figure S15. mPB immunoprobe diameter in media with increasing ionic strength for four different batches. Size measured by DLS and shown as (A) intensity size distributions in PBS and (B) intensity mean diameters. Error bars reflect measurement-to-measurement variance for a given batch. Black circles and arrow show corresponding samples in PBS.
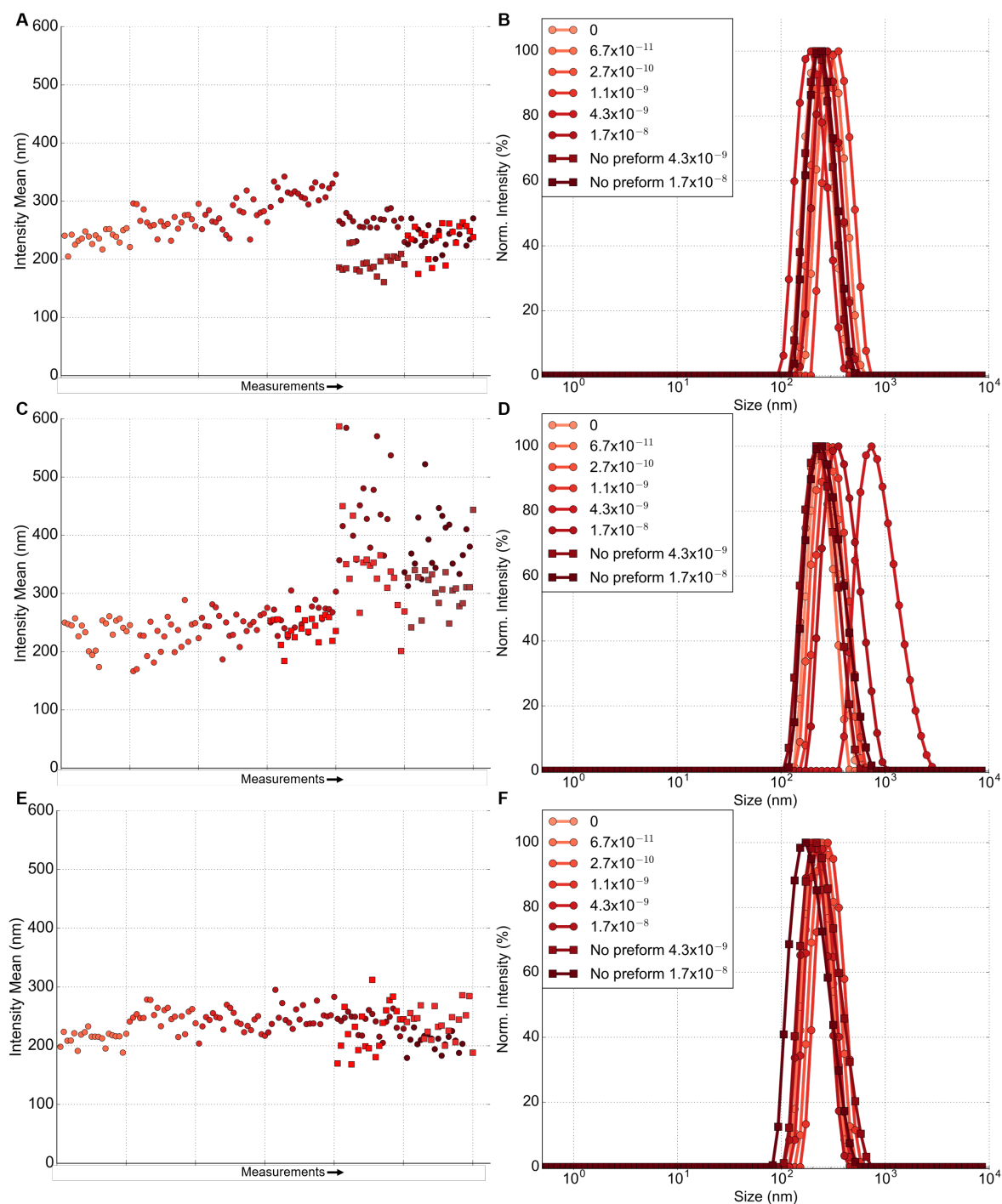
Figure S16. Antigen binding, in this case mouse IgG in DLS for three equivalent, but independent mPB immunoprobe batches with and without biomolecular corona preformation in HS. Figure shows the mean diameter increase and change in the distribution with antigen addition. (A and B) mPB1 and (E and F) mPB3 had minor increase in their size with antigen addition with and without corona preformation. (C and D) mPB2 increased in diameter with antigen addition.
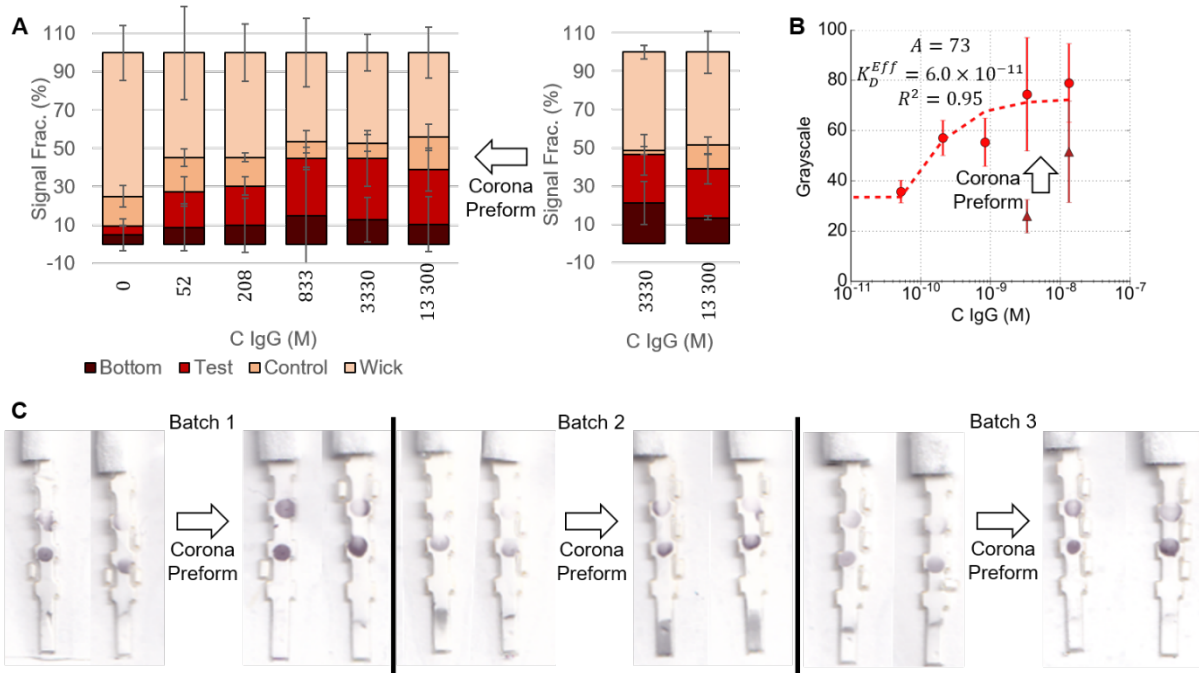
Figure S17. Preforming a biomolecular corona changes the performance of PB immunoprobes as measured by (A) their distribution on the dipstick, (B) antigen titration curve and (C) visual analysis of the dipsticks. All results are an average of three independent immunoprobe batches.
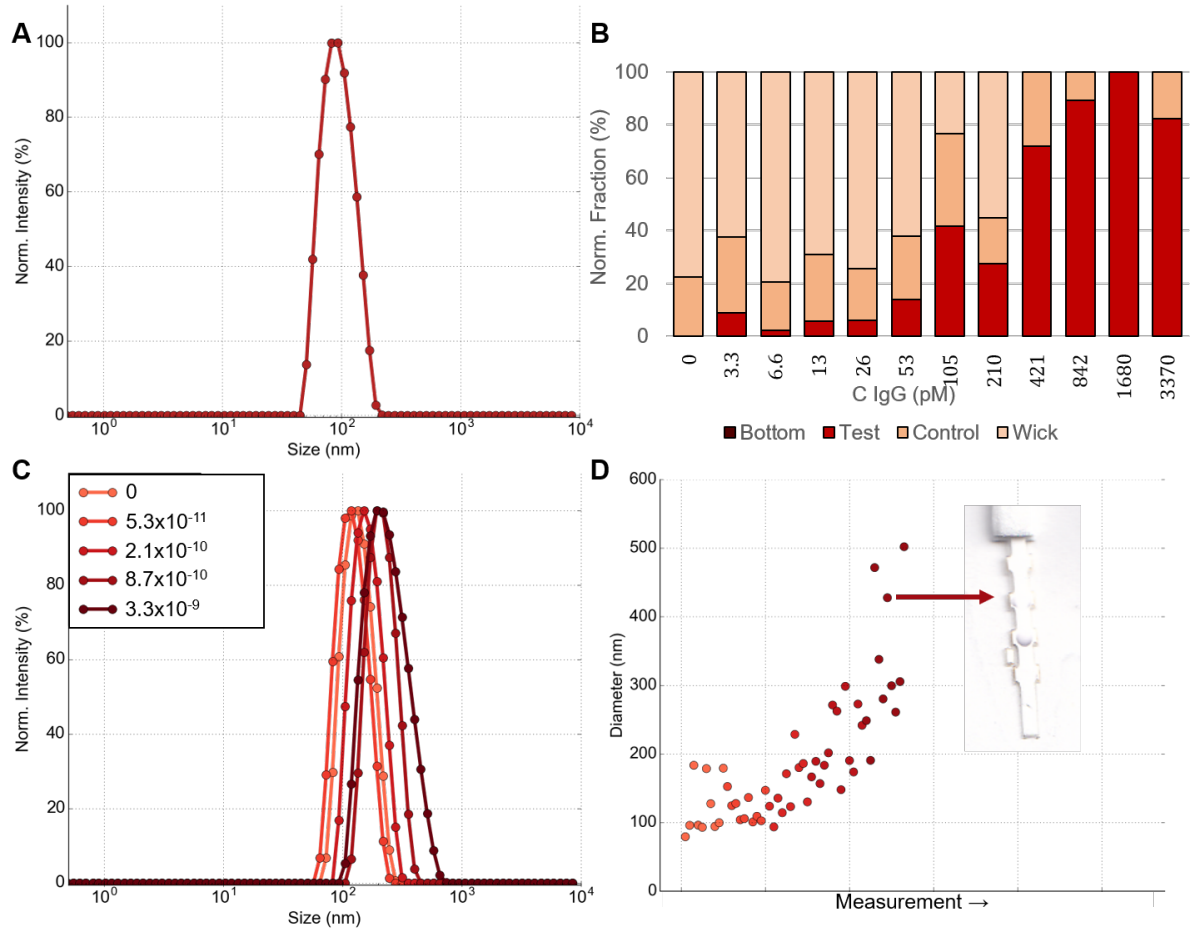
Figure S18. Characterization and binding of PEG backfill immunoprobes with four times the PEG added during synthesis. Figure shows (A) the immunoprobe distribution in PBS, (B) distribution on the dipstick assay during antigen titration, change in diameter with antigen binding in 30 mg/mL BSA shown by (C) size distribution and (D) intensity mean diameter.
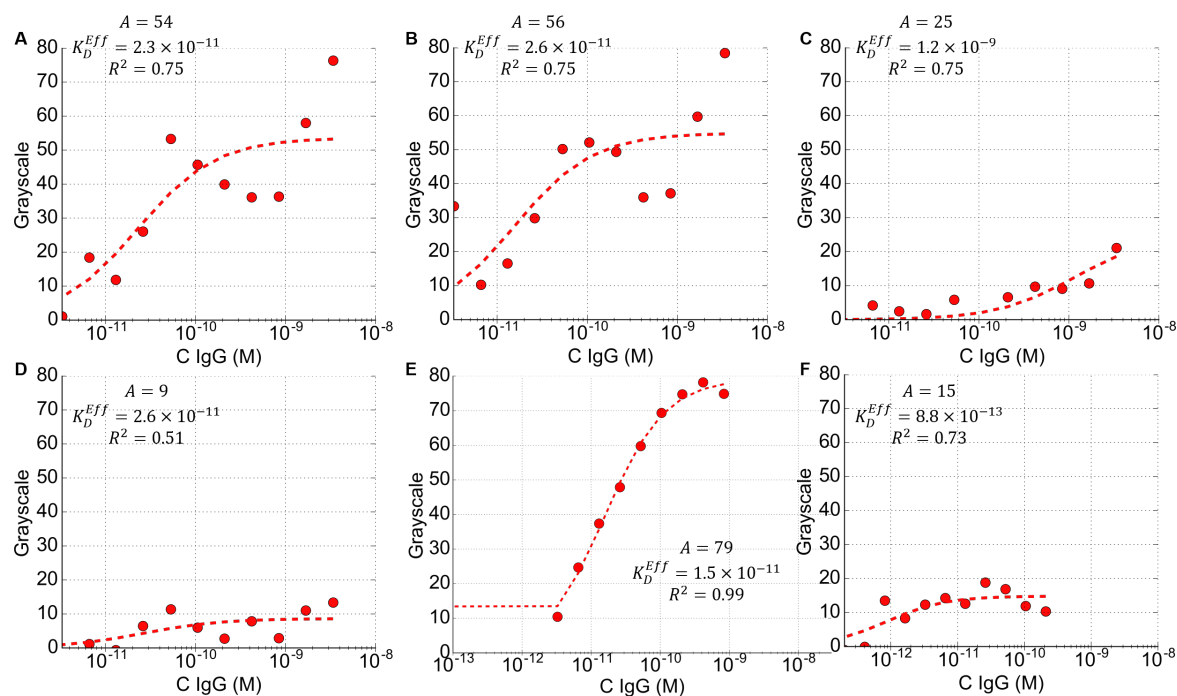
Figure S19. Antigen titration of (A) mPB (30 mg/mL BSA), (B) x4 PEG (30 mg/mL BSA), (C) x4 IgG (30 mg/mL BSA), (D) smaller Au NPs (30 mg/mL BSA), (E) smaller AuNPs in HEPES (HS) and (F) regular Au NPs in HEPES (HS) immunoprobes. All immunoprobes made with anti mouse IgG and titrated with mouse IgG.
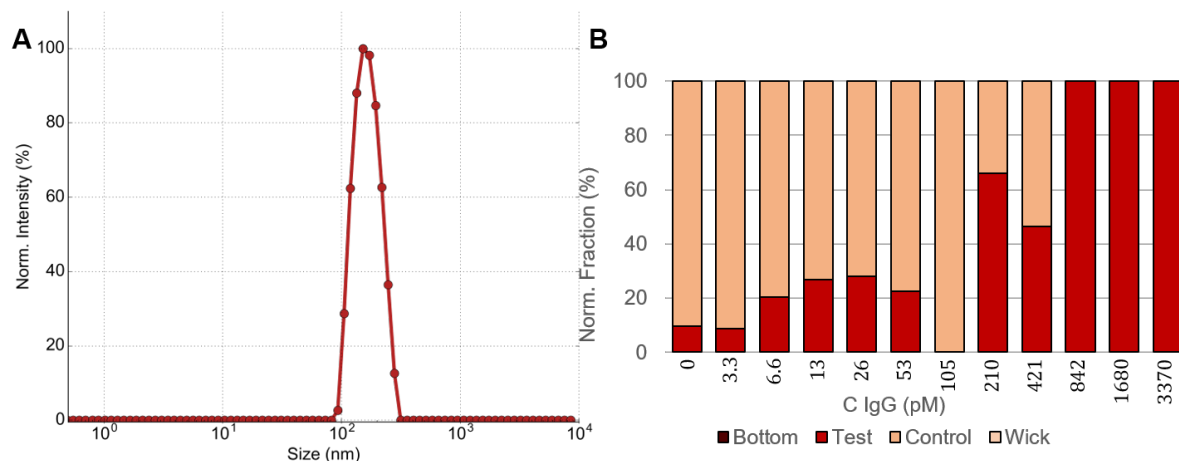

Figure S20. Characterization and binding of PEG backfill immunoprobes with four times the IgG added during synthesis. Figure shows (A) the immunoprobe distribution in PBS and (B) distribution on the dipstick assay during antigen titration.
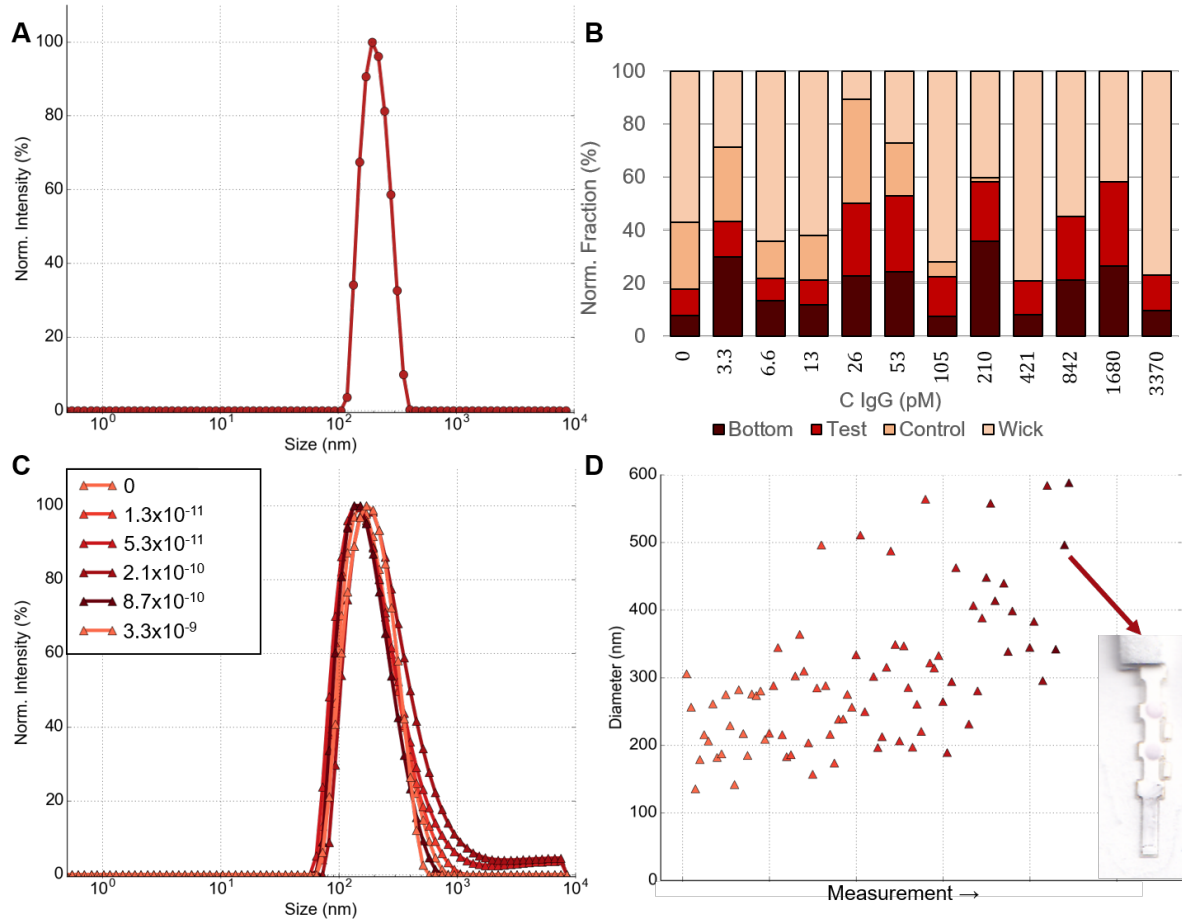
Figure S21. Characterization and binding of PEG backfill immunoprobes using small (~8 nm) Au NPs that were synthesized in a way comparable to the regular mPB immunoprobes. Figure shows (A) the immunoprobe distribution in PBS, (B) distribution on the dipstick assay during antigen titration, change in diameter with antigen binding in 30 mg/mL BSA shown by (C) size distribution and (D) intensity mean diameter.
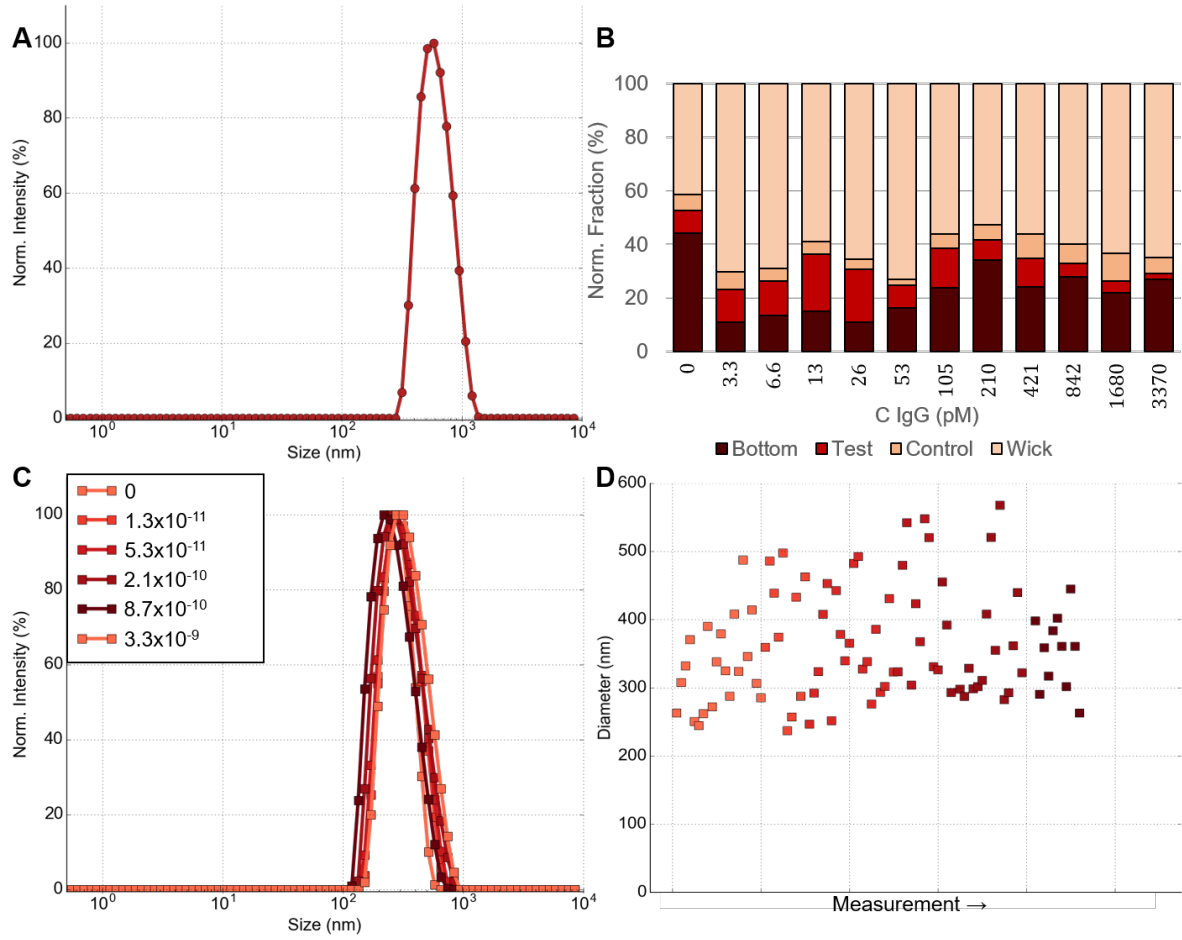
Figure S22. Characterization and binding of PEG backfill immunoprobes using regular (~20 nm) Au NPs where IgG binding and PEG backfill were done in HEPES pH 7.7. Figure shows (A) the immunoprobe distribution in PBS, (B) distribution on the dipstick assay during antigen titration, change in diameter with antigen binding in HS shown by (C) size distribution and (D) intensity mean diameter.
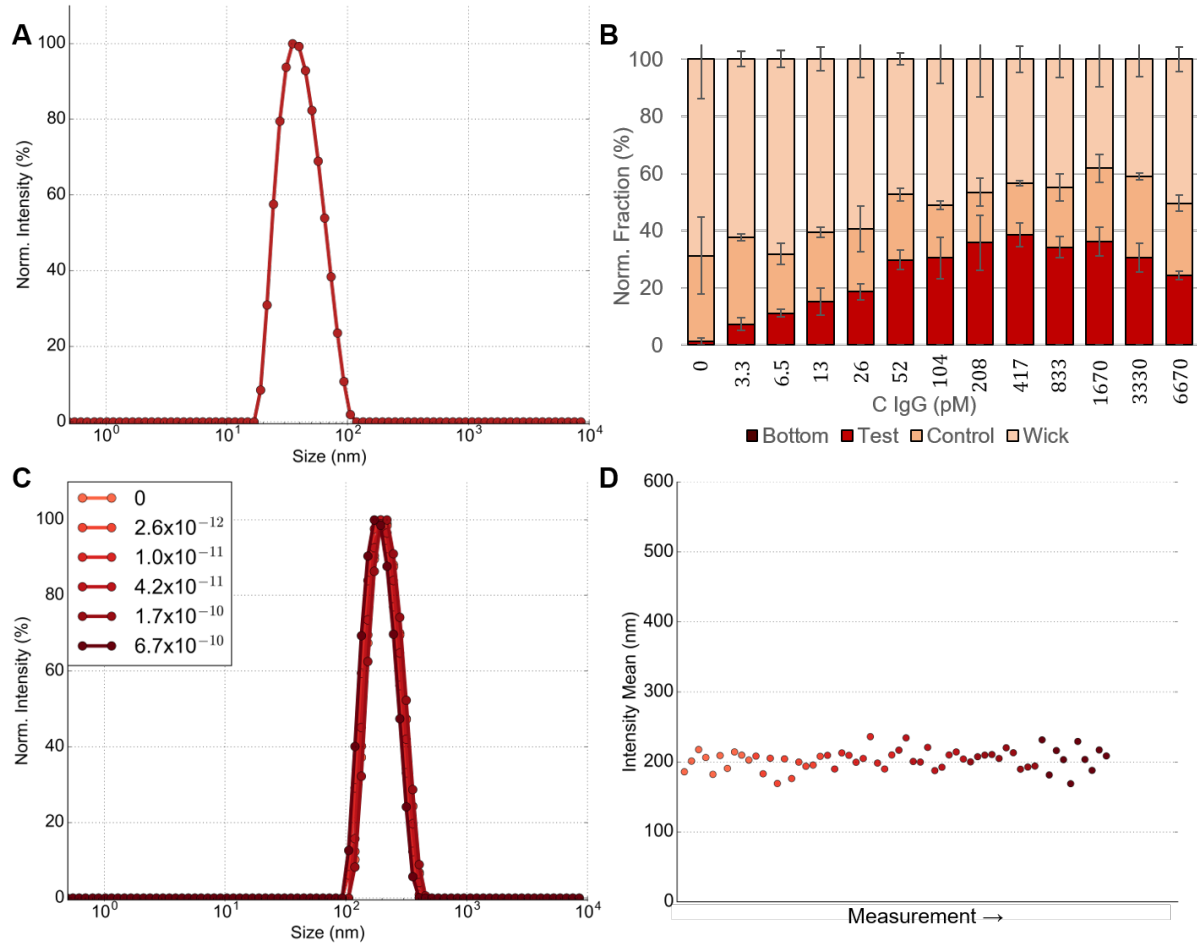
Figure S23. Characterization and binding of PEG backfill immunoprobes using small (~8 nm) Au NPs where IgG binding and PEG backfill were done in HEPES pH 7.7. Figure shows (A) the immunoprobe distribution in PBS, (B) distribution on the dipstick assay during antigen titration, change in diameter with antigen binding in HS shown by (C) size distribution and (D) intensity mean diameter.
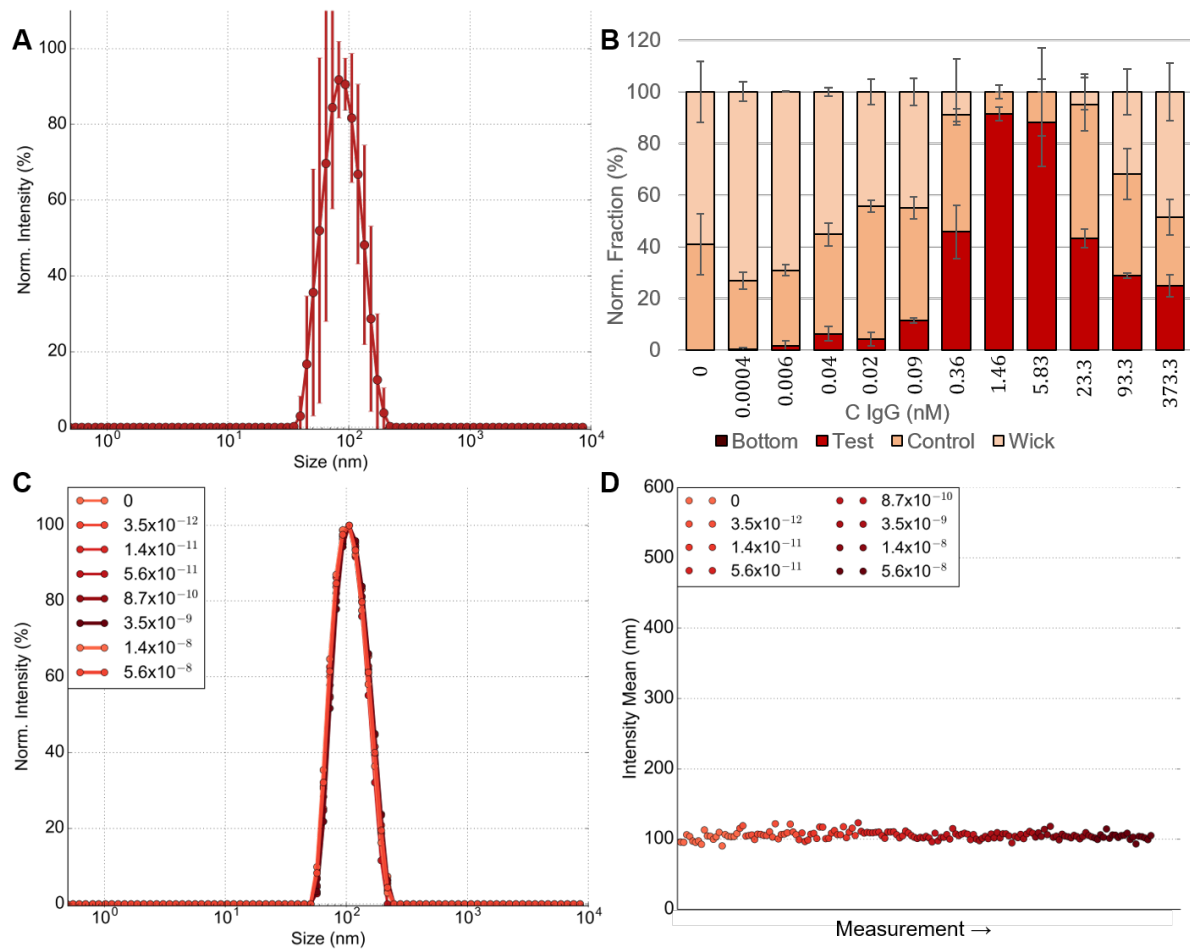
Figure S24. Characterization and binding of PEG backfill immunoprobes using regular (~20 nm) Au NPs where IgG binding was done without further changes, similarly to the regular mPB synthesis. However, particles were washed only once. Figure shows (A) the immunoprobe distribution in PBS, (B) distribution on the dipstick assay during antigen titration, change in diameter with antigen binding in HS shown by (C) size distribution and (D) intensity mean diameter.