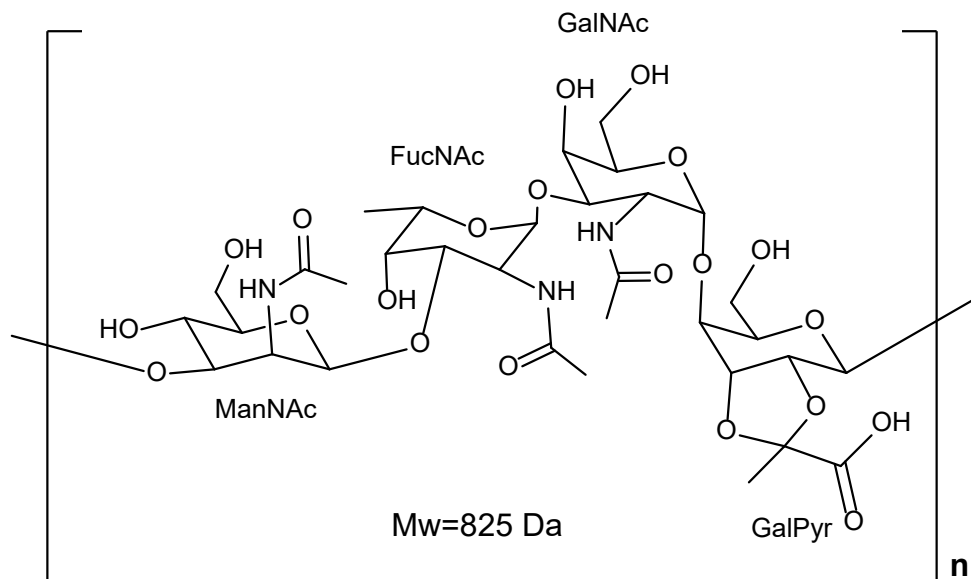


Supplementary information

Supplementary Figure 1. Example of pneumococcal Ps repeating unit structure (ST4)

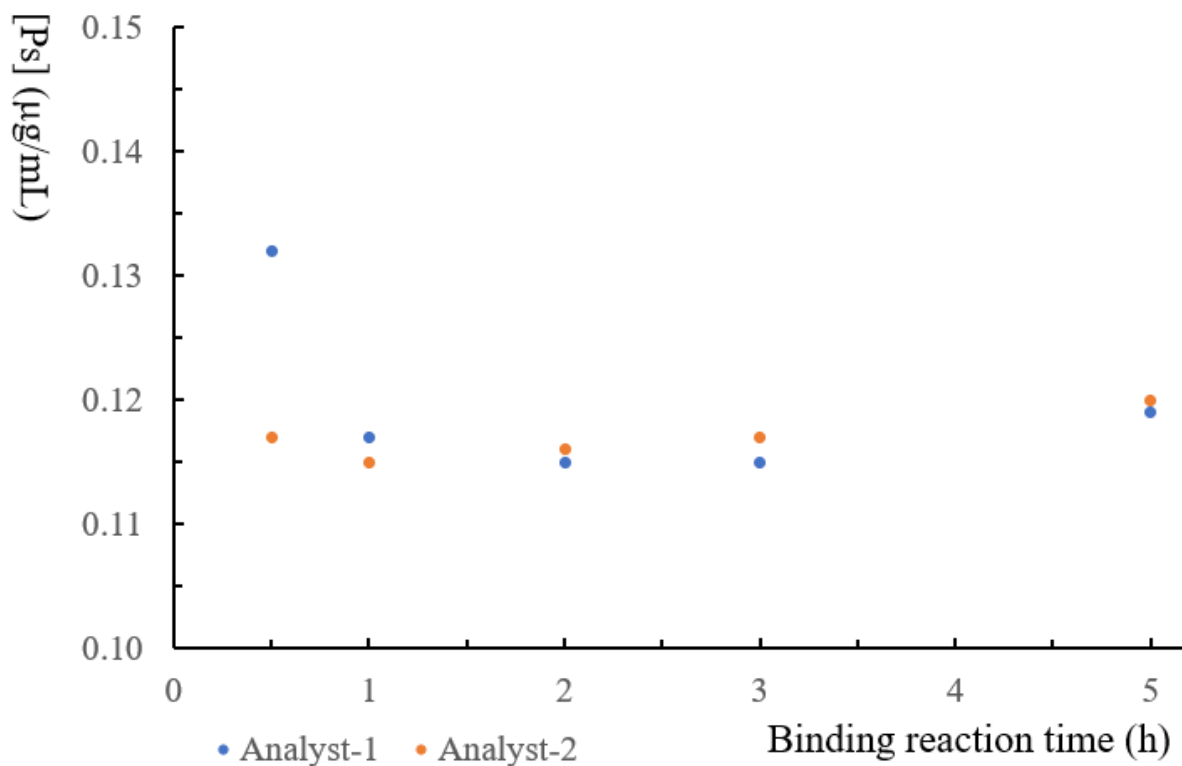
$[\rightarrow 3)\text{-}\beta\text{-D-ManpNAc-(1}\rightarrow 3)\text{-}\alpha\text{-L-FucpNAc-(1}\rightarrow 3)\text{-}\alpha\text{-D-GalpNAc-(1}\rightarrow 4)\text{-}\alpha\text{-D-Galp-2,3(S)Pyr(1}\rightarrow)]_n$



Supplementary Table 1. Antibody polysaccharide binding reactions for ST4 Ps standard. The first 5 Ps standards (STD1-5) demonstrated linear response to Ps concentration.

Binding reaction	1 $\mu\text{g/mL}$ ST4 Ps (μL)	0.1 mg/mL anti-ST4 mAb (μL)	Binding Buffer (μL)	Total Vol (μL)	mAb/Ps ($\mu\text{g}/\mu\text{g}$)	[Ps] ($\mu\text{g/mL}$)	APC FLR peak area	STD1-5 R^2
STD1	2	20	178	200	1000	0.01	40	0.9998
STD2	5	20	175	200	400	0.025	98	
STD3	10	20	170	200	200	0.05	212	
STD4	20	20	160	200	100	0.1	429	
STD5	30	20	150	200	67	0.15	655	
STD6	50	20	130	200	40	0.25	930	
STD7	75	20	105	200	27	0.375	1076	

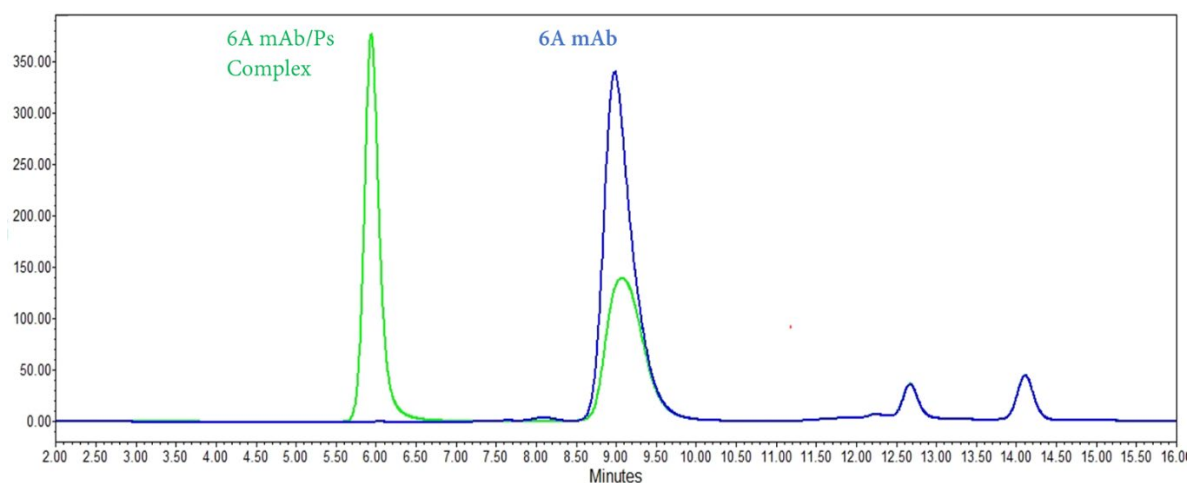
Supplementary Figure 2. Vaccine ST4 free polysaccharide concentration measured with at different binding reaction time points



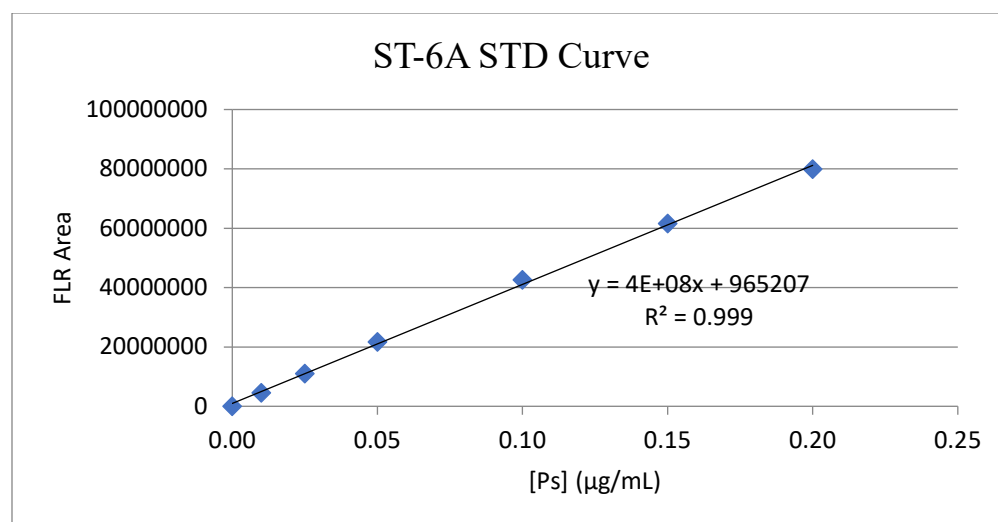
Supplementary Table 2. Vaccine ST4 free polysaccharide concentration measured with at different binding reaction time points

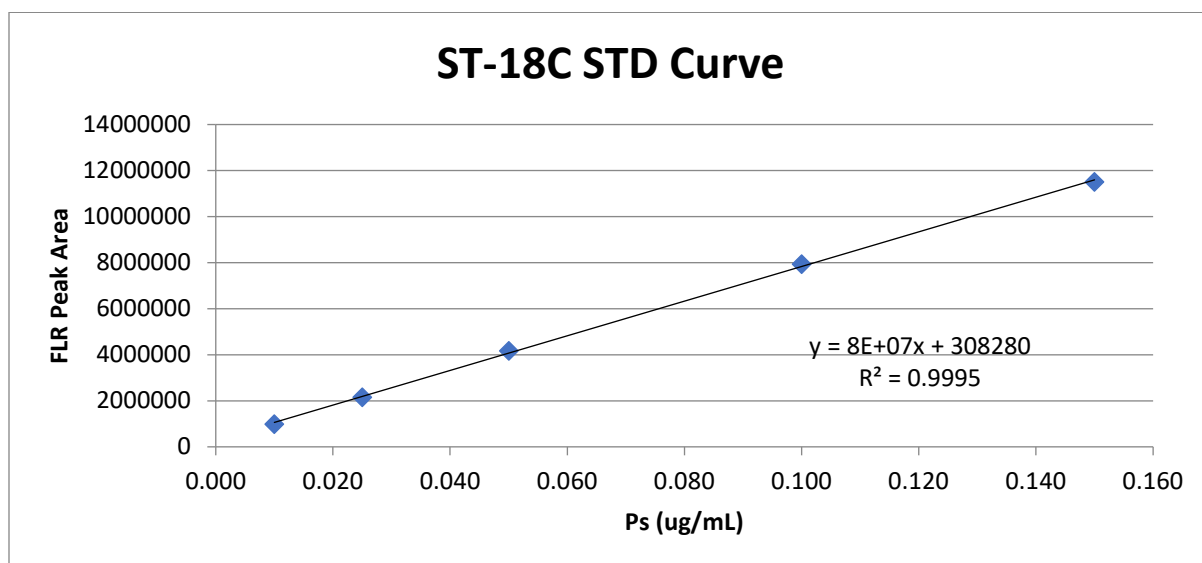
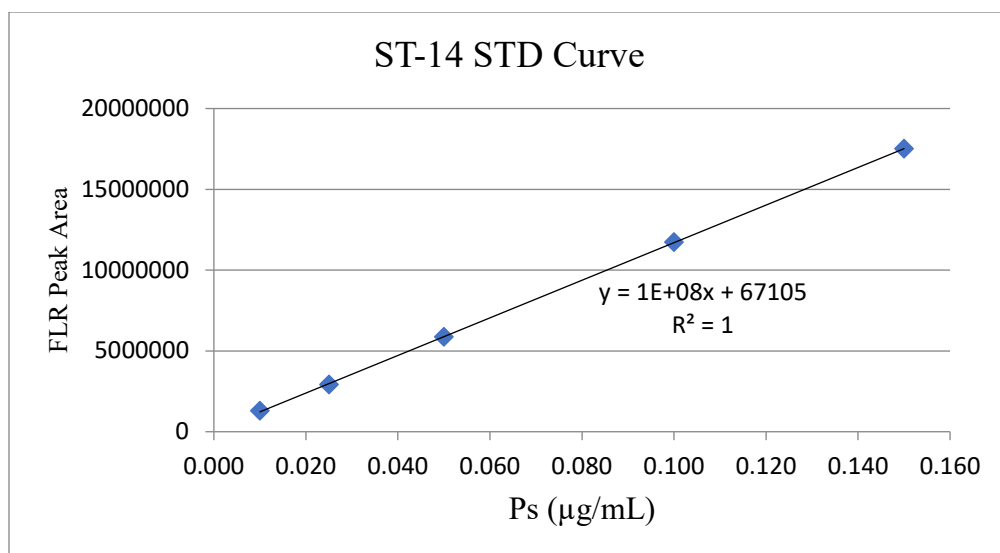
PCV15	Analyst-1/Instrument-1				Analyst-2/Instrument-2			
Binding time (h)	RSQ	PCV15 [ST4] (µg/mL)	Average (1-5h)	%CV (1-5h)	RSQ	PCV15 [ST4] (µg/mL)	Average (1-5h)	%CV (1-5h)
0.5	0.9980	0.132	Excluded		0.9999	0.117	Excluded	
1	0.9999	0.117	0.117	1.6	0.9998	0.115	0.117	1.8
2	0.9999	0.115			0.9998	0.116		
3	0.9998	0.115			0.9998	0.117		
5	0.9995	0.119			1	0.12		
Average [ST4] (µg/mL) (2 analysts)		0.117						
%CV (2 analysts)		1.6						

Supplementary Figure 3. Compare anti-6A mAb binding reaction with ST6A Ps (green trace) with binding reaction with ST6A knockout (KO) sample (blue trace) on SEC chromatography. Antibody polysaccharide complex (APC) was only observed on the binding reaction with ST6A Ps. Specificity was observed for all PCV15 anti-ST mAbs.



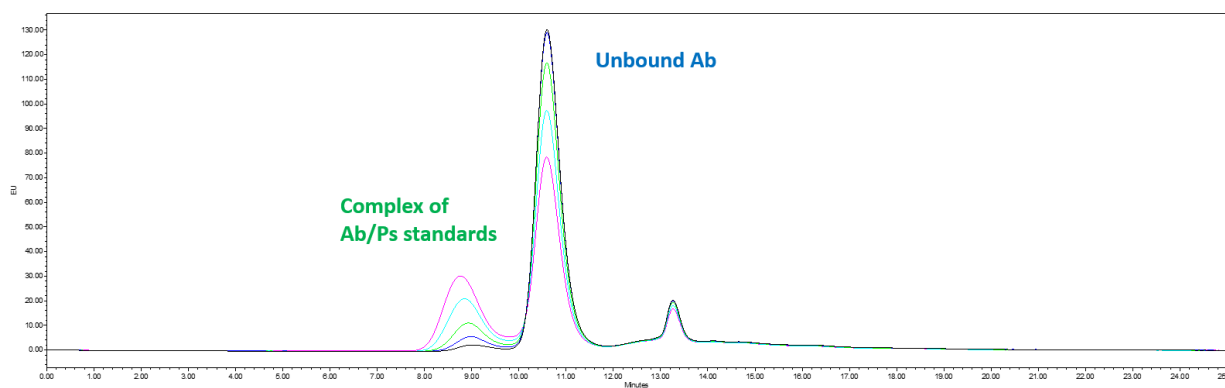
Supplementary Figure 4. Examples of assay standard curves





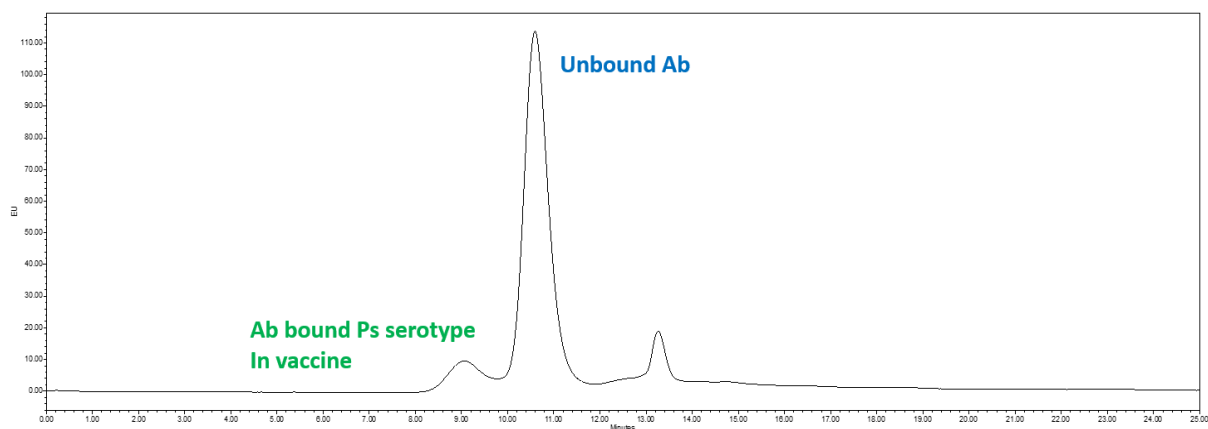
Supplementary Figure 5. Comparison of anti-ST mAb binding to Ps standards with binding to vaccine free Ps.

5-1a. Anti-ST14 mAb binding to ST14 standards

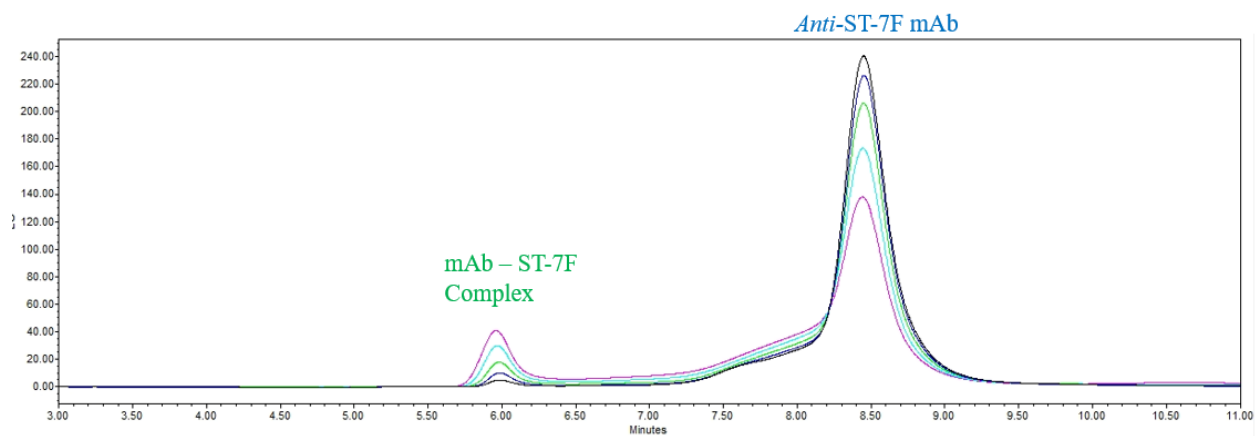


5-

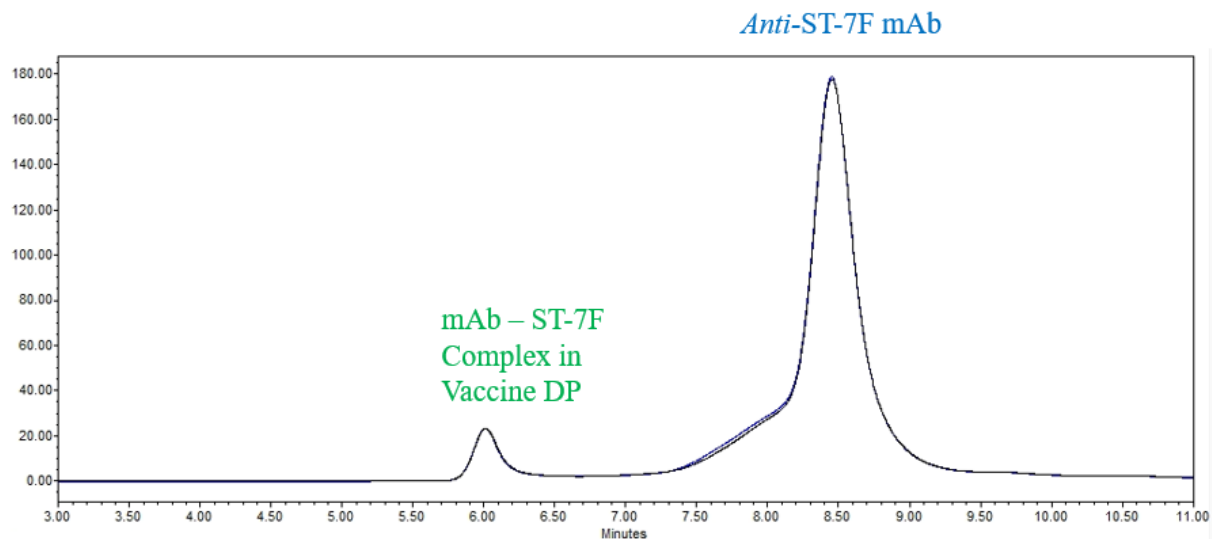
5-1b. Anti-ST14 mAb binding to PCV



5-2a. Anti-7F mAb binding to ST7F standards



5-2b. Anti-7F mAb binding to PCV15



Supplementary Table 3. Spike recovery of free Ps for six representative PCV15 serotypes

PCV15 Serotype	ST4	ST5	ST6A	ST9V	ST14	ST19A
STD curve R ²	0.9998	0.9999	0.9999	0.9994	0.9997	0.9999
STD curve intercept	-47562.14571	-76257.27711	853659.4819	-24030.67809	-257467	-305060
STD curve slope	272223467.8	106410195.2	581812530.1	181277138.5	156256712	215599880
Spike control FLR	6458648	2536243	14115115	3719461	2740630	8312623
LLOQ Spike FLR	9861719	4002479	22449281	6960319	5447363	11594188
Nominal Spike FLR	26282875	10561033	56552676	17525494	14864703	24152543
Spike control [Ps] (µg/mL)	0.0239	0.0246	0.0228	0.0207	0.0192	0.0400
LLOQ Spike [Ps] (µg/mL)	0.0364	0.0383	0.0371	0.0385	0.0365	0.0552
Nominal Spike [Ps] (µg/mL)	0.0967	0.1000	0.0957	0.0968	0.0968	0.1134
LLOQ Spike Recovered [Ps] (µg/mL)	0.0125	0.0138	0.0143	0.0179	0.0173	0.0152
Nominal Spike Recovered [Ps] (µg/mL)	0.0728	0.0754	0.0729	0.0762	0.0776	0.0735
LLOQ Spike (µg/mL)	0.015	0.015	0.015	0.015	0.015	0.015
Nominal Spike (µg/mL)	0.075	0.075	0.075	0.075	0.075	0.075
% Spike Recovery at LLOQ	83	92	95	119	115	101
% Spike Recovery at Nominal	97	101	97	102	103	98

Supplementary Table 4. Free Ps concentrations from antibody assisted HPLC, sandwich ELISA and expected from standard formulation.

PCV15 Serotype	1	3	4	5	6A	14	19A
Free [Ps] from Ab-HPLC (µg/mL)	0.33	0.39	0.11	0.43	0.13	0.14	0.76

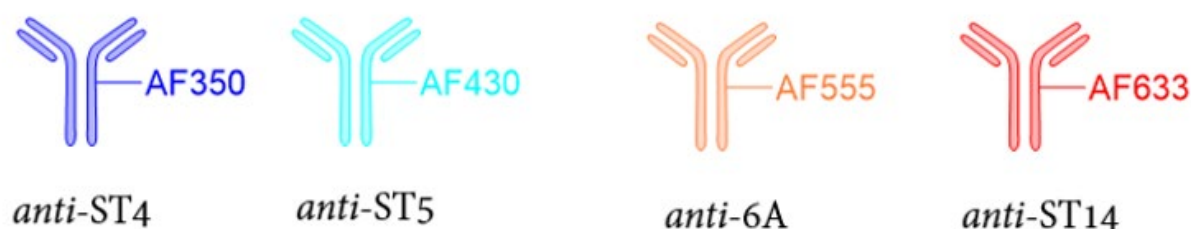
Free [Ps] expected from standard formulation ($\mu\text{g/mL}$) ^a	0.3	0.3	0.2	0.3	0.1	0.1	0.5
Free [Ps] from ELISA on a similar batch ($\mu\text{g/mL}$) ^b	0.29	0.32	0.32	0.38	0.08	0.08	0.71

a: Free [Ps] expected from standard formulation was calculated from theoretical free Ps input for a standard formulation batch.

b: Free [Ps] from ELISA was obtained from measurement of a similar PCV15 batch by sandwich ELISA. Sandwich ELISA was performed following procedure below.

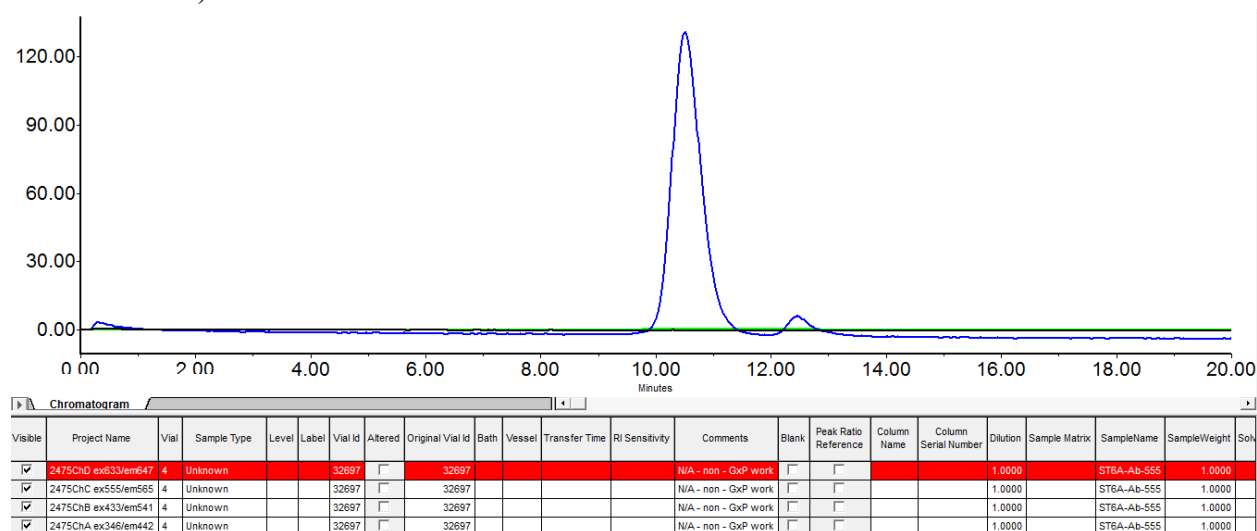
All antibodies were in-house generated serotype specific monoclonal antibodies used at optimized concentrations in the range between 1-3 $\mu\text{g/mL}$. Plates were washed 3X with wash buffer between each step (Tris-buffered saline + 0.05% polysorbate-20). Maxisorp high-binding 96-well plates (Nunc) were coated (100 $\mu\text{L/well}$) with serotype specific capture monoclonal antibodies (Merck & Co., Inc., Rahway, NJ, USA) in 1M $(\text{NH}_4)_2\text{SO}_4$ coating buffer. Following a 1 hour incubation with shaking, plates were blocked for 15 min with assay buffer comprised of 1% (w/v) BSA, 10 mM Tris, 0.15 NaCl, and 0.05 % (v/v) Ps-20, pH 8. References were applied as a serially diluted 11-point curve and samples were plated in 3 replicates (each replicate consisting of 4 pseudo-replicate wells). Samples and references were incubated for 1h at room temperature with shaking after which 100 $\mu\text{L/ well}$ of detection solution was added. The detection solution consisted of serotype specific detection antibodies and an alkaline-phosphatase-labeled anti-species secondary antibody (Jackson ImmunoResearch, cat #111-055-046). Binding was visualized by incubating plates for 35 min with 4-methylumbelliferyl phosphate substrate (Virolabs, XPHOS-100) and reading the resulting fluorescent signal with a Spectramax GeminiEM plate reader (Molecular Devices, CA) at 360 nm excitation and 440 nm emission.

Supplementary Figure 6. Alexa Fluor™ labeled anti-ST mAbs for multiplex HPLC assay



Supplementary Figure 7. Detection of Alexa Fluor™ 555 labeled anti-6A mAb (anti-ST6A-AF555) on four FLR channels (Ex/Em of 433nm/541nm, 555nm/565nm and 633nm/647nm).

Anti- ST6A-AF555 can only be detected on 555nm/565nm, not on any other three channels. The channel-specificity was also observed for all other three FLR labeled mAbs (anti-ST4-AF350 only detected at Ex/Em of 346nm/442nm, anti-ST5-AF430 at 433nm/541nm, anti-ST14-AF633 at 633nm/647nm).



Supplementary Table 5. Multiplex standard curve from binding of an anti-ST-AF cocktail with a multi-valent PCV standard that contains ST4, ST4, ST6A and ST14 conjugates

STD Curve	ST4	ST5	ST6A	ST14
[Ps] (µg/mL)	FLR350	FLR430	FLR555	FLR633
0.025	13862908	3271276	14629276	1191432
0.05	26449246	6062365	28413513	2344125
0.1	48912423	11303380	54599861	4446046
0.15	73535345	16928391	84152265	6757758
0.2	87356619	20278356	104312971	8126979
RSQ	0.991	0.993	0.997	0.993
Intercept	4951848	1137529	2465360	323634
Slope	429252002	99344993	521487784	40472705

Supplementary Table 6. Measurement of total Ps concentrations from 4 PCV24 serotypes in a single HPLC run

PCV24 total Ps	ST4	ST5	ST6A	ST14
STD curve R ²	0.9911	0.9926	0.9966	0.9928
Intercept	4951848	1137529	2465360	323634
Slope	429252002	99344993	521487784	40472705
FLR channel	FLR350	FLR430	FLR555	FLR633
Peak area	52581520	11054715	49747133	4163950

Binding Rx [Ps] ($\mu\text{g/mL}$)	0.111	0.100	0.091	0.095
Sample dilution	80	80	80	80
PCV24 total [Ps] ($\mu\text{g/mL}$)	8.9	8.0	7.3	7.6
%diff from target	11.0	0.2	9.3	5.1

Supplementary Table 7. Species and source of *anti* polysaccharide mAbs

Serotype	1	3	4	5	6A	6B	7F	9V
mAb Species	<i>Ms</i>	<i>Hu</i>	<i>Hu</i>	<i>Hu</i>	<i>Hu</i>	<i>Hu</i>	<i>Hu</i>	<i>Hu</i>
mAb Clone Source	UAB (Hyp1G5.1)	In-house	In-house	In-house	In-house	In-house	In-house	In-house
Serotype	14	18C	19A	19F	22F	23F	33F	
mAb Species	<i>Ms</i>	<i>Hu</i>	<i>Hu</i>	<i>Hu</i>	<i>Hu</i>	<i>Hu</i>	<i>Hu</i>	
mAb Clone Source	In-house	In-house	In-house	UAB (Hyp19FG5.1)	In-house	In-house	In-house	

In-house: from our in-house antibody generation/reagent teams

UAB: The University of Alabama at Birmingham

Ms: Mouse; *Hu*: Human

Supplementary Table 8. Dilution of *anti* polysaccharide mAbs before binding reactions

Anti Serotype	1	3	4	5	6A	6B	7F	9V	14	18C	19A	19F	22F	23F	33F
Target [mAb] (mg/mL)	0.10	0.10	0.10	0.10	0.10	0.30	0.05	0.10	0.10	0.10	0.15	0.10	0.15	0.10	0.15
Stock [mAb] (mg/mL)	4.60	4.30	4.81	4.40	0.99	11.52	1.01	4.00	3.40	1.40	4.16	6.59	1.04	1.02	1.05
Fold of dilution	46.0	43.0	48.1	44.0	9.9	38.4	20.2	40.0	34.0	14.0	27.7	65.9	6.9	10.2	7.0
Stock mAb volume (μL)	5.4	5.8	3.1	5.7	25.3	6.5	24.8	6.3	7.4	21.4	12.6	7.6	43.3	29.4	85.7
PBS (μL)	244.6	244	146.9	244.3	224.7	243.5	475	243.8	242.6	278.6	337.4	492	256.7	270.6	514
Total mAb solution volume (μL)	250	250	150	250	250	250	500	250	250	300	350	500	300	300	600

Supplementary Table 9a. Standard Binding Table

Complex	1 µg/mL ST Ps standard (µL)	0.1 mg/mL anti-ST mAb (µL)	Binding Buffer (µL)	Total Vol (µL)	[Ps] (µg/mL)
STD-1	2	20	178	200	0.01
STD-2	5	20	175	200	0.025
STD-3	10	20	170	200	0.05
STD-4	20	20	160	200	0.1
STD-5	30	20	150	200	0.15

Supplementary Table 9b. Vaccine Binding Table

Free Ps from Vaccine	Vaccine solution Vol (µL)	0.1 mg/mL anti-ST IgG mAb (µL)	Binding buffer (µL)	Total Vol (µL)	Dilution
Vaccine free Ps	20	20	160	200	10

Supplementary Table 10. Preparation standard curve for multiplex FLR labeled antibody assay

PCV reference standard reaction	1 µg/mL Ref STD (µL)	0.2 mg/mL ea of FLR-mAbs (µL)*	Binding Buffer (µL)	Total Vol (µL)	[Ps] (µg/mL)
PCV-REF-1	5	15	180	200	0.025
PCV-REF-2	10	15	175	200	0.05
PCV-REF-3	20	15	165	200	0.1
PCV-REF-4	30	15	155	200	0.15
PCV-REF-5	40	15	145	200	0.2

Supplementary Table 11. Preparation PCV24 for multiplex FLR labeled antibody assay

PCV24 reaction	PCV24 8x Dilution (µL)	0.2 mg/mL ea of FLR-mAb (µL)*	Binding Buffer (µL)	Total Vol (µL)	Total dilution
PCV24-8x dilution ^a	20	15	165	200	80

a: PCV24 was diluted 8-fold in binding buffer before added into the binding reaction.

*: FLR-mAb is a cocktail of four FLR labeled mAbs that consists of anti-ST4-AF350, anti-ST5-AF430, anti-ST6A-AF555 and anti-ST14-AF633. Concentration for each mAb is 0.2 mg/mL.

Supplementary Table 12. Chromatography Condition Table

Chromatography conditions	Chromatography condition-A	Chromatography condition-B
---------------------------	----------------------------	----------------------------

Column	TOSOH TSKGel GMPWxL (7.8 x 300 mm), 10 μ m	Shodex PROTEIN KW-803 (8.0 x 300 mm), 5 μ m
Flow Rate (isocratic) (mL/min)	0.8	1.0
Injection Volume (μ L)	80	
Mobile Phase	10 mM Bis-Tris-HCl, 300 mM NaCl, pH 7	
Column Temp ($^{\circ}$ C)	35	
Sample Tray ($^{\circ}$ C)	6	

Supplementary Table 13. APC binding reactions for SEC-UV-MALS-RI analysis

APC Complex Reaction	mAb/Ps (mg/mg)	1.0 mg/mL Anti-ST1 mAb (μ L)	0.25 mg/mL ST1 Ps (μ L)	Total reaction Vol (μ L)	HPLC INJ (μ L)
Complex-1 reaction	0.25	2.5	40	42.5	20
Complex-2 reaction	0.50	5.0	40	45.0	20
Complex-3 reaction	1.00	10.0	40	50.0	20