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# A homogeneous fluorometric assay platform based on novel synthetic proteins

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**Abstract**

Novel synthetic recombinant sensor proteins have been created to detect analytes in solution, in a rapid single-step "mix and read" noncompetitive homogeneous assay process, based on modulating the Förster resonance energy transfer (FRET) property of the sensor proteins upon binding to their targets. The sensor proteins comprise a protein scaffold that incorporates a specific target-capturing element, sandwiched by genetic fusion between two molecules that form a FRET pair. The utility of the sensor proteins was demonstrated via three examples, for detecting an anti-biotin Fab antibody, a His-tagged recombinant protein, and an anti-FLAG peptide antibody, respectively, all done directly in solution. The diversity of sensor–target interactions that we have demonstrated in this study points to a potentially universal applicability of the biosensing concept. The possibilities for integrating a variety of target-capturing elements with a common sensor scaffold predict a broad range of practical applications.


*Keywords:* Biosensor; Protein engineering; Homogeneous assay; Förster resonance energy transfer; Fluorescence

Homogeneous binding assays avoid the multiple binding and washing steps required in heterogeneous assays, and are thus simpler and faster, and more amenable to automation. Different principles could be used to develop homogeneous binding assays, including fluorescence polarization, scintillation proximity, enzyme/protein fragment complementation, fluorescence quenching, and resonant energy transfer [1]. Among these assay techniques, resonant energy transfer (especially FRET) is one of the more popular techniques for assays of macromolecules [1]. For instance, Ohiro et al. [2] reported an open sandwich FRET-based immunoassay technique, in which two single-chain variable antibody fragments (scFvs) that recognized distinct epitopes of a target antigen were, respectively, fused with a FRET donor protein or an acceptor protein followed by a leucine zipper motif. The acceptor and donor proteins were brought together in the presence of a target in solution and a FRET signal was generated as a result. In another FRET-based homogeneous noncompetitive immunoassay, a highly specific antibody against the immune complex formed between an anti-morphine antibody and morphine was labeled with a FRET donor while an anti-morphine antibody was labeled with a FRET acceptor. These labeled antibodies were used to detect morphine in a single-step homogeneous noncompetitive immunoassay based on FRET [3]. Both of these assays are however limited by requiring the use of antibodies as the detecting element.

In this study, we report a new sensor protein design that allows efficient FRET-based noncompetitive homogeneous assays of potentially a broad range of target molecules. Noncompetitive assays are generally more sensitive than competitive assays [2]. Furthermore, non-competitive assays do not require labeled target analogues that add complexity and cost and may not be readily available. The sensor proteins reported in this study comprise a protein scaffold inserted by genetic fusion between two fluorescent protein variants that form a FRET pair, such that the FRET pair is in close proximity to enable FRET.

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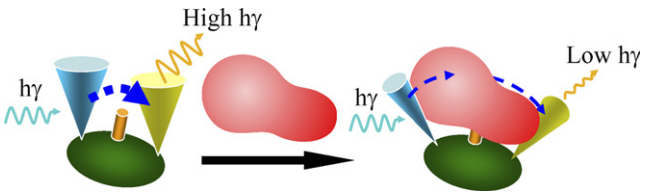


Fig. 1. Proposed working principle of the sensor protein is based on changes in FRET property upon binding to a target. The relative orientation and/or distance, or potentially the non-bulk refractive index between the FRET donor (cyan) and acceptor (yellow) on the protein scaffold (green) is altered upon binding of the target molecule (the red object). Here the target-capturing element is depicted by the orange stick. Cones depict rotation of donor and acceptor fluorophores around peptide bonds in the linkers connecting the fluorophores to the protein scaffold.

A specific target-capturing element is incorporated into the sensor scaffold at strategic locations. We postulated that binding of the target to the target-capturing element creates a steric effect to alter the distance and/or orientation, or potentially the non-bulk refractive index [4] between the FRET pair, leading to changes in FRET property of the sensor (Fig. 1). While the approach of flanking two fluorescent proteins (that form a FRET pair) on both ends of a binding protein has been employed to create a range of well-known FRET-based sensors, *e.g.,* the calcium-sensing Cameleon proteins [5] and the maltose-sensing FLIPmal proteins [6], all of these sensors require a binding protein moiety that, first of all, has a specific affinity for the target, and second, undergoes considerable conformational changes upon target binding to generate the sensor signal. Unlike these sensor proteins, a stable protein scaffold, which has no intrinsic affinity for the intended target, is used in the sensor protein reported here. The protein scaffold is used to display the target-capturing element (via chemical conjugation or genetic fusion) interposed between the FRET donor/acceptor pair, and it does not necessarily undergo conformational changes upon binding of the analyte to the sensor protein. Regarding this latter point, since the FRET pairs are connected to the scaffold via flexible covalent linkages, docking of the target analyte may push the fluorescent protein variants apart to alter the distance and/or orientation between the FRET pair fluorophore dipoles. In the sensor reported here, a mutated *Escherichia coli* maltose binding protein (MBP) is used as the protein scaffold which is sandwiched between a cyan fluorescent protein variant and a yellow fluorescent protein variant. It should be pointed out that even though MBP is a binding protein, it is used in our sensor construct not for its maltose-binding attribute, but rather for its desirable structural properties. To avoid potential interference from contaminating maltose in the sample, all tests reported here were conducted using sensors having the closed form of MBP by saturating the sensor with 1 mM maltose prior to incubating with the analytes. MBP is relatively small and its two termini are close enough to each other to allow efficient energy transfer [6]. It also tolerates a high degree of site mutations [7]. In the current work, selected amino acid residues of MBP were mutated to cysteine to allow site specific chemical conjugation with thiol-reactive target-capturing elements.

## Materials and methods

*Molecular cloning.* The sensor constructs that utilized enhanced cyan fluorescent protein (ECFP) and enhanced yellow fluorescent protein (EYFP) as the FRET pair were derived from the FLIPmal-25μ [6] construct which encodes a fusion protein with MBP sandwiched between ECFP and EYFP (generously provided by Dr. Wolf Frommer). Site directed mutagenesis to substitute cysteine at MBP position Q49 was performed using the Quick-Change site directed mutagenesis kit (Stratagene) with primers Q49C-front and Q49C-rear and template FLIPmal-25μ (Supplementary Table 1).

To create the CyPet/YPet [4] based sensor protein constructs, *malE* encoding MBP was amplified from the *E. coli* DH5α genome using malE-front primer which generates a BamHI restriction site and malE-rear primer which generates a SalI restriction site (Supplementary Table 1). The PCR product was cloned into the multiple cloning sites in pET21a(+) after double digestion with BamHI and SalI to create the pET21a(+)MBP construct. The CyPet [4] coding sequence was obtained from the plasmid pCyPet-His [4], using CyPet-front primer which generates a NheI site and CyPet-rear primer which generates a BamHI site. Similarly, the YPet [4] coding sequence was obtained from the plasmid pYPet-His [4], using YPet-front primer which generates a SalI site and YPet-rear primer which generates a XhoI site. Plasmids pCyPet-His and pYPet-His were kindly provided by Dr. Patrick Daugherty. The CyPet and YPet PCR products were fused into pET21a(+)MBP after digestion with BamHI/NheI and SalI/XhoI, respectively, to create the pET21a(+)CyPet-MBP-YPet construct. This resulting construct encodes the CyPet sequence, a flexible linker containing a BamHI restriction site, the sequence of the mature MBP protein, a flexible linker containing a SalI restriction site, followed by the YPet sequence and a hexa-histidine tag.

Site directed mutagenesis to mutate MBP positions E45, Q49, A52, and S73, individually, into cysteine was performed as described previously [8] and supplementary Table 1 shows the primers used for cloning. Site directed mutagenesis to create MBP double (Q49C/A52C) and triple (Q49C/A52C/S73C) mutants was performed by using pET21a(+)MBP as a template with Quick-Change site directed mutagenesis kit and primers Q49C/A52C-front, Q49C/A52C-rear, S73C-front, and S73C-rear (Supplementary Table 1). To express CyPet/YPet-based sensor proteins without the His tag, a stop codon was introduced into the pET21a(+)-CyPet-MBP-YPet plasmid right after the YPet coding sequence, creating the pET21a(+)CyPet-MBP-YPet-stop plasmid. Finally, the mutated pET21a(+)MBP constructs were double digested with SalI/BamHI and inserted into the pET21a(+)CyPet-MBP-YPet-stop plasmid digested with the same set of restriction enzymes.

Chloramphenicol acetyl transferase (CAT) gene was amplified from BL21(DE3)pLysS (Stratagene) using CAT-front primer which generates an NdeI restriction site and CAT-rear primer which generates a HindIII restriction site (Supplementary Table 1). The PCR product was cloned into the multiple cloning site in pET21a(+) after double digestion with NdeI and HindIII.

*Expression and purification of proteins.* Proteins were expressed in the host strain BL21(DE3)pLysS at 22 °C in Luria–Bertani (LB) media. His-tagged CAT and sensor proteins were purified using a $Ni^{2+}$-charged HiTrap™ Chelating HP column (Amersham Biosciences). The CyPet/YPet-based fusion proteins without the $His_6$-tag were purified using a two-step process modified from Peckham et al. [9] involving hydrophobic interaction chromatography (using a HiTrap™ Phenyl-HP column) followed by ion exchange chromatography (using a HiTrap™ Q-FF column).

*Conjugation of sensors.* Sensors were modified at the introduced cysteine residues using maleimide/thiol chemistry. Maleimide–$PEO_2$–biotin ((+)-biotinyl-3-maleimidopropionamidyl-3,6-dioxaoctanediamine) was obtained from Pierce, and maleimido-$C_3$-NTA (*N*-(3-maleimidobutylyl)-1-

carboxylpentylenediamine-*N*′-*N*′-diacetic acid) from Dojindo Laboratories. Conjugation reactions were conducted in 20 mM MOPS buffer, 0.5 mM EDTA, 0.4 mM tris-(2-carboxyethyl)phosphine (TCEP), pH 7.0. After 30-min incubation at room temperature an excess of maleimido-activated biotin or NTA reagent was added. After 5 h at 25 °C, the reaction was stopped by addition of 20 mM 2-mercaptoethanol and excess reagent removed either by dialysis or desalting. Conjugation of the FLAG peptide to the A52C mutant sensor protein was based on the formation of a stable hydrazone bond using a heterobifunctional crosslinker MTFB (1-*N*-((3-maleimido)propanamido)-13-*N*-((4-formyl)benzamido)-4,7,10-trioxotridecane) (Solulink Biosciences). After incubating with 50 mM TCEP for 30 min, the sensor protein A52C was modified with MTFB for 2 h at room temperature in 20 mM sodium phosphate, 150 mM NaCl, pH 7.0, followed by removing the free MTFB by desalting. The MTFB-activated sensor protein and the hydrazine-activated FLAG peptide were then mixed to yield the hydrazone-mediated conjugate. After conjugation, free FLAG peptide was separated from the sensor proteins using desalting cartridges followed by centrifugal filtration using Amicon Ultra-15 filters (Millipore). The formation of stable hydrazone was monitored and quantitated with a spectrophotometer at 354 nm. The peptide/sensor protein ratio was estimated to be approximately one. Conjugation of the FLAG peptide to the sensor protein scaffold was further confirmed by dot-blot analysis (data not shown).

*HABA [2-(4′-hydroxyazobenzene)-benzoic acid] assay.* The extent of modification of the sensors with biotin was verified using the HABA assay [10] with slight modification in which sodium bicarbonate (150 mM) was included in the assay buffer to counter the turbidity that was observed to develop in its absence [11]. Biotin was obtained from Sigma, avidin from Rockland, and HABA reagent from Fluka. Absorbance at 500 nm was measured using a TECAN Safire plate reader.

*Antibodies.* Rabbit anti-human intestine alkaline phosphatase antibody was from Rockland Inc. Affinity-purified goat anti-biotin Fab fragment was custom-made by the same company. Both were dialyzed against 20 mM sodium phosphate buffer, pH 7.0, and stored at 4 °C prior to use. Monoclonal anti-FLAG M2 antibody was purchased from Stratagene.

*FRET measurements.* For measuring anti-biotin Fab antibody, after mixing the biotin-conjugated sensor proteins (50 nM) with various concentrations of Fab antibody (0–250 nM), the reaction mixture was incubated at room temperature in the dark for 30 min, then measured in a Hitachi F-2500 fluorescence spectrophotometer. An excitation wavelength of 439 nm was used and donor/acceptor emission peaks were determined to calculate the FRET ratio. Standard deviations were calculated from 10 independent scans of the same sample. For $His_6$-tag protein detection, the NTA-conjugated sensor was reacted with $NiCl_2$ for 30 min and washed 3–4 times with 20 mM sodium phosphate buffer using Microcon YM30 filters (Millipore) to remove excess free nickel ions, then adjusted to 50 nM, before incubating with the CAT-His protein. The reaction mixture was incubated at room temperature in the dark for 30 min, before fluorescence measurements. A similar procedure was used to determine the FRET ratio of the FLAG-conjugated sensor protein in response to various concentrations of anti-FLAG M2 antibody. All dilutions were prepared using 20 mM sodium phosphate buffer, pH 7.0, with 0.1 mg/ml bovine serum albumin.

## Results and discussion

Initially ECFP and EYFP were used as the FRET pair, and position MBP-Q49 was selected for cysteine-substitution, since it is located between the N- and C- terminus of the MBP protein and exposed on the protein surface, which would allow efficient conjugation. As a proof-of-concept for the new sensor technology, we initially selected biotin as the target-capturing element, by conjugating maleimide–$PEO_2$–biotin to the sensor protein scaffold, for detecting a goat anti-biotin antibody Fab fragment. We intended to first verify the sensor response resulting from the formation of 1:1 sensor/analyte complex so we chose Fab fragment as the target for its monovalent binding property. As shown in Fig. 2A, the FRET ratio of the biotinylated sensor protein (normalized by dividing by the FRET ratio in the absence of the target analyte) decreased in response to increased concentrations of anti-biotin Fab antibody before reaching saturation at high Fab concentrations. FRET ratio is defined as the ratio of fluorescence intensities at the two emission peaks of the sensor, while the sensor is excited at the optimal donor excitation wavelength. The sensor response (expressed as the normalized FRET ratio, $\psi$, for a given target analyte concentration, $A$, and a sensor protein concentration, $P$) can be quantified to estimate the concentration of the target analyte in solution using a modified Scatchard equation [12] (*i.e.* $A = (\Psi - 1)\left(\frac{P}{(\Psi_S - 1)} + \frac{K_D}{(\Psi_S - \Psi)}\right)$, where $\psi_S$ and $K_D$ are the normalized FRET ratio at saturation and

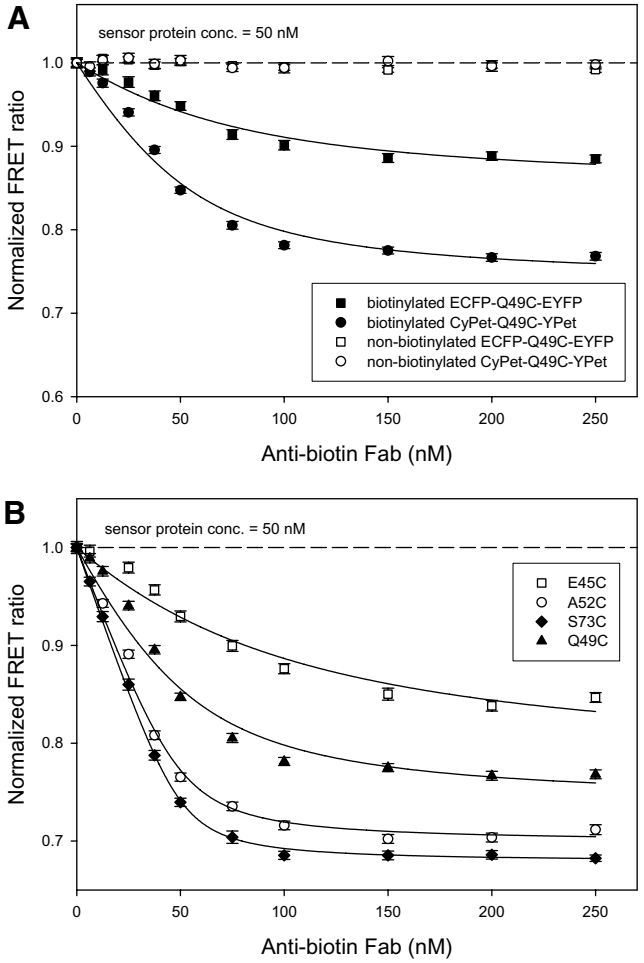


Fig. 2. (A) Comparison of the FRET responses between biotinylated MBP-Q49C sensors fused with ECFP-EYFP [6] or CyPet-YPet [4] protein pair for detecting anti-biotin Fab antibody. (B) Responses of biotinylated MBP-mutant sensors (E45C, Q49C, A52C, and S73C) fused with the CyPet-YPet FRET pair. The solid curves are obtained by data fitting using a modified Scatchard equation as described in the text.

equilibrium dissociation constant, respectively) by assuming that the change in FRET ratio is linearly proportional to the percentage of sensor proteins that are bound to the analyte. Least-squares regression was used to fit the modified Scatchard equation to the sensor-response data to generate the solid curves in Fig. 2A. As negative controls, no change in the FRET ratio was observed when the non-biotinylated sensor protein was incubated with anti-biotin Fab (Fig. 2A), or when the biotinylated sensor protein was incubated with an anti-alkaline phosphatase antibody, which is non-specific to MBP, fluorescent proteins, or biotin (data not shown).

The sensor was further improved by optimizing the fluorescent labels. The ECFP and EYFP in the initial sensor were replaced by CyPet and YPet, an engineered CFP/YFP pair optimized for FRET assays [4]. As a result, the FRET ratio of the sensor protein (in the absence of the anti-biotin Fab) was increased by 2.5-fold. In the presence of anti-biotin Fab, the maximum change in the normalized and un-normalized FRET ratio was increased by 2-fold (Fig. 2A) and 5-fold, respectively, hence generating a much more sensitive biosensor. To study the effect of the conjugation site of the target-capturing element, instead of MBP-Q49, we created three other sensor proteins containing MBP-E45C, -A52C, and -S73C mutation, respectively, while using CyPet/YPet as the FRET pair. These sensor proteins were conjugated with maleimide–$PEO_2$–biotin and analyzed for FRET in response to anti-biotin Fab. The biotin/sensor protein ratio was confirmed by the HABA assay [10] to be approximately one for all mutants. Fig. 2B compares the performance of these sensor proteins in detecting anti-biotin Fab. The best performance was seen with the two sensors bearing, respectively, the MBP-S73C and the MBP-A52C mutation, giving a maximum reduction in the normalized FRET ratio by 32% for the former (from a FRET ratio of 5.3 at zero Fab conc.) and 30% for the latter (from a FRET ratio of 5.2 at zero Fab conc.). The difference in the proximity and alignment of the mutation sites with respect to the fluorescent proteins at the MBP termini might have led to the variations in the maximum change in FRET ratio. Mutations S73C and A52C, which gave the highest maximum change in FRET ratio, are located on the surface of the protein and interposed approximately between the N- and C-terminus of MBP, whereas mutation E45C, which gave the lowest maximum FRET ratio change, is located farthest away from the axis connecting the MBP termini (and hence the FRET pair), compared to other mutations (see Supplementary Fig. 1).

Upon the initial verification of the sensor concept using biotin as a model target-capturing element, we then examined the technical feasibility of adapting the proposed sensor protein technology platform for rapid and accurate in-solution detection of oligo-histidine (His) peptide-tagged recombinant proteins. His-tag is widely used in research and large-scale protein manufacturing to aid recombinant protein purification. Currently, most detection methods of His-tagged proteins are based on solid-phase (heterogeneous) assays involving gel electrophoresis [13], Western blot [14], or surface plasmon resonance (SPR) [14]. Having a faster and simpler method for detection and quantification of His-tagged proteins directly in solution using a homogeneous assay, without running gels or using expensive instrument, should be quite useful. For this purpose, we have created a single (Q49C), a double (Q49C/A52C), and a triple mutant (Q49C/A52C/S73C) of MBP in which the mutation sites are in close vicinity of each other (Supplementary Fig. 1). Each of these MBP mutants was fused with CyPet/YPet to create the FRET-based sensors (note that there was no His-tag in these sensor protein constructs). We hypothesize that with more than one copy of target-capturing element conjugated to the sensor scaffold, the binding affinity toward their targets can be increased. This is particularly relevant with respect to the metal-chelating group that serves as the target-capturing element for His-tagged proteins. It was shown recently that the binding affinity of divalent vs. monovalent NTA chelator heads toward hexa-His tag peptides was almost 52-fold higher [15]. Maleimido-$C_3$-NTA, a sulfhydryl-reactive metal-chelator reagent with a hydrocarbon spacer, was chemically conjugated to the purified single, double, and triple biosensor mutants, at the mutated cysteine residues. After chelating the NTA conjugated sensor with $Ni^{+2}$, the sensor constructs were tested via FRET assay to detect His-tagged chloramphenicol acetyltransferase (CAT-His) and results are shown in Fig. 3. Increasing the number of Ni-chelated NTA groups per sensor protein molecule clearly led to improved sensor performance; the estimated $K_D$ value of the triple mutant sensor was about 90-fold lower than that of the single mutant sensor. The $Z'$ factor $(=1-3(\sigma_o+\sigma_s)/|R_s-R_o|$, where $R_s$ = mean FRET ratio at

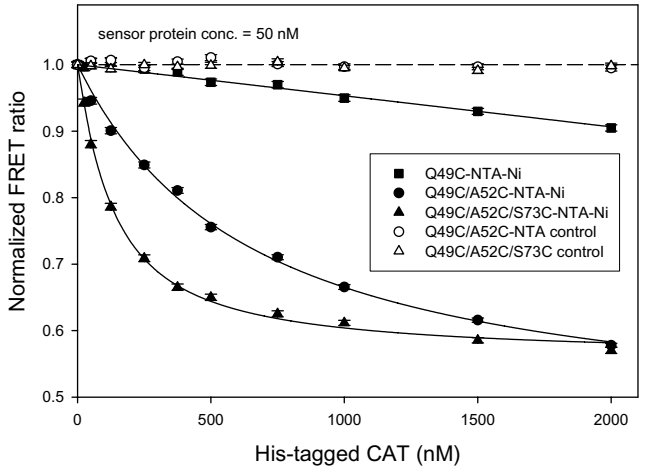


Fig. 3. Responses of Ni-NTA-conjugated CyPet-MBP-YPet sensor proteins containing single (Q49C), double (Q49C/A52C), or triple (Q49C/A52C/S73C) mutation, to increasing concentrations of His-tagged CAT proteins. The solid curves represent least-squares fitting using the modified Scatchard equation. As negative controls, double mutant sensor with NTA-conjugation but without $Ni^{+2}$-chelating and triple mutant sensor without NTA-conjugation were used.

saturation, $R_o$ = mean FRET ratio in the absence of the target analyte, and $\sigma_s$ and $\sigma_o$ are the corresponding standard deviations) [16], coefficient of variation (CV), and signal-to-noise ratio (S:N) for CAT-His measurement using the triple-mutant sensor are 0.94, 0.9%, and 71, respectively. The CV value was determined by repeating the assay six times using identical assay conditions. Assays producing $Z'$ values between 0.5 and 1 are considered robust (higher values indicating better assay performance) [16].

Synthetic peptide ligands such as Arg-Gly-Asp (RGD) cell-binding peptides, linear peptide epitopes, peptide aptamers, peptide substrates, peptide mimotopes, or peptide binders selected from phage libraries, are potential target-capturing elements useful in a wide variety of applications. In this study, as a proof-of-concept, we conjugated a FLAG peptide (DYKDDDDK) to the sensor protein scaffold carrying a single A52C mutation, and examined its utility for detecting anti-FLAG antibody. As shown in Fig. 4, the FLAG-conjugated sensor protein was able to detect anti-FLAG M2 monoclonal antibody in a manner similar to the other two sensors described above. Although divalent binding may have taken place especially at lower antibody/sensor ratios, it appeared that sensor/antibody complexes were predominantly formed at a 1:1 ratio. If an appreciable portion of the M2 antibody did exhibit divalent binding, we anticipate some extent of intermolecular FRET between the two sensor proteins that are bound to the same antibody molecule, potentially causing an increase in FRET. Since that was not observed, monovalent binding was assumed in the Scatchard equation for fitting the data in Fig. 4. For M2 antibody measurement, the $Z'$ factor, CV, and S:N are 0.88, 1.0%, and 37, respectively, indicating a reliable assay.

In conclusion, we have shown the feasibility of a new biosensor concept for homogeneous fluorometric assays. Its novelty lies in the usage of a protein scaffold having no intrinsic affinity for the intended target but which can be (chemically) modified to incorporate a target-binding moiety. Binding of the target molecule causes a change in FRET between the donor and acceptor fluorescent domains fused to the scaffold, as we demonstrated with an antibody against a peptide, an antibody Fab fragment against biotin, and a hexahistidine-tagged protein. The signal is proportional to the extent of target binding, and although at this stage the exact nature of the molecular change that leads to the change in FRET has not been determined, the diversity of sensor–target interactions that we have demonstrated points to a potentially universal applicability of the principle. Sensor sensitivity depends in part on the affinity between the target molecule and the target-capturing element, and for antibody–antigen interactions, sensitivities in the nanomolar range have been obtained. Further work is underway to examine the molecular mechanism underlying the signal change, and to further validate the concept with different targets and target-capturing moieties, including peptides genetically fused to the sensor scaffold.

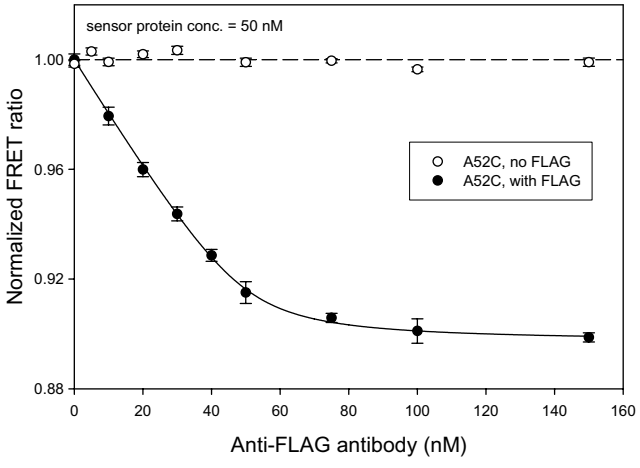


Fig. 4. Response of FLAG-conjugated CyPet-MBP-YPet sensor protein containing A52C mutation to increasing concentrations of anti-FLAG M2 antibody. The solid curve is from least-squares fitting using the modified Scatchard equation. The A52C mutant sensor protein without FLAG peptide conjugation was used as negative control.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.06.174.

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