

Supplementary Information

Efficient discovery of antibody binding pairs using a photobleaching strategy for bead encoding

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Table S1. Mixes of antibodies.

Mix number	Bead Type	Bleaching duration [hr]	Conjugated Ab Cat #	Clone	Vendor	Immunogen
Mix 1	488-multiplex beads (Quanterix)	24	71735	E6G6Q	CST	A monoclonal antibody is produced by immunizing animals with recombinant protein specific to the carboxy terminus of human Olig2 protein.
			sc-515947	H-10	Santa Cruz	AA 11-78
			NBP227301	Poly	Novus	A portion of amino acids 70-130 of human OLIG2
Mix 2	488-multiplex beads (Quanterix)	4	PA5-23456	Poly	Invitrogen	Synthetic peptide (88 SAAASSTKKDKKQMTEP E 105) of OLIG2 protein
			MA5-35964	O64	Invitrogen	A synthetic peptide corresponding to the N-terminus of the Human Olig-2
			MA5-42372	HL1072	Invitrogen	Recombinant protein encompassing a sequence within the N-terminus region of human OLIG2
Mix 3	750-multiplex beads (Quanterix)	3	76961-012	7074R	Antibodies.com LLC	Recombinant fragment, around amino acids 200–300, of human OLIG2 protein. The exact sequence is proprietary.
			NBP241269	Poly	Novus	Antibody was raised against a 15 amino acid peptide near the amino terminus of human OLIG2
			615952	W21023A	BioLegend	Recombinant fragment of the N-terminus of mouse Olig2
Mix 4	750-multiplex beads (Quanterix)	24	H00010215-M02	3D7	Abnova	AA 2-78
			100137-RP02-100	Poly	Sino	Produced in rabbits immunized with a synthetic peptide corresponding to the N-terminus of the Human Olig-2, and purified by antigen affinity chromatography
			MAB21134	2400	Abnova	Recombinant protein corresponding to amino acids 1-141 of human OLIG2
Mix	Bead Type	Bleaching	Conjugated	Clone	Vendor	Immunogen

number		duration [hr]	Ab Cat #			
Mix 5	750-multiplex beads (Quanterix)	0	ab220796	EPR2673	Abcam	Synthetic peptide. This information is proprietary to Abcam and/or its suppliers
			AF2418	Poly	R&D Systems	E. coli -derived recombinant human Olig2 Met1-Lys323; Accession # Q13516
			LS-B6029-GOS10	3C9	LS-Bio	AA 2-78
Mix 6	488-multiplex beads (Quanterix)	0	TA349037	Poly	Origene	OLIG2 antibody was raised against a 15 amino acid peptide near the amino terminus of human OLIG2
			ZRB1436-4X25UL	1K2	Sigma Aldrich	His-tagged recombinant fragment corresponding to 83 amino acids from the N-terminal region of human Olig2
			10215-RBM2-P1ABX	6695R	Neobiotechnologies	Recombinant fragment (around aa200–300) of human OLIG2 protein (exact sequence is proprietary)

Table S2. Bead Conjugation conditions for beads with mixes of antibodies.

Mix number	Bead Type	Capture Ab Cat #	clone	Capture Ab [µg]	Starting bead #	Batch size [µL]	Temp [°C]	EDC [µL]
Mix 1	488-multiplex beads (Quanterix)	71735	E6G6Q	25	3.98E+08	284	4	8.5
		sc-515947	H-10	25				
		NBP227301	Poly	25				
Mix 2	488-multiplex beads (Quanterix)	PA5-23456	Poly	25	2.99E+08	213	4	6.4
		MA5-35964	O64	25				
		MA5-42372	HL1072	25				
Mix 3	750-multiplex beads (Quanterix)	76961-012	7074R	25	5.22E+08	372	4	11.2
		NBP241269	Poly	25				
		615952	W21023A	25				
Mix 4	750-multiplex beads (Quanterix)	H00010215-M02	3D7	25	4.39E+08	313	4	9.4
		100137-RP02-100	Poly	25				
		MAB21134	2400	25				
Mix 5	750-multiplex beads (Quanterix)	ab220796	EPR2673	25	4.39E+08	313	4	9.4
		AF2418	Poly	25				
		LS-B6029-GOS10	3C9	25				
Mix 6	488-multiplex beads (Quanterix)	TA349037	Poly	25	3.88E+08	277	4	8.3
		ZRB1436-4X25UL	1K2	25				
		10215-RBM2-P1ABX	6695R	25				

Table S3. Bead Conjugation conditions for Simoa assays.

Bead Type	Bead number	Capture Ab Cat #	clone	Capture Ab [µg]	Starting bead #	Batch size [µL]	Temp [°C]	EDC [µL]
488-multiplex beads (Quanterix)	Beads 1	71735	E6G6Q	50	2.60E+08	178	4	5.3
	Beads 2	sc-515947	H-10	100	5.97E+08	409	4	12.3
	Beads 3	NBP227301	Poly	25	8.55E+07	61	4	1.8
	Beads 4	PA5-23456	Poly	50	3.20E+08	220	4	6.6
	Beads 5	MA5-35964	O64	50	4.23E+08	290	4	8.7
	Beads 6	MA5-42372	HL1072	50	2.23E+08	153	4	4.6
	Beads 7	76961-012	7074R	50	3.34E+08	239	4	7.2
	Beads 8	NBP241269	Poly	50	3.60E+08	257	4	7.7
	Beads 9	615952	W21023A	50	2.62E+08	201	4	5.6
	Beads 10	H00010215-M02	3D7	100	5.90E+08	406	4	12.0
	Beads 11	100137-RP02-100	Poly	50	2.65E+08	203	4	5.7
	Beads 12	MAB21134	2400	50	3.47E+08	267	4	7.4
	Beads 13	ab220796	EPR2673	50	2.66E+08	183	4	5.5
	Beads 14	AF2418	Poly	50	3.34E+08	256	4	7.2
	Beads 15	LS-B6029-GOS10	3C9	50	2.13E+08	152	4	4.6
	Beads 16	TA349037	Poly	50	3.14E+08	224	4	6.7
	Beads 17	ZRB1436-4X25UL	1K2	50	2.70E+08	193	4	5.8
	Beads 18	10215-RBM2-P1ABX	6695R	50	3.39E+08	242	4	7.3

Table S4. Detection antibodies.

	Detection Ab Catalog # (Biotinylated In-house)	Clone
DA 1	ab220796	EPR2673
DA 2	sc-515947	H-10
DA 3	MA5-42372	HL1072
DA 4	CST 71735	E6G6Q
DA 5	MA5-35964	O64
DA 6	PA5-23456	Poly
DA 7	H00010215-M02	3D7
DA 8	LS-B6029-GOS10	3C9

Table S5. Compensation matrix for cross-testing.

	Spillover into FL1-A :: B525-FITC-A	Spillover into FL9-A :: R660-APC-A	Spillover into FL11-A :: R763-APCA750-A
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FL1-A :: B525-FITC-A	100%	0%	0%
FL9-A :: R660-APC-A	0%	100%	26.68%
FL11-A :: R763-APCA750-A	0%	0%	100%

Table S6. Compensation matrix for generating 12-plex.

	Spillover into FL1-A :: B525-FITC-A	Spillover into FL10-A :: R712-APCA700-A	Spillover into FL11-A :: R763-APCA750-A	Spillover into FL9-A :: R660-APC-A
FL1-A :: B525-FITC-A	100%	0.04%	0.04%	0.03%
FL10-A :: R712-APCA700-A	1%	100%	31%	1%
FL11-A :: R763-APCA750-A	14%	4%	100%	2%
FL9-A :: R660-APC-A	0%	73%	32%	100%

Table S7. Cross-testing map. The assays in green were classified as positive in the multiplex assay and were further tested using Simoa.

	Bead number	Detection Ab Capture Ab	DA 1	DA 2	DA 3	DA 4	DA 5	DA 6	DA 7	DA 8
			ab220796	sc-515947	MA5-42372	CST 71735	MA5-35964	PA5-23456	H00010215-M02	LS-B6029-GOS10
Mix 1	Beads 1	71735	Assay 1	Assay 18	Assay 35	X	Assay 69	Assay 86	Assay 104	Assay 121
	Beads 2	sc-515947	Assay 2	X	Assay 36	Assay 52	Assay 70	Assay 87	Assay 105	Assay 122
	Beads 3	NBP227301	Assay 3	Assay 19	Assay 37	Assay 53	Assay 71	Assay 88	Assay 106	Assay 123
Mix 2	Beads 4	PA5-23456	Assay 4	Assay 20	Assay 38	Assay 54	Assay 72	Assay 89	Assay 107	Assay 124
	Beads 5	MA5-35964	Assay 5	Assay 21	Assay 39	Assay 55	X	Assay 90	Assay 108	Assay 125
	Beads 6	MA5-42372	Assay 6	Assay 22	X	Assay 56	Assay 73	Assay 91	Assay 109	Assay 126
Mix 3	Beads 7	76961-012	Assay 7	Assay 23	Assay 40	Assay 57	Assay 74	Assay 92	Assay 110	Assay 127
	Beads 8	NBP241269	Assay 8	Assay 24	Assay 41	Assay 58	Assay 75	Assay 93	Assay 111	Assay 128
	Beads 9	615952	Assay 9	Assay 25	Assay 42	Assay 59	Assay 76	Assay 94	Assay 112	Assay 129
Mix 4	Beads 10	H00010215-M02	Assay 10	Assay 26	Assay 43	Assay 60	Assay 77	Assay 95	X	Assay 130
	Beads 11	100137-RP02-100	Assay 11	Assay 27	Assay 44	Assay 61	Assay 78	Assay 96	Assay 113	Assay 131
	Beads 12	MAB21134	Assay 12	Assay 28	Assay 45	Assay 62	Assay 79	Assay 97	Assay 114	Assay 132
Mix 5	Beads 13	ab220796	X	Assay 29	Assay 46	Assay 63	Assay 80	Assay 98	Assay 115	Assay 133
	Beads 14	AF2418	Assay 13	Assay 30	Assay 47	Assay 64	Assay 81	Assay 99	Assay 116	X
	Beads 15	LS-B6029-GOS10	Assay 14	Assay 31	Assay 48	Assay 65	Assay 82	Assay 100	Assay 117	Assay 134
Mix 6	Beads 16	TA349037	Assay 15	Assay 32	Assay 49	Assay 66	Assay 83	Assay 101	Assay 118	Assay 135
	Beads 17	ZRB1436-4X25UL	Assay 16	Assay 33	Assay 50	Assay 67	Assay 84	Assay 102	Assay 119	Assay 136
	Beads 18	10215-RBM2-PIABX	Assay 17	Assay 34	Assay 51	Assay 68	Assay 85	Assay 103	Assay 120	Assay 137

Experimental

Calculation of the number of detector molecules per bead in the cross-testing assay:

The number of antibodies conjugated on beads assuming a conjugation yield of 60%:

45 μg of antibody is conjugated on ~ 400 million beads.

$$\frac{\text{Number of Ab \# conjugated}}{\text{bead}} = \frac{45 \mu\text{g} \cdot \mu\text{mol} \cdot 6.023 \times 10^{23} \text{Ab \#}}{150,000 \mu\text{g} \cdot 10^6 \mu\text{mol} \cdot 4 \times 10^8 \text{beads}} = 450,000 \frac{\text{Ab \#}}{\text{bead}}$$

Assuming 100% binding of OLIG2 to capture Ab:

At 1000 pg/mL of OLIG2:

$$\frac{\text{Number of OLIG2 \#}}{\text{bead}} = \frac{1000 \text{ pg} \cdot \text{pmol} \cdot 6.023 \times 10^{23} \text{Ab \#}}{32,000 \text{ pg} \cdot 10^{12} \text{ pmol} \cdot 150,000 \text{ beads}} = 12,547 \frac{\text{OLIG2 \#}}{\text{bead}}$$

Assuming each detection antibody binds two OLIG2 molecules:

$$\frac{\text{Number of detection \#}}{\text{bead}} = \frac{12547 \text{ OLIG2 \#} \cdot \text{detection Ab \#}}{2 \text{ OLIG2 \#}} = 6273 \frac{\text{detection Ab \#}}{\text{bead}}$$

Assuming one SA-647 molecule binds to each detection Ab:

$$\frac{\text{Number of detector \#}}{\text{bead}} = 6273 \frac{\text{detector \#}}{\text{bead}}$$

At 10000 pg/mL of OLIG2:

$$\frac{\text{Number of detector \#}}{\text{bead}} = 62,730 \frac{\text{detector \#}}{\text{bead}}$$

Photobleaching efficiency of different LEDs:

A stock of two million magnetic beads (488 or 750-dyed beads) in 2 mL Bead diluent was put in a 35mm dish and photobleached for different durations. Samples of 200,000 beads were taken out at each time point and tested on the flow cytometer. The percentage of photobleaching was calculated as:

$$\% \text{ photobleaching}_{(t)} = 100\% - \left(\frac{\text{MFI}_t}{\text{MFI}_0} \cdot 100\% \right)$$

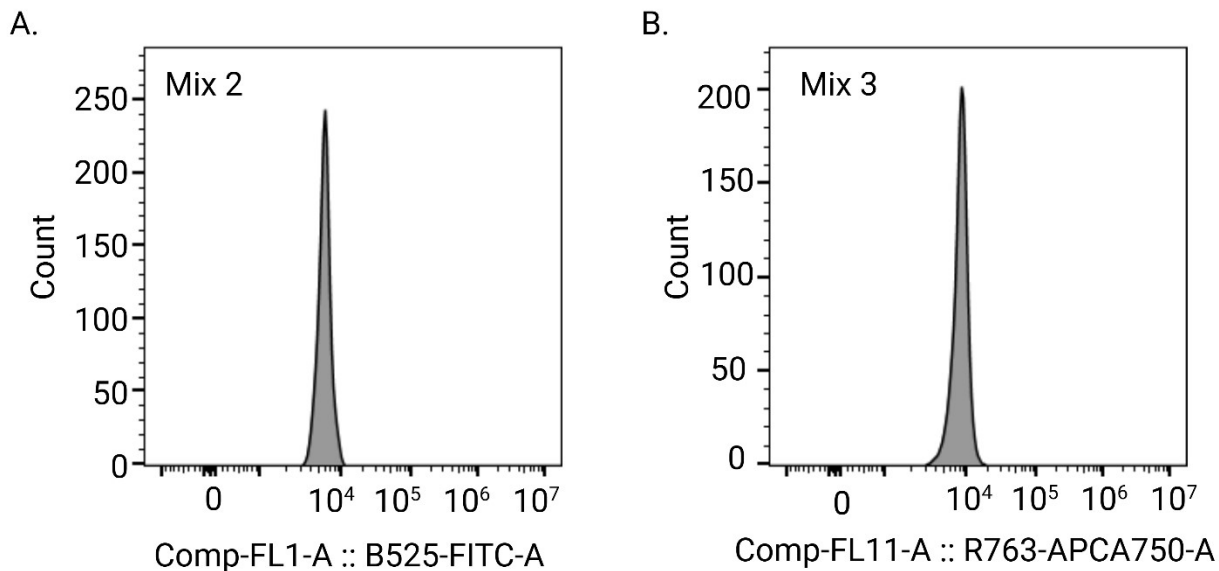
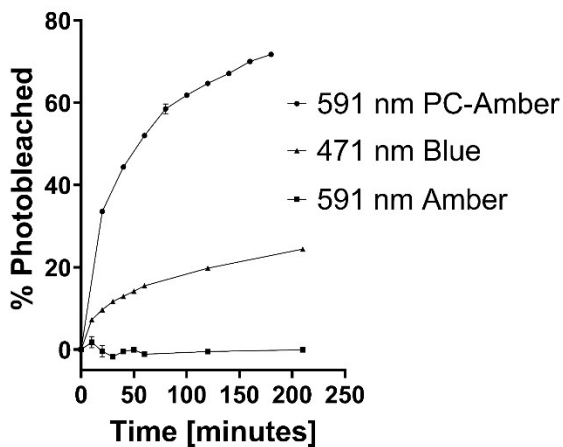


Figure S1. Histograms of bead mixes after photobleaching. Histograms show the distribution of photobleached beads. (A) Mix 2 (488 nm dye-encoded) photobleached for 4 hours. (B) Mix 3 (750 nm dye-encoded) photobleached for 3 hours.

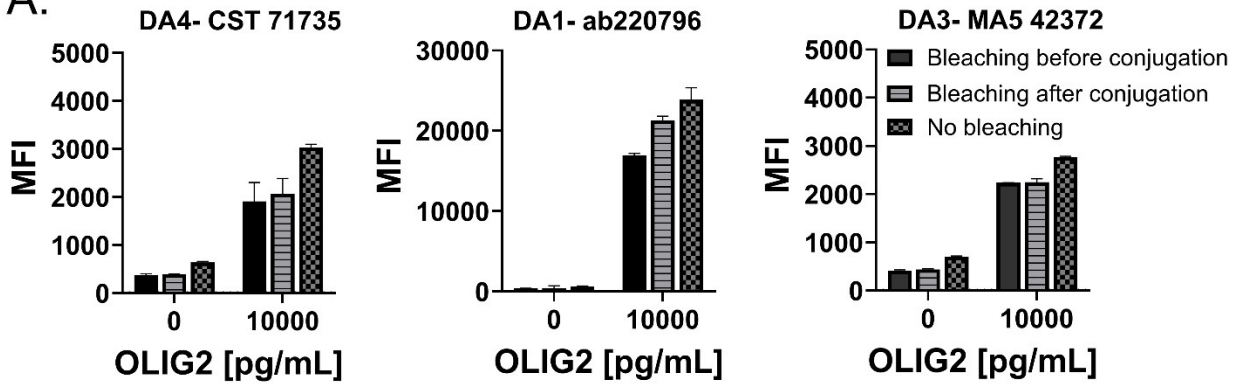


LED peak wavelength [nm]	Power intensity [W/cm ²]	Beads photobleached	Typical spectral half-width [nm] *	Driver current [mA]
471 Blue	0.372	750-dyed beads	20	1000
591 Amber	0.066	488-dyed beads	20	700
591 PC Amber	1.15	750-dyed beads	80	700

* DS68 LUXEON Rebel Color Line Product Datasheet
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Figure S2. Photobleaching efficiency using different LEDs. Magnetic beads were photobleached (488-dyed beads with 591 nm Amber and 750-dyed beads with 471 nm Blue and PC-Amber LEDs), and the % photobleaching over time is presented. Results represent the average of two replicates. Error bars represent the standard deviation.

A.



B.

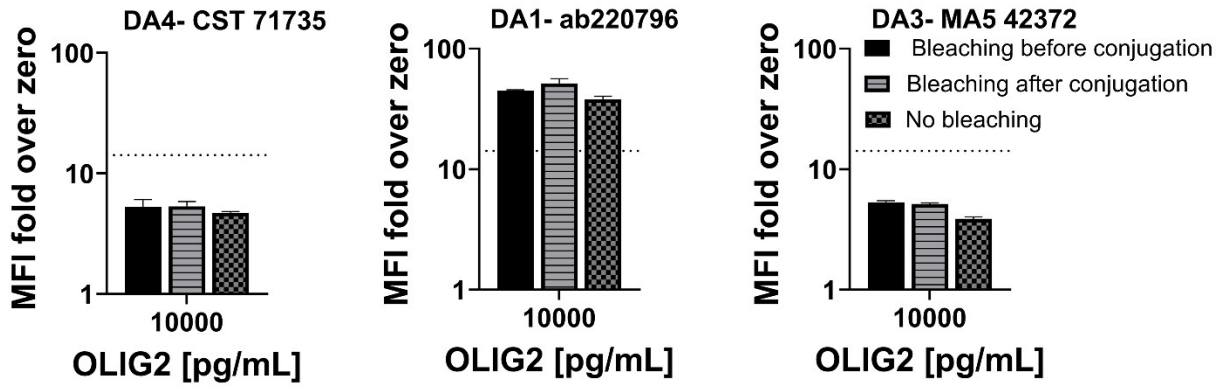


Figure S3. Comparison of cross-testing results with beads photobleached before and after conjugation. 488 nm dye-encoded paramagnetic beads were photobleached for 24 hrs before and after conjugation with antibodies for “Mix 1” or were conjugated with non-photobleached beads. The beads were cross-tested with detection antibodies 4 (CST 71735), 1 (ab220796), and 3 (MA5-42372). (A) Results represent the MFI. Error bars represent the median of four replicates with 95% CI. (B) The threshold for this experiment was set to (*MFI fold over zero* > 14.2). Results represent the mean values of the “MFI fold over zero”. Error bars represent the standard deviation (STD) of four replicates.

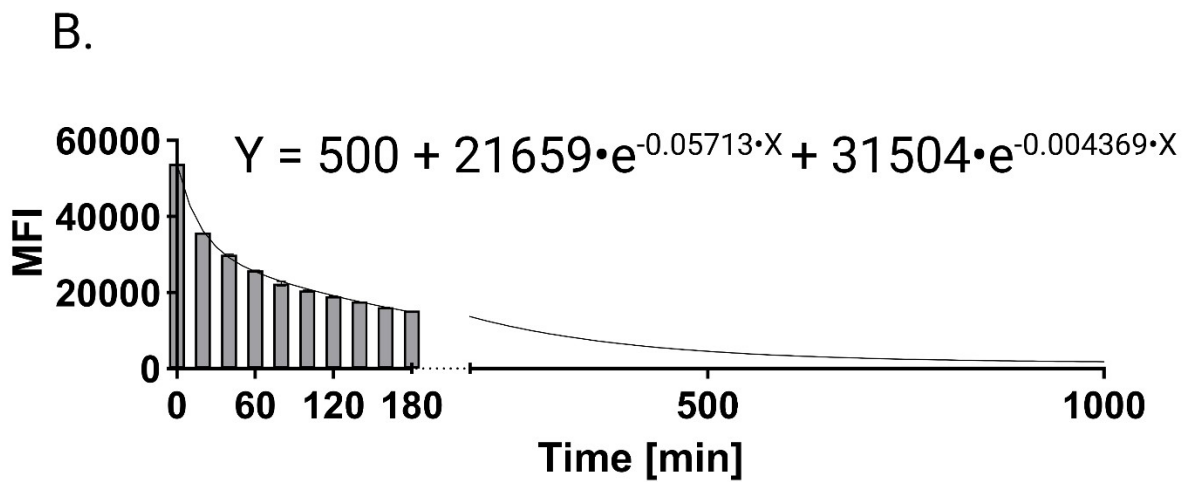
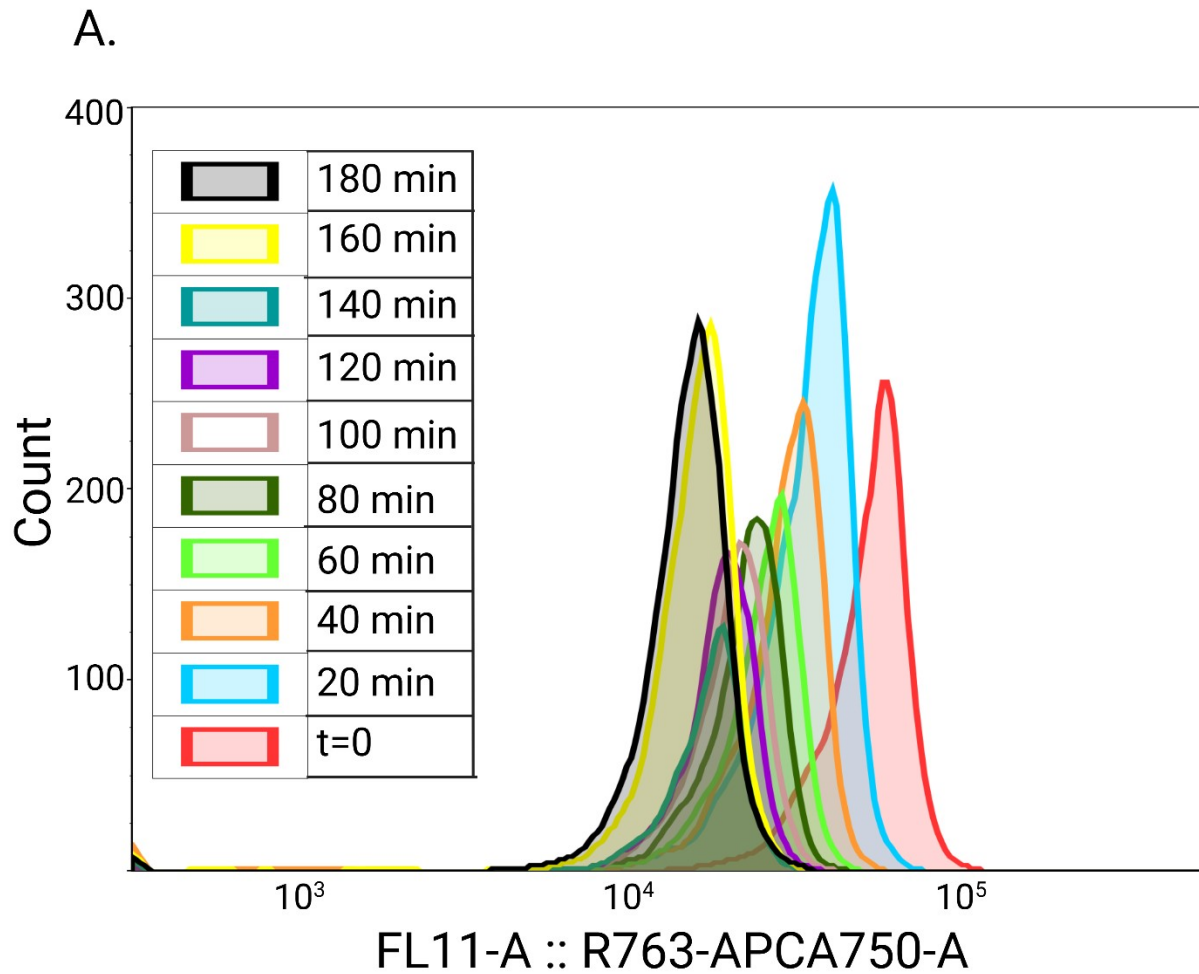


Figure S4. Photobleaching kinetics of 750 nm beads. (A) Histograms of the MFI after different photobleaching duration times. (B) Two-exponential decay curve fitted the photobleaching kinetics data of 750-dyed beads using GraphPad Prism 10.2.3. The plateau value was set to 1500 MFI.

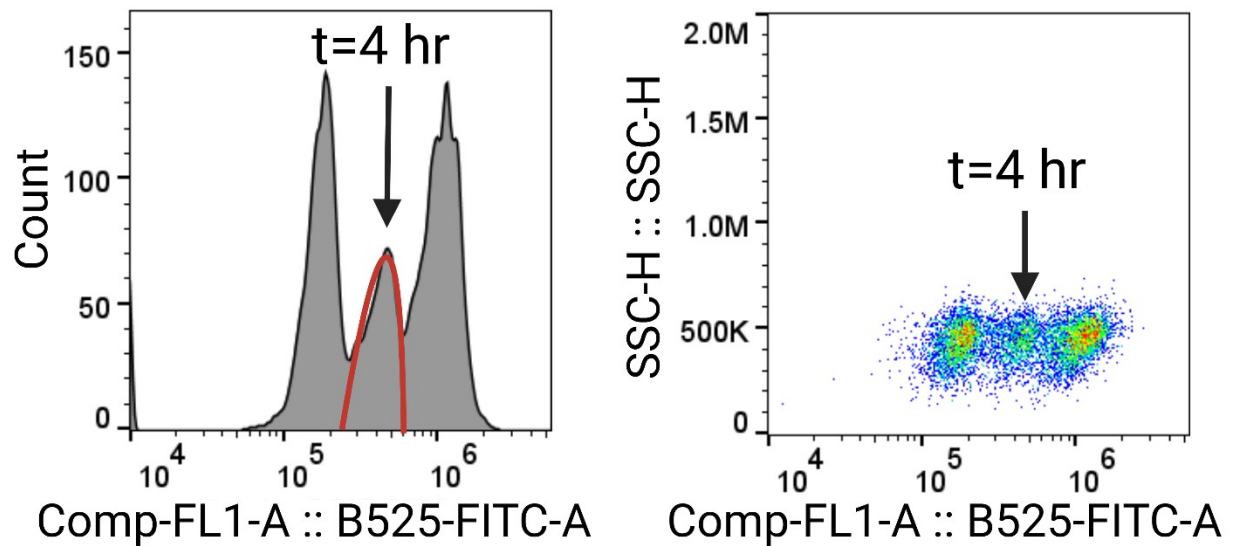
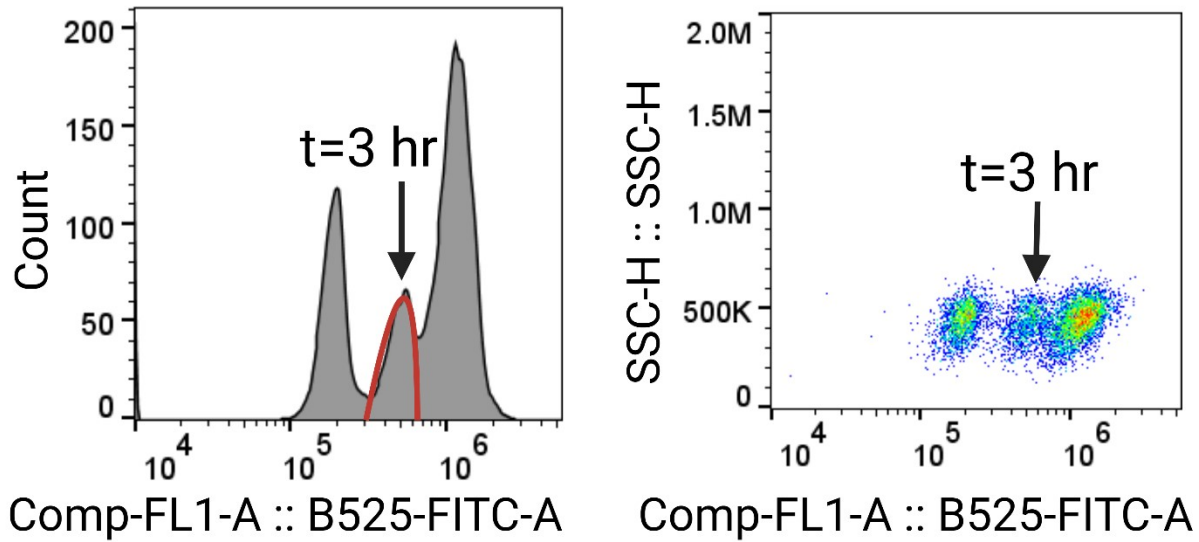


Figure S5. Photobleaching kinetics of Mix2 beads (488-dyed beads). Histograms and pseudo color plots after three and four hours of photobleaching.

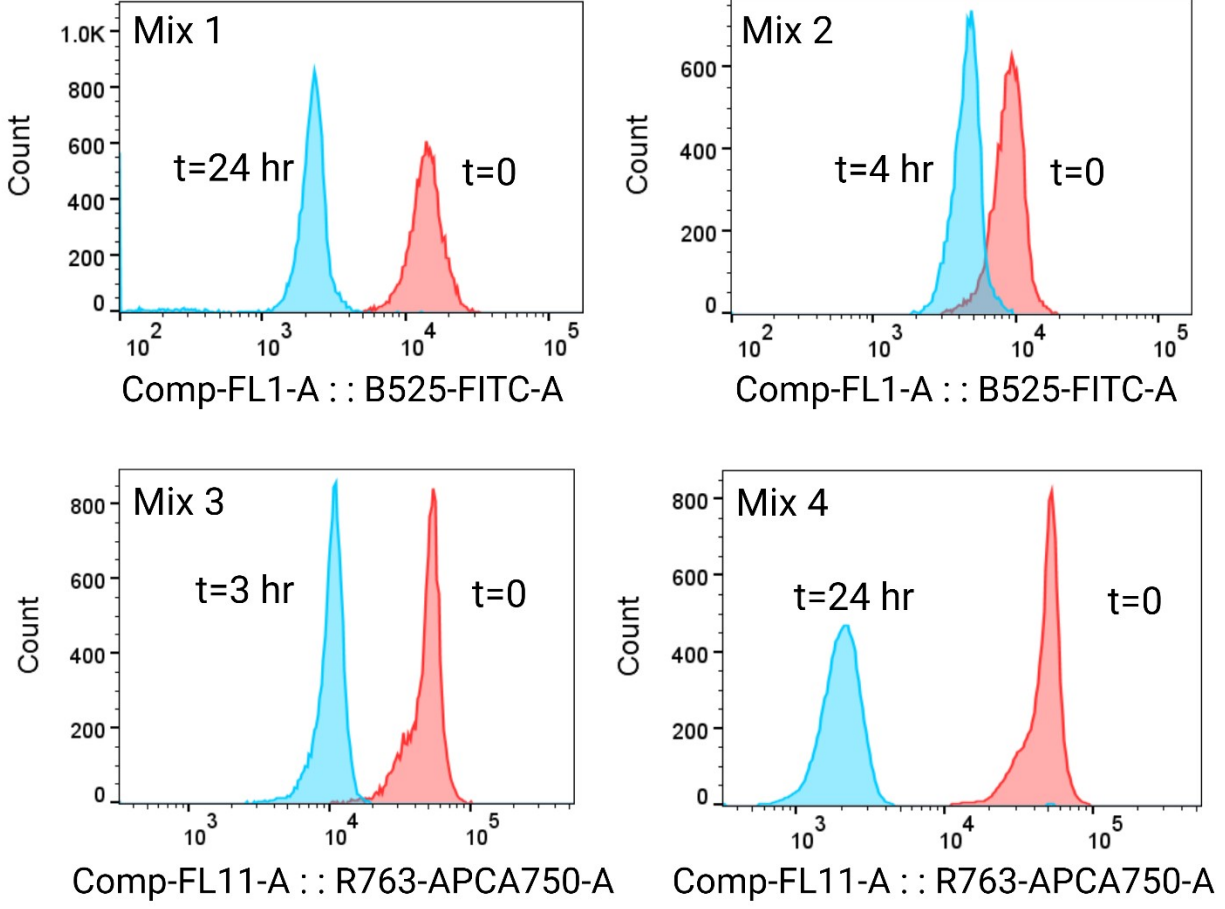


Figure S6. Histograms of mixes before and after photobleaching. Histograms of Mixes 1–4 MFI before and after photobleaching.

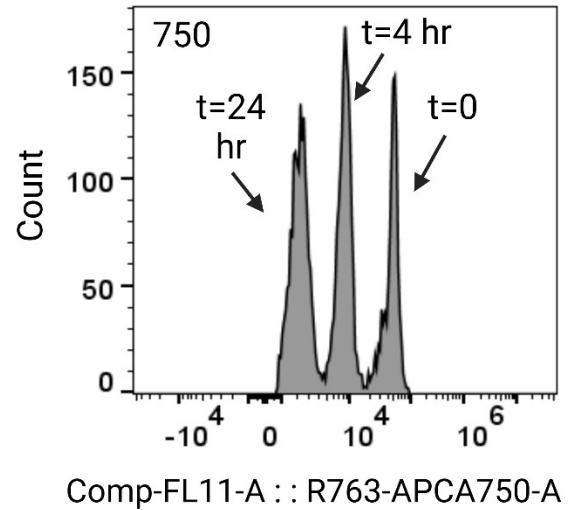
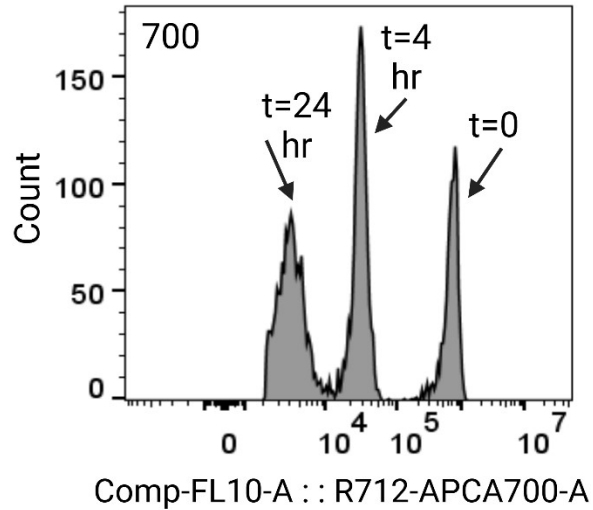
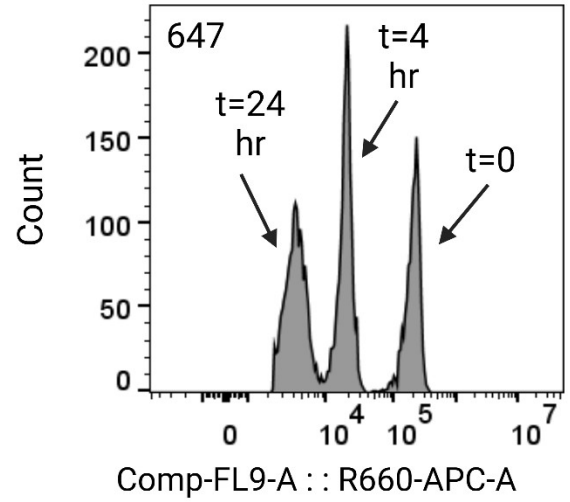
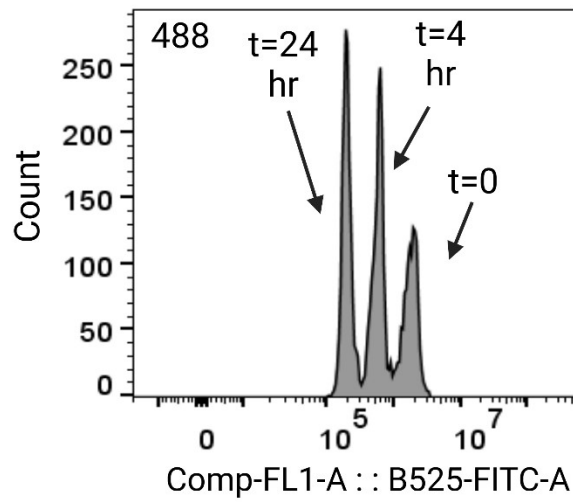
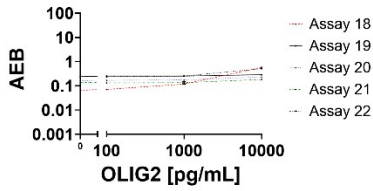
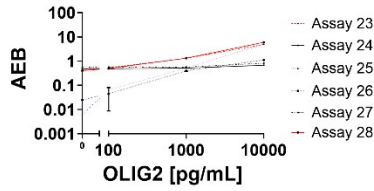


Figure S7. Histograms of beads before and after photobleaching for 12-plex. Histograms of 488, 647, 700, and 750-dyed beads MFI before and after photobleaching.

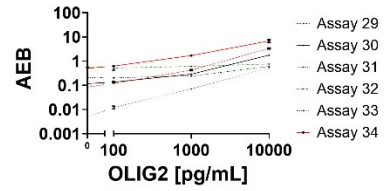
Mix 1,2 separate beads + DA2



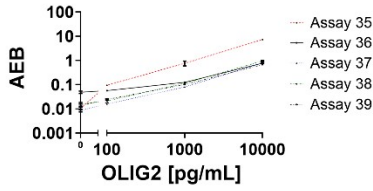
Mix 3,4 separate beads + DA2



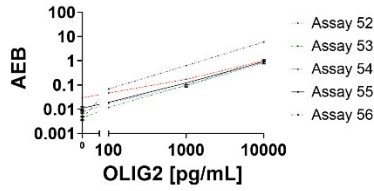
Mix 5,6 separate beads + DA2



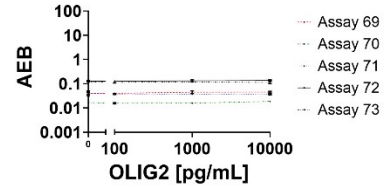
Mix 1,2 separate beads + DA3



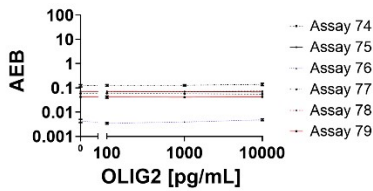
Mix 1,2 separate beads + DA4



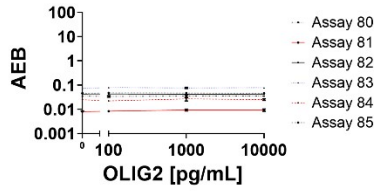
Mix 1,2 separate beads + DA5



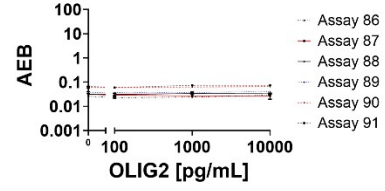
Mix 3,4 separate beads + DA5



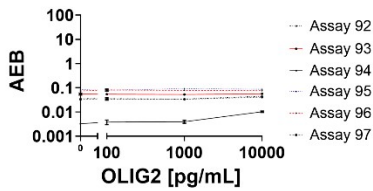
Mix 5,6 separate beads + DA5



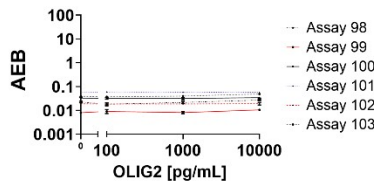
Mix 1,2 separate beads + DA6



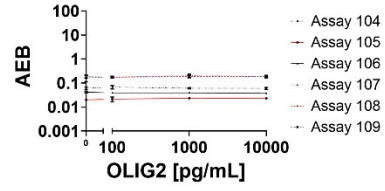
Mix 3,4 separate beads + DA6



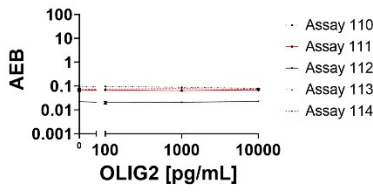
Mix 5,6 separate beads + DA6



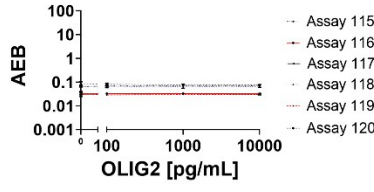
Mix 1,2 separate beads + DA7



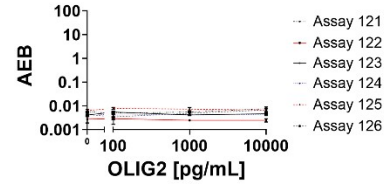
Mix 3,4 separate beads + DA7



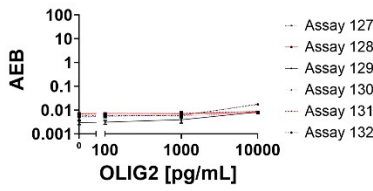
Mix 5,6 separate beads + DA7



Mix 1,2 separate beads + DA8



Mix 3,4 separate beads + DA8



Mix 3,4 separate beads + DA8

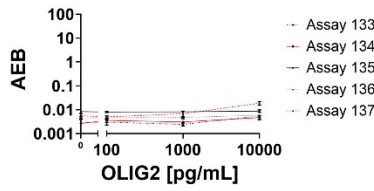


Figure S8. Testing negative cross-tested mixes using Simoa. Error bars represent the STD of two replicates.

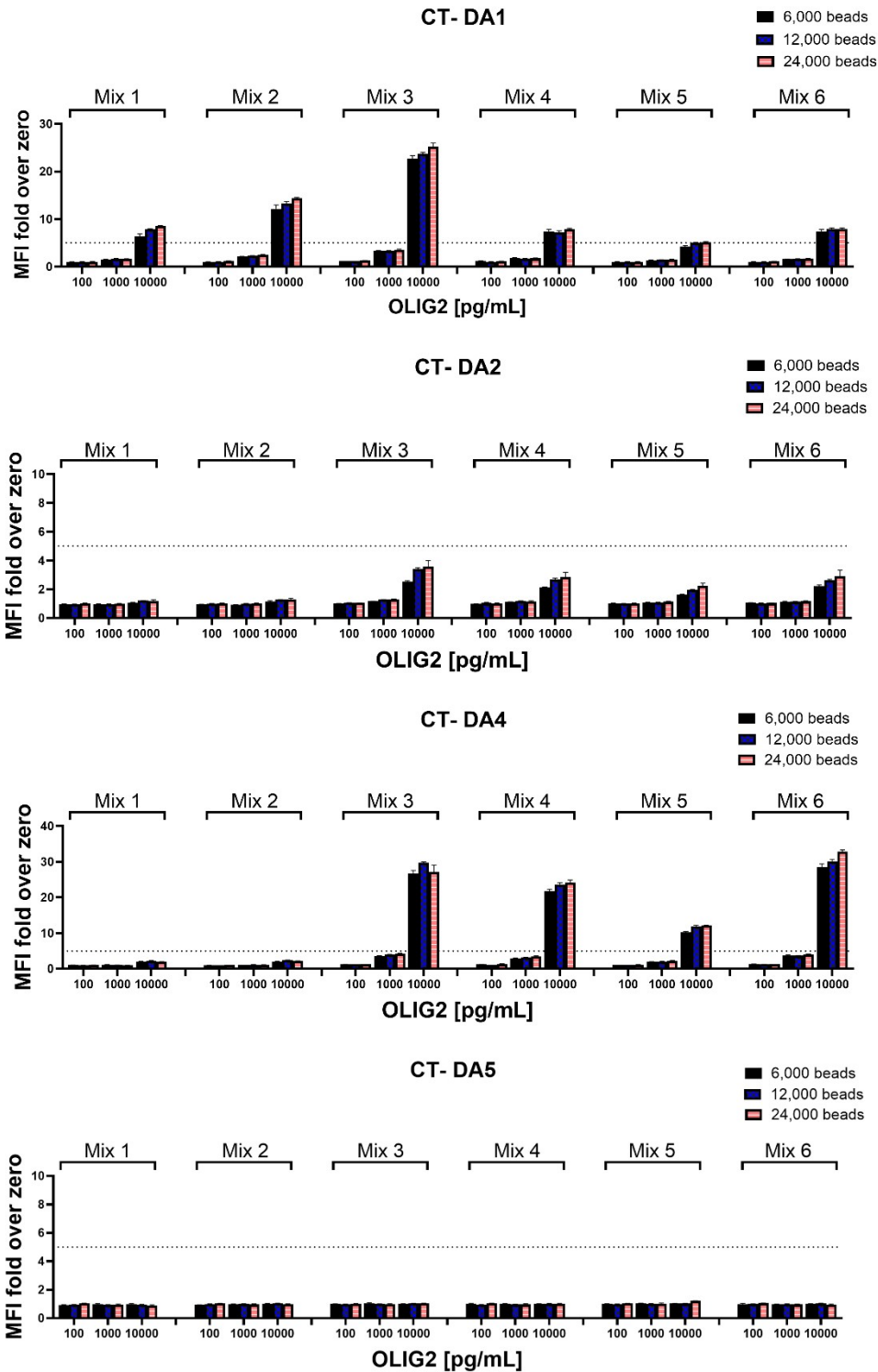


Figure S9. Recording different bead numbers (DA 1–2, 4–5). Cross-testing (CT) results of all mixes with detection antibodies 1,2,4, and 5, using the same number of beads, while recording 6,000, 12,000, or 24,000 beads. The error bars represent the standard error of the mean ($n = 6$ for the experiment with 12,000 beads, $3 \leq n \leq 5$ for experiments with 6,000 and 24,000 beads).

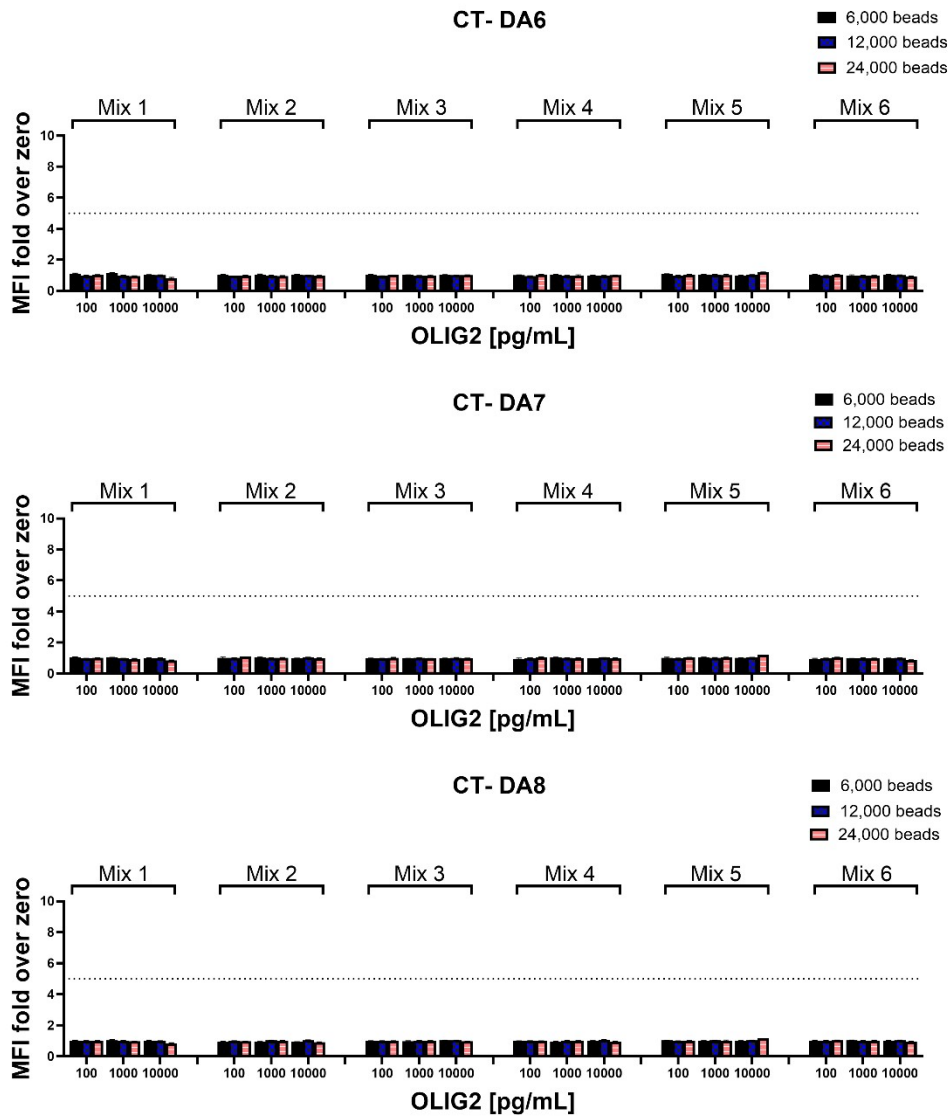


Figure S10. Recording different bead numbers (DA 6–8). Cross-testing (CT) results of all mixes with detection antibodies 6,7, and 8 using the same number of beads while recording 6,000, 12,000, or 24,000 beads. The error bars represent the standard error of the mean ($n = 6$ for the experiment with 12,000 beads, $3 \leq n \leq 5$ for experiments with 6,000 and 24,000 beads).