Supplementary Information for

Affimer-enzyme-inhibitor switch sensor for rapid wash-free assays of multimeric proteins

Hope Adamson[1,2], Modupe O. Ajayi[2,3], Emma Campbell[1,2], Erika Brachi[1,2], Christian Tiede[2,3], Anna A. Tang[2,3], Thomas L. Adams[2,3], Robert Ford[4], Alex Davidson[4], Matt Johnson[4], Michael J. McPherson[2,3], Darren C. Tomlinson[2,3,*] and Lars J.C. Jeuken[1,2,*]

[1] School of Biomedical Sciences, University of Leeds, Leeds, LS2 9JT, UK
[2] Astbury Centre for Structural Molecular Biology, University of Leeds, Leeds, LS2 9JT, UK
[3] School of Molecular and Cellular Biology, University of Leeds, Leeds, LS2 9JT, UK
[4] Avacta Life Sciences Ltd, Unit 20, Ash Way, Thorp Arch Estate, Wetherby, LS23 7FA, UK
* Correspondence to D.C.Tomlinson@leeds.ac.uk; L.J.C.Jeuken@leeds.ac.uk

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Experimental Section

Affimer selection and validation

Affimer binders against Herceptin and CPMV have previously been selected and validated.[1,2] Affimer binders against hCRP were selected as follows.

hCRP Target QC

Recombinant human CRP was purchased from Lee Biosolutions (140-11R). Target quality and purity was assessed by SDS-PAGE, then biotinylation was performed and confirmed by western blot, as previously described.[2]

Phage Display

Phage display was performed using three rounds against biotinylated human CRP. 1 x PBS-Tween20 (0.1% v/v) pH 7.4 was used as buffer. Pan 1 was subjected to 3 x 40 mins pre-panning against streptavidin coated plates before immobilising biotinylated hCRP at 10 µg ml^{-1} and incubating the phage for 2 h at RT. Unbound phage were removed by washing 27 times before being eluted by first reducing the pH using glycine pH 2.2 (followed by neutralisation with 1 M Tris pH 9.1) then by increasing the pH using triethylamine (and neutralisation with 1 M Tris pH 7.0) and finally using 0.1 mg ml^{-1} bovine trypsin. Pan 2 was performed with biotinylated hCRP (10 µg ml^{-1}) immobilised on Dynabeads™ MyOne™ Streptavidin T1 (ThermoFisher), with 2 x 1 h pre-panning and a 1 h incubation at RT of phage eluted from pan 1. Unbound phage were washed off using the KingFisher Flex Magnetic Particle Processor for 4 x 1 min. Elution was also carried out by the KingFisher, using identical conditions as described in Pan 1. Pan 3 was performed with biotinylated hCRP immobilised on a neutravidin coated Maxisorp plate, with 4 x 1 h pre-panning and 30 min target incubation. Unbound phage were removed by washing 27 times before being eluted as before.

Primary Screen

The Affimer coding regions resulting from panning rounds 2 and 3 were sub-cloned into pEtLECTRA vector cHA-His6, expressed in 1 ml cultures, purified using Ni-NTA resin and the 192 variants were screened using the iQue Screener (IntelliCyt), as previously described.[2] Briefly, biotinylated targets and deselection targets were immobilised onto QSH DevScreen iQue beads and Affimer binding quantified using Alexa488-conjugated Anti-HA antibody (BioLegend). Clones were also sequenced to identify unique sequences.

Affimer Protein Expression

Medium scale recombinant Affimer production (50 – 100 ml) was performed to generate 1 – 2 mg of Affimer material, as previously described.[2] Briefly, 100 ml Terrific Broth (Melford) with 50 µg ml^{-1} kanamycin was inoculated with 1 ml starter culture, grown at 37°C, 220 rpm until OD_{600} = 0.6 - 0.8, then induced with 1 mM IPTG and incubated at 25°C, 220 rpm overnight. Cells were harvested (10000 g, 10 min, 4°C), resuspended in lysis buffer (100 mM sodium phosphate, 150 mM NaCl, 10 % SoluLyseTM (Genlantis), Benzonase(R) (Novagen; 25 U/mL), 20 mM Imidazole, pH 8), sonicated for 5 min (10 s on, 10 s off) and filtered (0.45 µm). Protein was purified using Ni-NTA beads in an automated process (washed in 50 mM imidazole, eluted in 400 mM imidazole) and then buffer exchanged into PBS pH 7.4 + 0.02 % sodium azide for storage.

ELISA Validation

Selected Affimer binders were tested with a validation ELISA, as previously described.[2] Affimer binders selected from the validation ELISA were then tested in a sandwich ELISA. They were passively adsorbed onto Maxisorp plates at 1 µg ml^{-1} overnight at 4°C. Plates were

washed with 1x PBS-T (PBS diluted from 10x PBS; Gibco + 0.05 % Tween 20; Sigma. 3 x 300 µL per well on a BioTek 405 plate washer) and blocked with 1 x casein blocking buffer (diluted from 10x casein blocking buffer; Sigma) for 2 h at room temperature with gentle agitation, before incubation with titrated target (2-fold dilution from 20 µg ml^{-1}). The plate was washed again and bound target was detected with biotinylated Affimer (1 µg ml^{-1}) and streptavidin poly-HRP (Pierce), then visualised using TMB (Surmodics). TMB incubation time was 10 mins, and ODs were read at 450 and 630 nm.

Validation of binding to hCRP from human plasma

Four positive clones were identified from the prior ELISA validation (25, 27, 81, 90) and Affimer binding to hCRP from human plasma (in addition to recombinant hCRP) was confirmed by further ELISA analysis. The Affimer coding regions were subcloned into a pet11a vector, with c-terminal cysteine and $(His)_8$-tag, then expressed, purified and biotinylated for analysis, as previously described.[3] All protein concentrations were measured by BCA assay using Pierce BCA protein assay reagent (ThermoFisher).

Direct ELISA. A 50 µl aliquot of hCRP from human plasma (Sigma) at 10, 1 or 0 µg ml^{-1} in PBS was adsorbed onto a Nunc Maxisorp plate (ThermoFisher) for 1 h then washed thrice with PBS buffer containing 0.05 % v/v Tween 20 (PBST). Wells were then blocked with 1X casein (Sigma) in PBST (blocking buffer) for 1 h before washing thrice with PBST. Biotinylated Affimer in blocking buffer (50 µl, 2 µg ml^{-1}) was applied for 1 h then washed thrice with PBST before 25 µl of 1 µg ml^{-1} Streptavadin-HRP (Pierce high sensitivity, Thermo) in blocking buffer was added for 1 h. Wells were then washed six times with PBST before addition of 50 µl 3,3',5,5'-tetramethylbenzidine (TMB liquid substrate system, Sigma). Absorbance was measured at 650 nm after 14 min with a plate reader (Multiskan FC, Thermo).

Sandwich ELISA. A 50 µl Affimer aliquot at 10 or 0 µg ml^{-1} in PBS was adsorbed on a Nunc Maxisorp plate for 1 h then washed thrice with PBST. Blocking buffer was applied for 1 h, then washed thrice with PBST before addition of 50 µl of 10 or 0 µg ml^{-1} hCRP from human plasma (Sigma) in blocking buffer for 1 h. Wells were washed thrice with PBST and then biotinylated Affimer, Streptavidin-HRP and TMB were applied exactly as in the direct ELISA. Absorbance was measured at 650 nm after 13 min.

Surface plasmon resonance (SPR)

Affimer affinities for their target analyte were determined by surface plasmon resonance (SPR) using a BIAcore 3000 (GE Healthcare Europe GmbH). Herceptin, hCRP and CPMV were covalently immobilized on separate channels of a CM5 sensor chip with amine-coupling chemistry. The chip was activated with 50 mM N-Hydroxysuccinimide (NHS) and 200 mM 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) before target injection under optimised conditions (Herceptin, 2 µg ml^{-1} in 10 mM acetate pH 5.5; hCRP, 5 µg ml^{-1} in 10 mM acetate pH 5.0; CPMV 10 µg ml^{-1} in 10 mM acetate pH 4.0). Remaining reactive groups were deactivated with ethanolamine (1 M, pH = 8.5). Biacore experiments were performed at 25°C in PBST buffer (PBS pH 7.4, containing 150 mM NaCl and 0.05 % Tween 20). Affimers were injected at 6.25, 12.5, 25, 50, 100, 200, 400, and 800 nM at a flow rate of 5 µl min^{-1}, followed by 3 min stabilization and 10 min dissociation. The on- and off- rates and K_d parameters were obtained from a global fit to the SPR curves using a 1:1 langmuir model with drifting baseline, using the BIAevaluation software. Quoted K_d values are the mean ± SEM of three replicate runs.

Sensor cloning and mutagenesis

All primers (Integrated DNA Technologies) used for cloning and mutagenesis are shown in the Supplementary Information (Tables S1 to S4). The synthetic DNA sequence encoding BB_HA (Supplementary Information) was purchased from Genscript in a pET28a vector. Restriction cloning was used to introduce Affimer A (with L1) using SpeI / SalI, introduce Affimer B (with L3) using NheI / NotI and exchange linker 2 using SalI / NheI. The sensor construct vector was digested with the appropriate restriction enzymes (NEB), dephosphorylated with antarctic phosphatase (NEB), separated on an agarose gel and then purified. All DNA was purified using the Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare). Affimer insert DNA was PCR amplified with primers to introduce the appropriate restriction sites and L1 = TSA, TSAA, TSAASS or L3 = AAA, SAAA, ASSAAA linkers. DpnI was then used to remove parental vector DNA. DNA encoding inserts L2 = $(GSG)_5$ and $(GSG)_7$ was generated by PCR of two overlapping primers. PCR amplified DNA was digested with the appropriate restriction enzymes (NEB) and then purified. DNA encoding insert L2 = $(GSG)_{20}$ was purchased from Genscript in pUC57, cut from the vector with the appropriate restriction enzymes (NEB), separated on an agarose gel and then purified. The digested vector and insert were ligated with T4 DNA ligase (NEB) and transformed into *E. coli XL-1* competent cells (Agilent Technologies). The proline rich (L1 = AP, APAP, APAPEPAP, $(APAPEP)_2$) or helical (L1 = $(EAAAK)_2G$, $(EAAAK)_3P$) linkers were introduced using appropriate primers and the Q5 Site-Directed Mutagenesis Kit (NEB), according to the manufacturer's instructions. This kit was also used to generate a plasmid encoding TEM1-β-lactamase E104D (BLA) only, by introducing a stop codon after the BLA gene, using the appropriate primers. Plasmid DNA was purified using the ChargeSwitch Pro Plasmid Miniprep Kit (Invitrogen) and successful cloning and mutagenesis was confirmed by sequencing the full sensor construct (Genewiz).

Sensor expression and purification

All sensor constructs were produced and purified according to a modified version of the method outlined by Banala et al,[4] exploiting both His-tag and Strep-tag purification steps. BLA was purified using only His-tag purification. The appropriate pET28a vector was transformed into *E. coli* BL21 (DE3) cells. A 10 ml starter culture was added to 500 ml LB media (with 50 µg ml^{-1} kanamycin) and grown at 37°C before induction at OD_{600} ca. 0.6 with 0.3 mM isopropyl-β-D-thiogalactoside (IPTG) and overnight growth at 15°C. Cells were harvested at ca. 4000 *g* for ca. 20 min and then periplasmic protein was extracted by osmotic shock, as follows. The cell pellet was resuspended in 15 ml TSE buffer (30 mM Tris, 20 % w/v sucrose, 1 mM EDTA, pH 8) and placed on a roller mixer at 4°C for 15 min. Cells were pelleted at ca. 17000 *g* for 20 min and the supernatant retained as periplasmic fraction 1 (P1). The pellet was resuspended in 15 ml of ice cold 5 mM $MgSO_4$ and placed on a roller mixer at 4°C for 20 min. Cells were pelleted at ca. 17000 *g* for 20 min and the supernatant retained as periplasmic fraction 2 (P2). Fractions P1 and P2 were combined, adjusted to 50 mM Tris, 150 mM NaCl, 20 mM imidazole, pH 7.4 by addition of small amounts of concentrated stock solution and incubated with 500 µl Super Ni-NTA resin (Generon) on a roller mixer for 1 h at room temperature. The resin was washed thrice with 20 ml wash buffer (50 mM Tris, 150 mM NaCl, 20 mM imidazole, pH 7.4) and protein eluted with 4 X 500 µl elution buffer (50 mM Tris, 150 mM NaCl, 250 mM imidazole, pH 7.4). Protein-containing eluates were pooled and sensor constructs further purified using the Strep-Tactin spin column kit (IBA). Purified protein was then buffer exchanged into 50 mM Tris, 150 mM NaCl, pH 7.4 for storage using Zeba spin desalting columns (ThermoFisher). Protein concentration was determined by BCA assay and aliquots stored at -80°C.

Sensor characterisation

Targets. Antibody targets were anti-HA-tag antibody (HA Tag Mouse anti-Tag, Clone: 2-2.2.14, Invitrogen), Herceptin (Roche) and non-specific IgG mix from human serum (Sigma). Human C-reactive protein (hCRP) from human serum and control lysozyme protein from chicken egg white were purchased from Sigma. Cowpea mosaic virus (CPMV) was prepared as previously described[1] and protein concentration measured by BCA assay.

ELISAs. A 40 µl aliquot of hCRP at 20, 5 or 0 µg ml^{-1} in PBS was adsorbed onto a Nunc Maxisorp plate for 1 h then washed thrice with PBST. Blocking buffer was applied for 1 h before washing thrice with PBST. Sensor at 10 or 5 µg ml^{-1} in blocking buffer (40 µl) was applied for 1 h then washed thrice with PBST before 25 µl of 1 µg ml^{-1} Streptactin-HRP (IBA) in blocking buffer was added for 1 h. Wells were then washed six times with PBST before addition of 50 µl TMB. Absorbance was measured at 650 nm with a plate reader (Multiskan FC, Thermo). All steps were performed at room temperature.

Nitrocefin assays. In a non-binding surface 96-well assay plate (Corning) and a final volume of 200 µl assay buffer (50 mM sodium phosphate, 100 mM NaCl and 1 mg ml^{-1} BSA, pH 7), 2 nM sensor was incubated with serial dilutions of target for the stated period of time. Nitrocefin (Merck) was then added (at 50 µM) and absorbance monitored at 551 nM on a plate reader (Multiskan FC, Thermo). All assays were performed at room temperature. A four-parameter logistic (4PL) regression was fit to dose response curves using OriginPro 2016 or GraphPad Prism 8 software. The definitions outlined by Armbruster and Pry were used to calculate the limit of detection (LoD) = $\text{mean}_{\text{blank}}$ + 1.645(SD_{blank}) + 1.645($\text{SD}_{\text{low conc. test}}$).[5] Blank measurements with both non-specific and zero target were included in this calculation. Rather than giving an extrapolated LoD concentration, the lowest test concentration explicitly measured above this is given. The fold-activity gain of the sensor with target compared to without is calculated at a particular time = *t* using Equation 1.

$$\frac{A_{551}(target,\ t) - A_{551}(target,\ t=0)}{A_{551}(zero\ target,\ t) - A_{551}(zero\ target,\ t=0)} = \frac{\Delta A_{551}(target)}{\Delta A_{551}(zero\ target)} \quad (1)$$

Patient sample assays. Ten human plasma samples in K3-EDTA anticoagulant were obtained commercially from Clinical Trials Laboratory Services. In a non-binding plate and a final volume of 200 µl assay buffer, 2 nM BB_hCRP was incubated for 10 min with 2 µl patient plasma or 2 µl hCRP-free human serum (Aviva Systems Biology) spiked with hCRP. Nitrocefin was then added (at 50 µM) and absorbance monitored at 486 nM on a plate reader (Tecan Spark). The mean hCRP-spiked measurements were fit to a 4PL regression and for each individual measurement the concentration was interpolated back from the curve using GraphPad Prism 8 software. Accuracy and precision were assessed by recovery (%) = (mean interpolated concentration / nominal concentration) X 100 % and coefficient of variation (% CV) = (standard deviation of interpolated concentration / mean interpolated concentration) X 100 %. Patient plasma hCRP concentrations were also interpolated from the curve and compared to measurements with an ELISA kit (CRP human ELISA kit, Invitrogen), performed to manufacturer's instructions. The hCRP concentration for calibrations was confirmed by BCA assay and A_{280} measurement (DeNovix DS-11) using an absorption coefficient of 1.75 (1 mg ml^{-1}, 280 nm, 1 cm path length).[6]

CCF2-FA assay. In a non-binding plate and a final volume of 200 µl assay buffer with 0 or 10 % hCRP-free human serum, 2 or 20 nM BB_hCRP was incubated with serial dilutions of hCRP.

Then CCF2-FA (Fisher scientific) was added (at 2 µM) and fluorescence monitored at Ex. 409 nM, Em. 447 nM on a plate reader (Tecan Spark).

Leaf sample assay. CPMV-infected and uninfected negative control *N. benthamiana* leaves were prepared as previously described.[1] Crude leaf extracts were prepared using the P-PER plant protein extraction kit (ThermoFisher), according to manufacturer instructions. These samples were tested blind as labelled samples numbered 1-12. In a non-binding plate and a final volume of 200 µl assay buffer, 2 nM BB_CPMV was incubated with leaf extract (5 % v/v) for 15 min. Then nitrocefin was added (at 50 µM) and absorbance monitored at 551 nM on a plate reader (Multiskan FC, Thermo). Following the production of assay results the identity of the samples as either healthy or infected was revealed.

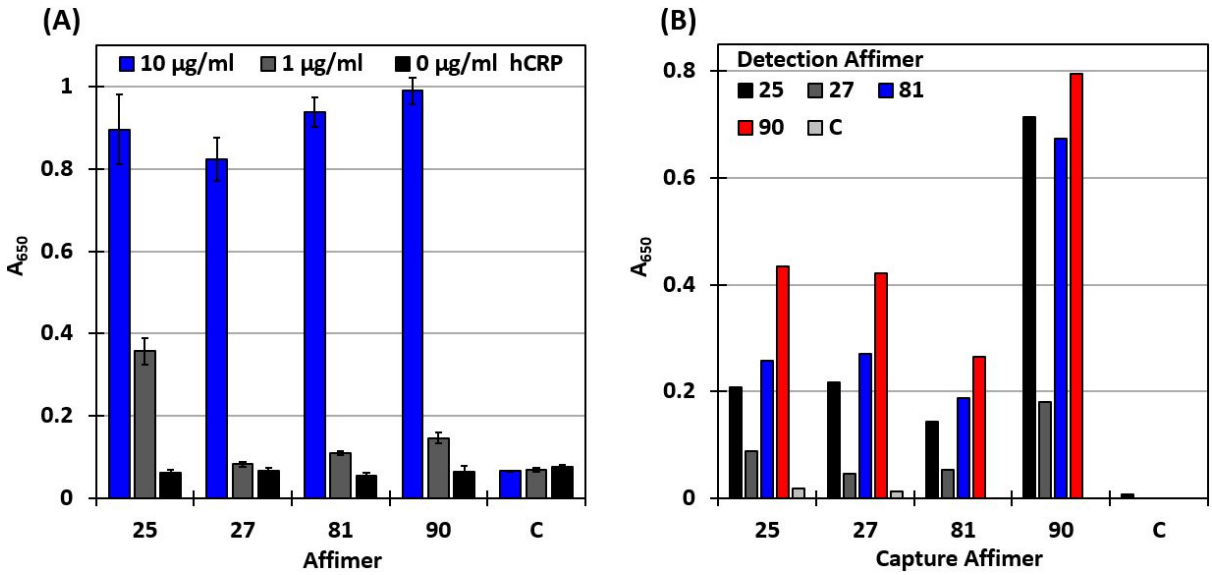

Figure S1. ELISAs to assess binding of hCRP to Affimers 25, 27, 81, 90 and non-specific control Affimer C (A) Direct ELISA to confirm binding of 2 µg ml^{-1} biotinylated Affimer to hCRP, absorbed to a maxisorp plate at the stated concentrations. Detection with streptavidin-HRP and visualisation with TMB, read at 650 nm after 14 minutes. Data are the mean of triplicate measurements on the same plate and error bars indicate standard deviation from the mean. **(B) Sandwich ELISA to assess pairwise binding** of "capture" Affimer adsorbed to a maxisorp plate at 10 µg ml^{-1} and 2 µg ml^{-1} biotinylated "detection" Affimer with 10 µg ml^{-1} hCRP. Detection with streptavidin-HRP and visualisation with TMB, read at 650 nm after 13 minutes. Absorbance values are corrected for the background absorbance with 0 µg ml^{-1} hCRP.

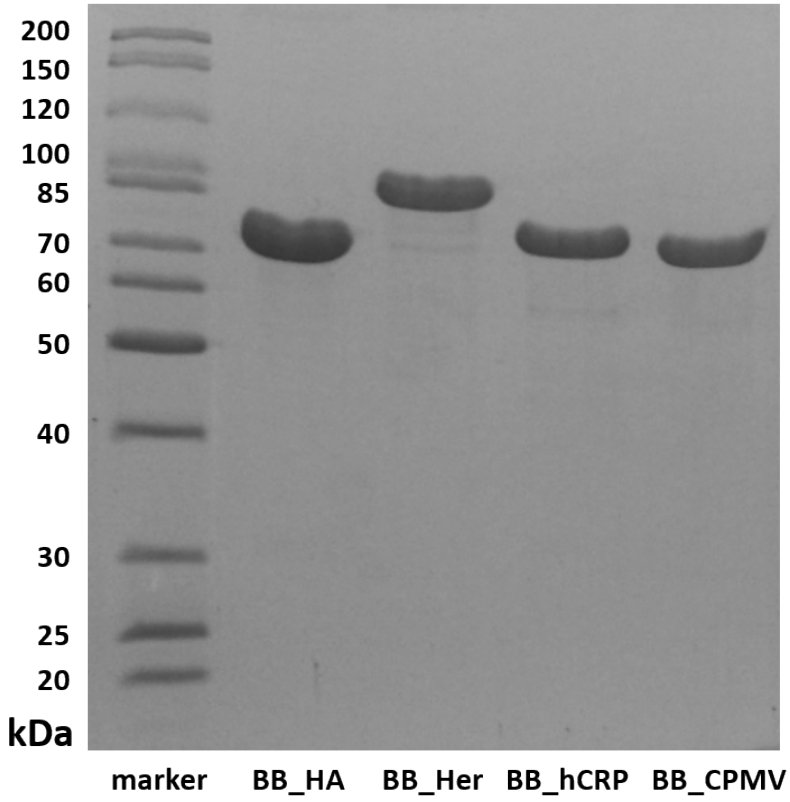

Figure S2. SDS-PAGE gel of ca. 2.5 µg sensor (BB_HA, BB_Her, BB_hCRP, BB_CPMV)

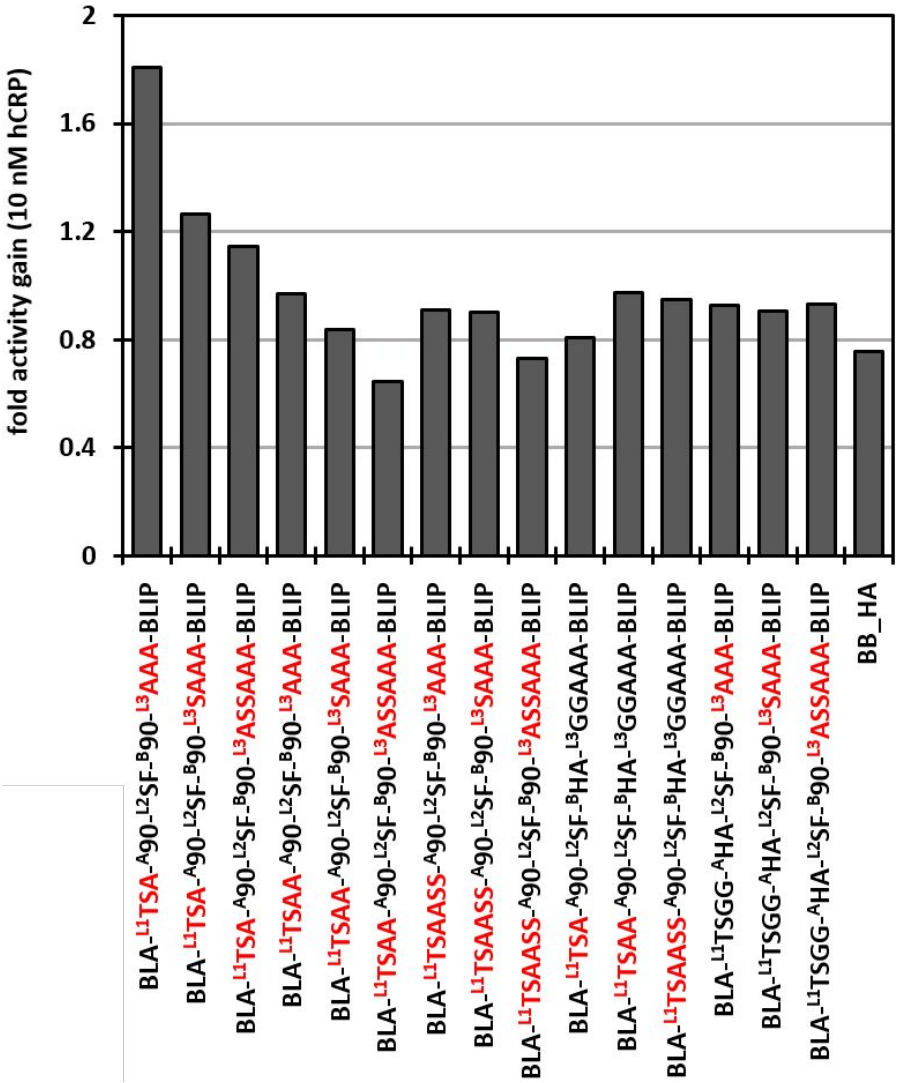

Figure S3. Effect of L1 and L3 and inclusion of one or two Aff-hCRP90 (90) Affimers on the fold activity gain of sensors with 10 nM hCRP 2 nM sensor, 0 or 10 nM hCRP, 30 minutes incubation, 50 µM nitrocefin addition, fold activity gain = ΔA_{551} (10 nM hCRP) / ΔA_{551} (0 nM hCRP) read after a further 30 minutes.

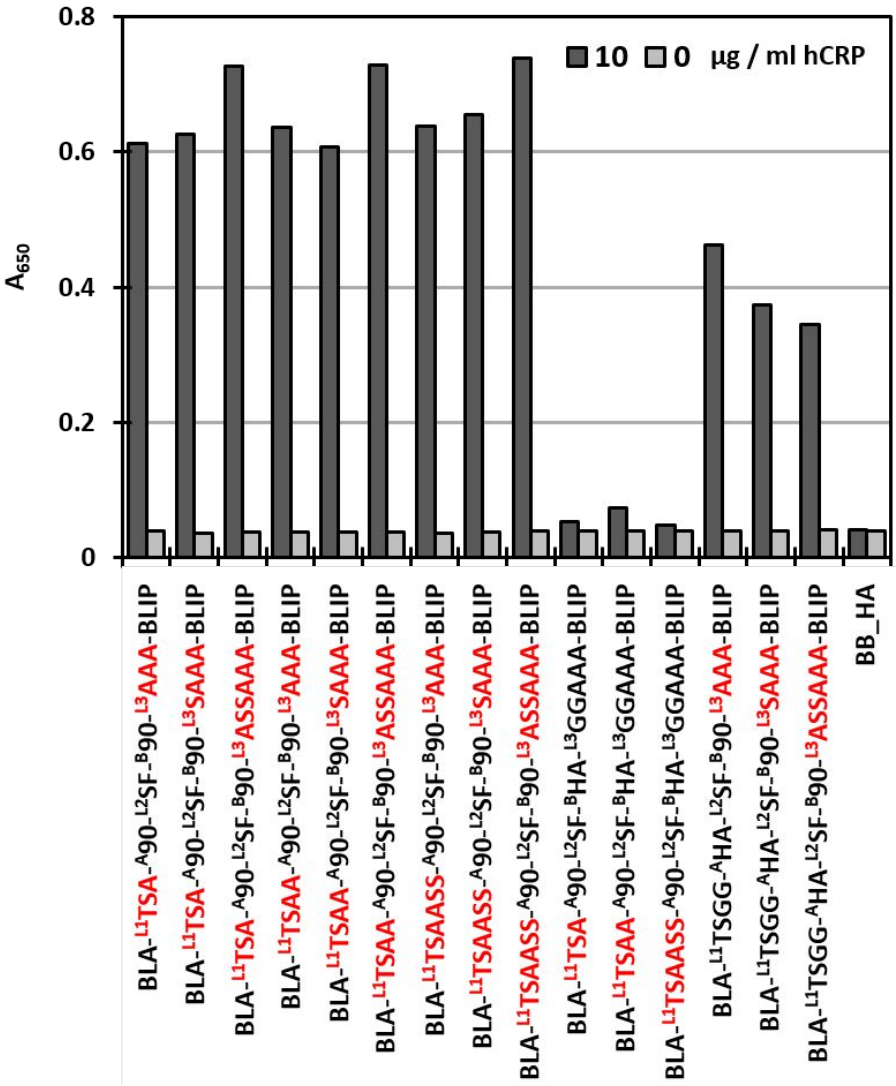

Figure S4. ELISA analysis of the effect of L1 and L3 and inclusion of one or two Aff-hCRP90 (90) Affimers on construct binding to hCRP 10 or 0 µg ml^{-1} hCRP adsorbed to maxisorp plate, 5 µg ml^{-1} sensor bound, detection with 1 µg ml^{-1} streptactin-HRP, visualised with TMB and read at 650 nm after 5 minutes.

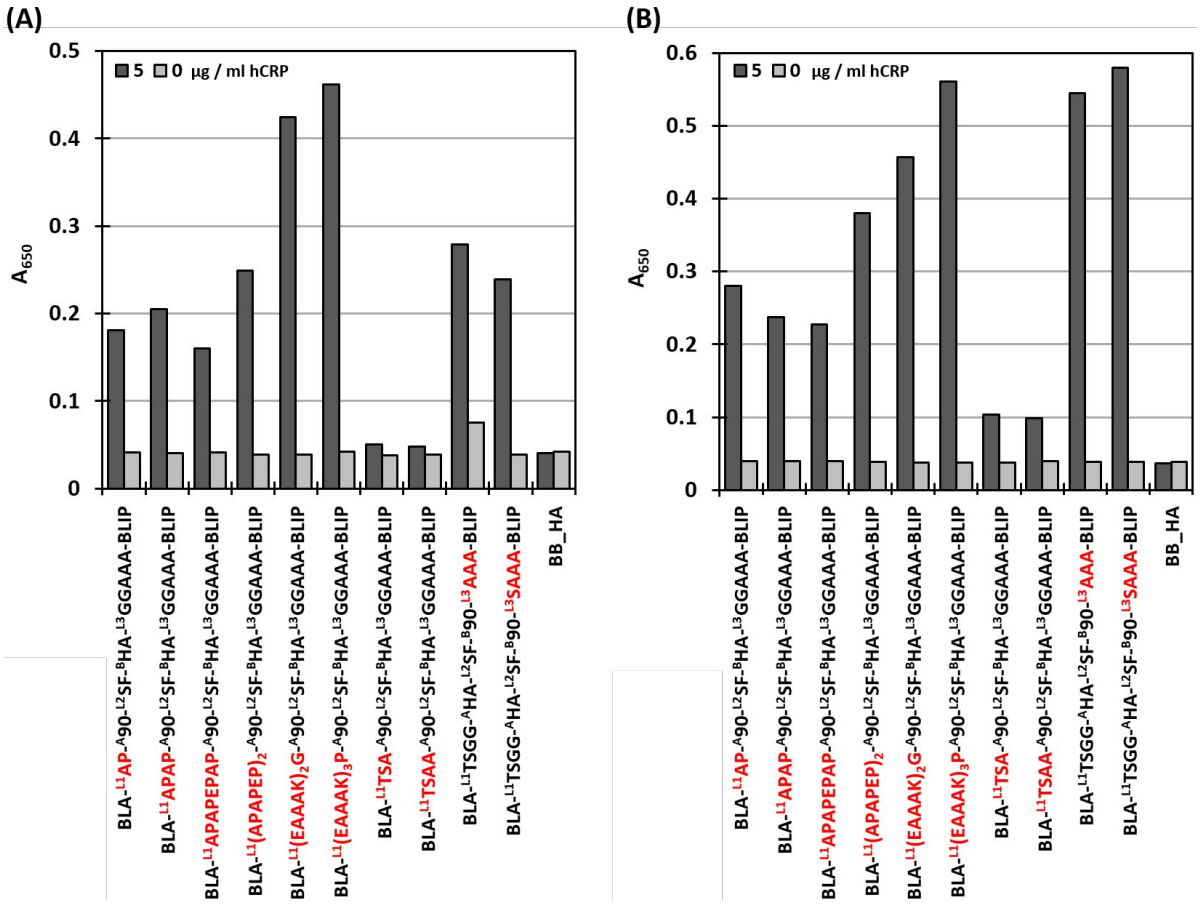

Figure S5. ELISA analysis of the effect of L1 on hCRP binding to Affimer site A using (A) streptactin-HRP and (B) anti-His-HRP detection 5 or 0 µg ml^{-1} hCRP adsorbed to maxisorp plate, 5 µg ml^{-1} sensor, 1 µg ml^{-1} detection, visualised with TMB and read at 650 nm after 10 minutes.

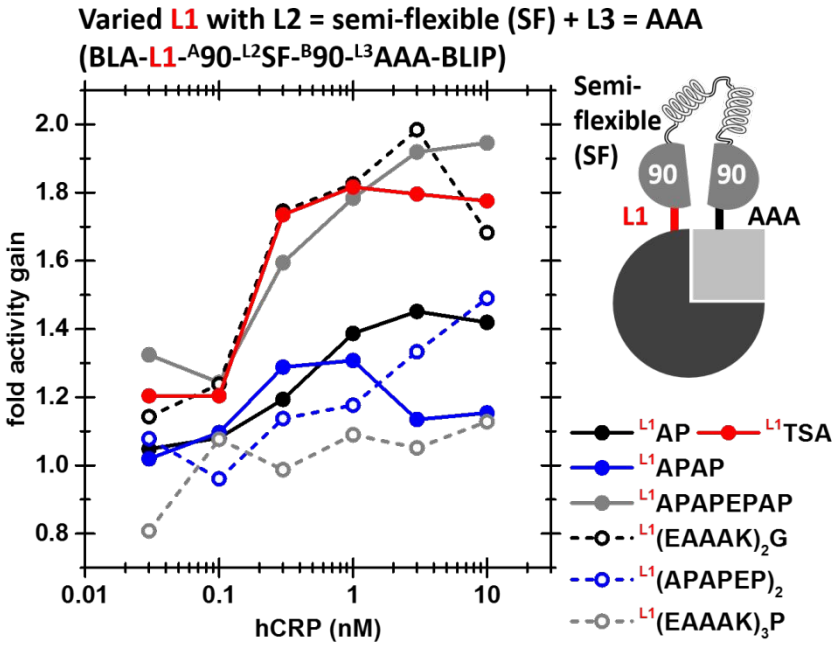

Figure S6. Effect of L1 on dose response of sensors to hCRP 2 nM sensor, 30 minutes incubation, 50 µM nitrocefin addition, fold activity gain = ΔA_{551} (hCRP) / ΔA_{551} (0 nM hCRP) read at 10 minutes.

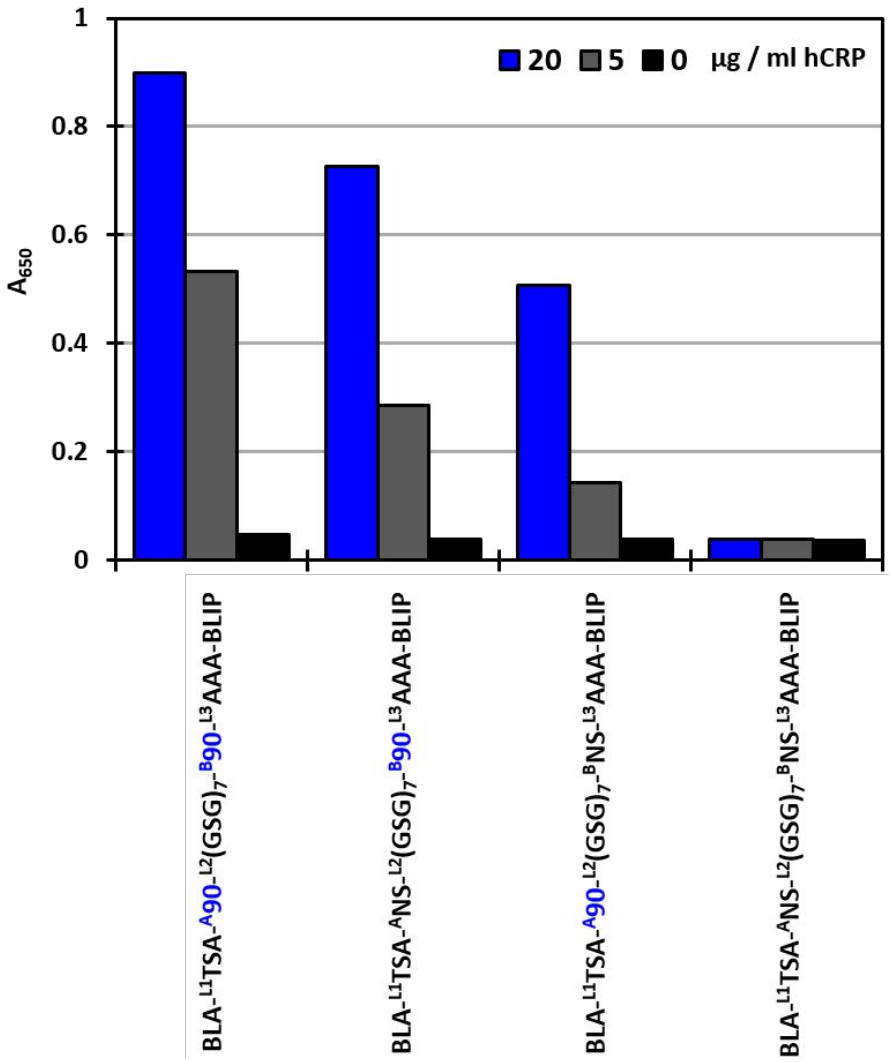

Figure S7. ELISA analysis of hCRP binding to constructs with L2 = $(GSG)_7$ and two, one or zero Aff-hCRP90 (90) Affimers 20 µg ml^{-1} hCRP (blue), 5 µg ml^{-1} hCRP (grey) and 0 µg ml^{-1} hCRP (black) adsorbed to maxisorp plate, 10 µg ml^{-1} construct bound, detection with 1 µg ml^{-1} streptactin-HRP, visualised with TMB and read at 650 nm after 5 minutes.

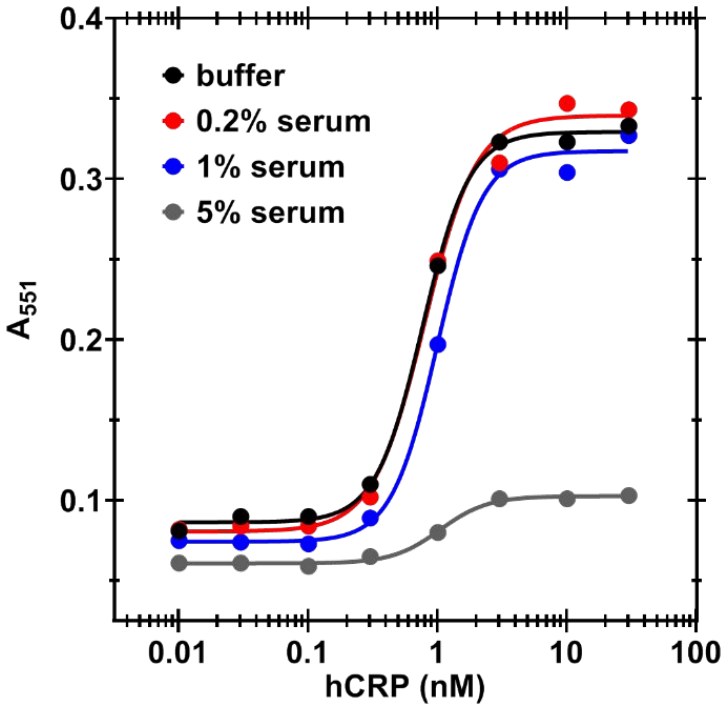

Figure S8. Effect of human serum on the dose response of BB_hCRP to hCRP 2 nM BB_hCRP, 0 minutes incubation, 50 µM nitrocefin addition, A_{551} read after 4 minutes.

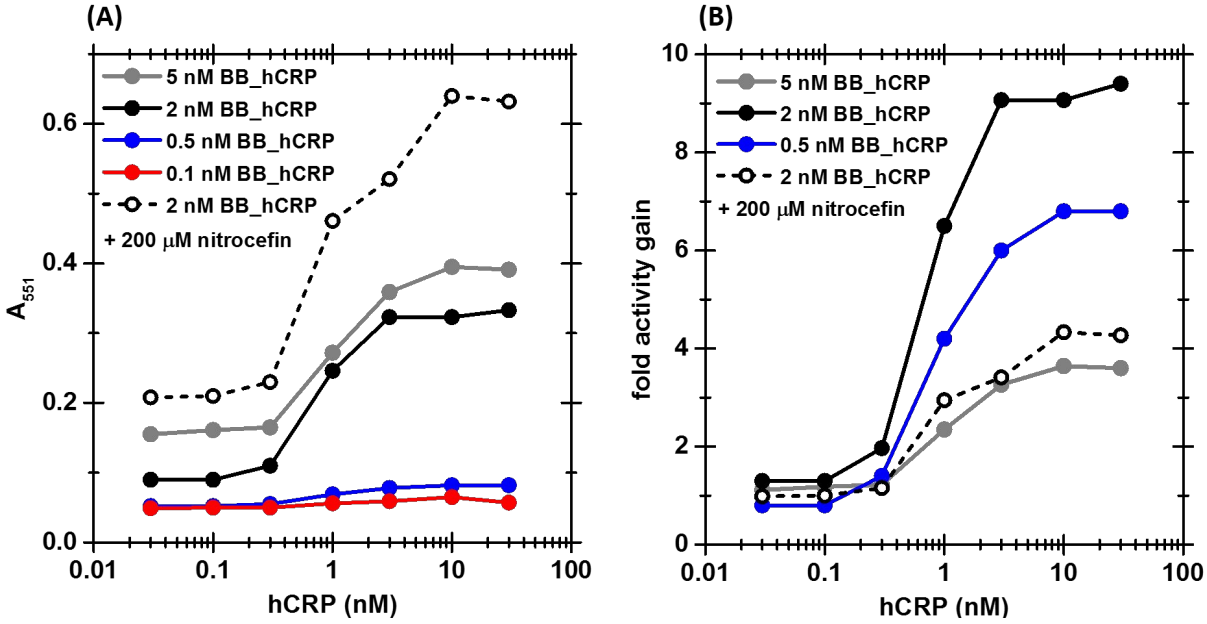

Figure S9. Effect of sensor and nitrocefin concentration on the dose response of BB_hCRP to hCRP with (A) A_{551} measurement (B) Fold activity gain = ΔA_{551} (hCRP) / ΔA_{551} (0 nM hCRP) 0.1 – 5 nM BB_hCRP, 0 minutes incubation, 50 µM nitrocefin (solid lines) or 200 µM nitrocefin (dash line) addition, A_{551} read after 4 minutes.

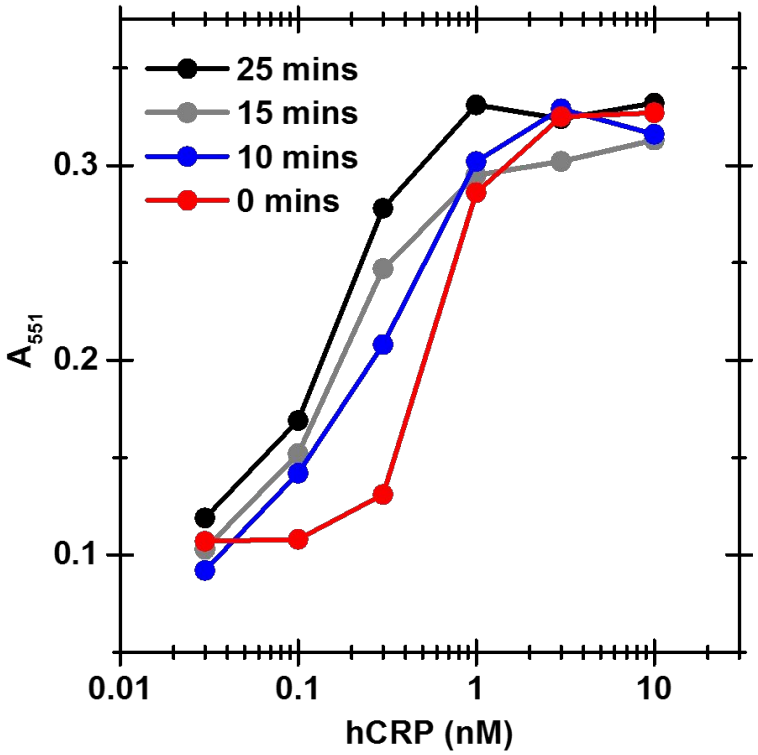

Figure S10. Effect of incubation time on the dose response of BB_hCRP to hCRP 2 nM BB_hCRP, 0 - 25 mins incubation with hCRP before 50 µM nitrocefin addition, A_{551} read after a further 10 minutes.

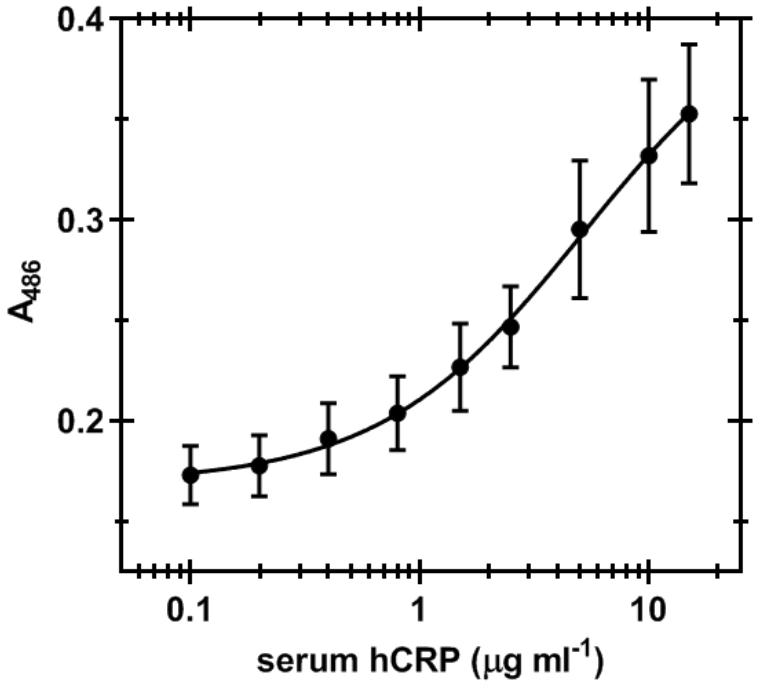

Figure S11. Calibration curve for quantification of hCRP spiked in human serum 2 nM BB_hCRP incubated with hCRP spiked in hCRP-free serum (diluted 100 fold) for 10 minutes before 50 µM nitrocefin addition, A_{486} read after a further 10 minutes. Data are the mean of eight independent measurements and error bars indicate standard deviation from the mean.

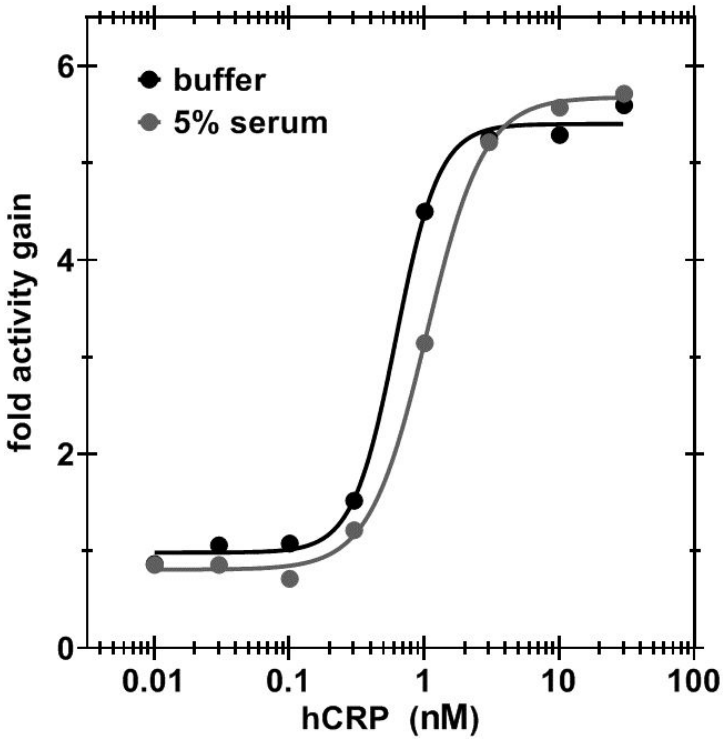

Figure S12. Dose response of BB_hCRP to hCRP in 5 % human serum matrix 2 nM BB_hCRP, 0 minutes incubation, 50 µM nitrocefin addition, fold activity gain = ΔA_{551} (hCRP) / ΔA_{551} (0 nM hCRP) read at 10 minutes.

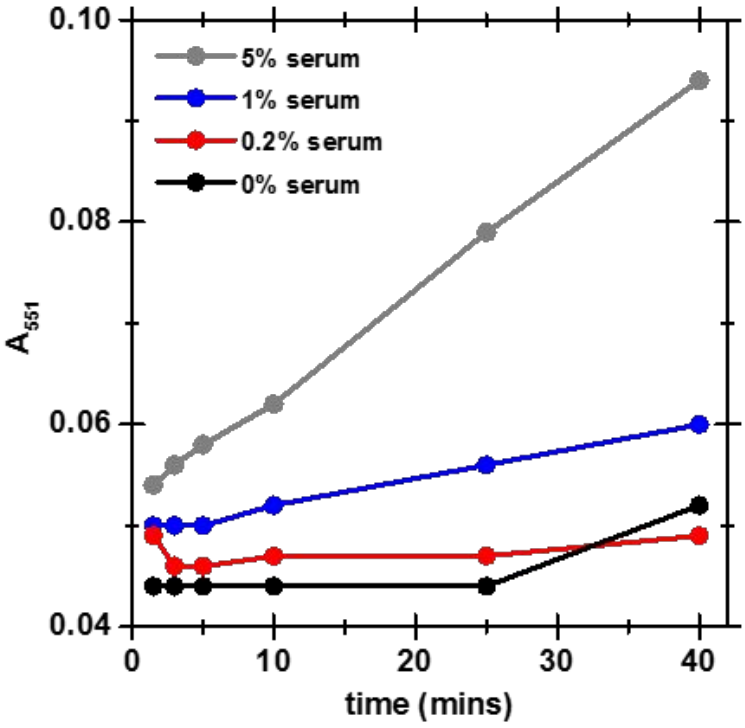

Figure S13. Breakdown of 50 µM nitrocefin in serum

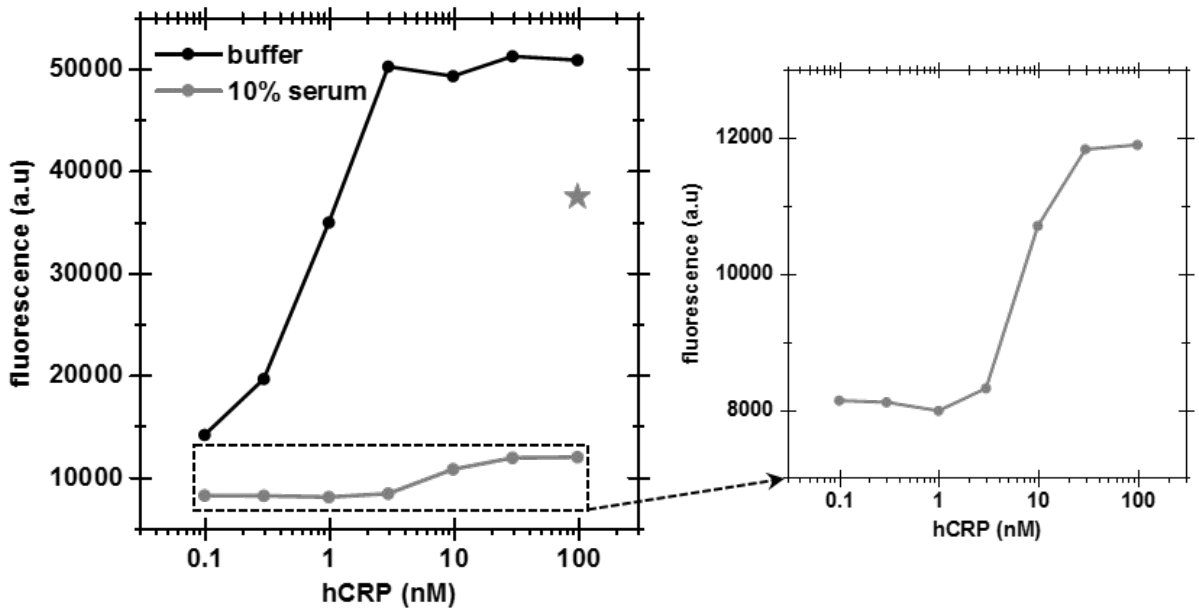

Figure S14. Dose response of BB_hCRP to hCRP in 10 % serum matrix with CCF2-FA substrate 2 nM BB_hCRP in buffer, 20 nM BB_hCRP in 10 % serum, 0 minutes incubation, 2 µM CCF2-FA addition, fluorescence (Ex 409 nm, Em 447 nm) read at 33 minutes. Grey star shows degree of quenching upon addition of 10 % serum to a solution with initial ~ 50000 a.u. fluorescence.

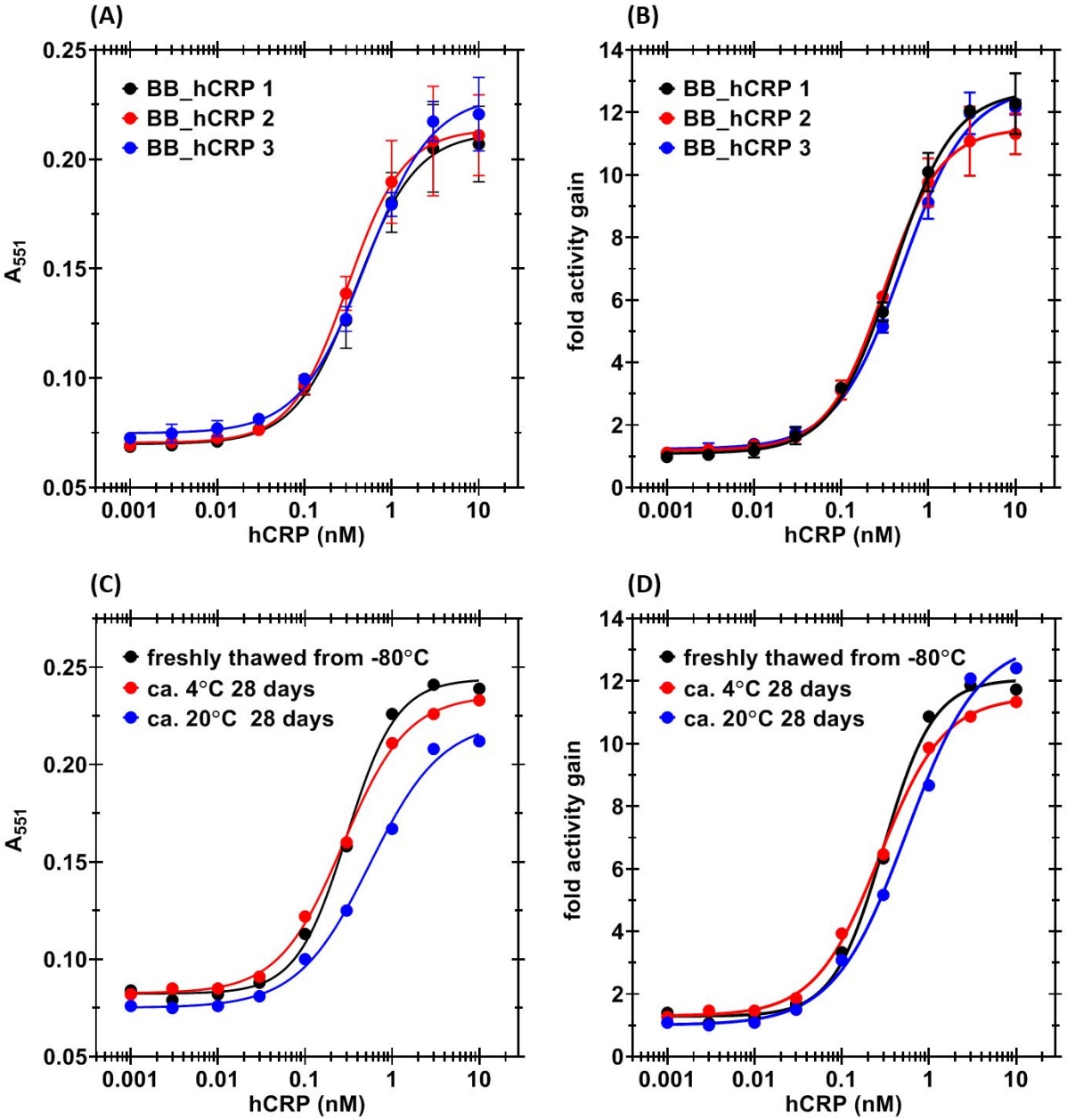

Figure S15. Batch-to-batch reproducibility and stability of BB_hCRP dose response to hCRP as (A), (C) A_{551} and (B), (D) Fold activity gain 2 nM BB_hCRP, 10 minute incubation, 50 µM nitrocefin addition, read at 5 minutes. Solid lines are 4PL regression fits. For (A) and (C) data points are the mean of triplicates in one assay and error bars indicate standard deviation from the mean.

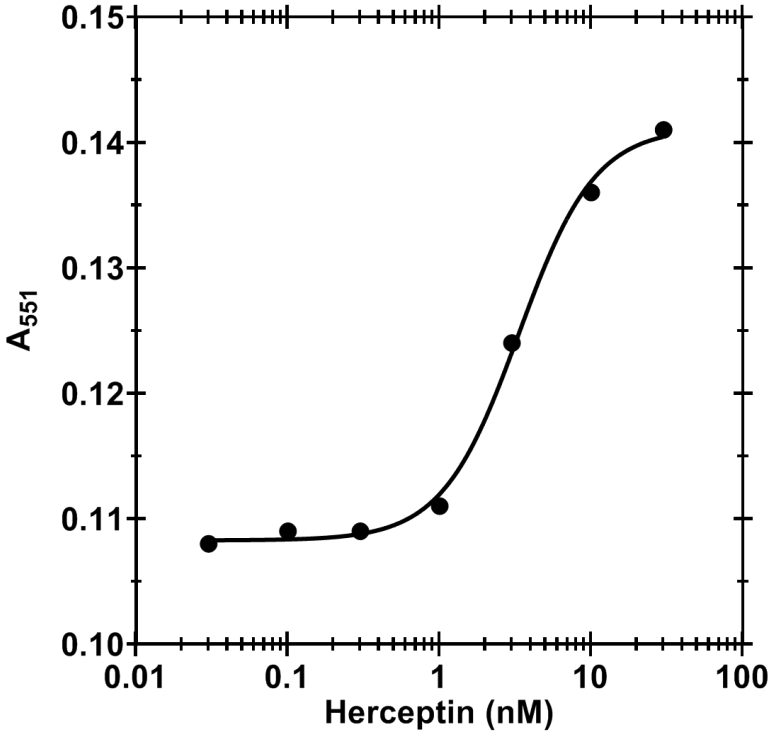

Figure S16. Dose response of BLA-^{L1}TSA-^{A}Aff-Her-^{L2}(GSG)$_7$-^{B}Aff-Her-^{L3}AAA-BLIP to Herceptin 2 nM sensor, 10 minute incubation, 50 µM nitrocefin addition, A_{551} read after a further 15 minutes. Solid line is a 4PL regression fit.

DNA and Protein sequence for BB_HA

Red – leader sequence
Blue – His-tag
Green – thrombin cleavage site
Yellow highlight – TEM1-β-lactamase (E104D)
Light grey highlight – linker 1 (L1)
Pink – HA-tag epitope
Green highlight – semi-flexible linker 2 (L2)
Dark grey highlight – linker 3 (L3)
Cyan highlight – β-lactamase inhibitor protein (E31A)
Orange – Strep-tag

DNA

ATGGCTTCTATCCAGCACTTCCGTGTTGCTCTGATCCCGTTCTTCGCTGCTTTCTGCCTGCCGGTTTTCGCTGCTCACCACCA
CCACCACCACCACCACCTGGTTCCGCGTGGTTCTCACCCGGAAACCCTGGTTAAAGTTAAAGACGCTGAAGACCAGCTGG
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Protein

MASIQHFRVALIPFFAAFCLPVFAAHHHHHHHHLVPRGSHPETLVKVKDAEDQLGARVGYIELDLNSGKILESFRPEERFPMMS
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GVTLSLSCFDVDGYSSTGFYRGSAHLWFTDGVLQGKRQWDLVGGLGGWSHPQFEK

DNA and Protein sequences

For the Herceptin and hCRP sensors the Affimer variable region sequences are denoted XXX. The sequence and plasmids will be shared with any academic who doesn't have a commercial interest, under an MTA.

BB_Her:

Red – leader sequence
Blue – His-tag
Green – thrombin cleavage site
Yellow highlight – TEM1-β-lactamase (E104D)
Light grey highlight – linker 1 (L1)
Pink – Herceptin Affimer
Green highlight – semi-flexible linker 2 (L2)
Dark grey highlight – linker 3 (L3)
Cyan highlight – β-lactamase inhibitor protein (E31A)
Orange – Strep-tag

DNA

ATGGCTTCTATCCAGCACTTCCGTGTTGCTCTGATCCCGTTCTTCGCTGCTTTCTGCCTGCCGGTTTTCGCTGCTCACCACCA
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CTAAAGTTGACTCTAAATCTCAGGAAAAACTGCTGGCTCCGTCTGCTCCGACCCTGACCCTGGCTAAATTCAACCAGGTTA
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ACCCGTCTACCGCTGGTGTTACCCTGTCTCTGTCTTGCTTCGACGTTGACGGTTACTCTTCTACCGGTTTCTACCGTGGTTCT
GCTCACCTGTGGTTCACCGACGGTGTTCTGCAGGGTAAACGTCAGTGGGACCTGGTTGGTGGTCTCGGTGGTTGGTCTCA
CCCGCAGTTCGAAAAA

Protein

MASIQHFRVALIPFFAAFCLPVFAAHHHHHHHHLVPRGSHPETLVKVKDAEDQLGARVGYIELDLNSGKILESFRPEERFPMMS
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AKEAAAKEAAAKEAAAKEAAAKEAAAKAGSGGSGGSGGSGGSGGSGAEAAAKEAAAKEAAAKEAAAKEAAAKEAAAKAGSG
GSGGSGGSGGSGGSGGASNSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQXXXXXXXXTMYYLTLEAKDGGKKKLYEAKV
WVKXXXXXXXXXXNFKELQEFKPVAAAAGVMTGAKFTQIQFGMTRQQVLDIAGAENCATGGSFGDSIHCRGHAAGDYYAYAT
FGFTSAAADAKVDSKSQEKLLAPSAPTLTLAKFNQVTVGMTRAQVLATVGQGSCTTWSEYYPAYPSTAGVTLSLSCFDVDGYSS
TGFYRGSAHLWFTDGVLQGKRQWDLVGGLGGWSHPQFEK

BB_hCRP:

Red – leader sequence
Blue – His-tag
Green – thrombin cleavage site
Yellow highlight – TEM1-β-lactamase (E104D)
Light grey highlight – linker 1 (L1)
Pink – hCRP Affimer
Green highlight – $(GSG)_7$ linker 2 (L2)
Dark grey highlight – linker 3 (L3)
Cyan highlight – β-lactamase inhibitor protein (E31A)
Orange – Strep-tag

DNA

ATGGCTTCTATCCAGCACTTCCGTGTTGCTCTGATCCCGTTCTTCGCTGCTTTCTGCCTGCCGGTTTTCGCTGCTCACCACCA
CCACCACCACCACCACCTGGTTCCGCGTGGTTCTCACCCGGAAACCCTGGTTAAAGTTAAAGACGCTGAAGACCAGCTGG
GTGCTCGTGTTGGTTACATCGAACTGGACCTGAACTCTGGTAAAATCCTGGAATCTTTCCGTCCGGAGGAGAGGTTCCCG
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CTGTGCTCTGCTGCTATCACCATGTCTGACAACACCGCTGCTAACCTGCTGCTGACCACCATCGGTGGTCCGAAAGAACTC
ACTGCGTTCCTGCACAACATGGGTGACCACGTTACCCGTCTGGACCGTTGGGAACCGGAACTGAACGAAGCTATCCCGAA
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CTTCTCGTCAGCAGCTGATCGACTGGATGGAAGCTGACAAAGTTGCTGGTCCGCTGCTGCGTTCTGCTCTGCCGGCTGGTT
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GTTTCACCTCTGCTGCTGCTGACGCTAAAGTTGACTCTAAATCTCAGGAAAAACTGCTGGCTCCGTCTGCTCCGACCCTGA
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CCTGGTCTGAATACTACCCGGCTTACCCGTCTACCGCTGGTGTTACCCTGTCTCTGTCTTGCTTCGACGTTGACGGTTACTC
TTCTACCGGTTTCTACCGTGGTTCTGCTCACCTGTGGTTCACCGACGGTGTTCTGCAGGGTAAACGTCAGTGGGACCTGGT
TGGTGGTCTCGGTGGTTGGTCTCACCCGCAGTTCGAAAAA

Protein

MASIQHFRVALIPFFAAFCLPVFAAHHHHHHHHLVPRGSHPETLVKVKDAEDQLGARVGYIELDLNSGKILESFRPEERFPMMS
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KRQWDLVGGLGGWSHPQFEK

BB_CPMV:

Red – leader sequence
Blue – His-tag
Green – thrombin cleavage site
Yellow highlight – TEM1-β-lactamase (E104D)
Light grey highlight – linker 1 (L1)
Pink – CPMV Affimer
Green highlight – $(GSG)_7$ linker 2 (L2)
Dark grey highlight – linker 3 (L3)
Cyan highlight – β-lactamase inhibitor protein (E31A)
Orange – Strep-tag

DNA

ATGGCTTCTATCCAGCACTTCCGTGTTGCTCTGATCCCGTTCTTCGCTGCTTTCTGCCTGCCGGTTTTCGCTGCTCACCACCA
CCACCACCACCACCACCTGGTTCCGCGTGGTTCTCACCCGGAAACCCTGGTTAAAGTTAAAGACGCTGAAGACCAGCTGG
GTGCTCGTGTTGGTTACATCGAACTGGACCTGAACTCTGGTAAAATCCTGGAATCTTTCCGTCCGGAGGAGAGGTTCCCG
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GGTTCATCGCTGACAAATCTGGTGCTGGTGAACGTGGTTCTCGTGGTATCATCGCTGCTCTGGGTCCGGACGGTAAACCG
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GAAAACTTCAAAGAACTGCAGGAGTTCAAACCAGTAGTCGACGGGTCCGGCGGTTCAGGCGGCTCTGGTGGCTCCGGTG
GGTCAGGTGGTTCTGGCGGGTCTGGCGCTAGCAACTCCCTGGAAATCGAAGAACTGGCTCGTTTCGCTGTTGACGAACAC
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CTGCTCACCTGTGGTTCACCGACGGTGTTCTGCAGGGTAAACGTCAGTGGGACCTGGTTGGTGGTCTCGGTGGTTGGTCT
CACCCGCAGTTCGAAAAA

Protein

MASIQHFRVALIPFFAAFCLPVFAAHHHHHHHHLVPRGSHPETLVKVKDAEDQLGARVGYIELDLNSGKILESFRPEERFPMMS
TFKVLLCGAVLSRVDAGQEQLGRRIHYSQNDLVDYSPVTEKHLTDGMTVRELCSAAITMSDNTAANLLLTTIGGPKELTAFLHN
MGDHVTRLDRWEPELNEAIPNDERDTTMPAAMATTLRKLLTGELLTLASRQQLIDWMEADKVAGPLLRSALPAGWFIADKSG
AGERGSRGIIAALGPDGKPSRIVVIYTTGSQATMDERNRQIAEIGASLIKHWTSANSLEIEELARFAVDEHNKKENALLEFVRVVK
AKEQRTRMNVYMGTMYYLTLEAKDGGKKKLYEAKVWVKAAENFKELQEFKPVVDGSGGSGGSGGSGGSGGSGGSGASNSL
EIEELARFAVDEHNKKENALLEFVRVVKAKEQRTRMNVYMGTMYYLTLEAKDGGKKKLYEAKVWVKAAENFKELQEFKPVAA
AAGVMTGAKFTQIQFGMTRQQVLDIAGAENCATGGSFGDSIHCRGHAAGDYYAYATFGFTSAAADAKVDSKSQEKLLAPSAP
TLTLAKFNQVTVGMTRAQVLATVGQGSCTTWSEYYPAYPSTAGVTLSLSCFDVDGYSSTGFYRGSAHLWFTDGVLQGKRQW
DLVGGLGGWSHPQFEK

Table S1. Primers used for Affimer amplification

Primer	Sequence
SpeI_TSA	AACGACTAGTGCAAACTCCCTGGAAATCGAAGAACTG
SpeI_TSAA	AACGACTAGTGCAGCTAACTCCCTGGAAATCGAAGAACTG
SpeI_TSAASS	AACGACTAGTGCAGCTTCAAGTAACTCCCTGGAAATCGAAGAACTG
SalI_VD	TAATGTCGACTACTGGTTTGAACTCCTGCAGTTCTTTG
NheI_AS	ATGCGCTAGCAACTCCCTGGAAATCGAAGAACTG
NotI_AAA	TAATGCGGCCGCTACTGGTTTGAACTCCTGCAGTTCTTTG
NotI_SAAA	ATTAGCGGCCGCACTTACTGGTTTGAACTCCTGCAGTTCTTTG
NotI_ASSAAA	ATTAGCGGCCGCACTTGAAGCTACTGGTTTGAACTCCTGCAGTTCTTTG

Table S2. DNA used for linker 2

Name	Forward primer	Reverse Primer	Final DNA sequence
$(GSG)_5$	TAATGTCGACGGTTCA GGCGGCTCTGGTGGCT CCGGTGGGTCAGGT	ATAAGCTAGCCCCAGA ACCACCTGACCCACCG GAGCCACCAGAGCC	TAATGTCGACGGTTCAGGCGGC TCTGGTGGCTCCGGTGGGTCA GGTGGTTCTGGGGCTAGCTTAT
$(GSG)_7$	TAATGTCGACGGGTCC GGCGGTTCAGGCGGC TCTGGTGGCTCCGGTG GGTCAGGT	ATAAGCTAGCGCCAGA CCCGCCAGAACCACCT GACCCACCGGAGCCAC CAGAGCC	TAATGTCGACGGGTCCGGCGG TTCAGGCGGCTCTGGTGGCTCC GGTGGGTCAGGTGGTTCTGGC GGGTCTGGCGCTAGCTTAT
$(GGS)_{20}$	N/A - DNA sequence purchased from Genscript		GTCGACGGTGGATCAGGTGGT TCCGGTGGGAGTGGTGGCTCA GGTGGATCTGGAGGGTCTGGT GGCTCCGGTGGCTCAGGTGGC TCAGGTGGATCCGGTGGATCTG GTGGGTCCGGTGGTAGCGGTG GATCAGGTGGATCTGGTGGCTC CGGTGGCTCCGGTGGTAGCGG TGGCTCAGGTGGATCCGCTAGC

Table S3. Primers used for introduction of proline rich or helical linker 1

Primer	Sequence
AP_f	GCACCGAACTCCCTGGAAATCGAAG
AP_r	CCAGTGTTTGATCAGAGAAG
APAP_f	GCACCGAACTCCCTGGAAATCGAAG
APAP_r	TGGAGCCCAGTGTTTGATCAGAGAAG
APAPEPAP_f	GAACCTGCTCCGAACTCCCTGGAAATCGAAG
APAPEPAP_r	CGGTGCAGGAGCCCAGTGTTTGATCAGAGAAG
$(APAPEP)_2$_f	GCTCCAGCTCCCGAACCTAACTCCCTGGAAATCGAAG
$(APAPEP)_2$_r	AGGTTCCGGTGCAGGAGCCCAGTGTTTGATCAGAGAAG
$(EAAAK)_2G$_f	AGCGGCTGCCAAAGGAAACTCCCTGGAAATCGAAG
$(EAAAK)_2G$_r	TCTTTTGCGGCGGCCTCCCAGTGTTTGATCAGAGAAG
$(EAAAK)_3P$_f	GCCAAAGAAGCTGCTGCCAAACCAAACTCCCTGGAAATCGAAG
$(EAAAK)_3P$_r	AGCTGCCTCTTTTGCGGCTGCCTCCCAGTGTTTGATCAGAGAAG

Table S4. Primers used for introduction of a stop codon after the TEM1-β-lactamase gene

Primer	Sequence
BLAstop_f	TAATAAACTAGTGGTGGTTACCCG
BLAstop_r	CCAGTGTTTGATCAGAGAAGC

References

(1) Hesketh, E. L.; Tiede, C.; Adamson, H.; Adams, T. L.; Byrne, M. J.; Meshcheriakova, Y.; Kruse, I.; McPherson, M. J.; Lomonossoff, G. P.; Tomlinson, D. C.; et al. Affimer reagents as tools in diagnosing plant virus diseases. *Sci. Rep.* **2019,** *9*, No. 7524.
(2) Adamson, H.; Nicholl, A.; Tiede, C.; Tang, A. A.; Davidson, A.; Curd, H.; Wignall, A.; Ford, R.; Nuttall, J.; McPherson, M. M.; Johnson, M.; Tomlinson, D. C. Affimers as anti-idiotypic affinity reagents for pharmacokinetic analysis of biotherapeutics. *Biotechniques* **2019,** *in press*, DOI: 10.2144/btn-2019-0089.
(3) Tiede, C.; Bedford, R.; Heseltine, S. J.; Smith, G.; Wijetunga, I.; Ross, R.; AlQallaf, D.; Roberts, A. P.; Balls, A.; Curd, A.; et al. Affimer proteins are versatile and renewable affinity reagents. *eLife* **2017,** *6*, No. e24903.
(4) Banala, S.; Aper, S. J.; Schalk, W.; Merkx, M. Switchable reporter enzymes based on mutually exclusive domain interactions allow antibody detection directly in solution. *ACS Chem. Biol.* **2013,** *8* (10), 2127-2132.
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(6) Okemefuna, A. I.; Stach, L.; Rana, S.; Buetas, A. J. Z.; Gor, J.; Perkins, S. J. C-reactive protein exists in an NaCl concentration-dependent pentamer-decamer equilibrium in physiological buffer. *J. Biol. Chem.* **2010,** *285* (2), 1041-1052.