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 SAAASSTKKDKKQMTEPE 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](https://us.vwr.com/store/supplier/id/0000041634/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 number** | **Bead Type** | **Bleaching duration [hr]** | **Conjugated Ab Cat #** | **Clone** | **Vendor** | **Immunogen** |
| **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 |
| 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.

|  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  |  |  | DA 1 | DA 2 | DA 3 | DA 4 | DA 5 | DA 6 | DA 7 | DA 8 |
|  | **Bead number** | Detection Ab    Capture Ab | **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-P1ABX | 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 of antibody is conjugated on ~400 million beads.

Assuming 100% binding of OLIG2 to capture Ab:

At 1000 of OLIG2:

Assuming each detection antibody binds two OLIG2 molecules:

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

At 10000 of OLIG2:

**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:

A comparison of a graph

Description automatically generated

**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.

A close-up of a computer

Description automatically generated

**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 group of graph of different sizes

Description automatically generated with medium confidence

**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 . Results represent the mean values of the “MFI fold over zero”. Error bars represent the standard deviation (STD) of four replicates.

A graph of a number of numbers and a graph of a number of numbers

Description automatically generated with medium confidence

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

A group of graphs showing numbers and numbers

Description automatically generated with medium confidence

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

A group of graphs with numbers and symbols

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

A graph of a number of numbers

Description automatically generated with medium confidence

**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.

A screenshot of a graph

Description automatically generated

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

A group of graphs showing different sizes and colors

Description automatically generated with medium confidence

**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 ( for the experiment with 12,000 beads, for experiments with 6,000 and 24,000 beads).

A diagram of a dna test

Description automatically generated with medium confidence

**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 ( for the experiment with 12,000 beads, for experiments with 6,000 and 24,000 beads).