Supplementary Information for

Rapid construction of fluorescence quenching-based immunosensor Q-bodies using α-helical coiled-coil peptides

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SI Materials and Methods

Materials

Escherichia coli XL10-Gold was purchased from Stratagene (La Jolla, CA, USA) and used for cloning, while SHuffle®T7 Express lysY from New England Biolabs (Ipswich, MA, USA) was used for protein expression. In-Fusion HD cloning kit was purchased from Takara-Bio (Shiga, Japan). Restriction enzymes were purchased either from Takara-Bio, Toyobo Biochemicals (Osaka, Japan), Roche Diagnostics (Tokyo, Japan), or New England Biolabs. Oligonucleotides were obtained from Eurofins Genomics (Tokyo, Japan) (Table S1). A PureYield plasmid miniprep kit was obtained from Promega Japan (Tokyo, Japan). Streptactin agarose was from IBA (Goettingen, Germany). 5-TAMRA-C6-maleimide (5-TAMRA-C6-mal.) was obtained from AAT Bioquest (Sunnyvale, CA, USA). Immunoblock was obtained from DS Pharma (Osaka, Japan). PentaHis HRP conjugate was obtained from Qiagen (Hilden, Germany). 3,3′,5,5′-Tetramethylbenzidine (TMBZ) was obtained from Sigma (St. Louis, MO, USA). Peptides used in this study were obtained from Lifetein (Somerset, NJ, USA) (Table S2). Other chemicals and reagents, unless otherwise indicated, were obtained from Wako pure chemicals. Ultrapure water was prepared with Milli-Q (Merck-Millipore, Tokyo, Japan).

Construction of E3-Fab and E4-Fab expression vectors

To generate dsDNA sequence encoding a peptide E3 sequence, primer pairs of Back1 and For1, Back2 and For2 were each annealed and amplified by PCR, respectively. Then, these products were purified and amplified with Back1 and For2. pEQ1H (Fab KTM219)st2, an expression vector encoding anti-BGP Fab fragment with Strep-tag at its each C-terminus was digested with *Nde*I and *Age*I. The generated E3 sequence was introduced into the digested vector using the In-Fusion HD cloning kit. Finally, pEQ1H (E3 Fab KTM219)st2 was obtained. pEQ1H (E4 Fab KTM219)st2 was also generated by In-Fusion reaction using an E4 sequence and the digested vector. To get the E4 sequence, the gene for E3 Fab was amplified with E4NdeBack and For2 primer pairs.

Expression and purification of proteins

E. coli SHuffle T7 Express lysY cells were transformed with the vector and cultured at 37 °C overnight in LBA medium (Luria-Bertani medium containing 100 μg/mL Ampicillin) and 1.5% agar. A single colony was picked up to inoculate a 4 mL LB liquid medium containing

100 μg/mL of ampicillin (LBA) and cultivated at 30 °C overnight. On the next day, 4 mL culture was added to 800 mL LBA and cultivated at 30 °C until OD_{600} reached 0.4 ~ 0.7. The culture was cooled down to 16 °C and isopropyl-thio-β-galactopyranoside (IPTG) was added to the culture at a final concentration of 0.4 mM to induce protein expression. The culture was then incubated at 16 °C for 19 h with shaking at 200 rpm. The cells were collected by centrifugation (8,000 g, 10 min, 4 °C) and resuspended in 8 mL of Strep wash buffer (100 mM Tris-HCl, 150 mM sodium chloride (NaCl), 1 mM ethylenediaminetetraacetic acid (EDTA), 0.1 % Brij35, pH 8.0). The pellet was disrupted on a One-shot cell disruptor (Constant Systems, Daventry, UK) followed by the collection of crude intracellular protein solution after centrifugation (8,000 g, 10 min, 4 °C). The supernatant was incubated with 0.2 mL of Streptactin agarose on a rotating wheel for 1.0 h at 4 °C. The beads were transferred to a Talon 2 mL disposable gravity column (Takara Bio, Otsu, Japan) and washed nine times with 1 mL of Strep wash buffer. After adding 0.4 mL of Strep elution buffer (Strep wash buffer, 2.5 mL desthiobiotin) and incubation at 25 °C for 5 min, the elution was collected. By an additional two elution processes (incubation at 25 °C for 3 min, 0.2 mL elution), 0.8 mL of the eluate was finally collected. The eluent was subjected to a gel filtration device (MicroSpin G-25 Columns, GE Healthcare, Amersham, UK) and equilibrated with PBS (10 mM phosphate, 137 mM NaCl, 2.7 mM potassium chloride, pH 7.4). The expression and purification of the protein were confirmed using SDS-PAGE analysis, and the concentration of the protein was determined using WSE-6100 Lumino-Graph I (ATTO, Japan) with the varied concentration of bovine serum albumin (BSA) as a standard.

SDS-PAGE analysis

The sample (2 μL) was mixed with 8 μL of SDS loading buffer (0.125 M Tris-HCl, 4% (w/v) SDS, 20% (w/v) glycerol, 0.01% (w/v) BPB, 100 mM DTT, pH 6.8), boiled at 95 °C for 5 min, and loaded to ready-made 10 - 20% polyacrylamide gel (SuperSep Ace; cat. 198-15041, Wako). The Precision Plus protein unstained standards (Bio-Rad) in which one-fiftieth of Dual Color marker was spiked were used as the molecular weight standards. The protein concentration was determined after electrophoresis (25 mA, 300 V, 55 min) and Coomassie Brilliant Blue staining by comparing with varied concentrations of BSA standard using WSE-6100 LuminoGraph I.

Enzyme-Linked Immunosorbent Assay

After 200 μL of streptavidin (10 μg/mL in PBS) was immobilized on the Costar3370 microplate for overnight at 4 °C, the well was filled with 200 μL of PBS that contains 20% Immunoblock for 2.5 h at 25 °C and washed three times with PBS containing 0.05% PBS (PBST). Subsequently, 50 μg/mL of biotinylated human BGP-C11 peptide in 100 μL of PBST was added and incubated for 1 h at 25 °C. Then 500 μM of biotin-PEG-amine (Tokyo Chemical Industry, Tokyo, Japan) in 100 μL of PBST was added and incubated for 1.5 h at 25 °C to block excess biotin-binding sites. After washing three times with PBST, 30 nM of the protein in 100 μL of PBST was added and incubated for 1 h at 25 °C. The well was washed thrice with PBST and the bound protein was probed with 0.1 μg/mL PentaHis HRP conjugate in PBST containing 5% Immunoblock for 30 min at 25 °C. The well was washed thrice with PBST and developed with 100 μL of substrate solution (100 μg/mL TMBZ, 0.06 μL/mL hydrogen peroxide in 100 mM sodium acetate, pH 6.0). After incubation for 3 min, the reaction was stopped with 100 μL of 10% sulfuric acid, and the absorbance was read at 450 nm with a reference at 650 nm using a microplate reader SH-1000 (Corona Electric, Ibaraki, Japan).

Fluorescence labelling of K4-C peptide

5-TAMRA-C6-mal. (1.1 μL of 10 mM stock in dimethylsulfoxide) was added to 200 μM of K4-C peptide in ultrapure water. After mixing using a rotating mixer CM-1000 (EYELA, Japan) with 2,000 rpm in dark for 2 h at 25 °C, the resulting solution was purified with RP-HPLC (Chromaster, Hitachi, Tokyo, Japan) and identified by MALDI-TOF-MS (AXIMA-CFR Plus, Shimadzu, Kyoto, Japan). FITC-labelled K4-C peptide (Lifetein) was also TAMRA-labelled by the same procedure.

Preparation of Coiled Q-body and fluorescence measurement

E4-Fab (1.0 μM) and less than 1 μM of fluorescence-labelled K4 peptide were mixed in PBST containing 0.1% BSA. After incubation in dark for 10 min at 25 °C, Coiled Q-body was obtained. The fluorescence of the coiled Q-body was measured using either a fluorescence spectrophotometer Model FP-8500 (Jasco, Tokyo, Japan) or a fluorescence microplate reader Clariostar (BMG Labtech Japan, Saitama, Japan). For fluorescence spectral measurements, 5.0 nM of Coiled Q-body in 250 μL of PBST containing 0.1% BSA, and BGP-C7 peptide was added by titration in a 5 × 5 mm^2 quartz cell (Starna Scientific, Hainault, UK). After each antigen addition, the solution was stirred for 10 min at 25 °C before the fluorescence spectral

measurements. As a control, the same volume of PBST containing 0.1% BSA was added to a reference cuvette for correcting the time-dependent attenuation of the signal during the titration due to increased volume and photobleaching. The measurement temperature, excitation/emission bandwidths, and scanning speed were set to 25°C, 5 nm, 200 nm/min, respectively. The excitation and emission wavelengths were chosen for each experiment. Dose-response curves were fitted to a 4-parameter logistic equation by using ImageJ software (http://rsbweb.nih.gov/ij/). The EC_{50} and limit of detection (LOD) were calculated from the fitted curve and as the concentration corresponding to the mean blank value plus 3 times the standard deviation for each assay, respectively.

Time course assay

The mixture of 50 μL of 1.0 μM E4-Fab (0.72 μM) and 19 μL of 1.9 μM K4-TAMRA (0.52 μM) was incubated for 10 min at 25 °C in dark. Afterwards, 12 mL of PBST was added to the solution (E4-Fab: 4.1 nM, K4-TAMRA; 3.0 nM respectively) and inverted a few times. As a zero-hour sample, the fluorescence measurement was carried out using a fluorescence microplate reader Clariostar. After the remaining mixture was put on an incubator at 20°C, the fluorescence intensity upon adding varied concentrations of BGP-C7 was measured at each time point.

Table S1. The DNA sequence of the primers used in this study (5′- 3′).

Oligo	Sequence
Back1	AAGGAGATATACATATGGCTGAAATTGCAGCCC
For1	TTTTCCAGCGCTGCTATTTCCTTTTCTAGGGCTGC
Back2	TAGCAGCGCTGGAAAAGGAAATCGCAGCACTTGAG
For2	GCTGTACCTCACCGGTGCCTCCCTTCTCAAGTGC
E4NdeBack	TATACATATGGCTGAAATCGCTGCACTTG AAAAGGAAATTGCAGCCCTAGAAAAG

Table S2. Peptides used in this study.

Peptide	Sequence	M.W.
BGP-C7	NH_2-RRFYGPV-COOH	894
bio-BGP-C11	bio-QEAYRRFYGPV-COOH	1629
K4-C	NH_2-KIAALKE KIAALKE KIAALKE KIAALKE C-COOH	3137
FITC-K4-C	FITC-KIAALKE KIAALKE KIAALKE KIAALKE C-COOH	3639
K4(Lys6FITC)-C	NH_2-KIAALK(FITC)E KIAALKE KIAALKE KIAALKE C-COOH	3526
K4(Lys13FITC)-C	NH_2-KIAALKE KIAALK(FITC)E KIAALKE KIAALKE C-COOH	3526
K4(Lys20FITC)-C	NH_2-KIAALKE KIAALKE KIAALK(FITC)E KIAALKE C-COOH	3526

Table S3. Stability of BGP CQ-body. Antigen-dependent fluorescence intensity change, EC_{50} and LOD of the CQ-body used in Fig. S4 at different incubation periods at 20°C.

Time	+Ag/-Ag (fold)	EC50 (nM)	LOD (nM)
0 h	3.0	23	6.9
1 h	3.0	19	7.7
3 h	3.0	23	6.4
5 h	3.0	24	5.2
24 h	2.6	25	11

Table S4. Comparison of FRET CQ-bodies with K4 at different fluorescein positions (1). Fluorescence peak ratio (TAMRA/Fluoresceine) and its antigen-dependency of each mixture used in Fig. S8.

	FK4T	K4(Lys6F)T	K4(Lys13F)T	K4(Lys20F)T
—	1.7	2.0	1.7	2.1
+ E4-Fab	0.45	0.54	0.84	1.8
+ E4-Fab + BGP-C7	1.1	1.5	1.8	3.2

Table S5. Comparison of FRET CQ-bodies with K4 at different fluorescein positions (2). Irradiation-based and FRET-based fluorescence responses. EC_{50} and limit of detection (LOD) of the CQ-bodies used in Fig. S9 are shown. An average of three independent measurements is shown.

Irradiation-based				FRET-based			
Peptide	**+Ag/-Ag (fold)**	**EC_{50} (nM)**	**LOD (nM)**	**Peptide**	**+Ag/-Ag (fold)**	**EC_{50} (nM)**	**LOD (nM)**
K4T	2.7	19	3.7	**K4T**	—	—	—
FK4T	2.3	14	3.7	**FK4T**	2.8	16	1.1
K4(Lys6F)T	2.1	12	3.4	**K4(Lys6F)T**	2.8	15	2.7
K4(Lys13F)T	2.1	11	3.5	**K4(Lys13F)T**	2.7	11	3.0
K4(Lys20F)T	1.5	11	4.2	**K4(Lys20F)T**	2.4	11	3.1

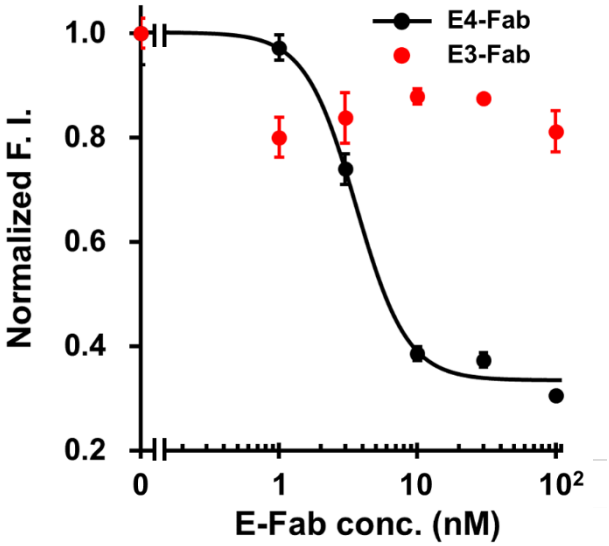

Figure S1. Dose-response curves for E3- or E4-Fab against 1 nM K4-TAMRA. The mixture of tagged Fab and K4-TMR was measured for the fluorescence intensity after 30 min incubation at 25°C.

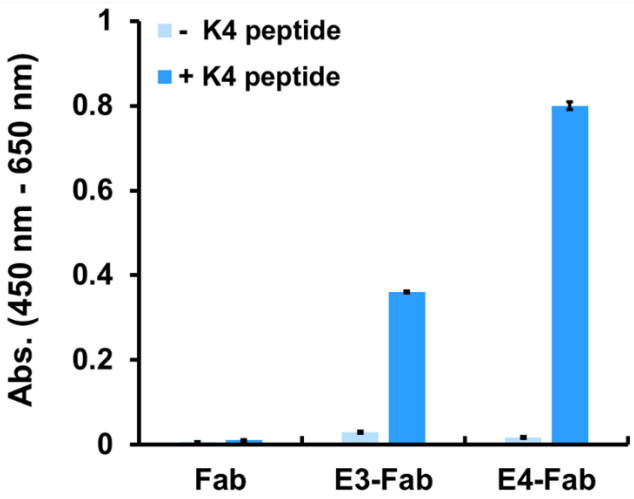

Figure S2. Binding activities of E3- and E4-Fab to K4-biotin evaluated by ELISA.

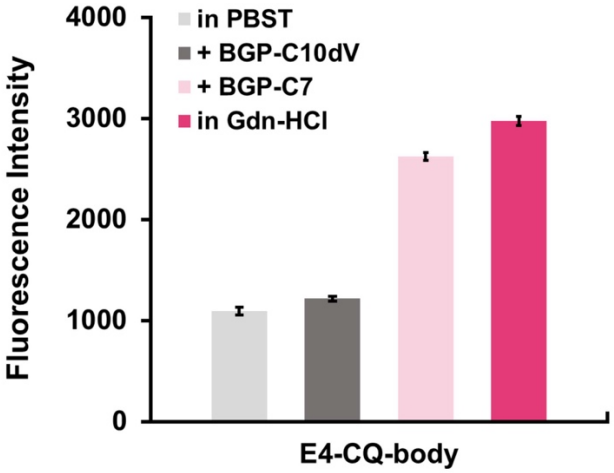

Figure S3. Antigen-specific fluorescence response of E4-CQ-body. Fluorescence intensities of BGP E4-CQ-body in the presence or absence of 1 μM BGP-C10dV, BGP-C7, and 7 M GdnHCl, 100 mM DTT are shown.

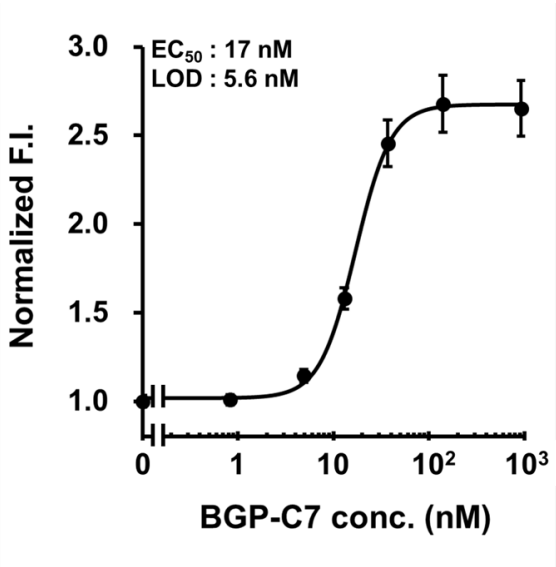

Figure S4. BGP-C7 dose-response of E4-CQ-body. The intensities are relative values to that in the absence of BGP-C7 (normalized fluorescence intensity).

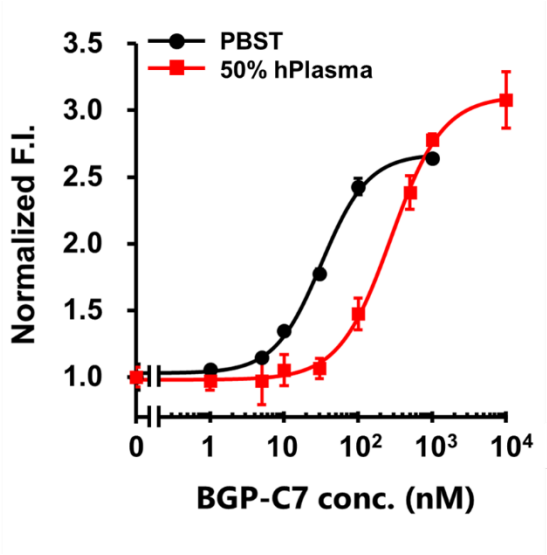

Figure S5. Microplate-based dose-response assay with BGP E4-CQ-body for BGP-C7 peptide either in PBST buffer or 50 % human plasma, 50% PBST.

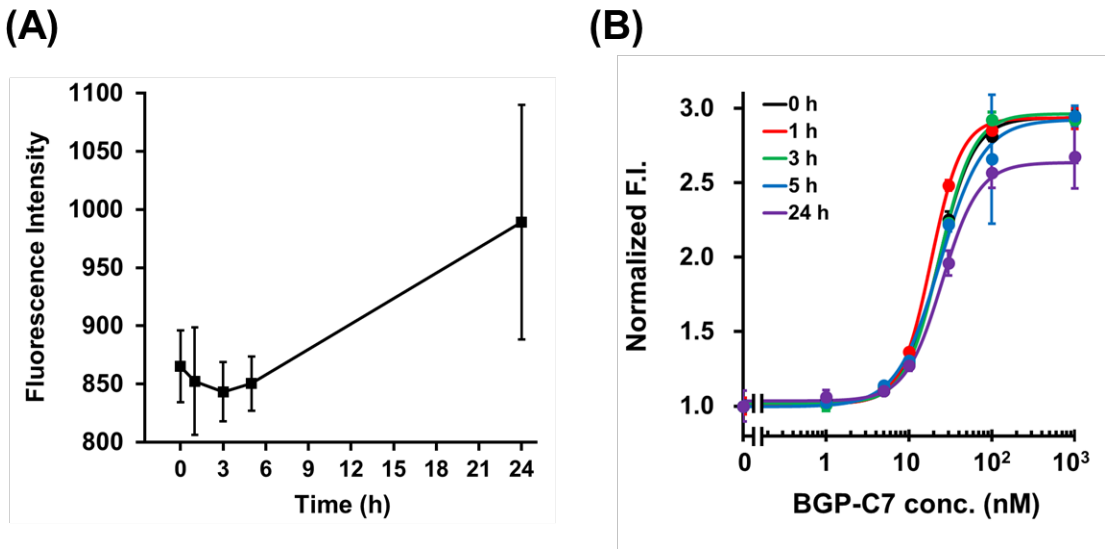

Figure S6. Stability of BGP E4-CQ-body (A) Fluorescence time course of the CQ-body. The fluorescence intensity at each time point indicates the value in the absence of antigen. (B) Microplate-based dose-responses for BGP-C7 using the CQ-body left for the indicated periods at 20°C.

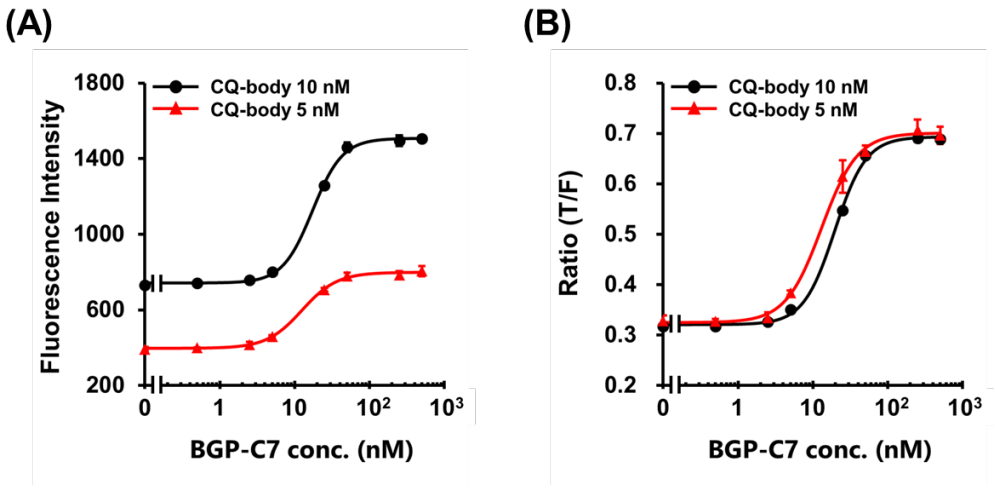

Figure S7. Dose-response curves using 10 or 5 nM dual (fluorescein and TAMRA)-coloured CQ-body for BGP-C7. Normalized values were not calculated. (A) Fluorescence intensity obtained with excitation at 545 nm. (B) Fluorescence intensity ratio obtained with excitation at 480 nm.

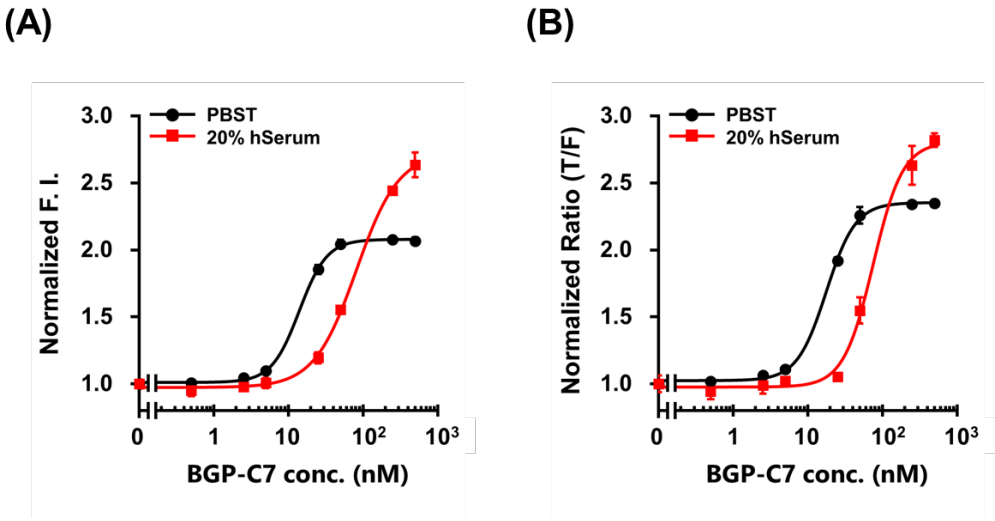

Figure S8. Microplate-based dose-response assay for BGP-C7 peptide either in PBST or in 20 % human serum / 80% PBST performed with FRET type CQ-body. **(A)** Normalized fluorescence intensity obtained with excitation at 545 nm. EC_{50} values were estimated as 14 nM and 82 nM in PBST and 20 % human serum, respectively. **(B)** Fluorescence intensity ratio with excitation at 480 nm. EC_{50} values were estimated as 18 nM and 75 nM in PBST and 20 % human serum, respectively.

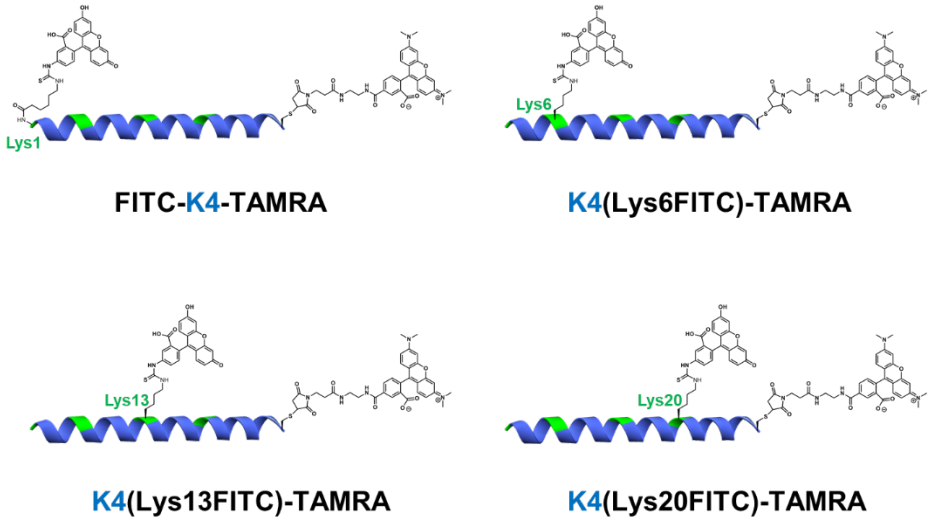

Figure S9. Schematic image of dual-coloured K4 variants.

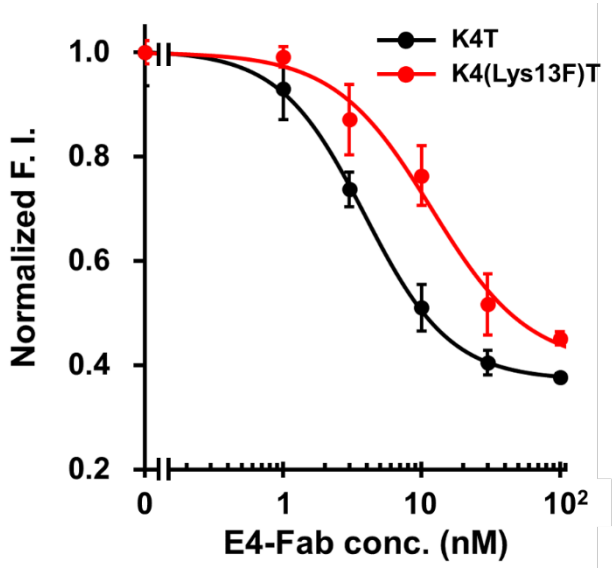

Figure S10. E4-Fab dose-dependent quenching of dual-coloured K4 variants. The mixture of E4-tagged Fab and K4T or K4(Lys13F)T (1 nM each) was measured for the fluorescence intensity after 30 min incubation at 25°C.

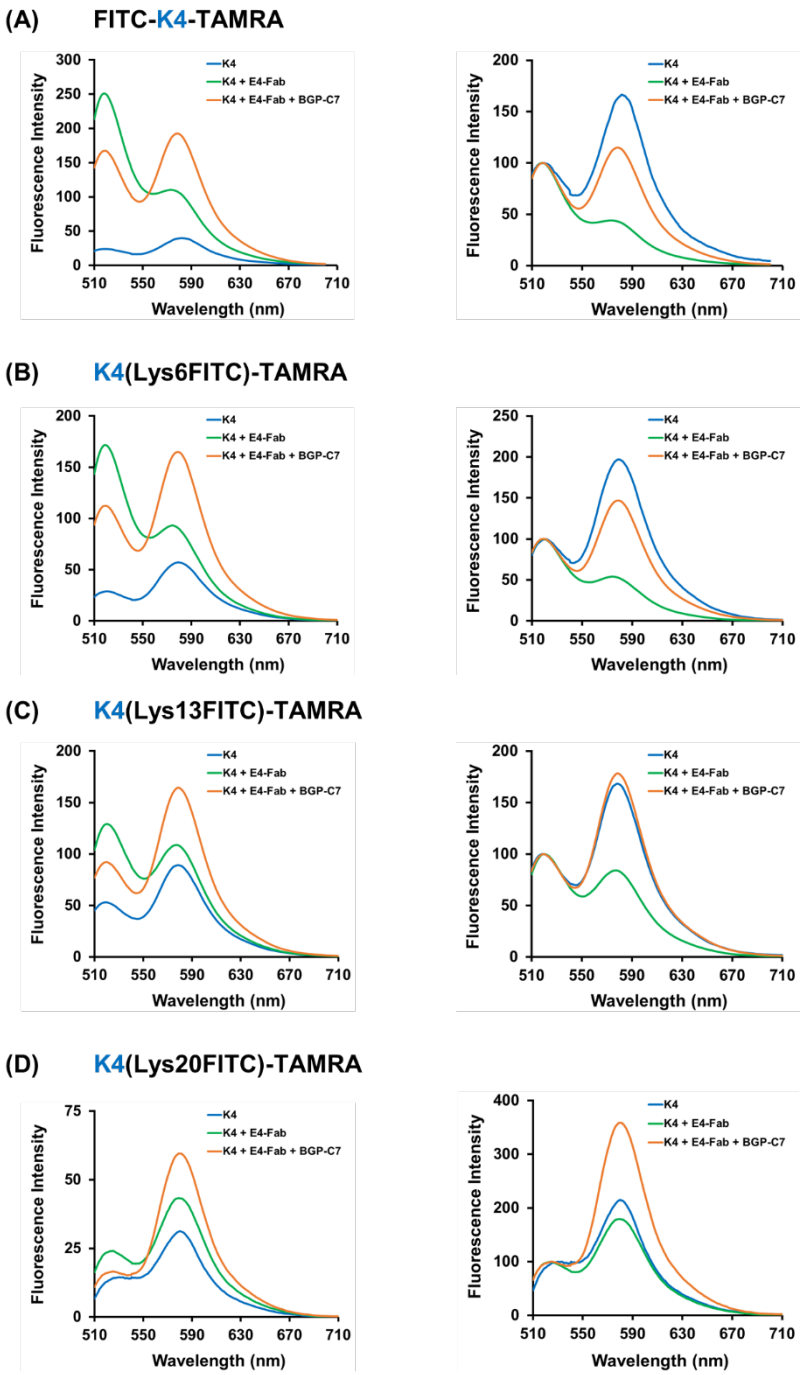

Figure S11. Fluorescence spectra of FRET CQ-bodies excited at 480 nm. Blue: peptide only; Green: Mixture with E4-Fab; Orange: FRET CQ-body with 1.0 μM BGP-C7. (A) FITC-K4-TAMRA (B) K4(Lys6FITC)-TAMRA. (C) K4(Lys13FITC)-TAMRA. (D) K4(Lys20FITC)-TAMRA. Left panels: raw data; Right panels: normalized at the fluorescein-derived peak.

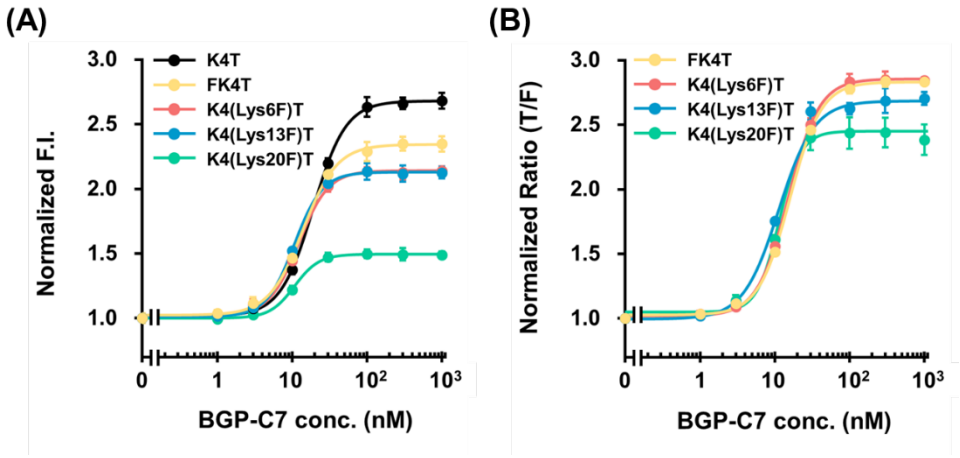

Figure S12. The effect of distance between Fluorescein and TAMRA on K4 peptide. Dose-responses of (A) irradiation-based and (B) FRET-based fluorescence. Averages ±1 SD are shown. Protein concentration: 5 nM.