

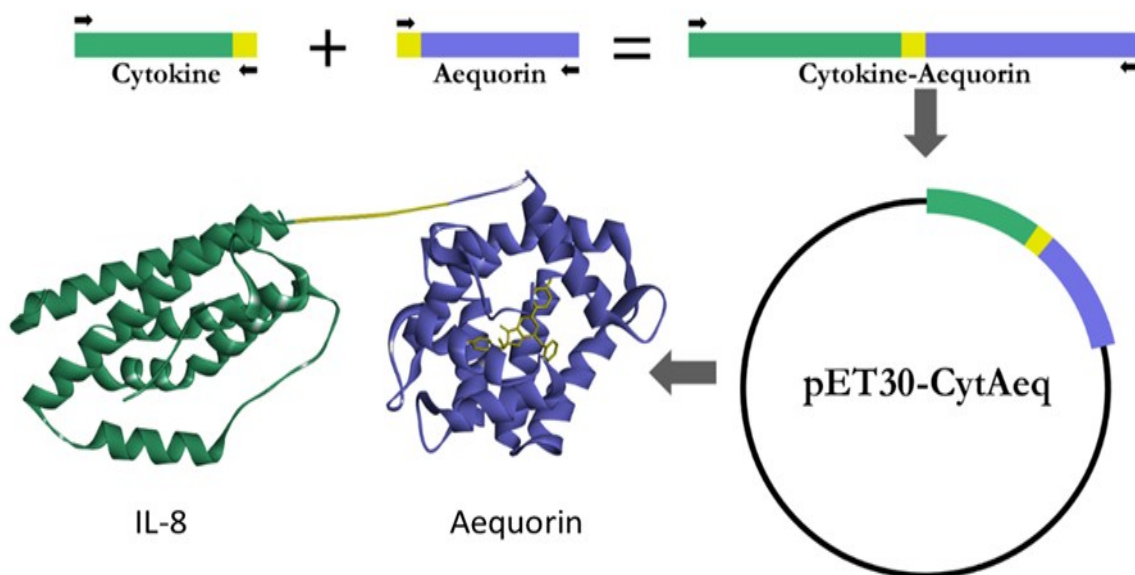
## Multiplexing Cytokine Analysis: Towards Reducing Sample Volume Needs in Clinical Diagnostics

XIAOWEN YU <sup>A,C</sup>, DANIEL SCOTT <sup>B</sup>, EMRE DIKICI <sup>A</sup>, SMITA JOEL <sup>A</sup>, SAPNA DEO <sup>A</sup>, SYLVIA DAUNERT <sup>\*A</sup>

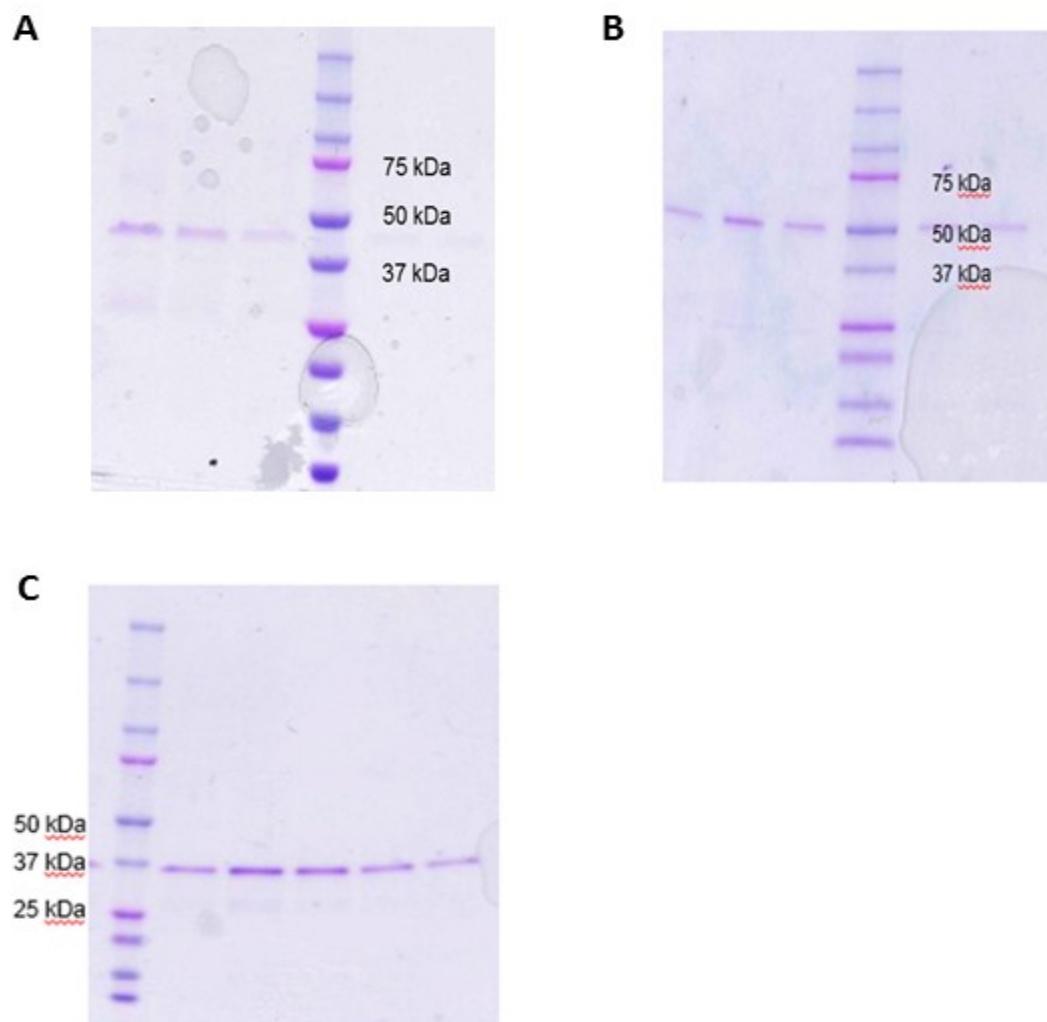
<sup>A</sup>Department of Chemistry, University of Miami, Miami, Florida 33124, United States

<sup>B</sup>Department of Chemistry, University of Kentucky, Lexington, KY 40506, United States

<sup>C</sup>Department of Biochemistry and Molecular Biology, Miller School of Medicine, University of Miami, Miami, Florida 33136, United States



**Figure S-1.** Schematic of the overlap extension PCR to create the three separate cytokine-aequorin fusion proteins. The green blocks are the genes of the cytokines. The blue blocks are the genes of the aequorin. The yellow blocks are the gene of the linker between cytokines and aequorin.



**Figure S-2.** SDS-PAGE of the three fusion proteins. (A) TNF $\alpha$ -Y82F (B) IL6-Y82F (C) IL8-F113W

**Table S-1.** Primer sequences for each overlap PCR for the creation of the cytokine-aequorin fusion proteins

Primer	Sequence (5'-3')
Aequorin Forward	TCTGGCGGTGGCGGTTCTGTGAAACTGACCAGCGACTTCGACAACCCAAGA
Aequorin Reverse <i>Bam</i> HI	AGAGGAGAGTTAGAGCCGGATCCTTAGGGGACAGCTCCACCGTAGAGCTTTTCGGAAGC
Aequorin Reverse <i>Hind</i> III	AGAGGAGAGTTAGAGCCAAGCTTTGATTGTTAGGGGACAGCTCCACCGTAGAGCTTTTC
TNF- $\alpha$ Forward	GGTATTGAGGGTCGCCATATGATGGTTCGTTCTAGCTCCCGTACCCCGTCTGACAAA
TNF- $\alpha$ Reverse	AGAACCGCCACCGCCAGACAGCGCGATGATACCGAAGTAAACCTGACCAGA
IL-6 Forward	GGTATTGAGGGTCGCGAATTCCAATCAATGAACTCCTTCTCCACAAGCGCCTTCGGTCCAGTTGCC

IL-6 Reverse	TTTCACAGAACCGCCACCGCCAGACATTTGCCGAAGAGCCCTCAG
IL-8 Forward	GGTATTGAGGGTCGCGAATTCCAATCAATGAGTGCTAAAGAACTTAGATGTCAGTGCATAAAG ACA
IL-8 Reverse	TTTCACAGAACCGCCACCGCCAGATGAGTTCTCAGCCCTCTTCAA

**Reagents.** Disodium ethylenediaminetetraacetate (EDTA) was purchased from Fisher Scientific (Fair Lawn, NJ). Luria-Bertani (LB) Agar was purchased from EMD Millipore (Darmstadt, Germany). LB Broth, pET-30 Xa/LIC Vector kit, KOD Hot Start DNA Polymerase, and Factor Xa Kits were purchased from Novagen (Darmstadt, Germany). LB Lennox was purchased from Alfa Aesar (WardHill, MA). Sodium dodecyl sulfate (SDS) was purchased from J. T. Baker. (Center Valley, PA). Plasmid mini-prep kits, gel extraction kits and Ni-NTA agarose were obtained from Qiagen (Valencia, CA). *EcoRI*, *HindIII*, and *BamHI*, restriction enzymes were purchased from New England BioLabs, Inc. (Ipswich, MA). Sodium phosphate, imidazole, calcium chloride, reduced and, oxidized glutathione and all the oligonucleotides were purchased from Sigma Aldrich (St. Louis, MO). Tween-20 was purchased from VWR (Radnor, PA). Isopropyl-beta-D-thiogalactoside (IPTG) was purchased from Gold Biotechnology (St. Louis, MO). Tris(hydroxymethyl)amino methane (Tris) free base, sodium chloride, glycerol and Urea were purchased from BDH Chemicals (distributed by VWR, West Chester, PA). Precision plus protein dual color standards were obtained from BIO-RAD (Hercules, CA). Bench top 1Kb DNA ladder was purchased from Promega (Madison, WI). Coelenterazine sampler kit was ordered from Biotium (Hayward, CA). The cDNA clones of human TNF- $\alpha$  was purchased from GeneCopoeia, Inc (Rockville, MD). The cDNA clones of human IL-6, and IL-8 were purchased from ATCC (Manassas, VA). Kanamycin sulfate and potassium chloride were obtained from AMRESCO (Solon, OH). Chloramphenicol and guanidinium chloride were purchased from Calbiochem (La Jolla, CA). The standard human TNF- $\alpha$  protein and human TNF- $\alpha$  monoclonal antibody were purchased from Abcam (Cambridge, MA). The standard human IL-6, and IL-8 proteins, human IL-6 monoclonal antibody, and human CXCL8/ IL-8 monoclonal antibody were purchased from R&D systems (Minneapolis, MN). The BL21(DE3)pLysS chemical competent cells were obtained from Invitrogen (Carlsbad, CA). The pooled human serum was obtained from Innovative research (Novi, MI). The Rosetta (DE3) competent cells suitable for electroporation were prepared in our lab. The protein-free (PBS) blocking buffer, BCA protein assay reagent (bicinchoninic acid) and Reacti-Bind goat anti-mouse IgG coated white 96-well plates and Pierce Protein Refolding Kit were from Pierce Biotechnology (St. Louis, MO). All chemicals were reagent grade or better and all aqueous solutions were prepared using 16 M $\Omega$  deionized distilled water produced by a Milli-Q water purification system (Millipore, Bedford, MA).

**Apparatus.** Polymerase chain reactions (PCR) were performed using an Eppendorf Mastercycler Personal thermocycler (Hamburg, Germany). DNA electrophoresis was performed using a FB105 Fischer Biotech Electrophoresis Power Supply (Pittsburgh, PA) and the gels were visualized using a UV Transilluminator (UVP, Upland, CA). OD<sub>600</sub> readings were taken using a Spectronic 21D UV-Vis Spectrophotometer (Milton Roy, Ivy Land, PA). Rosetta (DE3) cells were electroporated by Eppendorf Eporator (Hamburg, Germany). Bacteria employed for the expression of the cytokine-aequorin fusion proteins were incubated in a Fisher Scientific incubator orbital shaker and Fisher Scientific open air orbital shaker (Fair Lawn, NJ) and harvested by a Beckman J2-M1 centrifuge (Palo Alto, CA). The cells harvested were lysed using a Fisher Scientific 550 dismembrator (Pittsburgh, PA). Purity of the fusion proteins were verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using BIO-RAD 4-20% Tris-glycine gels in a BIO-RAD

Mini Protein Tetra Cell (Hercules, CA). Purified proteins were concentrated by Centrifugal Filter Units 10K from Millipore (Darmstadt, Germany). The concentrated proteins were dialyzed into the right buffers by Slide-A-Lyzer Dialysis Cassettes from Thermo Scientific (Rochford, IL). Bioluminescence measurements were made on a Polarstar Optima luminometer from BMG Labtech (Cary, NC) and decay kinetics analyzed with GraphPad Prism 5.0 (San Diego, CA). Emission spectra were taken on a custom-made SpectroScan instrument from Sciencewares (Farmingham, MA).

**Construction of Cytokine-Aequorin Mutant Fusion Proteins.** Three different fusion protein constructs were developed for assaying tumor necrosis factor alpha (TNF- $\alpha$ ), interleukins 6 (IL-6), and 8 (IL-8) simultaneously. Previously, aequorin mutants when combined with synthetic coelenterazine analogs have shown to exhibit altered emission spectra. For this study, the genetic sequence coding for the Y82F and F113W aequorin mutants were utilized. The cytokines coding sequences were genetically attached to the aequorin mutants of choice via overlap polymerase chain reaction (PCR). Each fusion protein was also designed to contain a SGGGS spacer sequence between aequorin mutant and cytokine. Aequorin mutant F113W was genetically fused to the 3' end of IL-8 (IL8-F113W) and aequorin mutant Y82F was genetically attached to the 3' end of both TNF- $\alpha$  (TNF $\alpha$ -Y82F) and IL-6 (IL6-Y82F).

For instance, the TNF- $\alpha$  genetic coding sequence was amplified with the TNF- $\alpha$  forward primer that also introduced a LIC/Xa cloning site and a *NdeI* restriction site on the 5' end, and the TNF- $\alpha$  reverse primer. Meanwhile, the aequorin mutant Y82F coding sequence was amplified using the aequorin forward primer and the *BamHI* aequorin reverse primer. This amplification introduced a *BamHI* restriction site and a LIC/Xa cloning site on the 3' end of the aequorin gene sequence. The two sequences were combined and then amplified with the TNF- $\alpha$  forward and the *BamHI* aequorin reverse primer to yield the TNF $\alpha$ -Y82F coding sequence. The PCR reaction conditions were started at 95 °C for 2 min for polymerase activation, and then repeated for 25 cycles for the next steps, 95 °C for 20 s, and 65 °C for 10 s, followed by 70 °C for 15 s/k pairs. Similar genetic manipulations were performed with the IL-6 coding sequence as a template and using the IL-6 forward and reverse primers. This introduced a LIC/Xa cloning site and an *EcoRI* restriction site on the 5' end of IL-6. The Y82F aequorin mutant was amplified similarly with aequorin forward primer and the *HindIII* aequorin reverse primer to introduce the *HindIII* restriction site and a LIC/Xa cloning site on the 3' end of the aequorin gene. The IL-6 and aequorin sequences were combined and amplified with the IL-6 forward and *HindIII* aequorin reverse primers to yield the IL6-Y82F coding sequence. Finally, the IL-8 forward and reverse primers were used in conjunction with the IL-8 coding sequence to introduce the LIC/Xa cloning site and the *EcoRI* restriction site on the 5' end. The aequorin forward and *BamHI* aequorin reverse primers were used to create a *BamHI* restriction site and a LIC/Xa cloning site on the 3' end of the F113W aequorin mutant. This sequence was combined with the newly created IL-8 coding sequence and the IL-8 forward and aequorin *BamHI* reverse primers were used to yield the IL8-F113W gene sequence.

All of the fusion protein coding sequences were then inserted into lac operator containing pET-30 plasmid by using Novagen pET-30 Xa/LIC Cloning Kits. The DNA sequencing was performed by the Sylvester Comprehensive Cancer Center Oncogenomics Core Facility at the University of Miami to confirm the gene sequences of the fusion proteins. The plasmids containing the gene for TNF $\alpha$ -Y82F and IL8-F113W were transferred into BL21(DE3)pLysS *Escherichia coli* cells and grown while shaking at 250 rpm at 37 °C in 500 mL LB broth containing 30  $\mu$ g/mL Kanamycin to an OD<sub>600</sub> ~ 0.6. The proteins were expressed in the cells after the addition of 1 mM IPTG. The cells expressing TNF $\alpha$ -Y82F were collected after eight hours' incubation at room temperature, while the cells expressing IL8-Y82F were collected after three hours'

incubation at 37 °C. The cell pellets were then resuspended in lysis buffer and lysed by using a dismembrator and then purified using immobilized metal affinity chromatography (Ni/NTA). The proteins were loaded into a gravity column containing Ni/NTA Agarose beads in 50 mM NaH<sub>2</sub>PO<sub>4</sub> pH 8.0, 300 mM NaCl, and 10 mM imidazole buffer. A sequential washing with buffers of 30 mM NaH<sub>2</sub>PO<sub>4</sub> pH 8.0, 300 mM NaCl, and 20, 50 and 70 mM imidazole, were then applied to the column. The fusion proteins were washed off by an elution buffer with 30 mM NaH<sub>2</sub>PO<sub>4</sub> pH 8.0, 300 mM NaCl, and 100 mM imidazole. 10 fractions of 1.5 mL volume were collected and fractions containing the highest concentration of the fusion proteins, determined by SDS-PAGE, were pooled. Similarly, the plasmid containing the IL6-Y82F gene was transformed into Rosetta (DE3) *Escherichia coli* cells and grown with shaking at 250 rpm at 37 °C in 500 mL of LB broth containing 30 µg/mL kanamycin to an OD<sub>600</sub> ~ 0.6. The proteins were expressed in the cells for after the addition of 1 mM IPTG. These cells were then collected after 8 hours' incubation at room temperature, lysed by sonication and then purified through Ni-NTA affinity chromatography under denaturing conditions. The proteins were loaded onto a Ni-NTA beads column in a 100 mM NaH<sub>2</sub>PO<sub>4</sub> pH 8.0, 10 mM Tris-Cl, and 6 M GuHCl buffer. A sequential washing with buffers of 100 mM NaH<sub>2</sub>PO<sub>4</sub>, 10 mM Tris-Cl, 8 M Urea with pH 6.3 and 5.9 adjusted by HCl, were performed followed by the elution of the fusion using an elution buffer with 100 mM NaH<sub>2</sub>PO<sub>4</sub> pH 4.5, 10 mM Tris-Cl, and 8 M Urea. Pure fractions were pooled and dialyzed in the refolding buffer (55 mM Tris pH 8.2, 21mM NaCl, 0.88mM KCl, 1 mM EDTA, 2 mM GSH and 1mM GSSG) for 24 hours at 4 °C followed by 2 hours at room temperature. Protein concentration was determined by the BCA protein assay from Pierce (St. Louis, MO). All purified proteins were stored at 4 °C until further use.

**Decay Half-Life of Cytokine-Aequorin Fusion Proteins.** The bioluminescence decay kinetics of each cytokine-aequorin fusion protein was examined with an array of coelenterazine analogues (coelenterazine *ntv*, *i*, *f*, *cp*, *hcp*, *fcp*, *n*, *fcp*, *h*). A three molar excess of each coelenterazine analog was added to aliquots of each of the three cytokine-aequorin fusion proteins and incubated 18 h at 4 °C. The fusion proteins were then diluted with 30 mM Tris/HCl pH 7.4, containing 2 mM EDTA, 150 mM NaCl (buffer A) until the maximal bioluminescent intensities were between 50,000 and 500,000 relative light units (RLUs) when analyzed on the Polarstar Optima luminometer. For the analysis, a volume of 10 µL of the coelenterazine bound fusion protein solution was added to the well of a microtiter plate and the bioluminescent light reaction was initiated by injecting 50 µL of 30 mM Tris/HCl pH 7.4 buffer containing 100 mM CaCl<sub>2</sub> (buffer B). The light intensity was then monitored for 50 s for semi-synthetic aequorin consisting with coelenterazine *i* and *n*, with readings taken every 200 ms. For the semi-synthetic aequorin consisting with other coelenterazine analogs, the light intensity was monitored for 10s, with readings taken every 40ms. The data was analyzed by GraphPad Prism 5.0 software using a non-linear, one phase exponential decay kinetics half-life equation.

**Bioluminescent Emission Spectra of Cytokine-Aequorin Fusion Proteins.** The bioluminescent emission spectral profile was observed for each of the fusion proteins with the array of coelenterazine analogues as used for the half-life study. The emission spectra were collected on SpectroScan, a custom made instrument based on the Thermo-Labsystems Luminoskan Ascent luminometer. The instrument is capable of recording the spectra of flash--type bioluminescence reactions in the range of 400-700 nm. A three molar excess of each coelenterazine was added to aliquots of each fusion protein and incubated for 18 h at 4 °C. For the analysis, a volume of 10 µL of the coelenterazine bound fusion protein solution was added to a well on a 96 well microtiter plate. 100 µL of buffer B was then injected in the well and the light was collected for 5.25 s.

**Concentration Optimization of Fusion Proteins.** Each fusion protein was incubated for 18 h at 4 °C with a three molar excess of a selected coelenterazine. The fusion proteins TNF $\alpha$ -Y82F, IL6-Y82F, and IL8-F113W was incubated with coelenterazine *f*, coelenterazine *i*, and coelenterazine *cp* respectively. The fusion proteins were then serially diluted with buffer A. A volume of 10  $\mu$ L of each protein of selected concentration was added to a well on a 96 well microtiter plate, 50  $\mu$ L of buffer B was injected into the well and bioluminescent signal was collected using Polarstar Optima. For the fusion proteins TNF $\alpha$ -Y82F and IL8-F113W, the Polarstar Optima collected light for 6 s. For IL6-Y82F, the Polarstar Optima collected light from 6-25 s.

**Binder Dilution Curves for Fusion Proteins with Cytokine Specific Antibody.** Binder dilution studies were performed in an identical fashion for all antibodies. All antibodies were serially diluted in Pierce protein-free (PBS) blocking buffer (buffer C) starting at an initial concentration of 25  $\mu$ g/mL antibody. Anti-mouse IgG precoated 96 well microtiter plates were used for all experiments performed with antibodies. Prior to incubation each well of the anti-mouse IgG precoated 96 well plate was washed three times with 200  $\mu$ L of buffer C. Next, an aliquot of 100  $\mu$ L of each antibody concentration was added to the wells of the plate and allowed to incubate at room temperature with shaking at  $\sim$ 500 rpm for 1 h. The antibody solution was removed from the wells and the wells were washed three times with 30 mM Tris-HCl pH 7.4 2mM EDTA, 150mM NaCl and 0.05% Tween-20 (buffer D). An aliquot of 100  $\mu$ L of the corresponding fusion protein solution was then added to the wells and allowed to incubate with shaking at  $\sim$ 500 rpm, room temperature for 1.0 h. For the TNF- $\alpha$  antibody a concentration of  $1.04 \times 10^{-8}$  M TNF $\alpha$ -Y82F was used,  $7.00 \times 10^{-8}$  M concentration of IL6-Y82F was employed with the IL-6 antibody, and  $5.81 \times 10^{-8}$  M of IL8-F113W fusion protein was used with the IL-8 antibody as determined from the concentration optimization studies of the fusion proteins. The wells were then emptied and washed three times with buffer D. The microtiter plate was then loaded into the respective instrument, and 50  $\mu$ L of buffer B was injected into each well, and the emitted luminescence light was collected using Polarstar Optima, 0-6 s for TNF $\alpha$ -Y82F and IL8-F113W, 6-25 s for IL6-Y82F. The collected bioluminescence light was then used to generate the binder dilution curves (Data not shown).

**Individual Cytokine Dose-Response Curves.** Anti-mouse IgG coated plates were employed for all experiments. Before use, each well was washed three times with 200  $\mu$ L of buffer C. An aliquot of 100  $\mu$ L of the respective antibody at the specified concentration (diluted with buffer C) was added to each well and allowed to incubate with shaking at  $\sim$ 500 rpm at room temperature for 1.0 h. The antibody concentration used for TNF- $\alpha$  and IL-6 is 1  $\mu$ g/mL, and for IL-8 is 0.5  $\mu$ g/mL. The antibody solution from each well was removed and washed three times with 200  $\mu$ L of 10 mM PBS pH 7.4 containing 2 mM EDTA and 0.05% Tween-20 (buffer E). Human interleukin standards TNF- $\alpha$  (Abcam, Cambridge, MA), IL-6, and IL-8 (Invitrogen, Carlsbad, CA) were serially diluted in 10 mM PBS buffer pH 7.4 containing 2 mM EDTA, 1  $\mu$ g/mL BSA (buffer F) and 100  $\mu$ L of the varied concentrations (in triplicate) were added to the wells and allowed to incubate with shaking at  $\sim$ 500 rpm at room temperature for 1.5 h. The wells were emptied and washed with 200  $\mu$ L of buffer D and 100  $\mu$ L of the corresponding cytokine-aequorin fusion protein was added to each well and allowed to incubate with shaking at  $\sim$ 500 rpm at room temperature for 1.0 h. Fusion protein concentrations used in the study were as follows: TNF $\alpha$ -Y82F with coelenterazine *f*-  $1.04 \times 10^{-8}$  M, IL6-Y82F with coelenterazine *i*-  $7.00 \times 10^{-8}$  M, and IL8-F113W with coelenterazine *cp*-  $5.81 \times 10^{-8}$  M. The wells were emptied and washed with 200  $\mu$ L of buffer D. Then an aliquot of 50  $\mu$ L of buffer B was added to each well and the bioluminescent intensity was measured using the Polarstar Optima through dual luminescence emission detection with two kinetic window. Channel A contained a 420 nm filter and

channel B housed a 520 nm filter (both of BMG Labtech (Cary, NC)). The first kinetic window consisted of the 0-6 s time frame after addition of the buffer B and the second window was 6-25 s.

**Simultaneous Multiplexed Cytokine Dose-Response Curves in Buffer and Human Serum.** For the dose-response curves in buffer, pre-coated anti-mouse IgG plates were washed three times with 200  $\mu$ L of buffer C. To each well, an aliquot of 100  $\mu$ L of the mixture of three anti-human cytokine antibodies in the same concentrations as used for the individual dose response plots listed above was added and allowed to incubate for 1.0 h with shaking at  $\sim$ 500 rpm at room temperature. The antibody solution was removed and the wells were washed three times with 200  $\mu$ L of buffer D. An aliquot of 100  $\mu$ L of each cytokine standard solution was then added to the wells. For each of the cytokines the response was examined in a dose-dependent manner while the concentration of the other two cytokines was held constant at 1000 pg/mL. The cytokine solutions were allowed to incubate with shaking at  $\sim$ 500 rpm at room temperature for 1.5 h. The solutions were then removed from the wells and the wells were washed three times with 200  $\mu$ L of buffer D. An aliquot of 100  $\mu$ L of each cytokine-aequorin fusion protein was then added to the wells and allowed to incubate for 1.0 h with shaking at  $\sim$ 500 rpm at room temperature. The wells were then drained and washed three times with 200  $\mu$ L of buffer D.

The same procedure was followed for the dose-response curve generated using human serum with the exception of the additions of the standard solutions. In order to establish a calibration plot, aliquots of human serum (Innovative research, Novi, MI) were spiked with the cytokines of interest to the final desired concentrations. One of the cytokines was added in the varying concentrations while the other two were held constant at 1000 pg/mL. A total volume of 100  $\mu$ L of the human serum with the desired cytokine concentrations was added to each well and allowed to incubate with shaking at  $\sim$ 500 rpm at room temperature for 1.5 h. The bioluminescent response was then measured on the Polarstar Optima after the addition of 50  $\mu$ L of buffer B to each well. Dual luminescence, with the 420 nm and 520 nm filters, was employed in the two distinct kinetic windows of 0-6 s and 6-25 s after the injections of buffer B into the well. All measurements were performed in triplicate with the mean value plotted.