Appendix B

Peptide-Based General Antibody Detection Agent

B.1 INTRODUCTION

Detecting the immune response to an infectious agent can provide useful *in vitro* diagnostic information. Assays of this nature require a general antibody detection agent, often a conjugated anti-Fc antibody or protein substrate, i.e., Protein A. However, most biological reagents have inherent variability, which can adversely influence the performance of the diagnostic test. Peptide capture agents have been shown to be highly robust and inexpensive to produce, and to have similar affinities and selectivities as antibodies. DeLano *et al.* reported the discovery of a cyclic 13-amino acid peptide (Fc-III) that bound the "consensus region" of IgG Fc by phage display affinity screening [1]. We explored possible improvements to the Fc-III peptide in terms of affinity and selectivity by employing *in situ* click screening to obtain a biligand, and in terms of stability by replacing the disulfide bridge of Fc-III, with the goal of using the modified peptide as a detection agent in diagnostic assays.

B.2 WELLS PEPTIDE

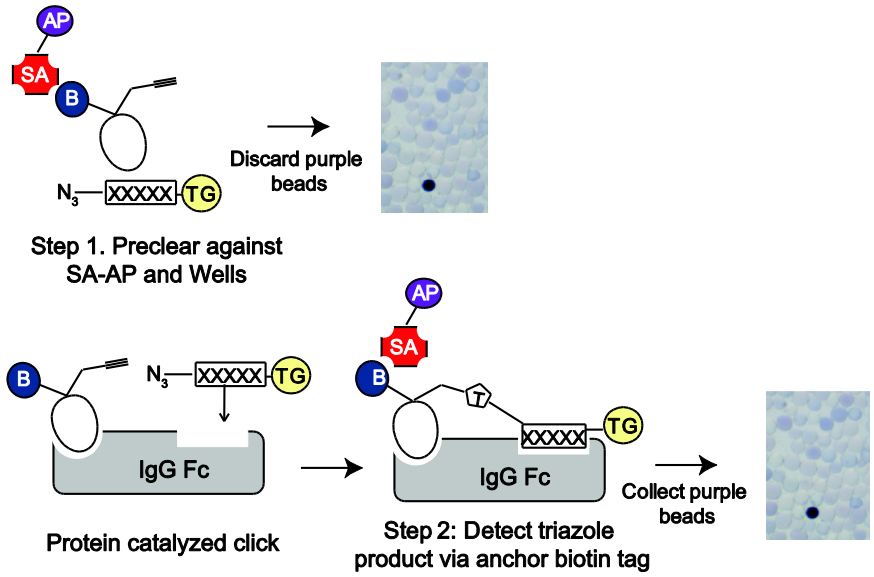
Using a cyclic phage display library of the form $X_iCX_jCX_k$ (where C is cysteine, X is a random amino acid, and $i + j + k = 18$), Delano *et al.* found the 13-residue Fc binding sequence Fc-III (DCAWHLGELVWCT) [1,2]. When synthesized, this peptide was found to inhibit the binding of Protein A Z-domain to Fc with a K_i of 25 nM, which is 200 fold greater affinity than an earlier, longer consensus sequence found by the group. The increased affinity with shorter length implied that the final sequence very efficiently

interacts with the Fc binding pocket. We added a propargyl glycine (Pra) residue at the N-terminus of Fc-III and tested the ability of this modified cyclic peptide to bind IgG Fc. The anchor peptide, monikered Wells peptide, was also C-terminally tagged with a polyethylene glycol (PEG) oligomer bridge and a biotin label.

B.3 BILIGAND SCREEN

The *in situ* click screen is illustrated in Scheme 2.1. The target IgG Fc is incubated with an excess of the selected anchor peptide and a large OBOC library at 4^{o}C for 6 hrs (see Materials and Methods). The OBOC library is synthesized on TentaGel resin (Rapp Polymere, Tuebingen, Germany), and is a comprehensive library of 5-mers with a 6th amino acid at the C-terminus presenting an azide functionality. The OBOC library is comprised of non-natural (D) stereoisomers of the natural amino acids, excluding cysteine and methionine. The *in situ* screen is designed to identify a secondary (2^{o}) peptide that, when coupled to the anchor, forms a biligand with increased selectivity and/or affinity for the target IgG Fc.

In Step 1 of the screen, the OBOC library is cleared of beads that exhibit non-specific binding to Wells peptide and alkaline phosphatase-conjugated streptavidin (SA-AP), which is used as a detection reagent in the next step. Step 2 is called a product screen, and is unique to sequential *in situ* click screens [3]. This screen detects the presence of *in situ* clicked reaction products, which are those hit beads containing the triazole-linked anchor peptide. A complete list of screen hits is given in Table B.1.



Scheme B.1. Screening strategy for selecting capture agents against human IgG Fc. The flow chart represents the use of the cyclic Wells peptide as an anchor ligand for in situ click screening against a large OBOC azide-presenting peptide library.

k	e	r	i	g
a	h	r	i	G
d	G	r	i	G
r	r	i	G	p
G	e	h	n	i
y	l	h	n	i
G	a	h	G	i
e	i	r	v	k
k	w	l	r	v
y	k	l	a	i
i	q	f	k	l
p	i	l	i	p
r	f	q	a	p
r	y	a	a	k
n	k	G	h	i

Table B.1. Biligand screen results. List of pentapeptide "hits" from OBOC biligand screens performed with Wells peptide and human IgG Fc. The selected secondary ligands corresponding to WA and WG are shown in blue and red respectively (shown in Figure B.1).

B.4 1,4 TRIAZOLE LINKED BILIGAND CHARACTERIZATION

The performance of these biligands was then characterized using an in-house developed an on-bead ELISA assay (data not shown). Sandwich ELISAs were used for affinity estimations of the two best performing biligands (Figure B.1) from the on-bead experiments. The affinity increase of the biligands over Wells is marginal. Hill fits to the ELISA data give K_d values of 18 nM for WA, 21 nM for WG, and 25 nM for Wells (Figure B.2).

B.5 1,5 TRIAZOLE LINKED BILIGAND CHARACTERIZATION

The in situ click process does not yield a stereospecific triazole product, and it is impossible to measure which click product variant the protein "catalyzed" during a screen. Therefore it can be useful to test the 1,5 triazole linked versions of potential biligands. Here, the biligand WA was synthesized using a 1,5 triazole small molecule linker and its performance compared to that of Wells by sandwich ELISA (Figures B.2 - B.3). The difference in affinity between the 1,5 triazole linked biligand and Wells was negligible.

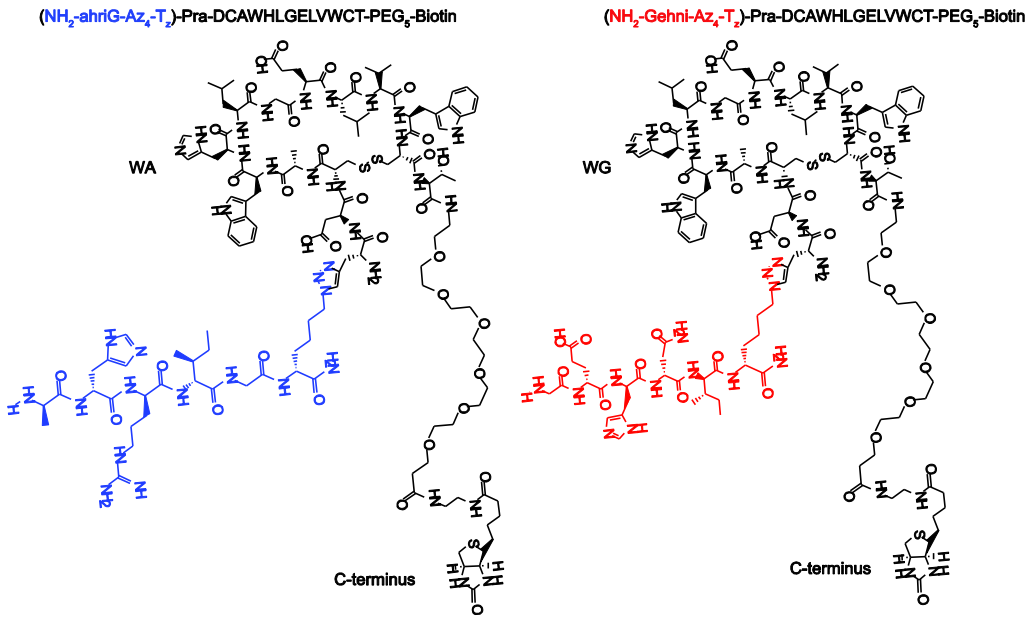


Figure B.1. Structures of peptide biligands identified in screen against IgG Fc. Acetylene-presenting anchor peptides (black) were derived from Fc-III. Wells-ahriG (WA) and Wells-Gehni (WG) were evolved from the original peptide appended with Pra at the N-terminus, and secondary ligand branches (colored) were identified from the *in situ* click screen of a 5-mer OBOC library presenting an azide functionality.

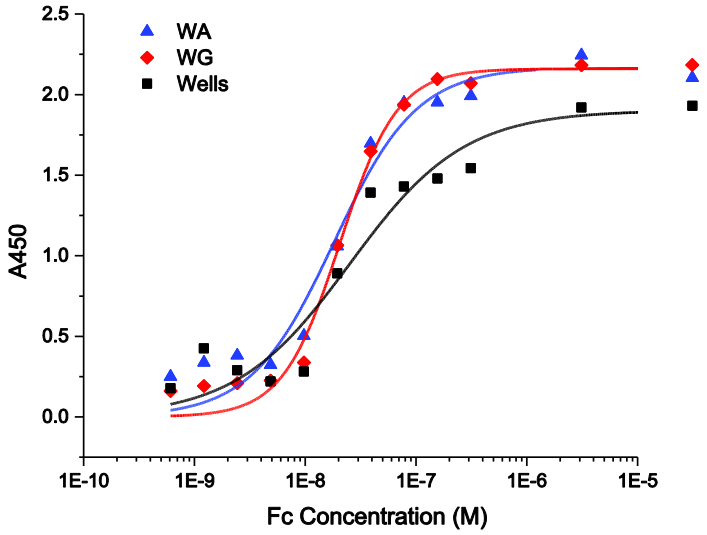


Figure B.2. Apparent affinity of Wells and biligands directed against Fc as determined by sandwich ELISA. Lines represent 1st order Hill fits to the affinity data. Wells: K_d = 25 nM, WA: K_d = 18 nM, WG: K_d = 21 nM.

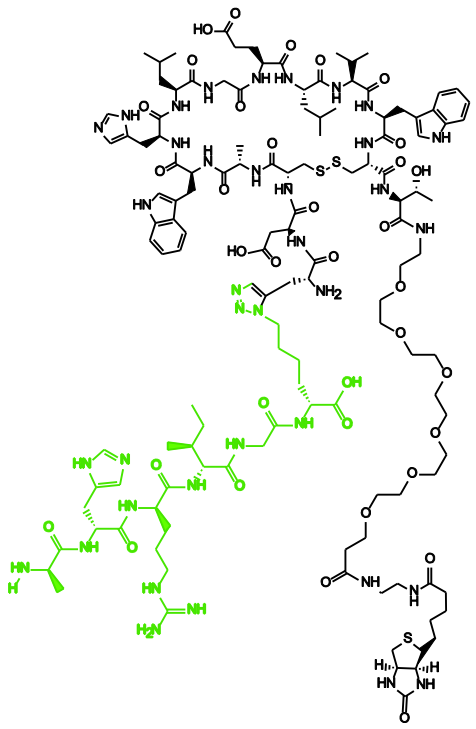


Figure B.3. Structure of 1,5 triazole linked biligand. Biligand peptide WA was synthesized with a 1,5 triazole linker.

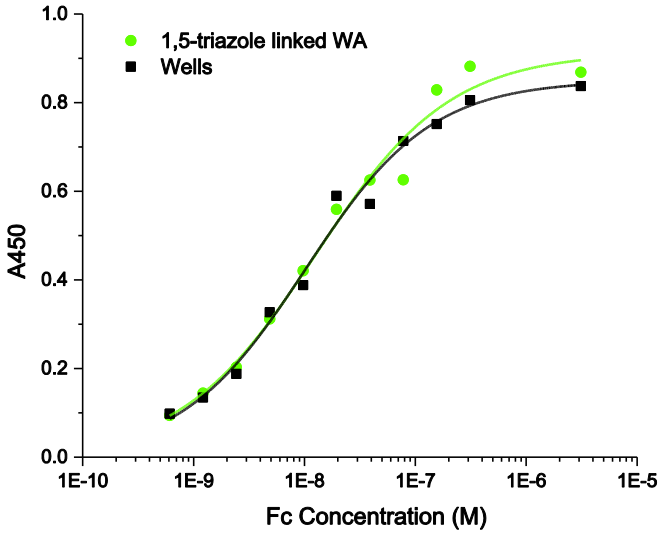


Figure B.4. Apparent affinity of Wells and 1,5 triazole linked WA directed against Fc as determined by sandwich ELISA. Lines represent 1st order Hill fits to the affinity data.

B.6 CLICK CYCLIZED WELLS

To enhance the stability of the Wells anchor peptide, we attempted to replace the disulphide bridge with a Cu(I) catalyzed click triazole. (D) or (L) azidoalanine (Az1) was substituted for Cys2, and Pra was substituted for Cys12, then the peptide was cyclized using Cu(I) catalyzed click chemistry (Figure B.5). Single point ELISAs showed that this approach to cyclization removed all affinity to IgG Fc (Figure B.6). This could be due to the change in confirmation of the peptide cycle when constrained by an alternative cyclization moiety. The use of both (D) and (L) Az1 was an attempt to circumvent that, but was unsuccessful.

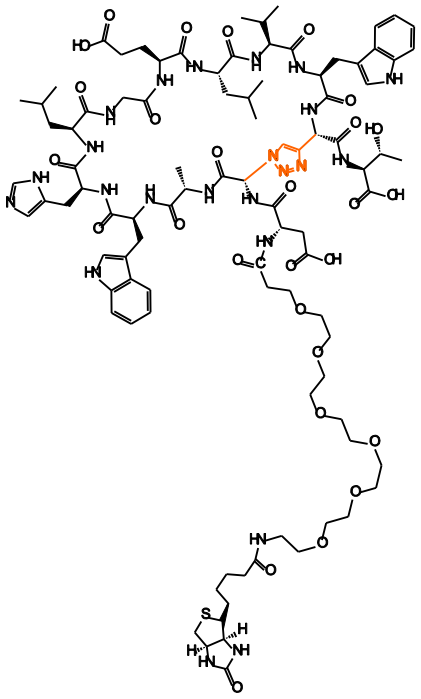


Figure B.5. Structure of Cu(I) click cyclized Wells peptide. The disulfide bond in Wells peptide was replaced with a Cu(I) catalyzed triazole formed from substitutions of Az1 and Pra for Cys2 and Cys12 respectively.

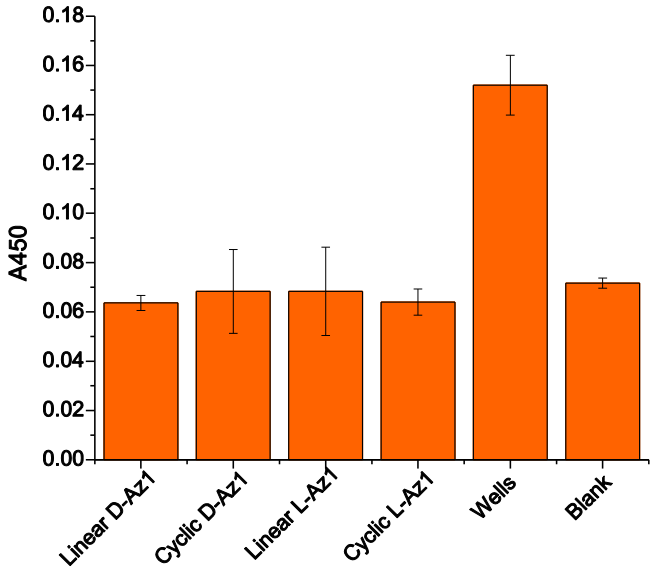


Figure B.6. Performance of click cylized Wells peptide variants compared to Wells peptide tested by sandwich ELISA. Fc (500 nM) was spiked into TBS, and captured antibody was detected by peroxidase-conjugated anti-human IgG antibody.

B.7 MATERIALS AND METHODS

B.7.1 Anchor Synthesis

The anchor Wells peptide was synthesized C → N on 200-300 mgs of Biotin NovaTag resin (EMD Biosciences, San Diego, CA) with a Titan peptide synthesizer using standard Fmoc chemistry [4]. The synthesis used Fmoc and side chain protected L amino acids (Aaptec, Louisville, KY), Fmoc protected propargylglycine (Pra) (Aaptec, Louisville, KY) and PEG_5 (EMD Millipore, Germany). The finished peptide was side-chain deprotected and cleaved from resin in a mixture of 95% v/v trifluoroacetic acid (TFA) (Sigma-Aldrich, St. Louis, MO), 5% v/v dH_2O, and 5% triethylsilane (TES) (Sigma-Aldrich, St. Louis, MO), and precipitated in diethyl either. $Cu(Phen)_3$ stock solution was prepared by mixing 150 mM Cu(II) sulfate pentahydrate (Sigma-Aldrich, St. Louis, MO) and 500 mM 1,10-phenanthroline (Sigma-Aldrich, St. Louis, MO) in 4:1 H_2O:Ethanol. The ether-precipitated ligand was cyclized by stirring the cleaved peptides dissolved in ~5 mL acetonitrile (ACN) overnight with 1 mL of the $Cu(Phen)_3$ stock solution. The cyclization mixture was lyophilized, and the final peptide product was purified using reverse phase (RP) HPLC on a Kinetex 5µ XB-C18 250 x 4.6 mm prep column (Phenomenex, Torrance, CA).

B.7.2 OBOC Screen

The screen used naïve one-bead-one-compound (OBOC) D-pentapeptide libraries on TentaGel resin (TG) (Rapp Polymere, Tuebingen, Germany) of the form NH_2-

$X_1X_2X_3X_4X_5$-Az_4-TG, excluding cysteine and methionine, where Az_4 is L-azidolycine. Libraries were synthesized on a Titan peptide synthesizer (Aaptec, Louisville, KY) using a split/mix method, and couplings were done using standard Fmoc SPPS chemistry in *N*-methylpyrrolidone (NMP).

Screening against Fc was carried out in two main steps: pre-clear, and product screen. ***Step 1 preclear****:* 200 mg of OBOC library was blocked in 5% w/v dry milk in Tris buffered saline (TBS) (25 mM Tris-HCl, 150 mM NaCl, 10 mM $MgCl_2$, pH 7.6) for 2 hr at room temperature, and then incubated with 1:10,000 phosphatase-conjugated streptavidin (SA-AP, Promega, Madison, WI) and 25 µM Wells peptide in 0.5% w/v dry milk in TBS for 1 hr. The beads were washed in high salt buffer (25 mM Tris-HCl, 750 mM NaCl, 10 mM $MgCl_2$, pH 7.6) for 1 hr, and then developed with BCIP/NBT (Promega, Madison, WI) in BCIP buffer (100 mM Tris-HCl, 150 mM NaCl, 1 mM $MgCl_2$, pH 9). After 1 hour, the reaction was quenched with conc. HCl, and the purple beads were discarded. The remaining library was decolorized in NMP, dried with methanol (MeOH) and dichloromethane (DCM), and then swelled and blocked in 5% w/v dry milk in TBS for 2 hr at room temperature. ***Step 2 product screen****:* 4 mL of a solution containing 25 µM A21 and 23 nM Fc in 0.5% w/v dry milk in TBS was added to the precleared OBOC library, and the *in situ* click reaction was allowed to proceed for 6 hours at 4°C. Click solution was drained and the beads were washed for 1 hr in high salt buffer. The beads were incubated for 1 hr at room temperature with 1:10,000 SA-AP in 0.5% w/v dry milk in TBS, washed for 1 hr with high salt buffer, and then developed with BCIP/NBT in BCIP buffer for 1 hour. The reaction was quenched with conc. HCl, and the purple beads

were retained and sequenced using a Procise CLC Edman Degradation Protein Sequencing System (Applied Biosystems, Kingston, RI).

B.7.3 Biligand Synthesis

1,4 Triazole linked WA and WG

The secondary ligands including C-terminal Az_4 for WA and WG were synthesized C → N on 200-300 mgs of Rink amide resin with a Titan peptide synthesizer using standard Fmoc chemistry. The synthesis used Fmoc and side chain protected D amino acids and L-Az_4 (Aaptec, Louisville, KY). The ligands were side-chain deprotected and cleaved from resin in 95:5:5 TFA:dH_2O:TES, then precipitated in diethyl either and purified using RP-HPLC on a Kinetex 5µ XB-C18 250 x 4.6 mm prep column. The purified secondary ligands were clicked to cyclized Wells peptide in solution using 2x molar excess of secondary, 10x Cu(I) iodide, 50x L-ascorbic acid, and 4x Tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine (TBTA) (Sigma-Aldrich, St. Louis, MO) in 5:1 NMP:dH_2O and stirring overnight at room temperature. The clicked biligand products were purified from the reaction mixture by RP-HPLC on a C18 semi prep column.

1,5 Triazole linked WA

The anchor peptide was synthesized C → N omitting Pra on 200-300 mgs of Biotin NovaTag resin (EMD Biosciences, San Diego, CA) with a Titan peptide synthesizer using standard Fmoc chemistry [4]. A pre-clicked Fmoc protected 1,5 triazole Pra-Az4 small molecule linker (InDi Molecular) was amide coupled to the N-terminal Asp of Wells

peptide, which after Fmoc deprotection served as the starting point for the synthesis of the secondary ligand. The finished peptide was side-chain deprotected and cleaved from resin in a mixture of 95% v/v trifluoroacetic acid (TFA) (Sigma-Aldrich, St. Louis, MO), 5% v/v dH_2O, and 5% triethylsilane (TES) (Sigma-Aldrich, St. Louis, MO), and precipitated in diethyl either. $Cu(Phen)_3$ stock solution was prepared by mixing 150 mM Cu(II) sulfate pentahydrate (Sigma-Aldrich, St. Louis, MO) and 500 mM 1,10-phenanthroline (Sigma-Aldrich, St. Louis, MO) in 4:1 H_2O:Ethanol. The ether-precipitated ligand was cyclized by stirring the cleaved peptides dissolved in ~5 mL acetonitrile (ACN) overnight with 1 mL of the $Cu(Phen)_3$ stock solution. The cyclization mixtures were lyophilized, and the final peptide product was purified using reverse phase (RP) HPLC on a C18 prep column (Phenomenex, Torrance, CA).

B.7.4 Click Cyclized Wells Synthesis

The anchor Wells peptide was synthesized C → N, including N-terminal PEG and biotin, on 200-300 mgs of Rink amid resin (AnaSpec, Fremont, CA) with a Titan peptide synthesizer using standard Fmoc chemistry [4]. The synthesis used Fmoc and side chain protected L amino acids (Aaptec, Louisville, KY), Fmoc protected propargylglycine (Pra) (Aaptec, Louisville, KY), Fmoc protected (D) and (L) azidoalanine (Iris Biotech, Germany), PEG_5 (EMD Millipore, Germany), and biotin (Sigma-Aldrich, St. Louis, MO). The finished peptide was side-chain deprotected and cleaved from resin in a mixture of 95% v/v trifluoroacetic acid (TFA) (Sigma-Aldrich, St. Louis, MO), 5% v/v dH_2O, and 5%

triethylsilane (TES) (Sigma-Aldrich, St. Louis, MO), and precipitated in diethyl either. A Cu(I) click cyclization solution was prepared by mixing 10x Cu(I) iodide, 50x L-ascorbic acid, and 4x Tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine (TBTA) (Sigma-Aldrich, St. Louis, MO) in 5:1 NMP:dH_2O. The ether-precipitated ligands were cyclized by stirring the cleaved peptides dissolved in ~2 mL Cu(I) click solution. The clicked biligand products were purified from the reaction mixture by RP-HPLC on a C18 semi prep column.

B.7.5 Assays

Biligand binding ELISA

Biotinylated WA, WG, and Wells peptides were immobilized on streptavidin-coated 96-well plates (Thermo Scientific Pierce, Rockford, IL) at a concentration of 400 nM in TBS. The plates were blocked with 5% w/v dry milk in TBS, and then the anchor peptides were incubated with varying concentrations of Fc in 0.5% w/v dry milk in TBS at room temperature for 1 hr. Bound antibody was probed with horseradish peroxidase (HRP)-conjugated mouse monoclonal antibody to human IgG Fc (Abcam, Cambridge, MA), diluted 1:10,000 in 0.5% w/v dry milk in TBS for 1 hr at room temperature. The colorimetric assay was developed with TMB substrate (KPL, Gaithersburg, MD), then quenched with 1 M H_2SO_4 and read at 450 nm.

1,5 triazole linked biligand binding ELISA

Biotinylated 1,5 triazole linked WA peptide was immobilized on streptavidin-coated 96-well plates (Thermo Scientific Pierce, Rockford, IL) at a concentration of 300

nM in TBS. The plates were blocked with 5% w/v dry milk in TBS, and then the anchor peptides were incubated with varying concentrations of Fc in 0.5% w/v dry milk in TBS at room temperature for 1 hr. Bound antibody was probed with horseradish peroxidase (HRP)-conjugated mouse monoclonal antibody to human IgG Fc (Abcam, Cambridge, MA), diluted 1:10,000 in 0.5% w/v dry milk in TBS for 1 hr at room temperature. The colorimetric assay was developed with TMB substrate (KPL, Gaithersburg, MD), then quenched with 1 M H_2SO_4 and read at 450 nm.

Click cyclized Wells ELISA

Biotinylated Cu(I) click cyclized Wells peptide was immobilized on streptavidin-coated 96-well plates (Thermo Scientific Pierce, Rockford, IL) at a concentration of 1 µM in TBS. The plates were blocked with 5% w/v dry milk in TBS, and then the anchor peptides were incubated with 500 nM of Fc in 0.5% w/v dry milk in TBS at room temperature for 1 hr. Bound antibody was probed with horseradish peroxidase (HRP)-conjugated mouse monoclonal antibody to human IgG Fc (Abcam, Cambridge, MA), diluted 1:10,000 in 0.5% w/v dry milk in TBS for 1 hr at room temperature. The colorimetric assay was developed with TMB substrate (KPL, Gaithersburg, MD), then quenched with 1 M H_2SO_4 and read at 450 nm.

B.8 CONCLUSION

Described in this appendix is an attempt to improve the previously discovered Fc binding peptide Fc-III in terms of affinity, selectivity, and stability. While a number of approaches were taken, the performance of the original Wells anchor peptide was not significantly improved. Preliminary immunoprecipitation data (not shown) indicated possible selectivity enhancement, but was not verified. The lack of improvement of Wells peptide with the addition of a secondary ligand may reflect the increased dwell time that such a strong binder has with its target during a screen; there are less significant constraints on the nature of the secondary ligand that clicks to the anchor in the *in situ* process due to the relatively constant presence of the anchor ligand at the target binding site.

B.9 ACKNOWLEDGEMENTS

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B.10 REFERENCES

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