Supporting Information

Screening One Bead One Compound Libraries Against Serum Using a Flow Cytometer: Determination of the Minimum Antibody Concentration Required for Ligand Discovery

Osayemwenre Erharuyi[†], Scott Simanski, Patrick J. McEnaney and Thomas Kodadek[*]

Department of Chemistry, The Scripps Research Institute,130 Scripps Way, Jupiter, Florida 33458, U.S.A.

[†]Current Address: Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Benin, Benin City, Nigeria.

Corresponding Author

*E-mail: kodadek@scripps.edu

Materials

All chemicals and solvents were of commercial source and used without further purification. All steps involving water utilized distilled water filtered through a Barnstead Nanopure filtration system (Thermo Scientific).

EXPERIMENTAL PROCEDURES

Ligand synthesis

The FLAG peptides were synthesized on 10 μm TentaGel M NH_2 resin (0.50 g, 0.21 mmol/g, loading, ~1.95×10^9 beads/g, catalog no. M30102, Rapp-Polymere, Tuningen, Germany). The beads were swollen in dimethylformamide (DMF) for 2 h before use. DMF was used as the solvent unless otherwise mentioned. For synthesis of the peptides, Fmoc-methionine-OH (5 mol. equiv./g bead) was anchored to beads using HOBt (1-hydroxybenzotriazole) (5 mol.equiv./g), HBTU [2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluroniumhexafluorophosphate] (5 mol.equiv./g), and N,N-diisopropylethylamine (DIEA) (5 mol. equiv./g) overnight. This was followed by Fmoc deprotection using 20% piperidine in DMF first for 5 min, washed with DMF and then for 20 min. Following the methionine coupling, the PEG 2-[2-(Fmoc-amino)-ethoxy]-ethoxy acetic acid linker and all Fmoc amino acid residues were coupled following peptide/amide bond formation procedure as previously described.[10] The final sequences for the peptides FLAG and FLAG-D4H were DYKDDDDK and DYKHNNYN respectively. KMS31 was synthesized as described previously,[22] but using 10 μm TentaGel beads.

Protein concentration determination

Total serum protein was determined spectrophotometrically using a Nanodrop instrument. Total IgG concentration was determined using the Ready-SET-Go ELISA kit (eBioscience).

Preparation of fluorescein-labeled beads

10 μm TentaGel beads were partitioned to protect the surface accessible amines with Fmoc using the procedure previously described.[18,28] Briefly, TentaGel 10 μm beads (5 mg, 0.23 mol/g) were incubated in water (12 h, RT). Beads were centrifuged (1 min, 500 x g) and the supernatant decanted. Fmoc-OSu (1.9 mg, 0.5 equiv.) was dissolved in 2.5 mL of a 1:1 mixture of diethyl

ether:dichloromethane (DCM). The solution was added to bead aliquots in a 5 mL centrifuge tube followed by extensive mixing (vortex 30 s, sonicate 30 s, vortex 30 s) and incubated (20 min, room temperature, rotating). The sample was centrifuged (500 x g, 1 min), washed (4x DMF) and re-equilibrated in DMF (3 h, RT, rotating).

To 1 mg of partitioned beads generated above Fluorescein-N-hydroxysuccinimide (Fluorescein-NHS) (211 nM, 150 µL, 0.032 nmols) in DMF with 1% TMP was added and incubated shaking (2 h, 37°C). Following labeling the beads were washed (3x DMF, 3x DCM, 1x DMF). After interior fluorescent labeling 0.05 mg of 160 µm TentaGel beads (0.43 mmol/g) were added as a synthesis control. The exterior Fmoc protecting group removed (20% piperidine in DMF, 5 min RT, 15 min RT).

Expression and purification of CLL BCR IgG

The cloning, expression, and purification of the monoclonal antibody was performed as described[29] Briefly, CLL014 monoclonal antibody was expressed as the IgG1 isotype from light chain (pIgG-lambda) and heavy chain (pIgG-gamma) expression plasmids. Heavy and light chain variable gene segments from CLL014 clones were amplified and sub-cloned into expression plasmids to construct pIgGlambda, or pIgG-gamma. The mammalian expression vectors were transiently transfected into HEK 293T cells (ATCC, CRL-11268) with OMNIfect transfection reagent (transomic technologies, cat. no. OTR1003). Transfected HEK 293T cells were cultured in X-vivo serum-free medium (Lonza, cat. no. 04-743Q) at 37°C in 5% CO_2 after adding neutridoma-SP (Roche, cat. no. 11011375001) (250 µL per 150 T culture flask). Following 5 days of transfection, the medium was collected after centrifugation (1000 x g, 10 min) to remove cells, the supernatants was incubated with recombinant Protein G beads over night at 4^{o}C (30 µL of packed beads per 50 mL of media). The beads were loaded into a 20mL affinity column (20mL of 100 mM Tris-HCl (pH 8.0) was used for column equilibration and washing) and eluted with IgG elution buffer following the manufacturer's instruction (Thermo Fischer Scientific, cat. no. 21004), and 1 M Tris-HCl (pH 9.0) was used for immediate neutralization. The neutralized eluate was concentrated with a 30 kDa cutoff centrifugal filter devices (Millipore) and dialyzed into 1×PBS. Purified CLL014 monoclonal antibodies were stored at -20 °C until use.

Antibody Incubations and Flow Cytometric Analysis

TentaGel beads displaying FLAG peptide and Fluorescein labeled beads were equilibrated in water for 2 h, followed by overnight incubation in 1X PBS containing 0.05% Tween 20. The beads were then incubated with the blocking buffer, PBS starting block (Thermo Scientific, cat. no. 37538) for 2 hours. After incubation in blocking buffer, approximately 1,000 of beads displaying FLAG peptide were doped into approximately 10,000 of fluorescein labeled beads. The bead mixture was added to mouse serum (45 mg/mL total protein, 18.1 mg/mL total IgG; Immunoreagents, Inc., Raleigh, NC, cat. no. SP-002-VX5) that had been diluted 250-fold into PBST buffer,the final volume was 200 µL. To this solution was added monoclonal anti-FLAG M2 antibody (Sigma-Aldrich, cat. no. F3165) to give the desired final concentration. After incubating for 1 h at 4^{o}C with rotation, the supernatant was removed (by brief centrifugation and decanting) and the beads were washed three times with PBST. The beads were then incubated with Alexa Fluor 647-labeled chicken anti-mouse IgG antibody (Invitrogen, cat. no. A21463) diluted 1:200 in blocking buffer 1 h at 4°C with rotation. After washing three times with PBST, the beads were re-suspended in PBST (200 µL) and transferred to FACS tubes.

The fluorescence intensities were measured using a flow cytometer (BD FACS Canto II, BD Bioscience).

The same procedure was repeated for the FLAG-D4H peptide.

For the KMS31 beads, the same protocol was followed except human serum obtained from a healthy volunteer (59 mg/mL total protein, 3.9 mg/mL total IgG) was substituted for the mouse serum, CLL014 IgG was doped into the sample and Alexafluor 647-labeled goat anti-human IgG (Invitrogen, cat. no. A21445) diluted 1:200 in blocking buffer was used to stain the beads.

Supplementary Figures

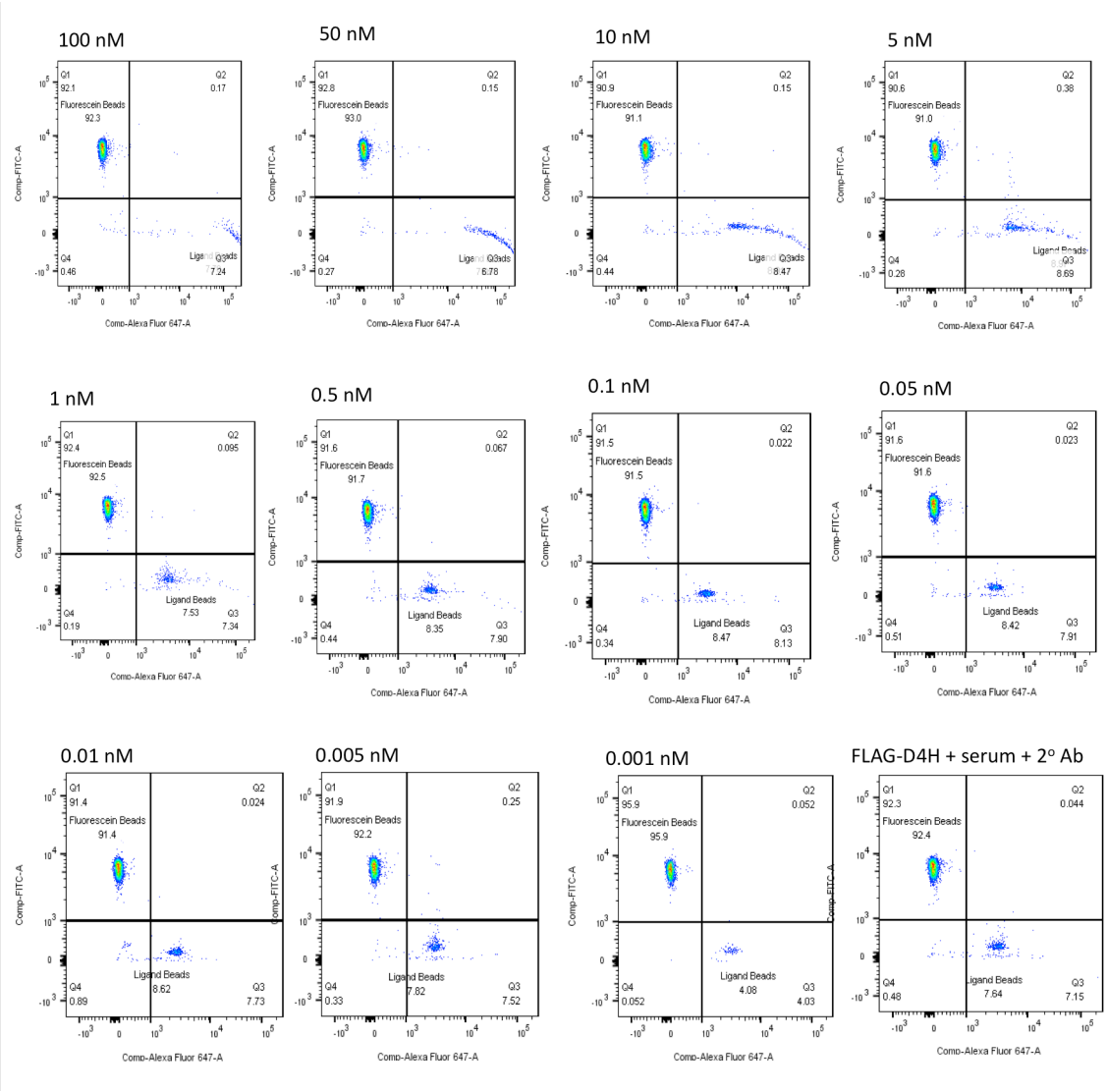

Figure S1: FACS Dot plots of FLAG-D4H against Anti-FLAG Ab in mouse serum. The concentration of the anti-FLAG antibody doped into the serum is shown above each plot.

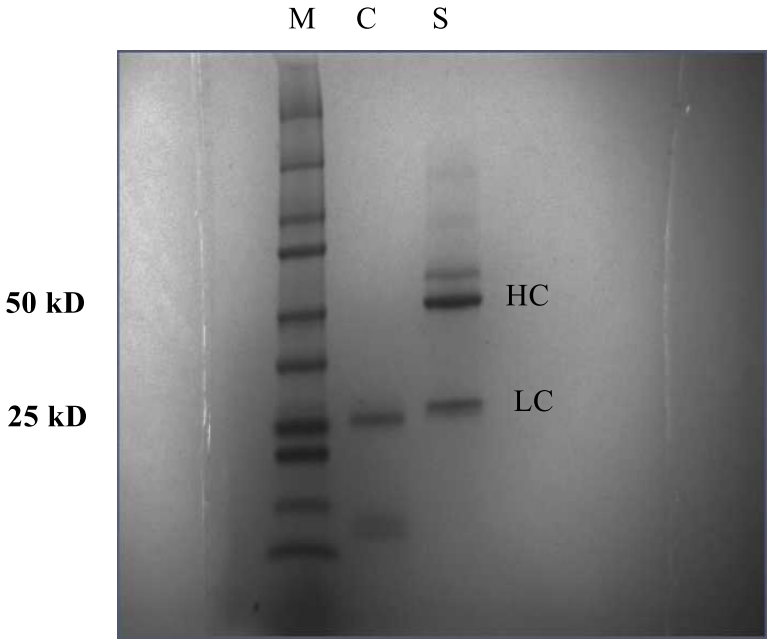

Figure S2: SDS PAGE of purified CLL014 monoclonal antibody. 10 µL of a 1:5 mixture of sodium dodecylsulphate (SDS) and purified antibody was added to each lane of a polyacrylamide gel in the electrophoresis tank, and run for 30 minutes at 200 V. Protein was detected using commassie stain. (M) Marker lane, (C) control lane, (S) sample lane. HC = Heavy chain, LC = Light Chain.

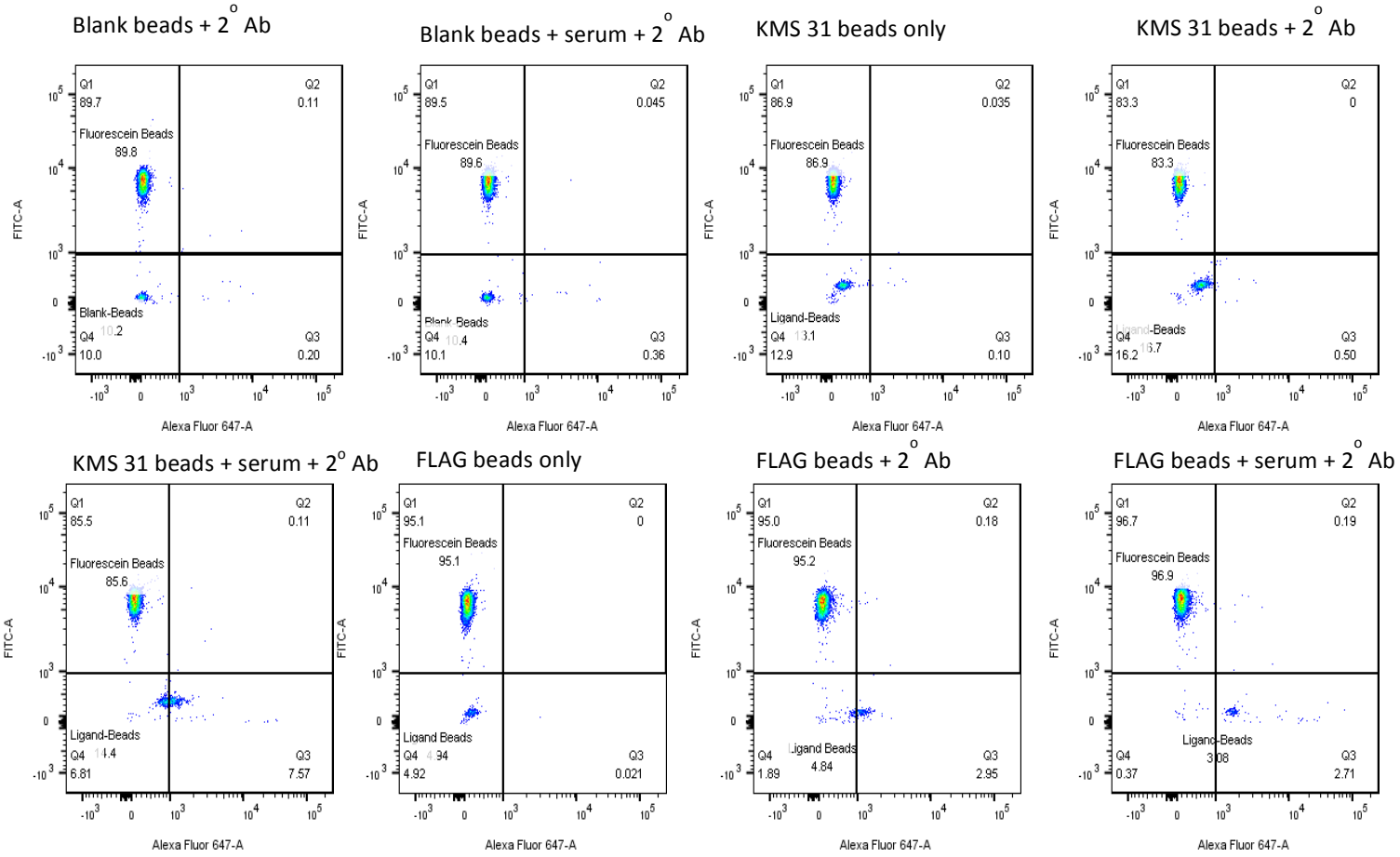

Figure S3: FACS Dot plots for the control experiments.

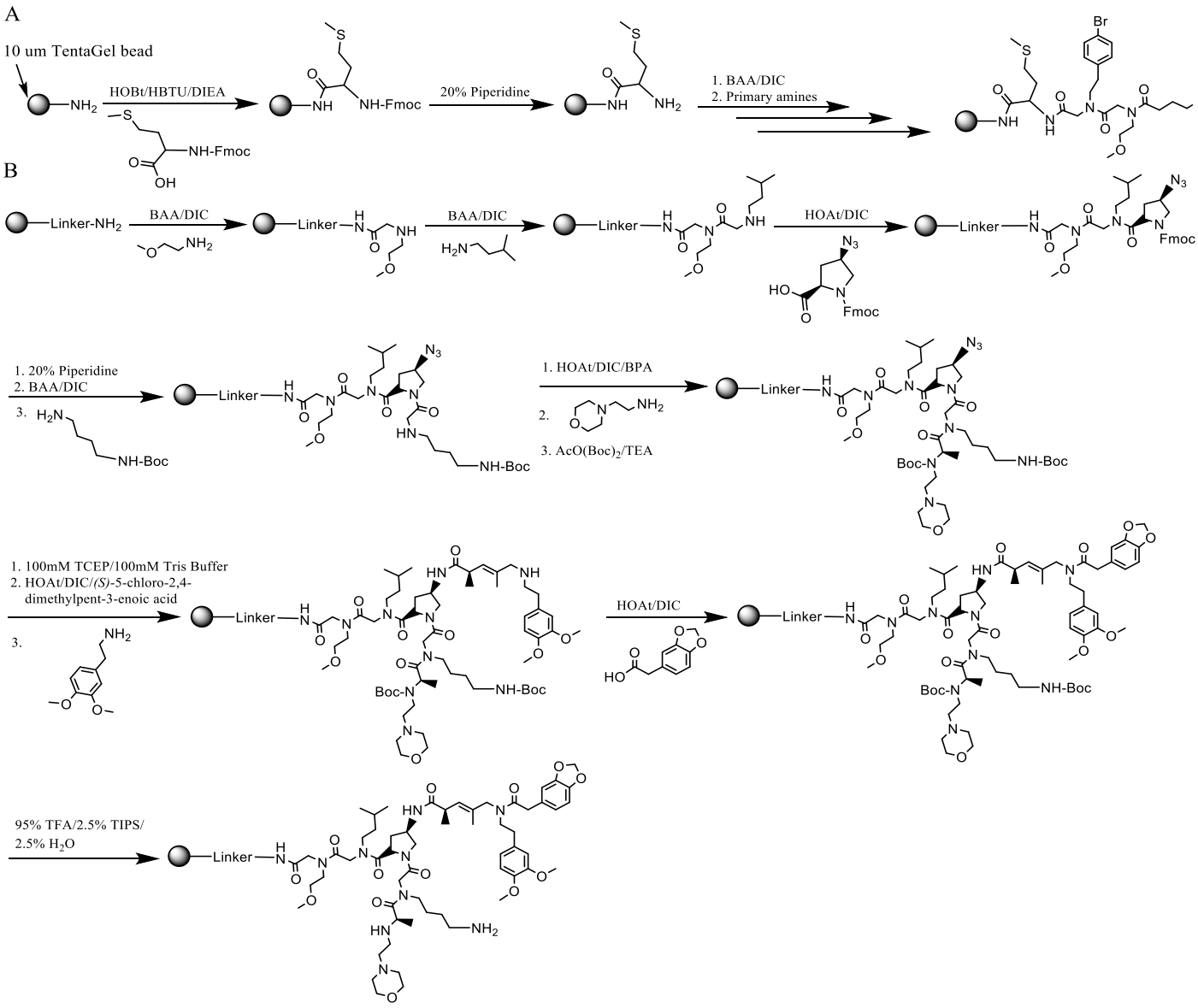

Figure S4: Synthetic scheme for KMS31.

Supplementary Tables

Table S1: Flow statistics of Anti-FLAG-FLAG Peptide Binding

Conc. of Anti-FLAG (nM)	Subset Name	Count	Freq. of Total	Freq. of Parent	Mean FITC FI	Mean Alexa Fluor 647 FI
0.001	Fluorescein Bead	4818	48.2	95.8	5982	177
	Ligand-Bead	209	2.09	4.16	133	5365
0.005	Fluorescein Bead	4292	48.0	95.5	5921	187
	Ligand-Bead	204	2.28	4.54	132	13950
0.01	Fluorescein Bead	5162	51.6	97.3	5987	178
	Ligand-Bead	142	1.42	2.68	139	40088
0.05	Fluorescein Bead	4877	48.8	96.1	6025	178
	Ligand-Bead	193	1.93	3.80	145	138378
0.1	Fluorescein Bead	4782	47.8	94.8	5962	180
	Ligand-Bead	262	2.62	5.19	141	188355
0.5	Fluorescein Bead	4870	48.7	94.7	6165	182
	Ligand-Bead	272	2.72	4.28	185	188601
1	Fluorescein Bead	4850	48.5	95.8	5937	185
	Ligand-Bead	211	2.11	4.17	150	238361
5	Fluorescein Bead	4679	46.8	95.5	5984	184
	Ligand-Bead	218	2.18	4.45	133	261417
10	Fluorescein Bead	4903	49.0	96.8	6096	186
	Ligand-Bead	159	1.59	3.14	167	261417
50	Fluorescein Bead	4329	43.3	95.6	5998	191
	Ligand-Bead	197	1.97	4.35	149	261417
100	Fluorescein Bead	4611	46.1	95.8	5993	185
	Ligand-Bead	199	1.99	4.14	135	261417

Table S2: Flow statistics of Anti-FLAG-FLAG-D4H Binding

Conc. of Anti-FLAG (nM)	Subset Name	Count	Freq. of Total	Freq. of Parent	Mean FITC FI	Mean Alexa Fluor 647 FI
0.001	Fluorescein Bead	3575	44.5	89.8	5596	136
	Ligand-Bead	326	4.06	8.19	268	2648
0.005	Fluorescein Bead	3738	46.5	88.6	5618	128
	Ligand-Bead	373	4.64	8.85	175	2004
0.01	Fluorescein Bead	3880	43.6	89.2	5644	133
	Ligand-Bead	381	4.28	8.76	174	2495
0.05	Fluorescein Bead	4042	45.5	89.5	5626	131
	Ligand-Bead	388	4.36	8.59	177	2610
0.1	Fluorescein Bead	4873	48.7	87.8	5644	128
	Ligand-Bead	519	5.19	9.35	234	2464
0.5	Fluorescein Bead	4090	42.5	89.3	5670	135
	Ligand-Bead	398	4.13	8.69	190	3266
1	Fluorescein Bead	3848	42.5	90.2	5697	137
	Ligand-Bead	331	3.65	7.76	246	4354
5	Fluorescein Bead	3810	45.2	88.9	5653	143
	Ligand-Bead	391	4.63	9.12	188	8745
10	Fluorescein Bead	4104	48.6	88.3	5660	134
	Ligand-Bead	455	5.39	9.78	169	12041
50	Fluorescein Bead	3575	37.6	89.2	5660	139
	Ligand-Bead	366	3.84	9.13	192	57551
100	Fluorescein Bead	3707	43.1	89.5	5665	135
	Ligand-Bead	340	3.95	8.21	176	117659

Table S3: Flow statistics of CLL 014-KMS Binding

Conc. of CLL 014 (nM)	Subset Name	Count	Freq. of Total	Freq. of Parent	Mean FITC FI	Mean Alexa Fluor 647 FI
0.01	Fluorescein Bead	4459	44.6	84.8	6501	182
	Ligand-Bead	800	8.00	15.2	372	1285
0.05	Fluorescein Bead	4339	43.4	85.9	6459	176
	Ligand-Bead	711	7.11	14.1	341	1200
0.1	Fluorescein Bead	4051	40.5	86.0	6501	177
	Ligand-Bead	657	6.57	14.0	370	1311
0.5	Fluorescein Bead	4188	41.9	86.7	6515	190
	Ligand-Bead	645	6.45	13.3	402	1507
1	Fluorescein Bead	3959	39.6	86.6	6483	170
	Ligand-Bead	611	6.11	13.4	334	1057
5	Fluorescein Bead	4330	43.3	85.7	6473	172
	Ligand-Bead	722	7.22	14.3	319	2108
10	Fluorescein Bead	4076	40.8	85.2	6438	157
	Ligand-Bead	705	7.05	14.7	388	2094
50	Fluorescein Bead	4081	40.8	87.4	6422	197
	Ligand-Bead	589	5.89	12.6	328	10238
100	Fluorescein Bead	4456	44.6	85.9	6466	188
	Ligand-Bead	729	7.29	14.1	322	14570