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Supplemental information

Preclinical evaluations of Pfs25-EPA and Pfs230D1-EPA in AS01 for a vaccine to reduce malaria transmission

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Supplementary Text - Methods and Results of Formulation Studies

METHODS

Formulation Studies

Compatibility studies between Pfs25-EPA and Pfs230-EPA nanoparticles and AS01 were performed to determine formulation stability. Assessments for nanoparticle, liposome, or final formulation integrity were performed using SDS-PAGE and a high throughput panel including differential scanning calorimetry (DSC), nephelometry, turbidimetry and dynamic light scattering (DLS) on samples prepared in duplicate. SDS PAGE samples monitored T0 and 24h timepoints; high throughput panel tests monitored T0, 2h, 4h, and 24h at 4°C, 25°C, and 40°C. Formulations were prepared in Eppendorf tubes and transferred to 96-well plates (100-200uL/well). The formulations for these tests were prepared by admixing the Pfs25-EPA or Pfs230-EPA nanoparticles in a 4 mM PBS buffer PBS pH7.4: 154 mM NaCl, 1.04 mM KH2PO4, 2.41 mM Na2HPO4, 0.56 mM NaH2PO4) with the AS01 adjuvant (100 ug/mL MPL and 100 ug/mL QS-21). The final concentration of these formulations ranged by test method from approximately 26-254 µg/mL of the Pfs25 or Pfs230 antigen content, but consistently contained a final AS01 concentration of 25 µg MPL/25 µg QS-21. Additionally, formulation samples were retained on each day of vaccination during preclinical animal studies and were monitored for particle size and antigen stability by SDS-PAGE of samples retained immediately post formulation (T=initial) and post vaccination (T=pv) after storage at 4°C.

Aspecific adsorption of antigen on vaccine vials. Vials composed of polypropylene, siliconized and unsiliconized glass were tested for holding 500 uL of 26 µg/mL (Pfs25) or 32 µg/mL (Pfs230) nanoparticle/AS01 formulations and analyzed in triplicate for aspecific adsorption of Pfs25-EPA and Pfs230-EPA at T=0 and T=24 hours after storage at 4°C using three methods: 1) protein content by UV, 2) Intrinsic Fluorescence Emission, and 3) protein content by microBCA.

Protein content by UV absorption near 280 nm was performed by calculating absorbance from a sample tested against a blank and measured at 280, 360 and 900-975 nm. The results are expressed in relative concentration directly proportional to the calculated absorbance using the formula (A280-A360)/A975-A900).

Intrinsic fluorescence (IF), a function of the emission of Tryptophan, Tyrosine and Phenylalanine content in each sample, was analyzed by evaluating the maximum emission peak after excitation at ~280 nm.

Protein content by MicroBCA was performed using the Thermo Scientific Pierce Micro BCA Protein Assay Kit.

Nanoparticle Integrity by SDS-PAGE. Integrity of the Pfs25-EPA and Pfs230-EPA nanoparticles integrity at 60 ug/mL final vaccine formulation was assessed for Pfs25- and Pfs230-EPA at T0h and T24h after storage at 4°C or 40°C using SDS-PAGE under identical analysis conditions for each antigen, run under reducing or non-reducing conditions. 4-12% Bis-Tris 12 well gels with MOPS buffer were run for 1 hour at 200V-120mA. Samples were loaded with 4X concentrated loading buffer, and gels were silver stained for visual analysis. Samples were run either centrifuged or not centrifuged prior to loading. For formulation stability analysis during the animal studies, AS01 and AS04 formulation samples were mixed with 4X loading buffer (Invitrogen™ NuPAGE™ LDS Sample Buffer, ThermoFisher Scientific) and 20% SDS in a 1:1:2 ratio, heated for 5 minutes at 95°C, then stored at -80°C until analyzed. Alhydrogel samples of 100 µL centrifuged at 4000 rpm for 5 minutes and 90 µL of supernatant was transferred to a new Eppendorf tube. Samples were stored at -80°C. All antigen formulations were loaded directly onto 3-8% Tris-Acetate gels with 1X Tris-Acetate buffer in ddH2O.

High Throughput: Aggregation by Nephelometry/Turbidimetry. For both the Pfs25-EPA and Pfs230-EPA formulations at 60 µg/mL, 100 µL samples were transferred to a Corning 3679 polystyrene flat bottom reading plate, and nephelometry and turbidimetry were monitored using the same reading plate. Turbidimetry was determined by measuring absorbance at 320 nm and 275 nm according to the spectrum of either Pfs25-EPA or Pfs230-EPA.

High Throughput: Liposome integrity by dynamic light scattering (DLS). Samples read for Nephelometry/Turbidity were also measured against a 100 nm nanoparticle control at 22°C with an acquisition time of 180 seconds.

Stability by differential scanning calorimetry (DSC). Samples were diluted in either the AS01 liposome buffer at pH 6.1 or the Pfs25-EPA and Pfs230-EPA buffer (4 mM PBS, pH 7.4) in a 1:1 ratio by volume for a final concentration of 254 µg/mL antigen content in 400 µL sample volume. Samples were then placed on a plate stored at 4°C and scanned using a ramp from 10 to 95°C at a rate of 90°C/min.

Liposome integrity by hemolysis. QS-21 lytic activity was assessed on sheep red blood cells. The reaction was performed in a 96-well microplate, with 125 µL per well. A standard curve of QS-21 was first prepared; water was used as a positive control and AS01B was used as the negative quenched control. 13 µL of red blood cells (diluted 10 times in DPBS buffer) were then added, and samples gently mixed manually and left to sit at room temperature for 30 minutes. After centrifugation at 1500 RPM for 5 minutes, the OD of the supernatant was read at 405 nm, and the hemolytic activity of QS-21 determined by comparing to the standard curve.

Particle size analysis of vaccine during animal studies. On each day of vaccination, the AS01 formulations in Studies 1, 2, 4 and 5 were analyzed by DLS within 4 hours from initial formulation, directly post group vaccinations (Tpv). 10 µL of final formulation was diluted in 990 µL of Gibco™ distilled water and evaluated on a Zetasizer Nano-ZS, ZEN 3600 (Malvern Panalytical, Inc.).

ELISpot analysis of bone marrow plasma cells.
Bone marrow plasma cells were recovered from the 3 µg dose groups on Days 42 and at the end of study (Day 223 and Day 230) (Study 1). The mouse IgG ELISpot was performed using reagents from Mabtech, Inc (Cincinnati, OH). Cells were obtained by flushing the bone marrow from the femurs with DPBS using a 28G needle and syringe. The bone marrow was strained through a 40 mm filter to remove bone debris and to create a single-cell suspension. Erythrocytes were lysed with lysing buffer and the remaining immune cells were centrifuged and placed in IMDM media supplemented with 10% FBS, HEPES, and pen/strep. 96-well ELISpot plates were coated with Pfs25 antigen (10 mg/ml) overnight, followed by blocking for 2 hours with TBS/milk. Cells were plated at concentrations ranging from 250,000 per well to 31,250 per well and incubated for 16 hrs in a 37° C incubator. Cells were washed 5 times with PBS and anti-IgG-biotin detection antibody was incubated (1 mg/ml) for 2 hours at room temperature. Plates were washed 5 times and streptavidin-ALP (1:1,000) was added and incubated for 1 hour at room temperature. After washing, substrate was added and spots were allowed to develop for 15-20 minutes, followed by washing with water to stop the reaction. After plates were dry, images were captured using an Immunospot analyzer (CTL, Shaker Heights, OH). Spots were counted manually and results were reported as antibody-secreting cells (ASCs) per million cells.

RESULTS
Aspecific adsorption. For both of the lower dose concentrations of Pfs25- and Pfs230-EPA (26 µg/mL and 32 µg/mL respectively) there was no difference between the different vial types (polypropylene, siliconized or unsiliconized glass vials, polystyrene microplate) after 24h +4°C storage, thus aspecific adsorption is not vial type dependent. However, protein content in solution slightly decreased over time as measured by IF and UV (but not BCA) as compared to a fresh dilution used as the reference.

Nanoparticle integrity by SDS-PAGE. The observation of Pfs25-EPA and Pfs230-EPA nanoparticle smears in all samples without apparent intensity fluctuation over time suggests that these antigens are not subject to degradation or chemical modification after being incubated for 24h at 40°C. AS01 and its buffer have no impact on either antigen during this period of time and at this temperature.

Aggregation by Nephelometry/Turbidimetry. Nephelometry results indicate that no major aggregation of either Pfs25- nor Pfs230-EPA was observed after incubation for 24h at 40°C at a clinical dose. Moreover, there was no major difference between the nanoparticle (Pfs25- or Pfs230-EPA) in its buffer or in the AS01 buffer, suggesting that the AS01 buffer has no impact on the aggregation of either antigen.

Antigen intrinsic absorbance and intrinsic fluorescence exhibited no major differences between the different conditions, suggesting that AS01 has no impact on Pfs25- or Pfs230-EPA aggregation.

Stability by DSC. DSC preliminary results show that both Pfs25- and Pfs230-EPA nanoparticles undergo some transitions before 40°C as expected.

Liposome integrity. For both Pfs25- and Pfs230-EPA, DLS showed no modification of liposome size after 24h at 40°C. In all conditions, there seems to be no influence of the buffer composition (Pfs25- or Pfs230-EPA buffer or AS01 buffer) on the integrity of liposomes.

After testing liposomal samples for hemolytic activity on sheep red blood cells, results show that QS-21 remained quenched in the liposomes with free QS-21 under the maximum limit of 5-6 µg/mL after 24h at 40°C. Pfs25- or Pfs230-EPA and their buffers have no impact on the adjuvant during this period of time and at these temperatures.

Dilution studies pre-mixing with AS01. The pH and the aggregation state of both Pfs25- and Pfs230-EPA were studied after dilution in different buffers. For Pfs25-EPA at 32 µg/ml and Pfs230-EPA at 26 µg/ml, turbidimetry, nephelometry and DLS showed that no major aggregation issue occurred at these concentrations and after maximum 24h at 25°C.

Formulation stability during immunogenicity studies. Formulations remained stable as analyzed by SDS-PAGE and particle sizing during the vaccination period on each day of vaccination Studies 1, 2, 4 and 5. Final AS01 formulations had a particle size range of 109.3 to 128.3 d.nm during the <4 hour period from formulation to vaccination, throughout the course of the 4 animal studies, and all formulations maintained one peak around 100 d.nm. Nanoparticle evaluation by SDS-PAGE and silver stain resulted in extracted antigens retaining their pre-formulation migration pattern and appearance as compared with a non-formulated reference on each day of vaccination. Alhydrogel formulations from Studies 1, 2, 3 and 4 were prepared by centrifugation with supernatants evaluated by SDS-PAGE and silver staining. The Alhydrogel formulation samples did not show any bands on the gels, indicating that the nanoparticle remained ~100% bound as expected for these TBV/Alhydrogel formulations.[1]

Acknowledgements
The authors are grateful to GSK for providing the Adjuvant Systems AS01 and AS04, as well as for preliminary stability testing shown in these Supplementary Materials, support and guidance for its formulation and use for the preclinical studies.

References

1. Zhu, D., Wu, Y., McClellan, H., Dai, W., Rausch, K., Jones, D., Aebig, J., Barnafo, E., Butler, B., Lambert, L., et al. (2017). Accelerated and long term stability study of Pfs25-EPA conjugates adjuvanted with Alhydrogel(R). Vaccine *35*, 3232-3238. 10.1016/j.vaccine.2017.04.067.

Supplementary Table 1: Summary of Pfs25-EPA and Pfs230D1-EPA formulation studies. Prior to studies described in **Figures 1** and **2** (Pfs25+Pfs230 combo mouse study figures, ELISA and SMFA), formulations were prepared as described in the table below and assessed for stability of interim and final formulations to ensure proper preparation and storage of formulations on days of immunization. Of note, the formulations were stable for the duration of the immunizations, and were held at 2-8˚C prior to administration within 4 hours of injection.

Protein concentration: 60 µg/mL antigen (Pfs25 or Pfs230 content) unless otherwise stated.

Antigen in AS01B buffer (10 mM PO4 (Na2/K) – 150 mM NaCl pH 6.1)
AS01 (100 µg/mL MPL/100 µg/mL QS-21) diluted in nanoparticle buffer (154 mM NaCl, 1.04 mM KH2PO4, 2.41 mM Na2HPO4, 0.56 mM, NaH2PO4 pH 7.4)

Final formulations composed of aqueous nanoparticle diluted 1:1 by volume with AS01 (100 µg/mL MPL/100 µg/mL QS-21)

Formulations were prepared and tested in duplicate.
200 µL per well in 96 well plate for differential scanning calorimetry (DSC), nephelometry, turbidity and dynamic light scattering (DLS), high throughput (HT) analysis) unless otherwise stated

Tests performed at T=0, 2h, 4h, and 24h
4˚C, 25˚C 40˚C or a subset of these parameters as stated for each assay type.

Tests performed on Pfs230D1-EPA and Pfs25-EPA nanoparticles		**Parameters tested**	**Details for data collection**	**Results**
Evaluations for aspecific adsorption of nanoparticle to container types used during formulation preparation and storage.	Aspecific adsorption by UV	Protein Concentration: 26 µg/mL Pfs230 content or 32 µg/mL Pfs25 content in protein buffer: 154 mM NaCl, 1.04 mM KH_2PO_4, 2.41 mM Na_2HPO_4, 0.56 mM NaH_2PO_4, pH 7.4 Vial types: 3 mL Siliconized glass (S+), 3 mL Unsiliconized glass (S-), 15 mL Falcon polypropylene tube 1.5 mL Eppendorf polypropylene tube (500 µl/vial) 100 µl/well: Plate Corning 3679 (UV plate, polystyrene flat bottom) -Tested in triplicate - Incubation time: T0 and 24h 4°C	Absorbance measured at ~280 Abs – 360 and at 900-975 nm Concentration calculated using: (A280-A360nm)/(A975-A900nm) Results expressed in relative concentration directly proportional to calculated absorbance.	Aspecific adsorption occurs at 24 hours at 4°C at this lower vaccine dilution, but is not related to container type. *(See Figure S1.)*
	Aspecific adsorption Protein content by BCA		MicroBCA (Thermo Scientific Pierce Micro BCA Protein Assay Kit) Measure of total protein concentration of dilute protein solutions (A562nm, 0.5 to 20 µg/mL)	
	Aspecific adsorption Intrinsic Fluorescence		λex: ~ 280nm (Tyr/Trp) and emission spectrum to determine the λem max.	No major differences existed over time between the different temperatures for any of the samples tested. *(See Figure S4)*
Examination of final formulations for any modification or precipitation of nanoparticles due to adjuvant or adjuvant buffer during storage for up to 24 hours at 4˚C and 40˚C.	Chemical integrity by SDS-PAGE	T=0, 2h, 4h, and 24h 4˚C and 40˚C Centrifuged or not centrifuged samples. Reducing and Non-Reducing conditions	Criterion XT 4-12% Bis-Tris, 12 well gels 0.3 ug antigen loaded per well MOPS diluted 20X in MilliQ water 1 hour at 200V-120mA Sample buffer: 4X with or without β–mercaptoethanol (reducing or non-reducing) Silver Stain	No evident differences in migration pattern existed between any of the non-reduced or reduced conditions, centrifuged or not centrifuged for any of the Pfs230D1 samples. The nanoparticles are stable in AS01 in the conditions tested.

Evaluation for effects of Adjuvant buffer on nanoparticles	Antigen Stability by DSC	254 ug/mL Pfs230 + AS01 (200 uL Pfs230 + 200 uL AS01) prepared in either Pfs230 or AS01 buffer	Scan 10-95C at 90C/min. Plate storage temp: 4C Volume/well = 400 uL	Some transitions occur prior to 40˚C with buffer+antigen, thus vaccine will be stored below 40˚C.
	Antigen Aggregation by Nephelometry and turbidity	100uL of 60 ug/mL in Pfs230 buffer or AS01 buffer T=0, 2h, 4h, and 24h 4C, 25C 40C Controls: 100nM nanoparticles for DLS Successive formazine dilutions (10-200 FNU) for nephelometry Study repeated for 26 ug/mL Pfs230 and 32 ug/mL Pfs25 samples after dilution in 154 mM NaCl to mimic preparation of lower dosage formulation intended for clinic.	Turbidity performed for Pfs230 only: measure absorbance at 320 nM and 275 nM (intrinsic absorbance of aromatic residues) according to the spectrum of the antigen Nephelometry: NU measured in the range of 250-4000	At 60 µg/mL, no aggregation of Pfs230 was observed at any temperature or timepoint tested, or between centrifuged or not centrifuged samples. There is no major difference between the stability of the Pfs230 in it's own or AS01 buffer suggesting AS01 buffer has no impact on the antigen. *(See Figures S2 and F3)* Data for lower concentration samples resulted in similar observations (data not shown).
Evaluation for effects of Adjuvant buffer on nanoparticles and for effects of nanoparticles on adjuvant by particle sizing analysis.	Liposome integrity by Dynamic Light Scattering (DLS)	Dynapro II DLS control: 100 nm Nanoparticle 60 ug/mL Pfs230 or Pfs25 26 ug/mL Pfs230 and 32 ug/mL Pfs25 in nanoparticle buffer, and after dilution in 0.9% NaCl and admixed with adjuvant buffer	100 uL sample per well 22C for 180 seconds with maximum laser power (100%)	Nanoparticle samples diluted in adjuvant buffer exhibit possible aggregation based on mean particle size after centrifugation referenced to uncentrifuged samples. Formulations with AS01 exhibit the expected particle size for the liposomes, thus the integrity of the AS01 did not change. Samples of the diluted antigen did not result in changes to the particle size for either nanoparticle (data not shown).
Evaluation of nanoparticle and nanoparticle buffer effects by measuring free QS-21 to determine liposome integrity.	Liposome integrity by sheep red blood cell hemolysis	60 ug/mL Pfs230 or Pfs25 Sheep Red Blood Cell (SRBC) lytic activity 4C and 40C temperatures tested. Standard Curve of QS-21 prepared: 0-100 ug/mL in DPBS buffer. Water s positive control for hemolysis. AS01 is negative quenched control.	13 uL SRBC diluted 10 times in DPBS added to samples and gently mixed. Incubated for 30 min RT. Centrifuged 5 min 1500 rpm OD of supernatants read at 405nM Hemolytic activity of QS-21 determined by comparing to standard curve.	QS-21 remains quenched in liposomes after 24h at 40C, thus the nanoparticles have no deleterious effects on the AS01 in the final clinical formulation.
Monitoring any effects of pH from adjuvant buffer on nanoparticle stability in final formulations for potential lowest dose prepared for clinical evaluations.	pH	26 ug/mL Pfs230 and 32 ug/mL Pfs25 in nanoparticle buffer, and after dilution in 0.9% NaCl and admixed with AS01 buffer 4 and 25C T=0 and 24 h	Measurement of pH following incubation	Formulations of nanoparticles initially are ~7.29 regardless of temperature or time. After dilution in NaCl, pH values range from 6.81 to 6.74. After admixing diluted samples with AS01 buffer, pH values range are ~6.27. There is no visual change noted.

A

Pfs230D1-EPA

Cc in µg Prot /ml	Mean of inter-days tests			%age difference between fresh and 24h preparations		
	Average Iintrinsic Fluorescence	Average UV	Average µBCA	IF	UV	µBCA
Pfs230 T0 (fresh dilution)	**55**	**57**	**45**	**0**	**0**	**0**
Pfs230 T24h +4°C plate 96 wells	47	46	43	16	20	6
Pfs230 T24h +4°C glass vial (siliconized) S+	52	47	42	6	18	7
Pfs230 T24h +4°C glass vial (unsiliconized) S-	52	49	40	6	15	12
Pfs230 T24h +4°C Eppendorf 1.5	52	48	43	7	17	6
Pfs230 T24h +4°C Falcon 15ml	50	47	39	10	18	14

B

Pfs25-EPA

Cc in µg Prot /ml	Mean IF	Mean UV	Mean µBCA	%age difference between fresh and 24h preparations		
				IF	UV	µBCA
Pfs25 T0 (fresh dilution)	**72**	**71**	**69**	**0**	**0**	**0**
Pfs25 T24h +4°C plate 96 wells	66	64	71	7	10	-3
Pfs25 T24h +4°C glass vial S+	59	62	69	18	13	0
Pfs25 T24h +4°C glass vial S-	63	68	70	12	4	-1
Pfs25 T24h +4°C Eppendorf 1.5	66	64	71	8	10	-3
Pfs25 T24h +4°C Falcon 15ml	67	65	69	6	9	0

Figure S1. The aspecific adsorption of Pfs230D1 and Pfs25 nanoparticles, related to STAR Methods.
Summary of aspecific adsorption testing performed for **(A)** Pfs230D1-EPA and **(B)** Pfs25-EPA nanoparticles at 26 µg/mL Pfs230 or 32 µg/mL Pfs25 content (the lowest intended clinical preparation) using intrinsic fluorescence, UV or microBCA assays. All samples are compared with a fresh dilution of the respective antigen (top row, green). Color changes from green to red indicate a small to larger difference from the freshly diluted sample. A decrease of the protein content after 24 hours at 4˚C storage (from 6-20%) was observed. No major differences between the different types of containers was observed, thus aspecific adsorption does not depend on these tested container types.

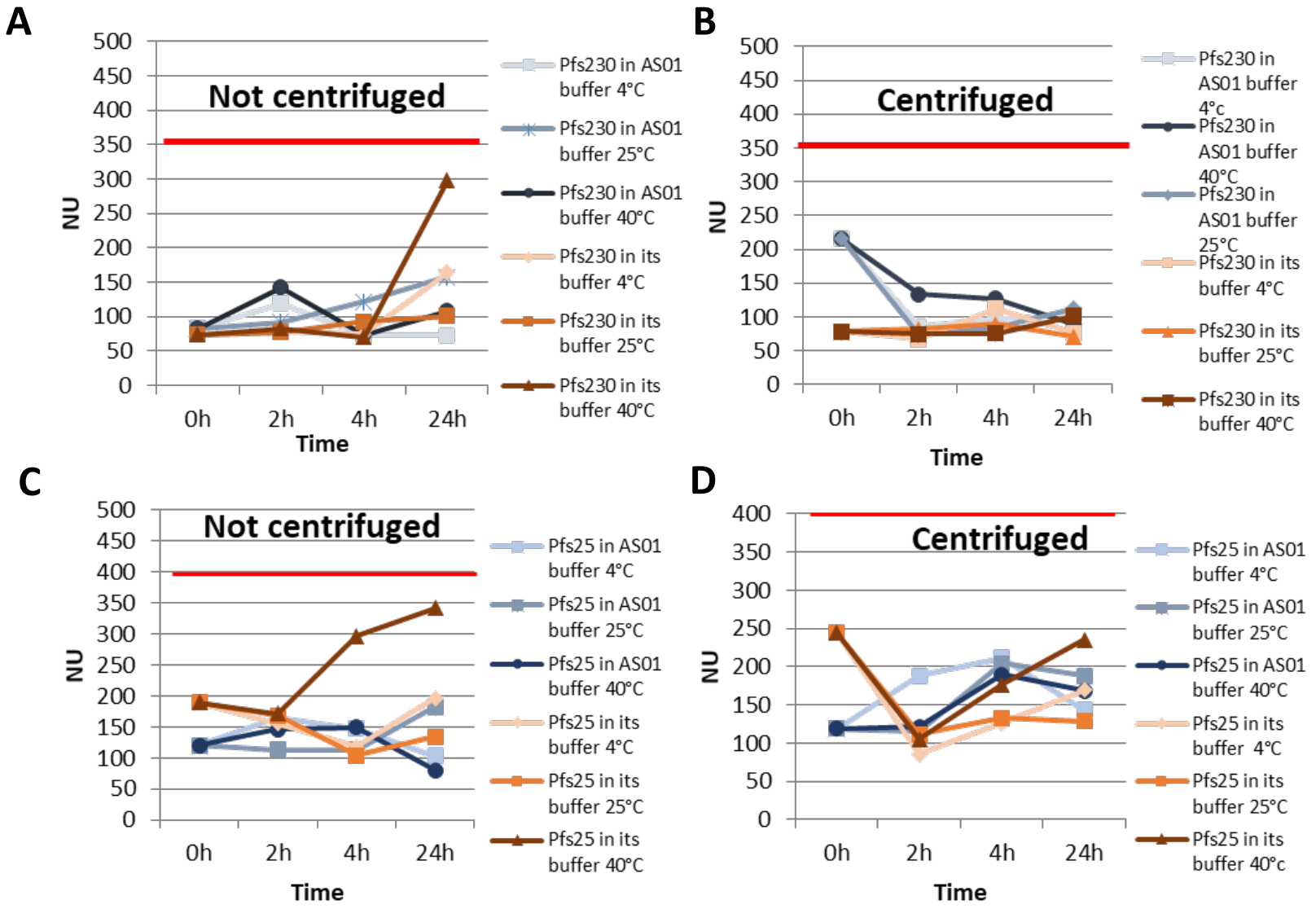
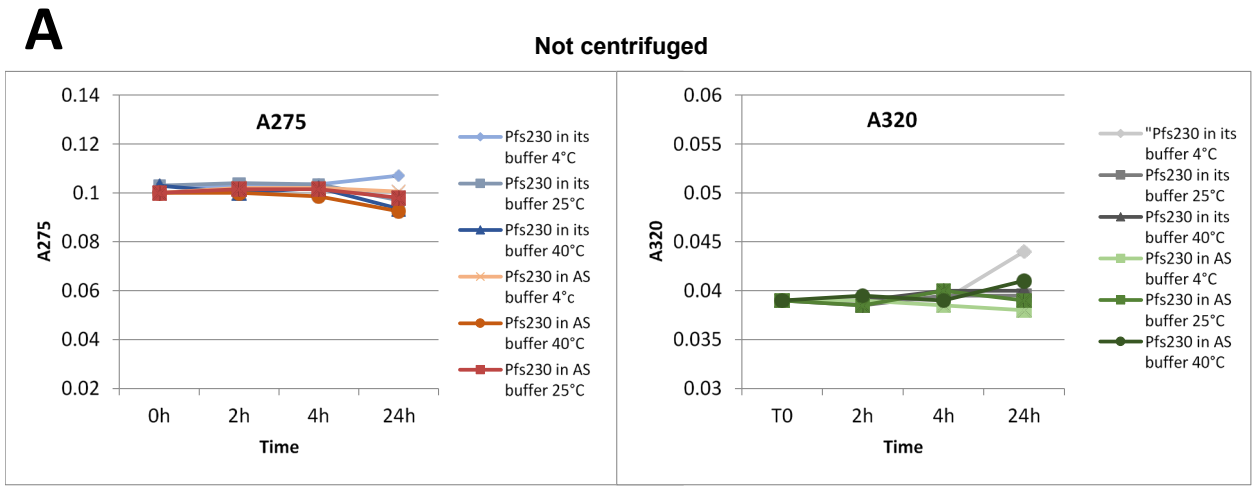
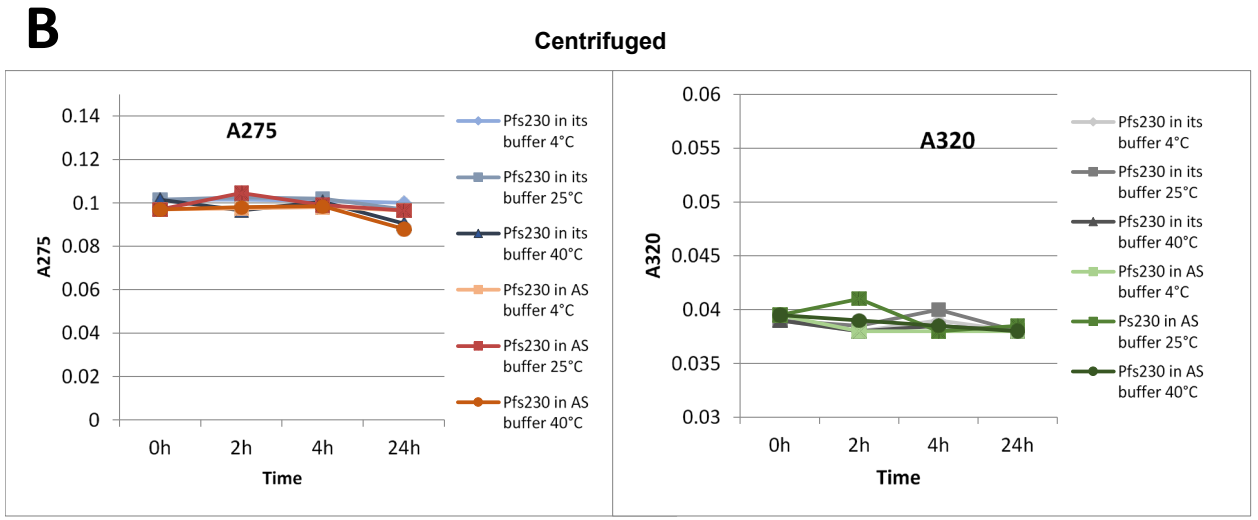


Figure S2. The nephelometry results of Pfs230D1 and Pfs25 nanoparticles, related to STAR Methods. Formazine dilutions were prepared (10-200 FNU) and measured in turbidity and nephelometry. For nephelometry, values of NU= 250-4000 are valid. The 10 FNU, NU=357 (red line) is the lower formazine dilution. As all samples for either Pfs230D1 or Pfs25 nanoparticles fall below this value, this suggests that at the 60 ug/mL antigen concentrations no major aggregation is occurring in any of the time/temperature conditions with either nanoparticle or AS01 buffer, whether samples are centrifuged or not centrifuged. The Pfs230D1 and Pfs25 nanoparticles are stable and the AS01 buffer does not impact their stability.





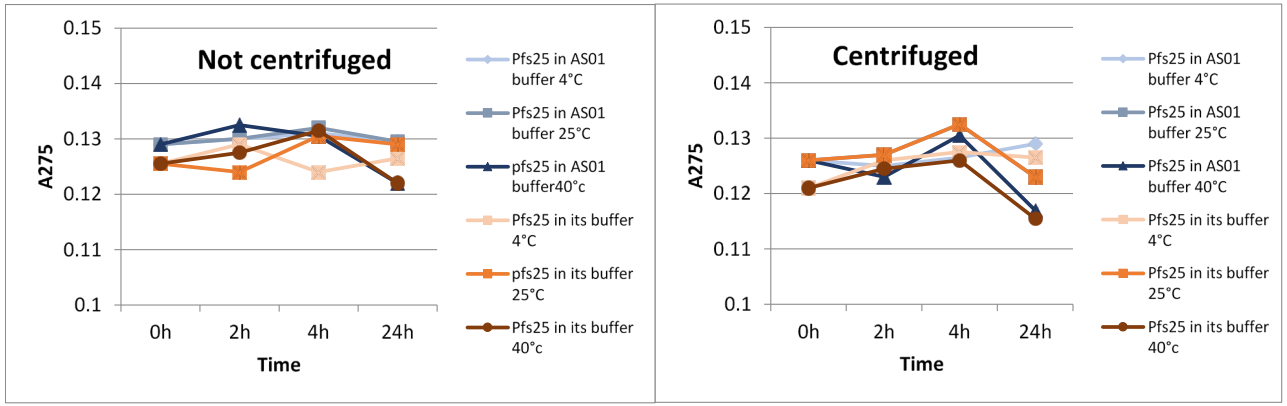


Figure S3. The turbidimetry at 320nm and absorbance at 275 nm for Pfs230D1 nanoparticles, related to STAR Methods. Turbidity measurements occurred at absorbance of 320 nm with the formazine dilutions used for nephelometry and are dependent on formazine concentration being linear at A320, with the assumption being valid for absorbance values of A320= 0.2 – 0.4. As the signal is weak for all samples, and is inferior to the 10 FNU (A320 = 0.06) lowest formazine dilution, this suggests that no major aggregation events occurred for any of the Pfs230D1 samples at the times and temperatures tested, in either the Pfs230D1 or AS01 buffer. Turbidity was not captured for the Pfs25 nanoparticle, but the absorbance at A275 was measured as shown for centrifuged and non-centrifuged samples, with similar results to the Pfs230D1 nanoparticle.

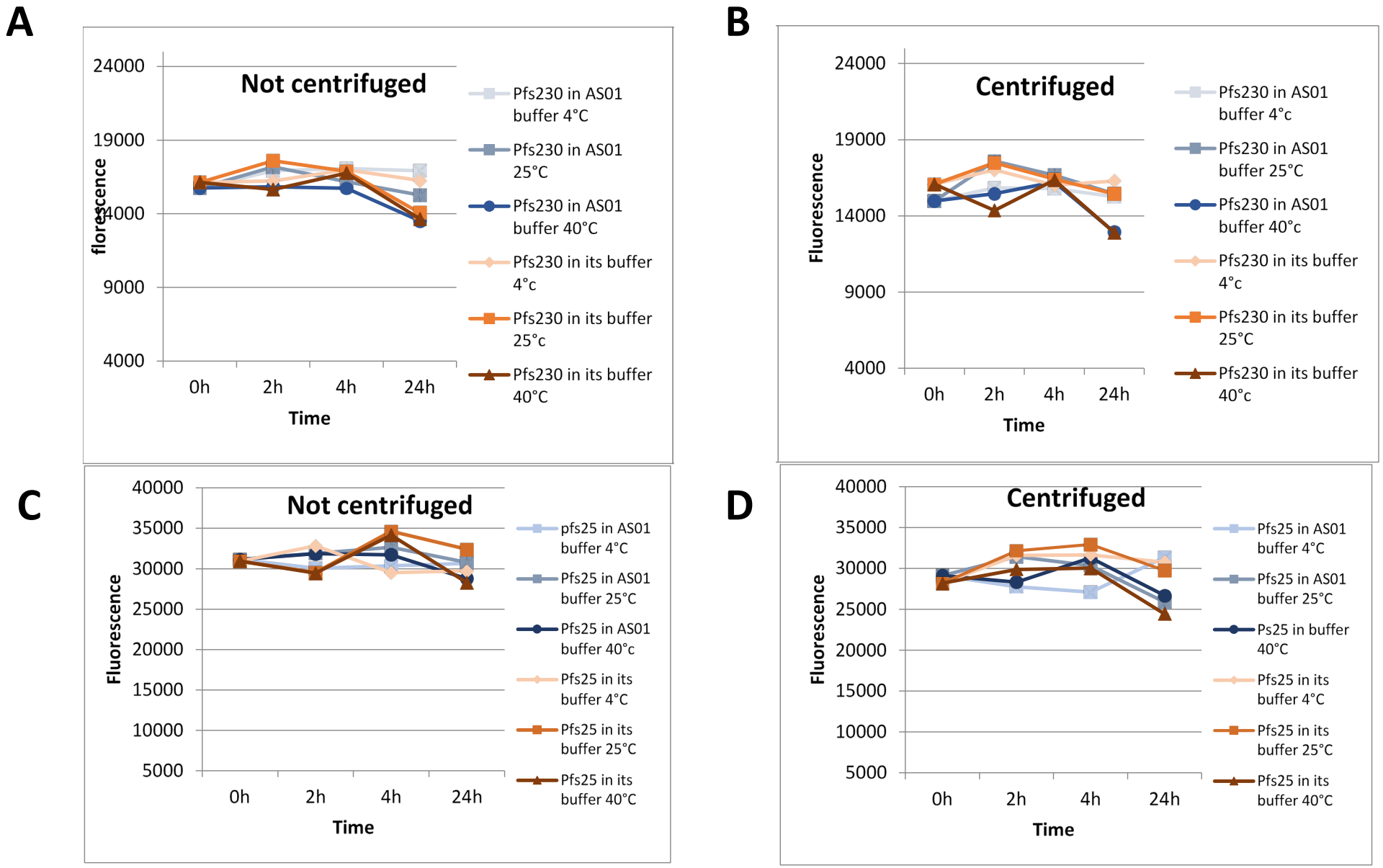


Figure S4. The intrinsic fluorescence of Pfs230D1 and Pfs25 nanoparticles, related to STAR Methods. Intrinsic fluorescence did not indicate any differences over time between the different temperatures for each sample tested, and no major differences were noted between centrifuged and non-centrifuged samples for either nanoparticle.

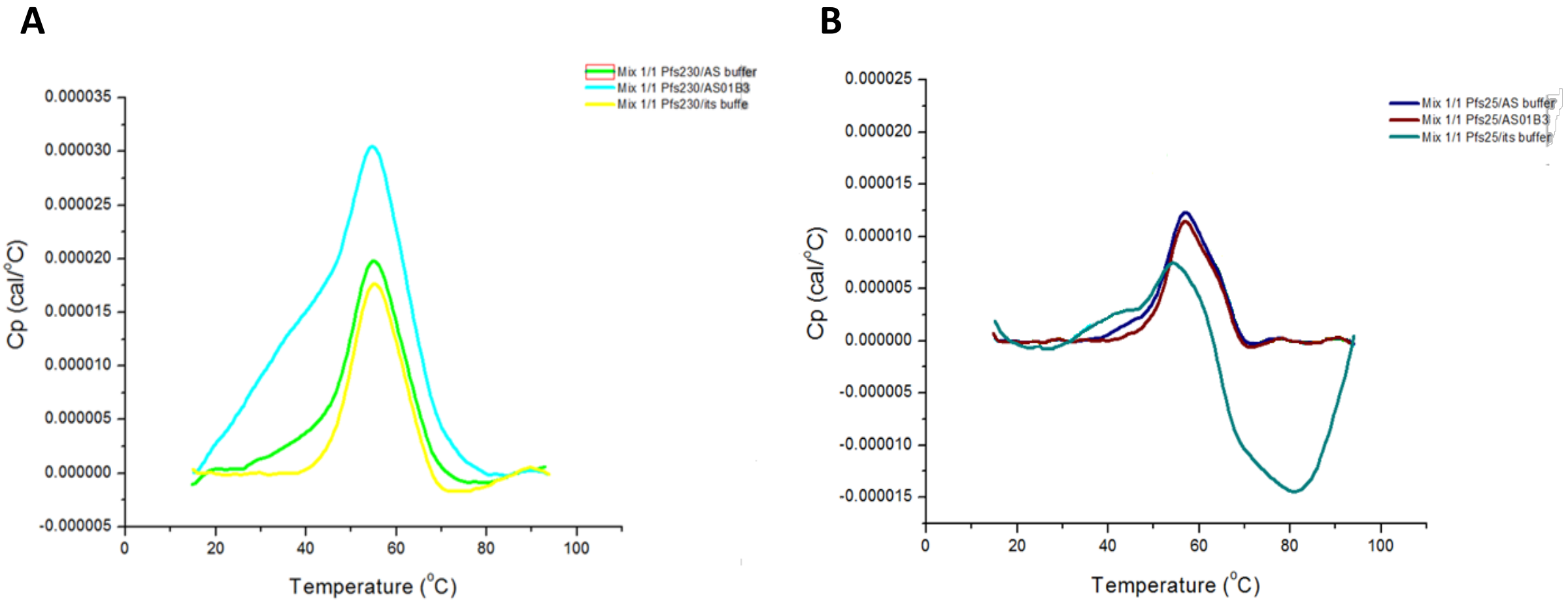
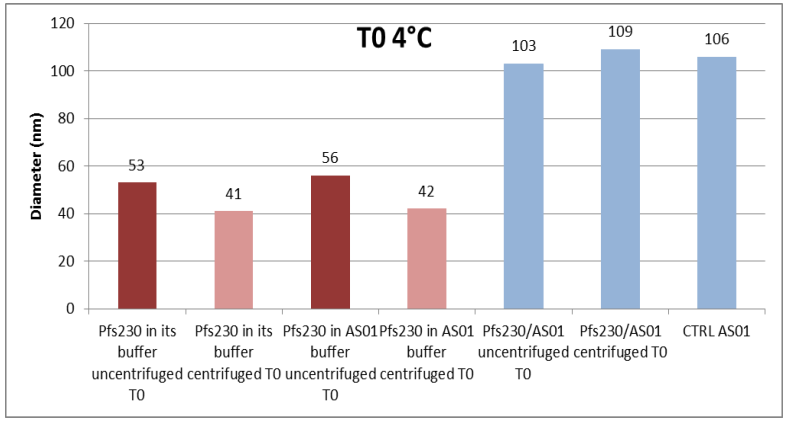
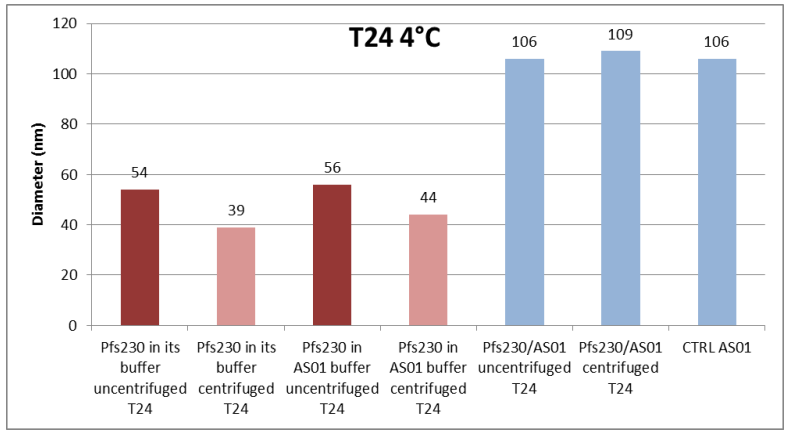
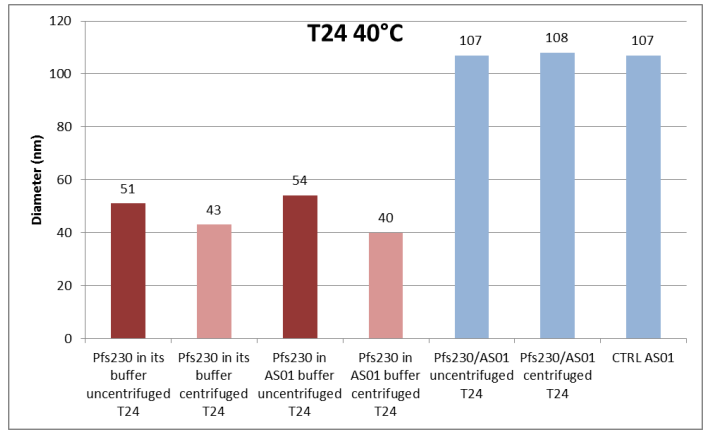
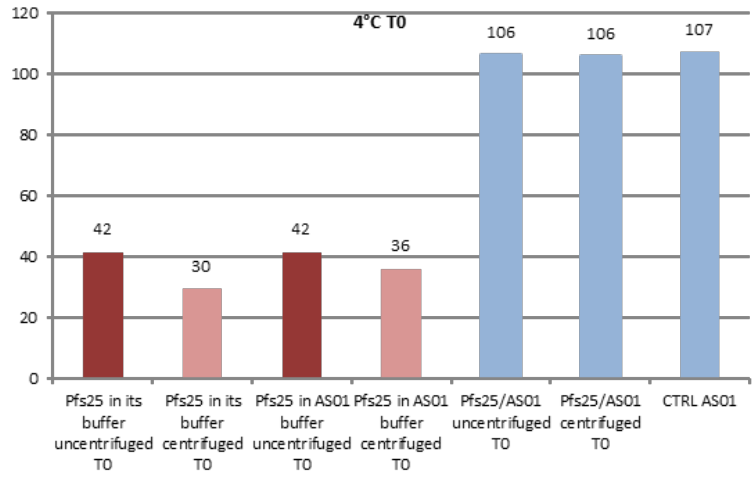


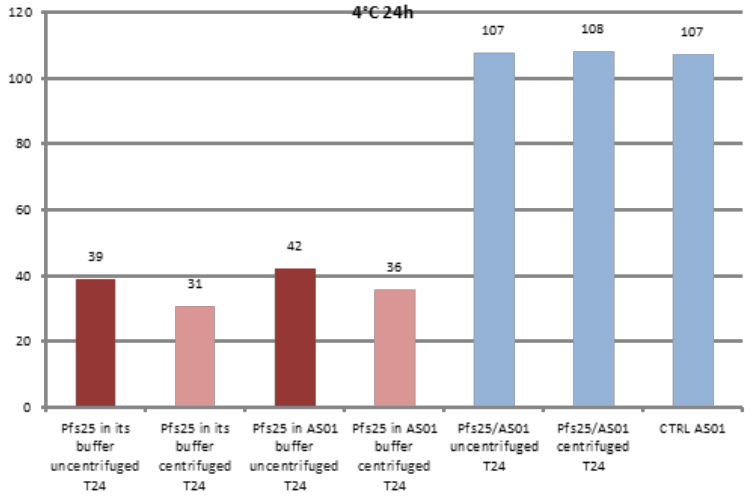
Figure S5. The capillary-DSC results for Pfs230D1 and Pfs25 nanoparticle preparations, related to STAR Methods. (A) Pfs230D1 nanoparticle results: yellow, green and aqua thermograms (Pfs230D1/AS01 buffer, Pfs230D1/Pfs230D1 buffer, and Pfs230D1/AS01 respectively). **(B)** Pfs25 nanoparticle results: dark blue, red and green thermograms (Pfs25/AS01 buffer, Pfs25/Pfs25 buffer, and Pfs25/AS01 respectively). Nanoparticles in buffer results show a slight effect of the nanoparticle/AS01 buffer on the nanoparticle prior to reaching 40˚C.











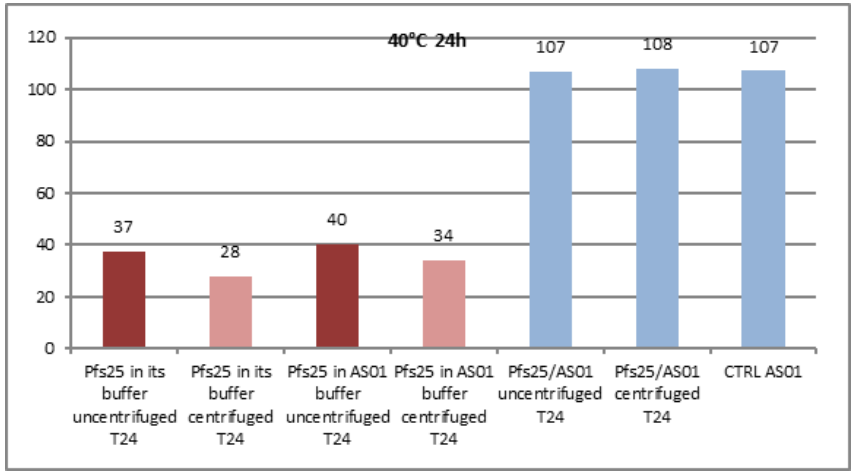
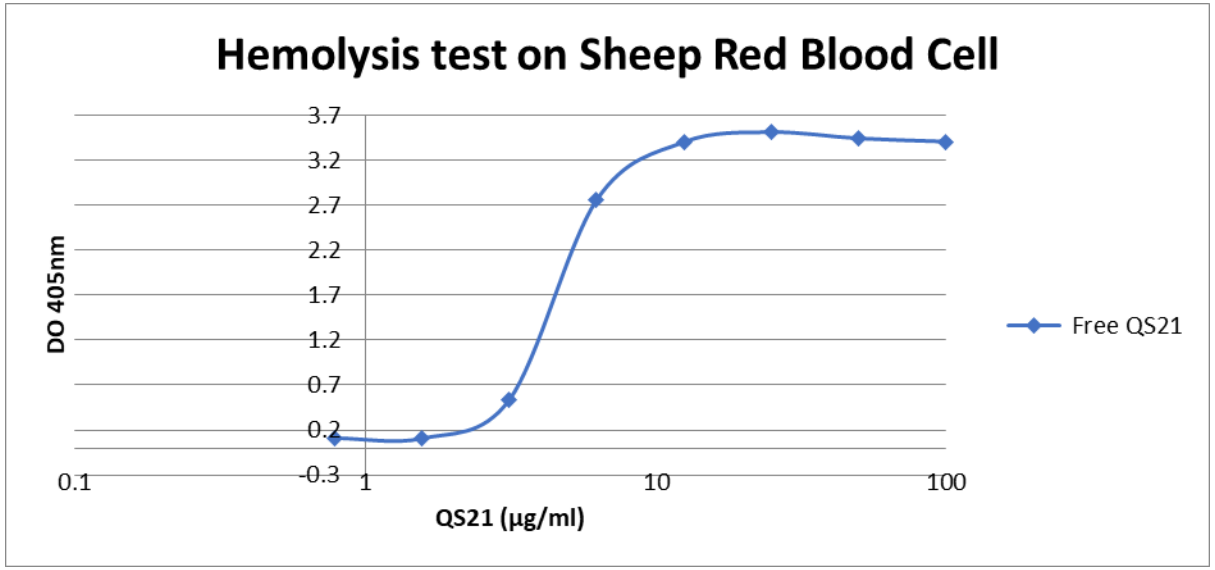


Figure S6. The dynamic light scattering (DLS) profiles of Pfs230D1-EPA and Pfs25-EPA nanoparticles formulated with AS01, related to STAR Methods. Dynamic Light Scattering determines the hydrodynamic particle size. The results below correspond to the Z-average diameter calculated by the cumulants method. A subset of samples are presented, as other timepoints/temperatures exhibited the same profile. Presented in red are nanoparticle samples in buffer which have the same mean size at all temperatures and timepoints. However, the mean size decreases after centrifugation, suggesting the presence of a few aggregates in the uncentrifuged samples for both nanoparticles. All samples formulated with AS01 measure the expected size of the liposomes, approximately 103-110 nm in diameter. There is no loss of integrity of the AS01 after formulation with Pfs230D1-EPA or Pfs25-EPA nanoparticles.



	Pfs230 Samples	QS21 (µg theoretical)	QS21 quenched (%)	Free QS21 (µg/ml)	OK/KO
Pfs230 + AS01	T0	*50*	99	0,7	OK
	T2h +4°C	*50*	99	0,7	OK
	T4h +4°C	*50*	99	0,7	OK
	T24h +4°C	*50*	99	0,7	OK
	T2h +40°C	*50*	98	1,0	OK
	T4h +40°C	*50*	98	1,1	OK
	T24h +40°C	*50*	97	1,4	OK
Pfs230 buffer + AS01	T0	*50*	98	0,8	OK
	T2h +4°C	*50*	99	0,8	OK
	T4h +4°C	*50*	99	0,8	OK
	T24h +4°C	*50*	98	0,8	OK
	T2h +40°C	*50*	98	1,0	OK
	T4h +40°C	*50*	98	1,1	OK
	T24h +40°C	*50*	98	1,3	OK

	Pfs25 Samples	QS21 (µg theoretical)	QS21 quenched (%)	Free QS21 (µg/ml)	OK/KO
Pfs25 + AS01	T0	*50*	98	1.0	OK
	T2h +4°C	*50*	98	1.0	OK
	T4h +4°C	*50*	98	0.9	OK
	T24h +4°C	*50*	98	0.8	OK
	T2h +40°C	*50*	98	1.0	OK
	T4h +40°C	*50*	98	1.2	OK
	T24h +40°C	*50*	98	1.2	OK
Pfs25 buffer + AS01	T0	*50*	98	1.0	OK
	T2h +40°C	*50*	98	1.2	OK
	T4h +40°C	*50*	97	1.3	OK
	T24h +40°C	*50*	97	1.5	OK

Figure S7. The hemolysis of sheep red blood cells to test stability of Pfs230D1-EPA and Pfs25-EPA/AS01 formulations, related to STAR Methods. All measurements reflect that the QS-21 is under the limit (5-6 ug/mL). The QS-21 remained quenched in all samples tested, indicating stable Pfs230D1-EPA/AS01 and Pfs25-EPA/AS01 formulations. The amount of free QS-21 remains below the limit

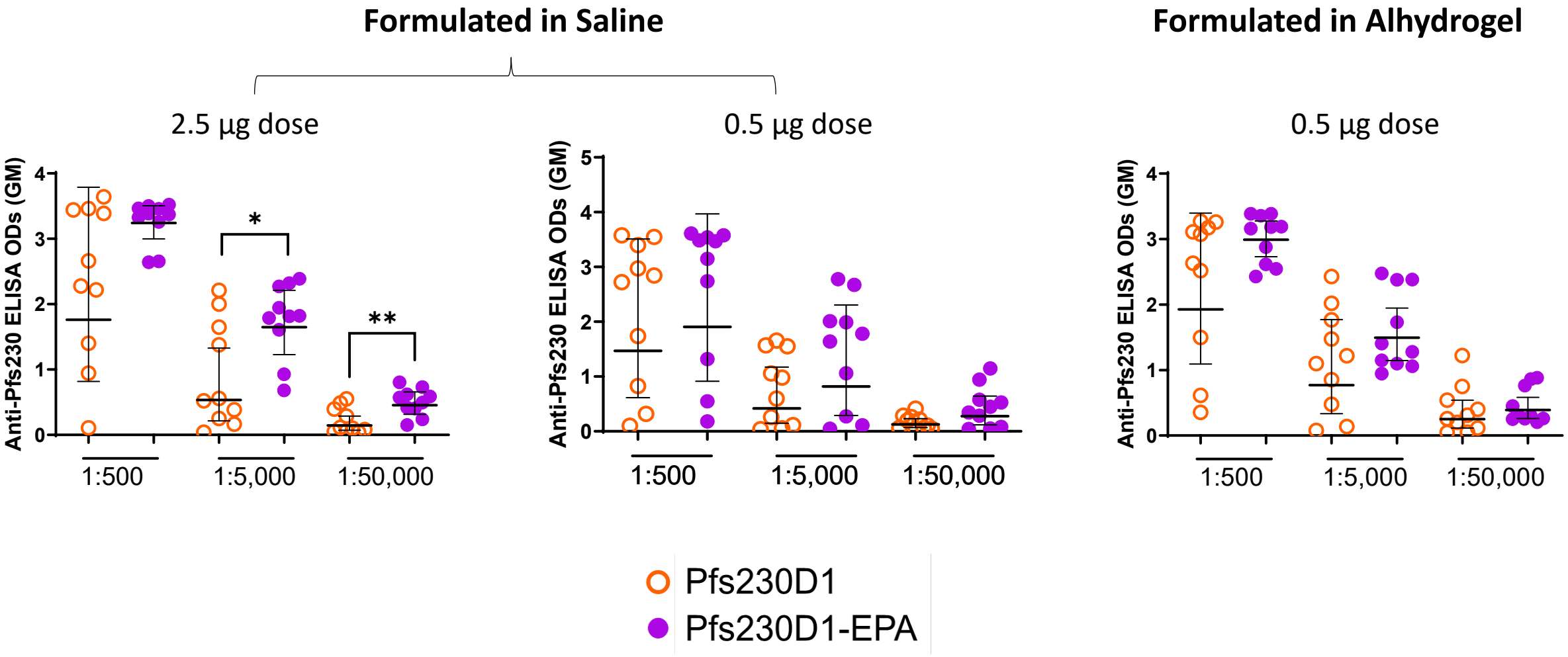


Figure S8. Conjugation to EPA enhances anti-Pfs230D1 antibody responses, with or without adjuvants, related to to Figure 4 (Study 3). CD-1 mice were immunized via intramuscular injection on days 0 and 28 with unconjugated Pfs230D1 or Pfs230D1 conjugated to EPA, formulated in saline or Alhydrogel (900 µg/mL). Anti-Pfs230 antibody levels were measured in CD-1 mice 42 days after immunization. Significant differences were analyzed between the two groups at each dilution, using Mann-Whitney test. Asterisks indicate p-values: *p<0.05; **p<0.01. Error bars indicate geometric mean with 95%CI.

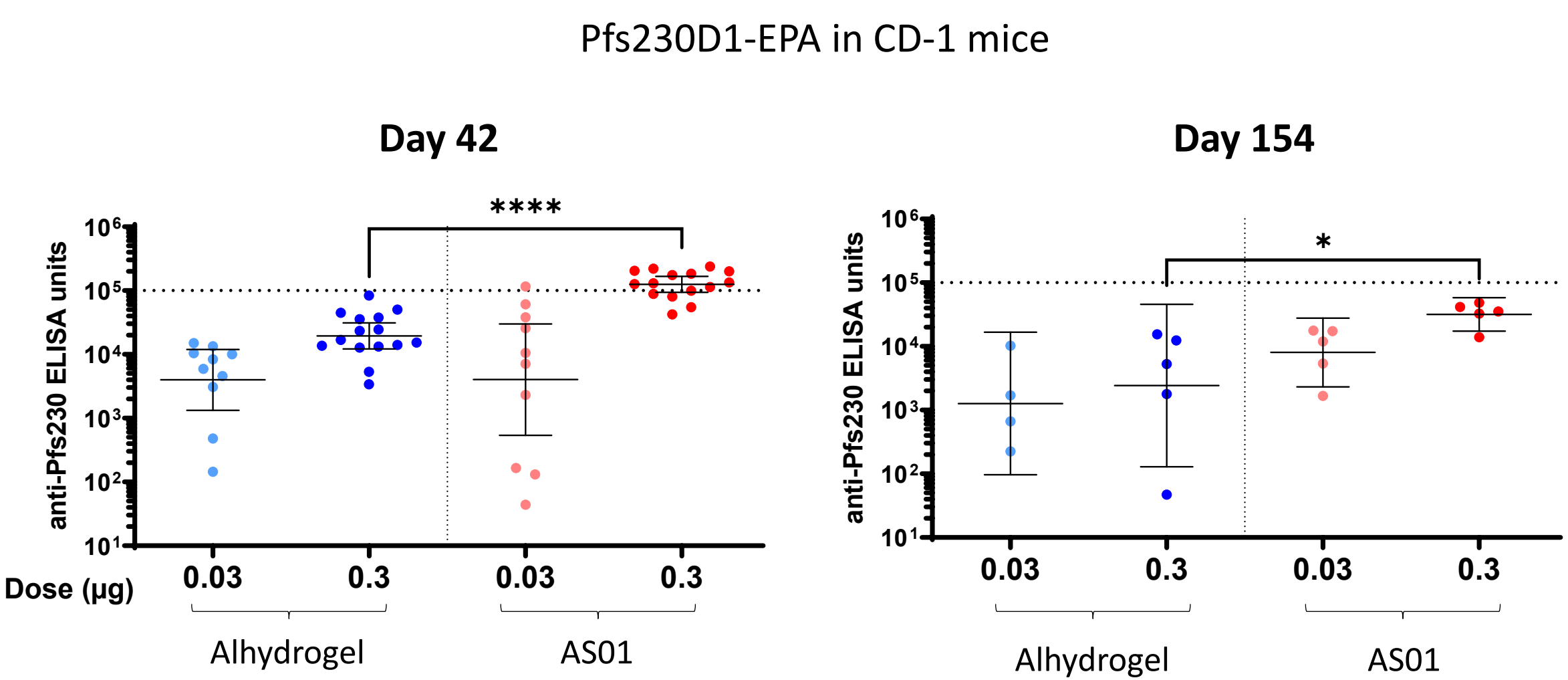


Figure S9. Superior antibody responses were induced by Pfs230D1-EPA formulated in AS01 versus Alhydrogel in CD-1 mice, related to Figure 5 (Study 4). CD-1 mice were immunized on Days 0 and 28 with Pfs230D1-EPA formulated in AS01. ELISAs were conducted on days 42 and 154. Presented are individual ELISA data for each dose at each timepoint for AS01 and Alhydrogel formulations. Differences between adjuvant formulations were analyzed by Mann-Whitney test; asterisks indicate significant p-values: ****p<0.0001; * p<0.05. Error bars indicate geometric mean with 95%CI.

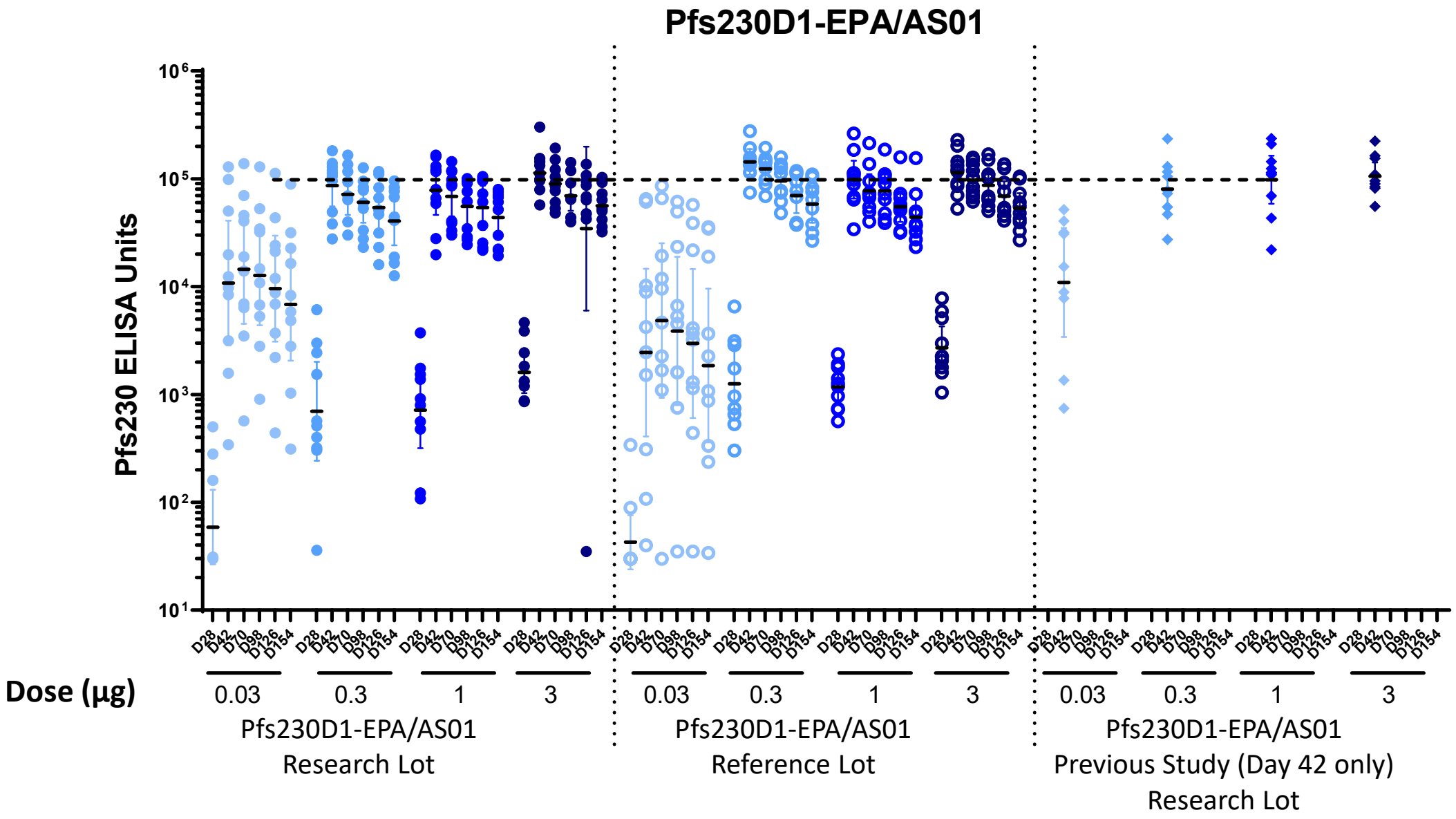


Figure S10. The immunogenicity of Pfs230D1-EPA/AS01 Research and Reference lots in CD-1 mice, related to Figure 4 (Study 5). CD-1 mice were immunized on Days 0 and 28 with Pfs230D1-EPA research lot (left panel) and reference lot (middle panel) formulated in AS01**.** ELISAs were conducted on Days 28, 42, 70,98,126 and 154 (study termination). Presented are individual ELISA data for each dose at each timepoint for research and reference lots of Pfs230D1-EPA/AS01. In addition, Day 42 sera ELISA results from **Figure 4** are added here (right panel) to illustrate the comparability between studies of this formulated vaccine at different doses. Error bars indicate geometric mean with 95%CI.

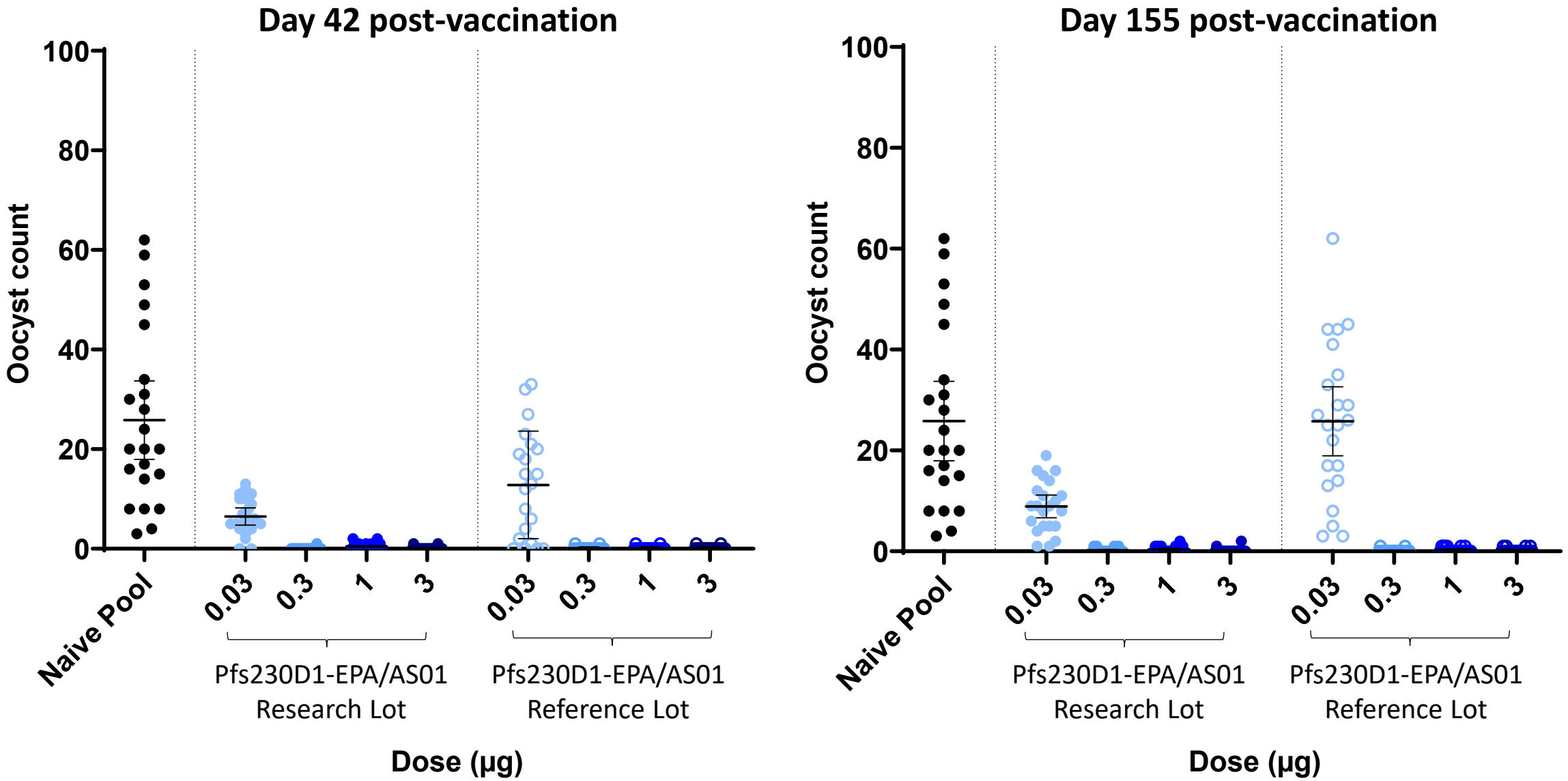
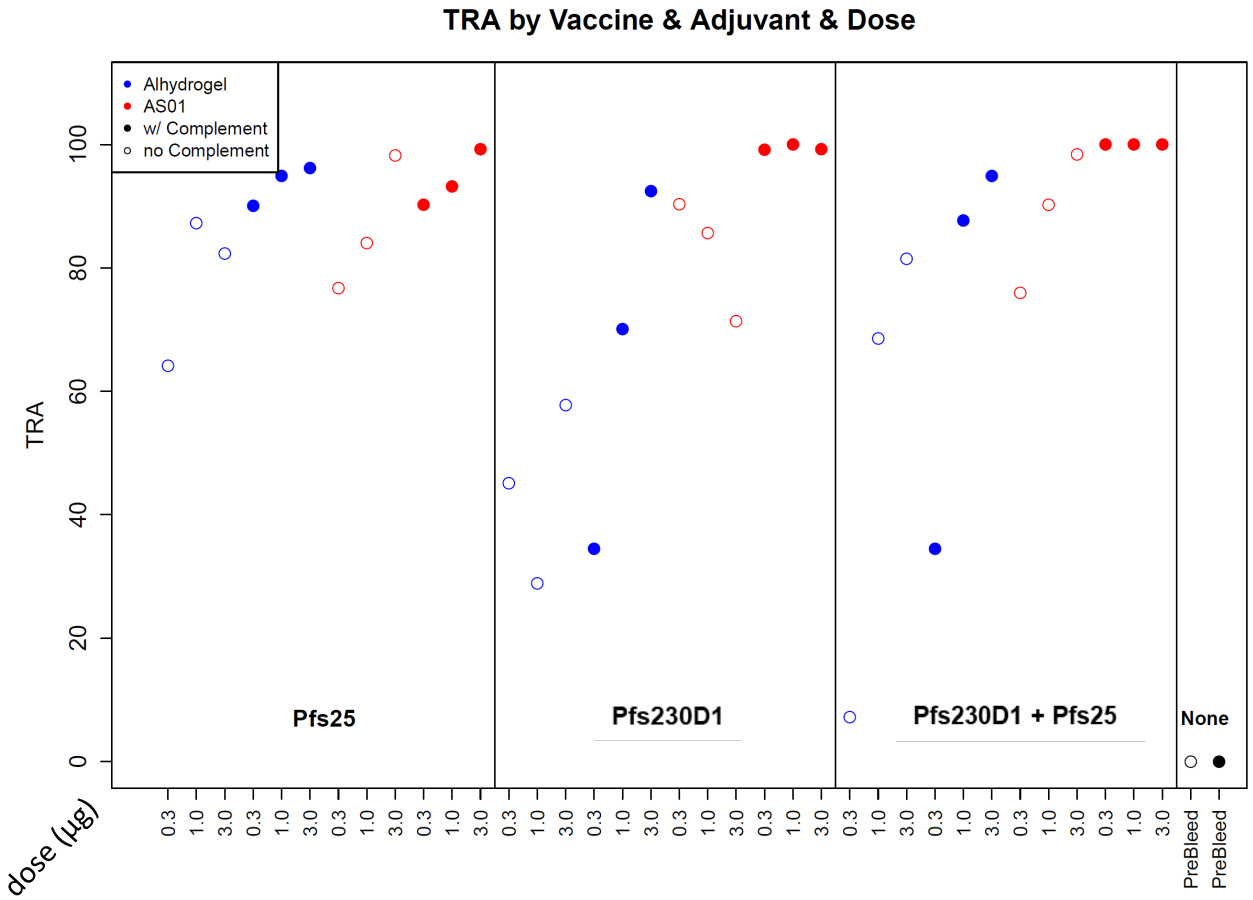


Figure S11. The functional activity of Pfs230D1-EPA/AS01 Research and Reference lots in CD-1 mice, related to Figure 6 (Study 5). Standard membrane feeding assays (SMFA) were performed to evaluate Pfs230D1-EPA/AS01 vaccine-induced transmission-reducing activity (oocyst reduction in mosquito midguts). All samples were run in the same assay in the presence of intact human serum to assess complement-dependent activity; baseline points are identical in both panels. Error bars indicate mean with 95%CI.



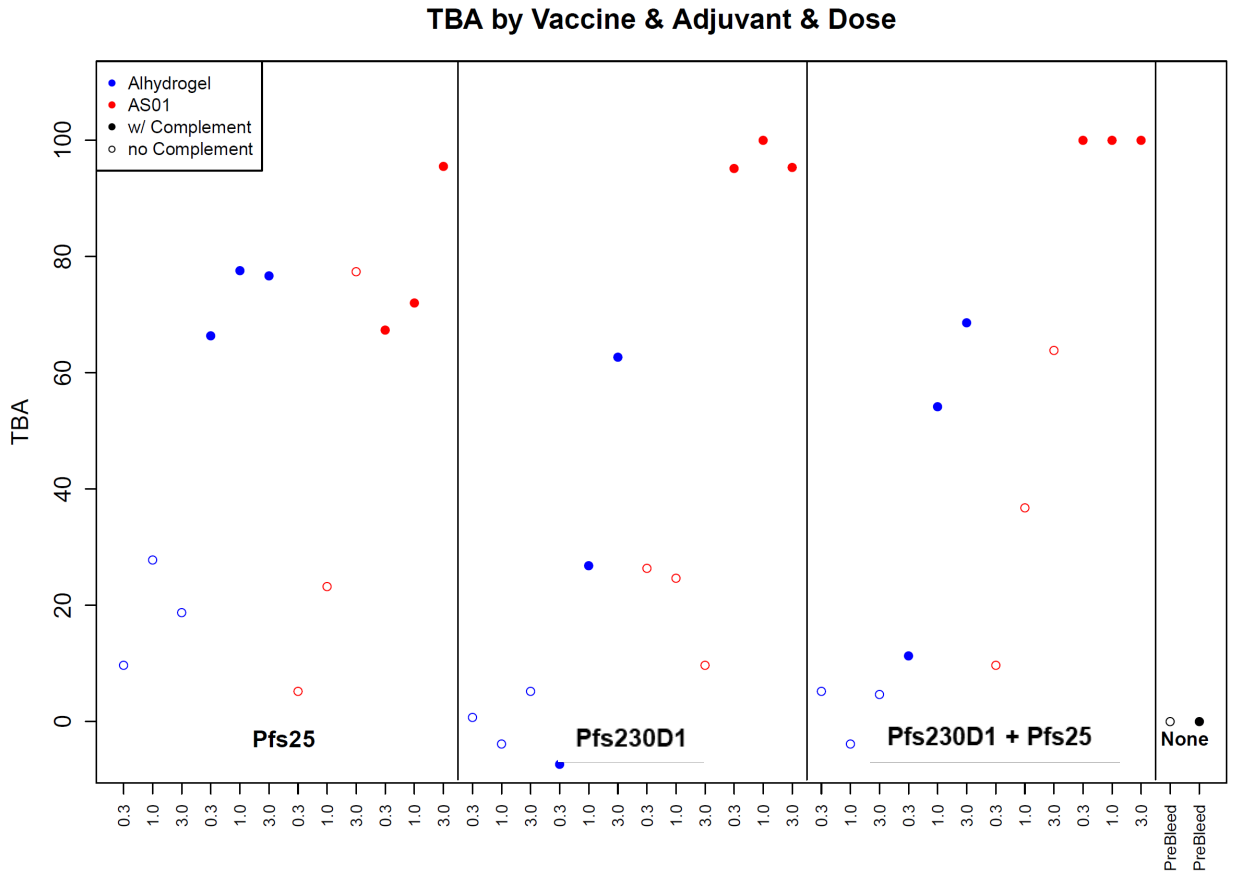


Figure S12. The transmission-reducing and transmission-blocking activity as analyzed by vaccine, adjuvant, and dose, related to Figure 6 (Study 5). Transmission reducing activity (TRA, reduction in infection intensity) and Transmission blocking activity (TBA, reduction in infection prevalence) of the sera were tested by an ex vivo standard membrane feeding assay (SMFA). TBA and TRA are calculated by the following formulas:

$$TRA = 100\ x \left(\frac{Mean\ Oocyst\ Number_{neg\ ctrl} - Mean\ Oocyst\ Number_{test}}{Mean\ Oocyst\ Number_{neg\ ctrl}} \right)$$

and

$$TBA = 100\ x \left(\frac{Mean\ No.Inf.Mosquito_{neg\ ctrl} - Mean\ No.Inf.Mosquito_{test}}{Mean\ No.Inf.Mosquito_{neg\ ctrl}} \right)$$

where the negative control (*neg ctrl*) feed used pooled pre-vaccination sera from all animals.