Supplementary Material:

Efficient Selection of DARPins with Subnanomolar Affinity using SRP Phage Display

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Supplementary Results: Development of the „optimized selection protocol“

This section describes the development of an optimized selection protocol for our DARPin phage library, based on experiences from the different selection approaches using members of the ErbB receptor family as target proteins. For both ErbB1 and ErbB4, the extracellular domains I-III of the respective receptor fused to huIgG1_Fc were used as targets (see **Table 1** of the publication). For the first panning we had performed three rounds of selection on immobilized target protein as described in the **Material and Methods** section of the publication. During the second and third selection rounds we added an excess of soluble huIgG_Fc (1 μM) to the selection reaction to counter-select binders against the Fc domain. From each selection round we analyzed the amplified pool of phage particles by phage ELISA (**Supplementary Figure 3(a)** and **3(b)**). For ErbB1 good enrichment of phage particles displaying target specific DARPins was observed for the second and third round, with a slight

enrichment of phage particles showing unspecific background binding (binding of non-target-coated matrix) (**Supplementary Figure 3(a)**). For ErbB4 the enrichment of such background binding phage particles was much stronger, and almost equal signals were obtained on neutravidin, streptavidin and immobilized target protein (**Supplementary Figure 3(b)**). This background binding could be reduced by applying extensive prepanning steps on a non-target-coated matrix before performing the selection round on the target protein, but still not to the level required for efficient screening of single clones (data not shown). Sequence analysis of some of these background binders revealed defective DARPins of which part of the DARPin framework is not consensus due to multiple deletions or insertions (members of the non-functional part of the initial library). The C-terminal part of all of the clones analyzed was in frame with the phage coat protein, a prerequisite for display on phage particles.

Selection on target protein in solution was previously reported to result in less background enrichment when compared to selections on immobilized target proteins.[1] Thus, to avoid enrichment of phage particles with such non-specific binding properties we performed another three rounds (starting with a fresh library aliquot) of selection on ErbB1 and ErbB4 in solution as described in the **Material and Methods** section of the publication. During the second and third selection rounds soluble huIgG1_Fc was added for competition as described above. Analysis of the amplified pools of phage particles by phage ELISA showed for both selections highly specific binding signals on the respective target protein in round three (**Supplementary Figure 3(c)** and **3(d)**), without enrichment of background binding phage particles. When comparing the results obtained for selection on immobilized ErbB1 (**Supplementary Figure 3(a)**) and ErbB1 in solution (**Supplementary Figure 3(c)**), a faster enrichment of binders is revealed when performing the selection on immobilized target protein, however, with the disadvantage of a higher tendency for the enrichment of background binding phage particles.

As a consequence, we combined both methods, performing the first round of selection on immobilized target protein (high recovery of phage particles needed in order not to irreversibly lose binders), followed by two rounds on target protein in solution. This turned out to be the most effective selection strategy and was used as optimized selection protocol for our phage DARPin library. Results obtained by using this protocol for selection of binders against ErbB1 (**Supplementary Figure 3(e))** do show fast enrichment of target specific binders without any enrichment of background binding phage particles (see also selections on huIgG1_Fc, TNFα and Her2-509 described in the results section of the publication).

Supplementary Discussion: Target protein dependent selection approaches

Many methods of target protein presentation have been used for phage selections[2] including panning on whole cells, on tissues or even using living organisms, but if available, purified target protein is preferentially used. The biophysical properties of a purified target protein do strongly influence the selection, and, therefore, the chance of successful selection of binders from a given library. For “well behaved” target proteins, such as the huIgG1_Fc domain, the selection is simple and straightforward (**Figure 1(a)** of the publication). For more complicated target proteins (e.g. membrane proteins or proteins with low stability) the careful design of the selection procedure is essential to guide the selection into the desired direction in order to obtain specific binders. Background enrichment, meaning the accumulation of binders that unspecifically bind to the non-target coated matrix, was highly target-protein dependent in our hands. When comparing selections on immobilized ErbB1 and ErbB4, much lower background enrichment was observed for ErbB1 (**Supplementary Figure 3(a)**), than for ErbB4 where background binding signals were almost equal to the specific binding signals on the target protein (**Supplementary Figure 3(b)**). Prepanning on non-target coated matrix only slightly improved the result. Performing the selections on the same target protein in

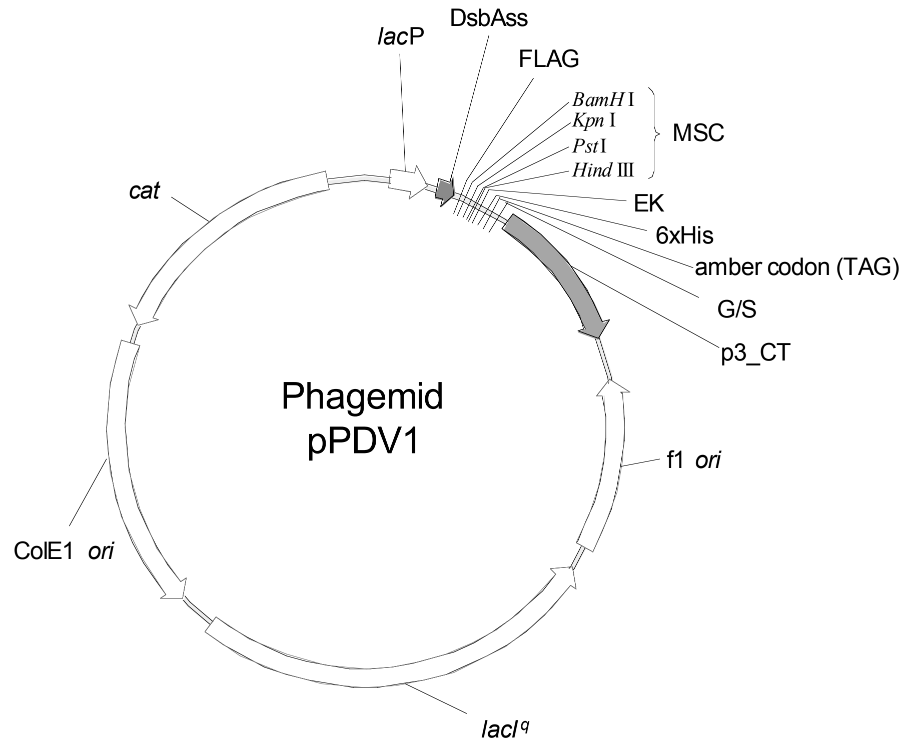
solution completely abolished unspecific background binding signals (**Supplementary Figure 3(c)** and **3(d)**). This might be attributed to the short capturing step and the possibility to change the reaction vial during the selection process.

Target protein presentation not only has an influence on background enrichment but also on enrichment factors. Even if accompanied by background enrichment we observed a faster enrichment of specific binders when performing selections on immobilized target protein, compared to selections on soluble target protein (**Supplementary Figure 3(a)** and **3(c)**). One possible explanation is that the immobilization of the target protein on solid plastic support leads to a locally very high concentration of the target protein, favorable for the capturing of all potential binders, which is especially important for the first selection round.

From the observations described above we used for further selections our optimized selection protocol composed of a first selection round on immobilized target protein, followed by two rounds of selection on soluble target protein. In the case of ErbB1 this lead to the very fast enrichment of specific binders (**Supplementary Figure 3(e)**) without any detectable background enrichment and was further successfully used for the selection on huIgG1_Fc, TNFα and ErbB2-509.

References

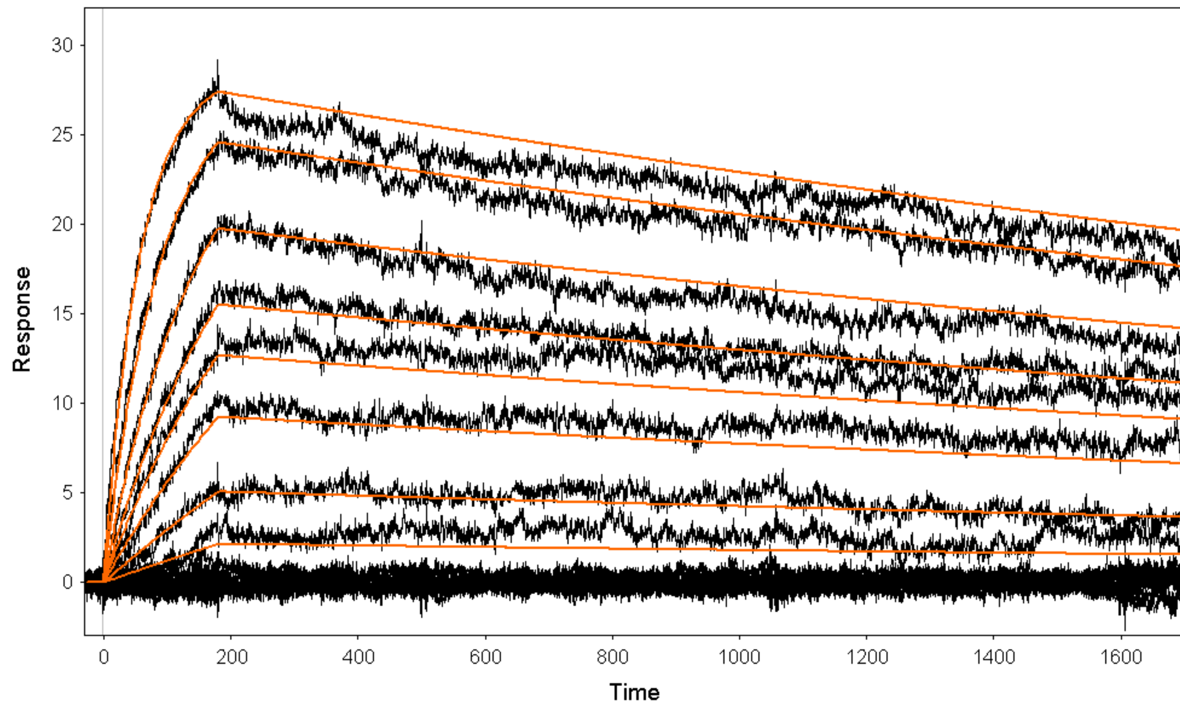
1. Dennis, M.S. in Phage display in biotechnology and drug discovery. (ed. S.S. Sidhu) 143 - 164 (CRC, Boca Raton; 2005).
2. Bradbury, A.R. & Marks, J.D. Antibodies from phage antibody libraries. *J. Immunol. Methods* **290**, 29-49 (2004).



Supplementary Figure 1. Schematic representation of the phagemid pPDV1. The expression cassette is composed of a *lac* promoter/operator element (*lac*P), the coding sequences for the signal sequence of *E. coli* DsbA (DsbAss) directing the fused protein to SRP dependent translocation, the multiple cloning site (MCS) to introduce the gene encoding the protein of interest (POI) with the restriction sites *Bam*HI, *Kpn*I, *Pst*I, and *Hind*III depicted, a suppressable stop codon (amber codon (TAG)), the coding sequences for a flexible glycine/serine linker (G/S) and for the C-terminal domain (amino acids 250-406) of protein 3 of filamentous phage M13 (p3_CT), mediating incorporation of the fusion protein into the phage particle. The coding sequence of the POI is flanked by DNA sequences encoding a FLAG-tag (FLAG), an enterokinase site (EK) and a His_6 tag (6xHis). In addition, the vector carries a f1 origin of replication (f1 *ori*) to permit production of virions using an appropriate helper phage, such as VCS M13; a plasmid origin (ColE1 *ori*), the *lacI^q^* gene providing high levels of the *lac* repressor for the control of the lacP *in cis* and an antibiotic resistance marker (the gene for chloramphenicol acetyl transferase (*cat*), providing resistance to chloramphenicol) to allow selection as a plasmid in *E. coli*

Supplementary Figure 2: Affinity determination of selected DARPins. Surface plasmon resonance (SPR) was measured using a BIAcore 3000 instrument (BIAcore, Uppsala Sweden). All measurements were performed in HBS-T buffer (20 mM HEPES, 150 mM NaCl, 3 mM EDTA pH 7.4, 0.005% Tween-20) at a flow rate of 50 µl/min. The following amounts of biotinylated target protein described in **Table 1** of the man publication were immobilized on SA chips (BIAcore): ErbB1 (1000 RU), ErbB2 (509) (400 RU), ErbB4 (600 RU), huIgG1_Fc (500 RU). For the determination of kinetic data, the interactions were measured as follows: five minutes initial buffer flow, followed by a 2 to 15 min injection of DARPin at different concentrations (1 nM to 250 nM) and a final off-rate measurement of 10 to 75 minutes with buffer flow. The signal of an uncoated reference cell and buffer response was always subtracted from the sensograms (double referencing). The kinetic data of the interaction were evaluated with a global fit using Scrubber 2 (BioLogic Software Ltd). For each DARPin analyzed a representative set of curves with the corresponding fits are shown below. The statistical errors in the parameters given are those obtained from the best fit error and underline data quality and the good description of the data by the model. However, the true error of the kinetic parameters, estimated from measurements of independent protein preparations will be at least ±20%. Results are shown for DARPins binding ErbB1: (a) E_01, (b) E_67, (c) E_68 and (d) E_69, ErbB2: (e) 9_16, (f) 9_26 and (g) 9_27, ErbB4: (h) B4_01, (i) B4_02, (j) B4_50 and (k) B4_58 and huIgG1_Fc: (l) I_02, (m) I_07, (n) I_11 and (o) I_19.

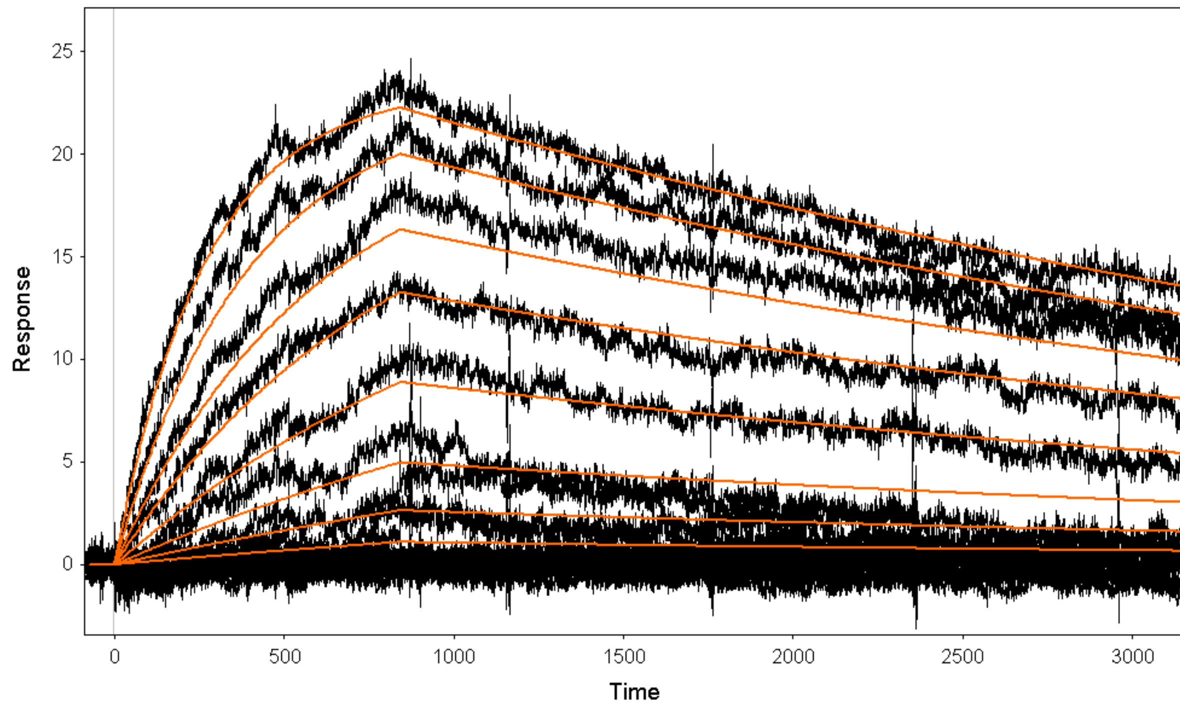
a) E_01



$K_D = 496 \pm 2$ pM $k_{on} = (44.2 \pm 0.2) \times 10^4\ M^{-1}s^{-1}$ $k_{off} = (0.2194 \pm 0.0007) \times 10^{-3} s^{-1}$

DARPin concentrations [nM]: 1, 2.5, 5, 7.5, 10, 15, 25, 40

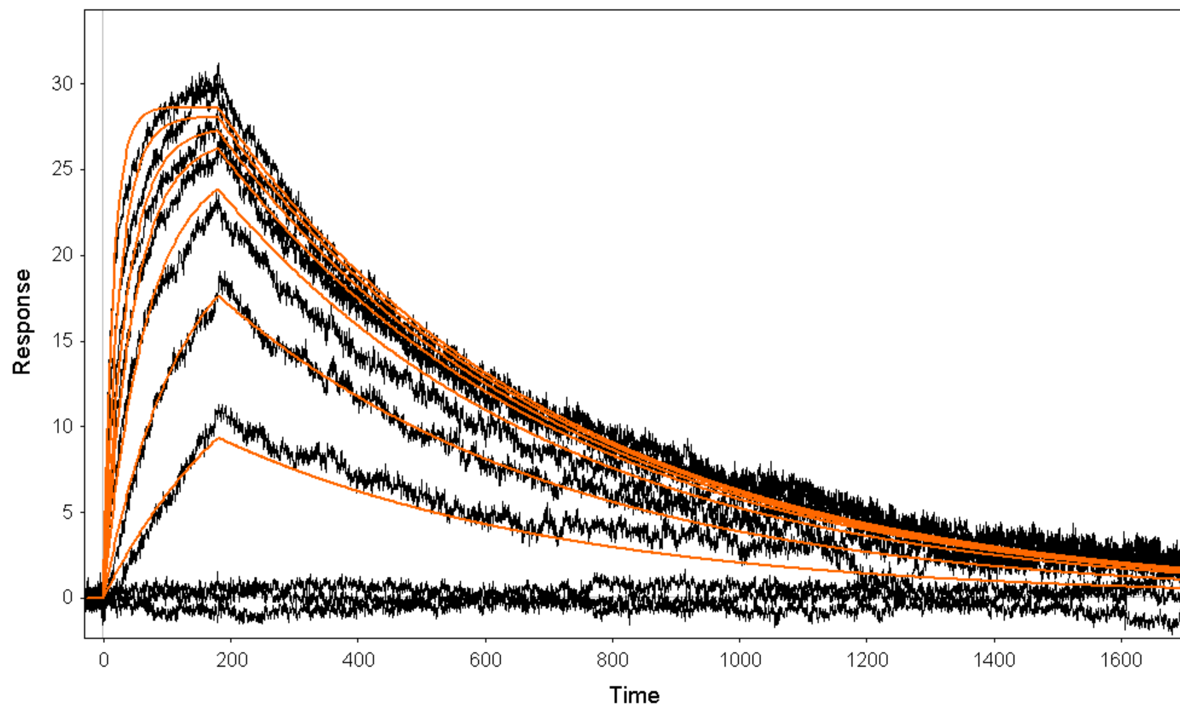
b) E_67



$K_D = 7.3 \pm 0.1$ nM $k_{on} = (2.95 \pm 0.04) \times 10^4\ M^{-1}s^{-1}$ $k_{off} = (0.215 \pm 0.002) \times 10^{-3} s^{-1}$

DARPin concentrations [nM]: 2, 5, 10, 20, 35, 50, 80, 120

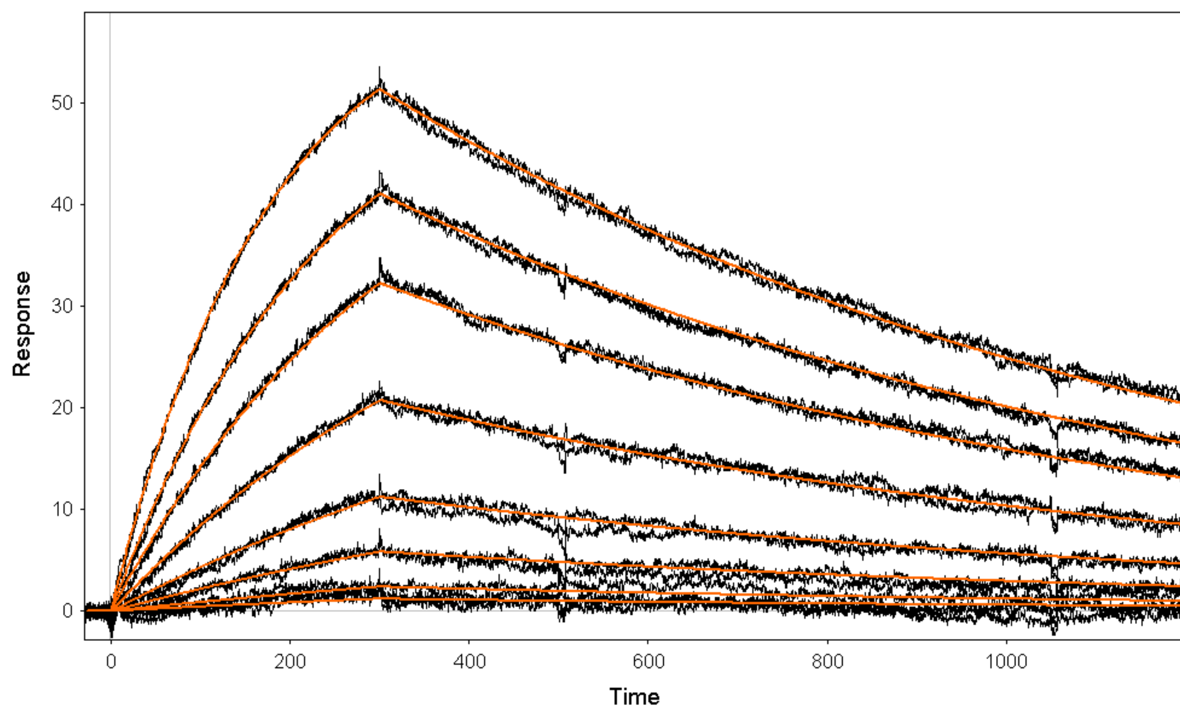
c) E_68



$K_D = 724 \pm 3$ pM $k_{on} = (258.6 \pm 0.8) \times 10^4\ M^{-1}s^{-1}$ $k_{off} = (1.873 \pm 0.006) \times 10^{-3}s^{-1}$

DARPin concentrations [nM]: 1, 2.5, 5, 7.5, 10, 15, 25

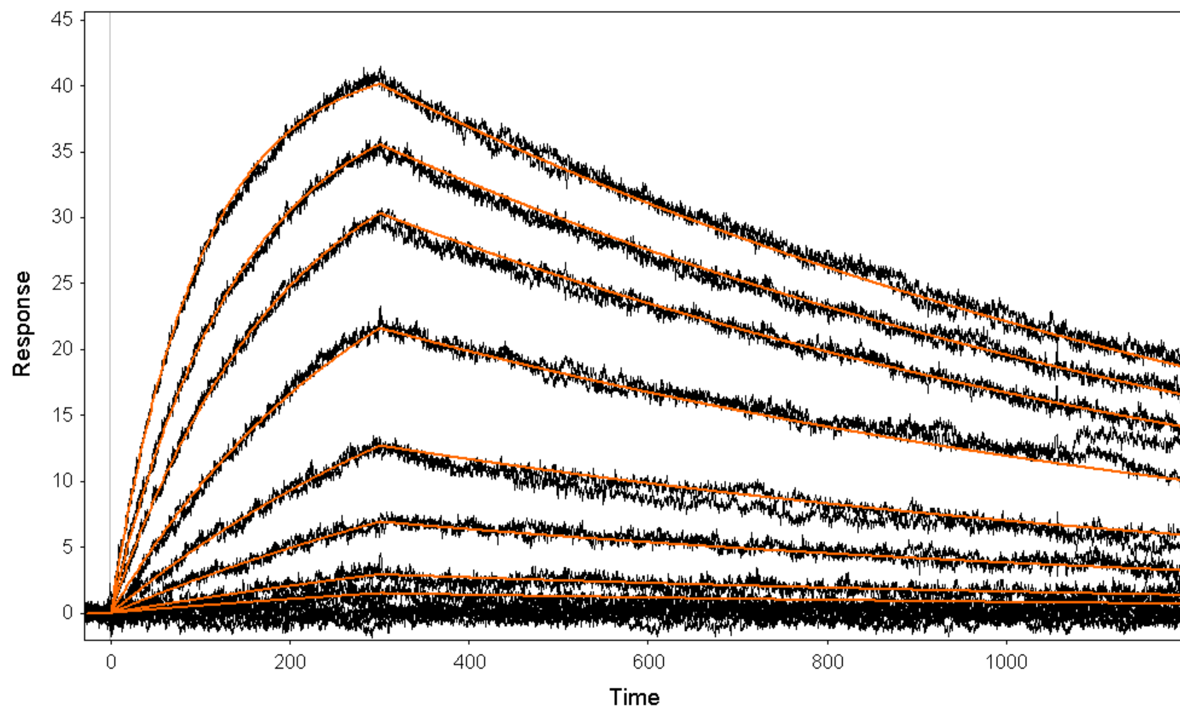
d) E_69



$K_D = 15.20 \pm 0.08$ nM $k_{on} = (7.32 \pm 0.03) \times 10^4\ M^{-1}s^{-1}$ $k_{off} = (1.113 \pm 0.003) \times 10^{-3}s^{-1}$

DARPin concentrations [nM]: 1, 2, 5, 10, 20, 35, 50, 75

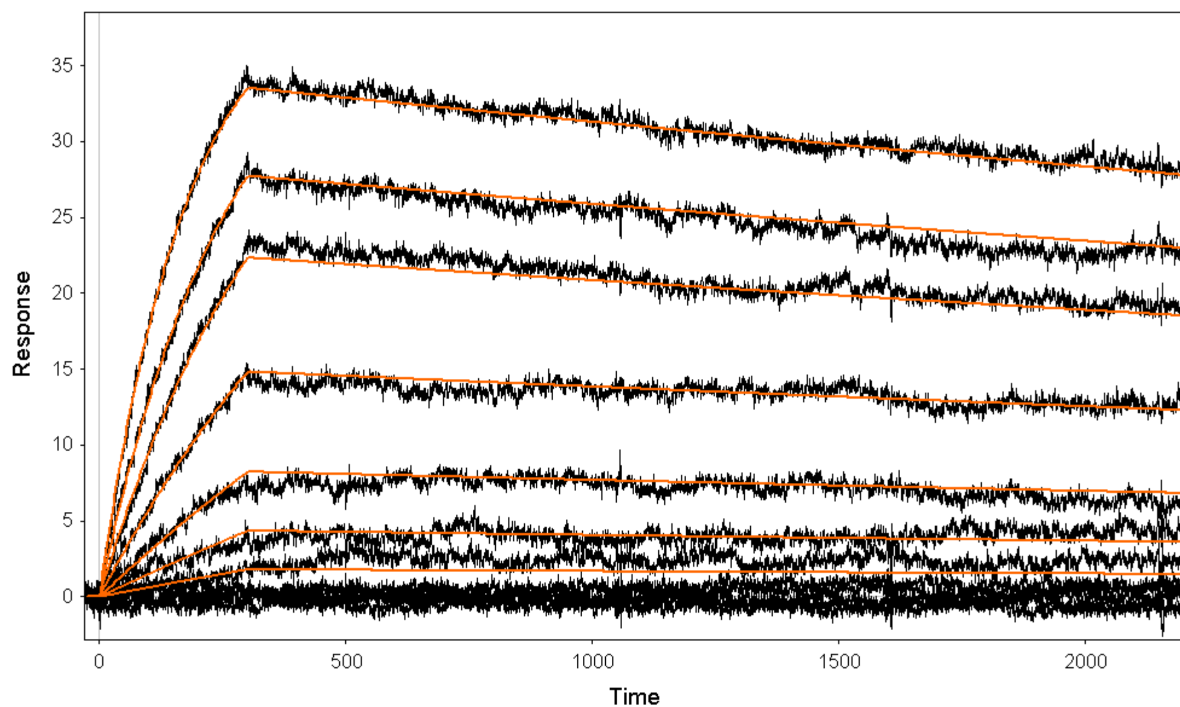
e) 9_16



$K_D = 6.90 \pm 0.02$ nM $k_{on} = (12.36 \pm 0.01) \times 10^4\ M^{-1}s^{-1}$ $k_{off} = (0.853 \pm 0.003) \times 10^{-3}s^{-1}$

DARPin concentrations [nM]: 1, 2, 5, 10, 20, 35, 50, 75

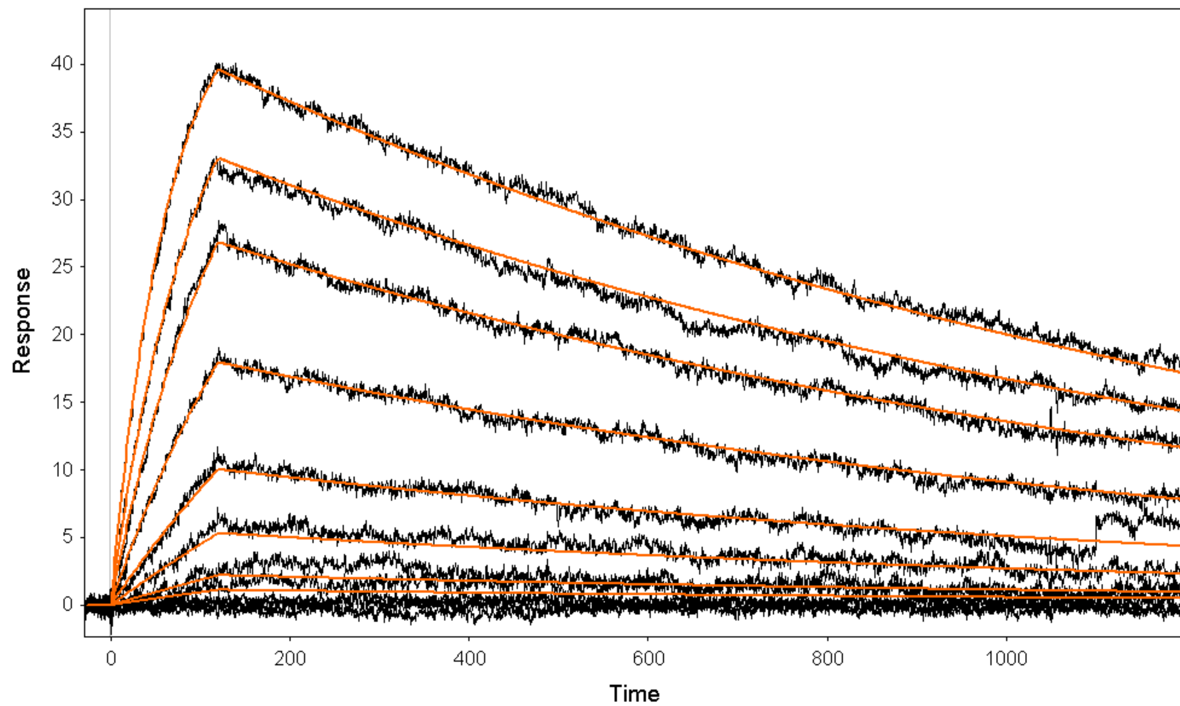
f) 9_26



$K_D = 1.36 \pm 0.03$ nM $k_{on} = (7.38 \pm 0.01) \times 10^4\ M^{-1}s^{-1}$ $k_{off} = (0.101 \pm 0.002) \times 10^{-3}s^{-1}$

DARPin concentrations [nM]: 2, 5, 10, 20, 35, 50, 75

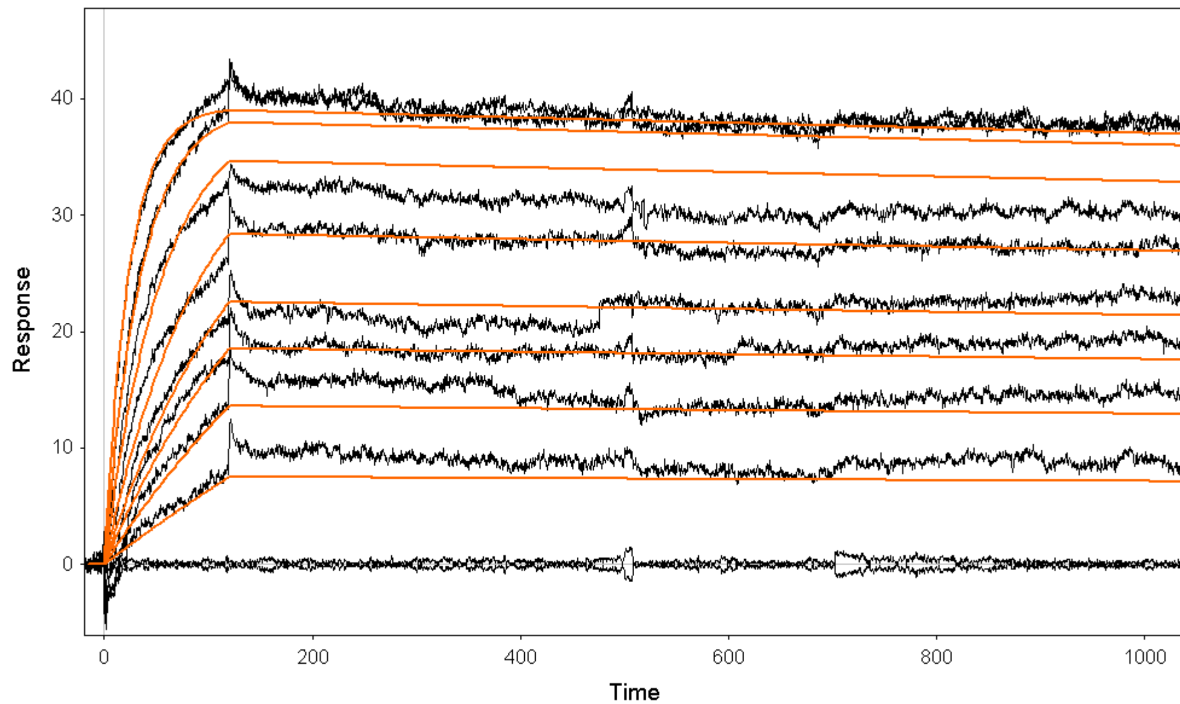
g) 9_29



$K_D = 3.83 \pm 0.03$ nM $k_{on} = (20.25 \pm 0.04) \times 10^4\ M^{-1}s^{-1}$ $k_{off} = (0.776 \pm 0.007) \times 10^{-3}s^{-1}$

DARPin concentrations [nM]: 1, 2, 5, 10, 20, 35, 50, 75

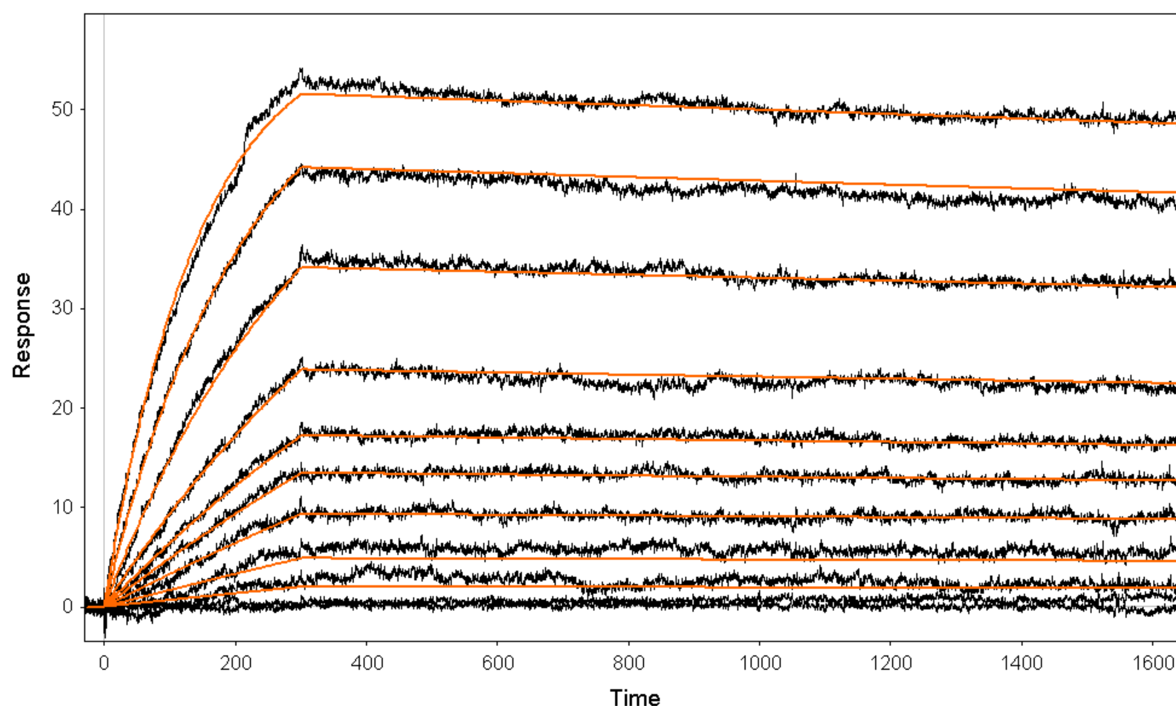
h) B4_01



$K_D = 80 \pm 2$ pM $k_{on} = (72.8 \pm 0.5) \times 10^4\ M^{-1}s^{-1}$ $k_{off} = (0.058 \pm 0.001) \times 10^{-3}s^{-1}$

DARPin concentrations [nM]: 2.5, 5, 7.5, 10, 15, 25, 40, 60

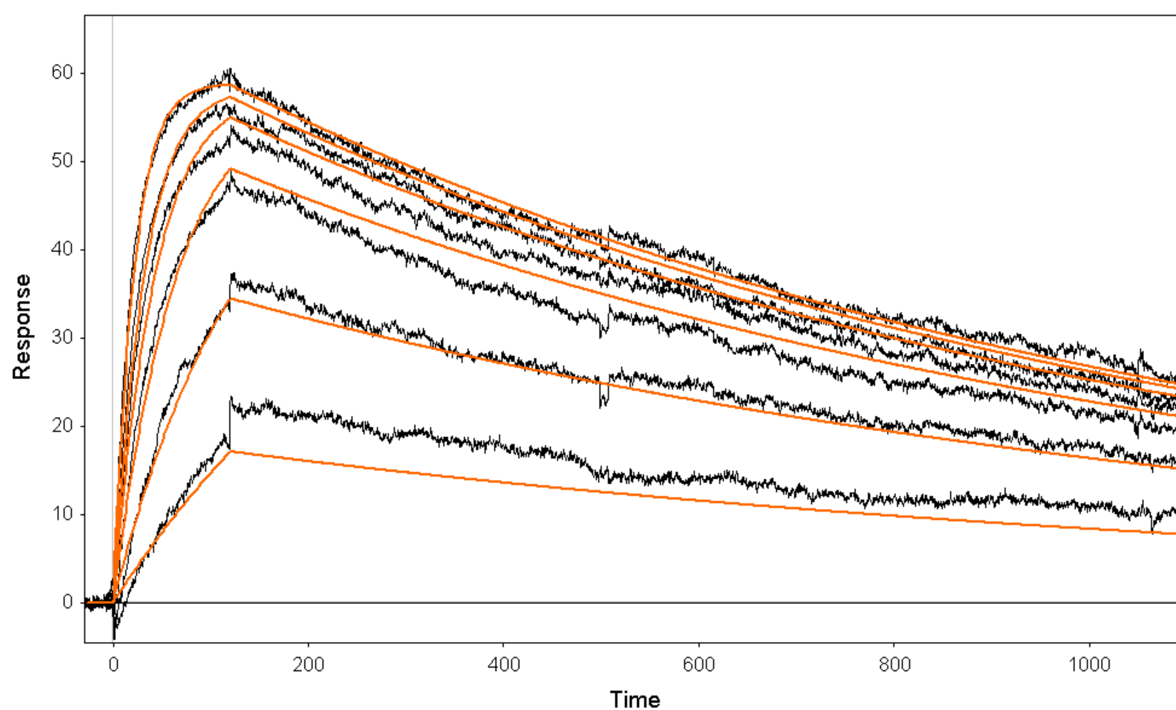
i) B4_02



$K_D = 387 \pm 3$ pM $\quad k_{on} = (11.63 \pm 0.06) \times 10^4\ M^{-1}s^{-1}$ $\quad k_{off} = (0.045 \pm 0.004) \times 10^{-3}s^{-1}$

DARPin concentrations [nM]: 1, 2.5, 5, 7.5, 10, 15, 25, 40, 60

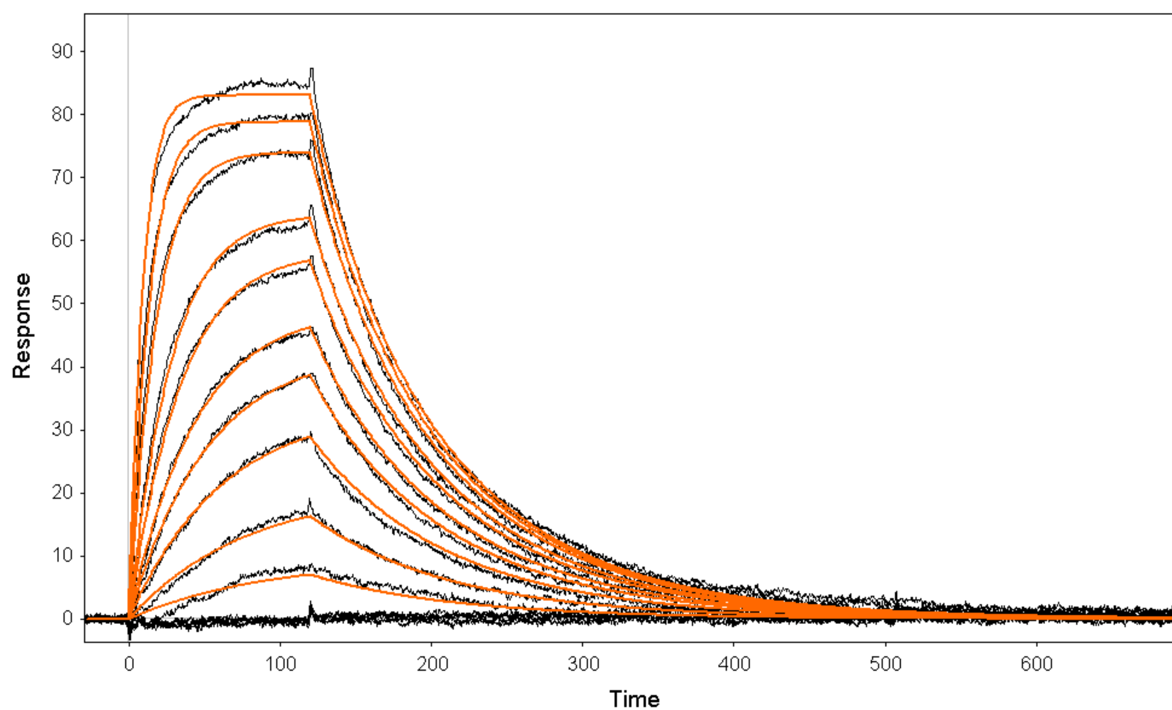
j) B4_50



$K_D = 273.7 \pm 0.7$ pM $\quad k_{on} = (348 \pm 2) \times 10^4\ M^{-1}s^{-1}$ $\quad k_{off} = (0.952 \pm 0.003) \times 10^{-3}s^{-1}$

DARPin concentrations [nM]: 1, 2.5, 5, 7.5, 10, 15

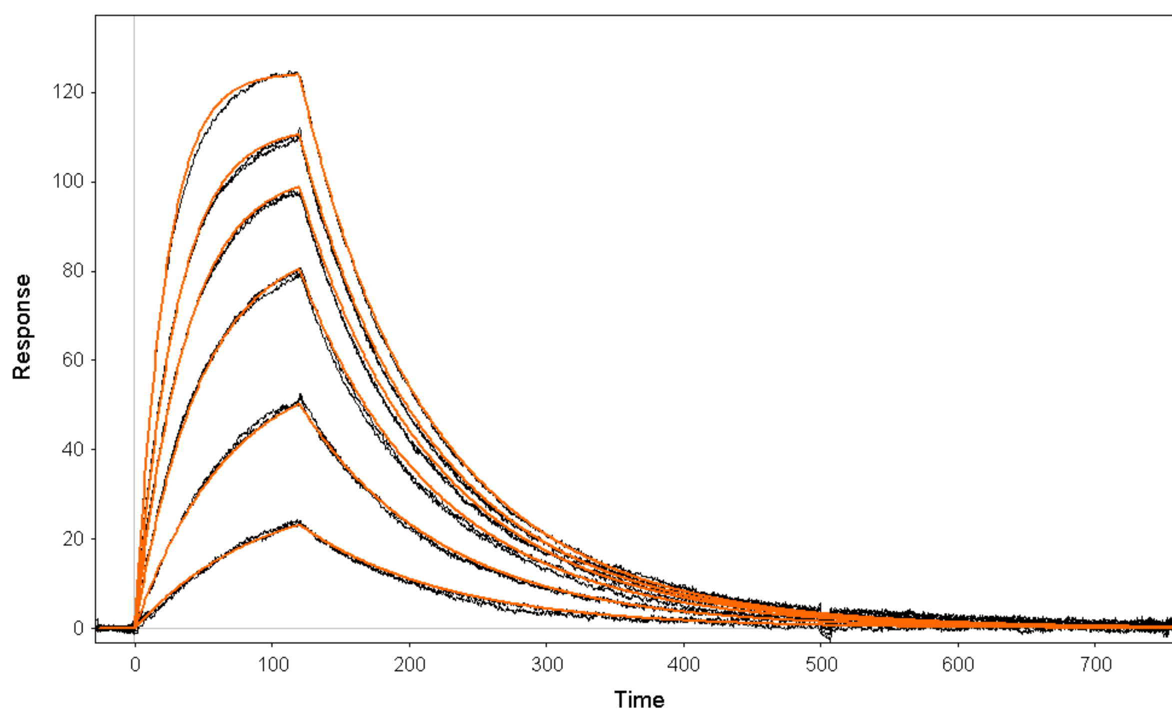
k) B4_58



$K_D = 9.00 \pm 0.01$ nM $k_{on} = (159.3 \pm 0.05) \times 10^4\ M^{-1}s^{-1}$ $k_{off} = (14.34 \pm 0.05) \times 10^{-3}s^{-1}$

DARPin concentrations [nM]: 1, 2.5, 5, 7.5, 10. 15, 20, 35, 50, 75

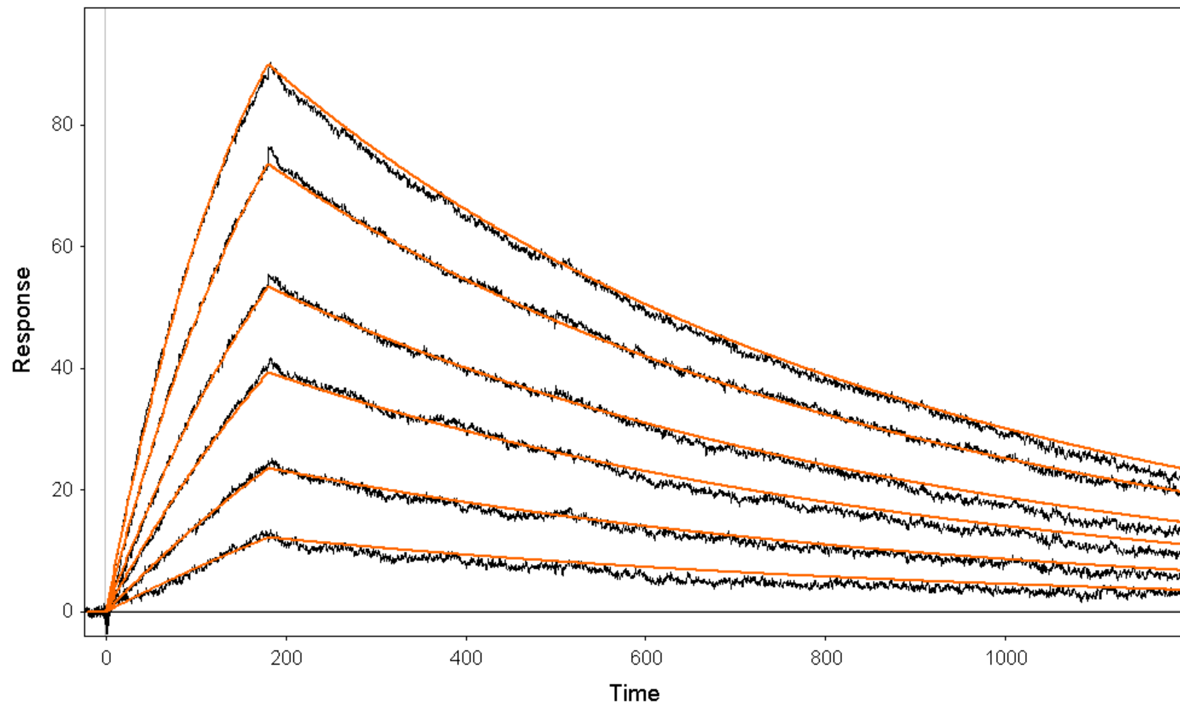
l) I_02



$K_D = 41.1 \pm 0.1$ nM $k_{on} = (28.29 \pm 0.06) \times 10^4\ M^{-1}s^{-1}$ $k_{off} = (11.62 \pm 0.02) \times 10^{-3}s^{-1}$

DARPin concentrations [nM]: 10, 25, 50, 75, 100, 150

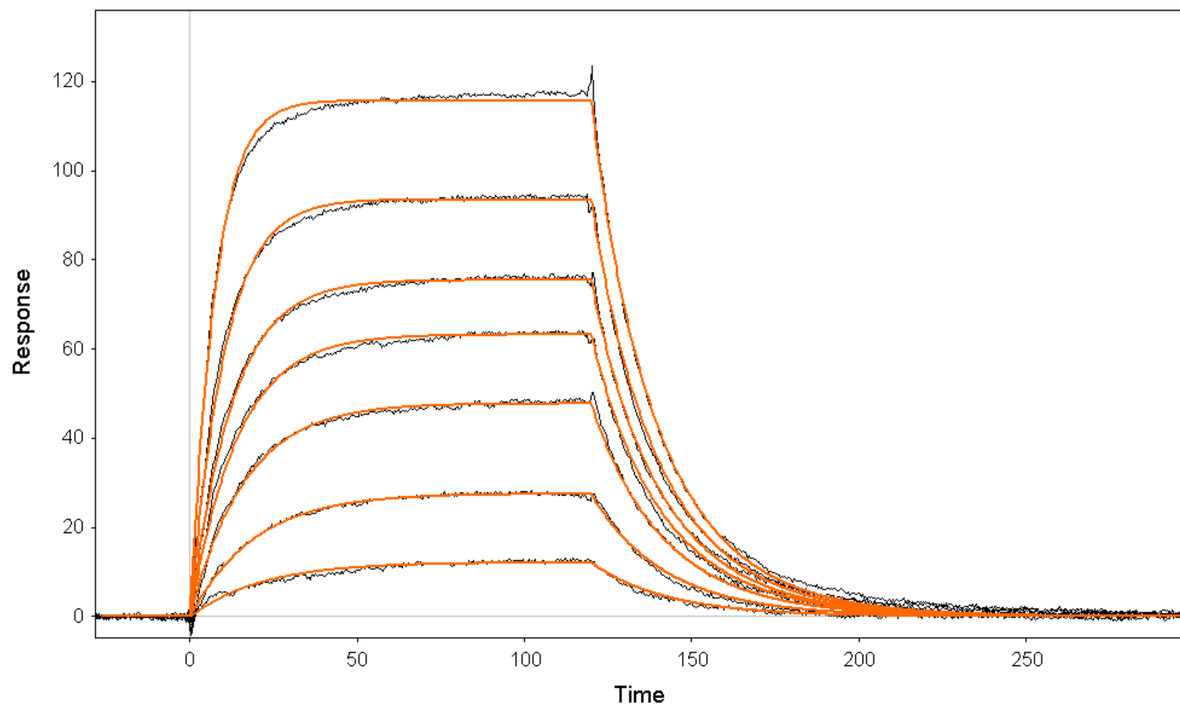
m) I_07



$K_D = 24.5 \pm 0.1$ nM $k_{on} = (7.03 \pm 0.03) \times 10^4\ M^{-1}s^{-1}$ $k_{off} = (1.723 \pm 0.007) \times 10^{-3}s^{-1}$

DARPin concentrations [nM]: 10, 20, 35, 50, 75, 100

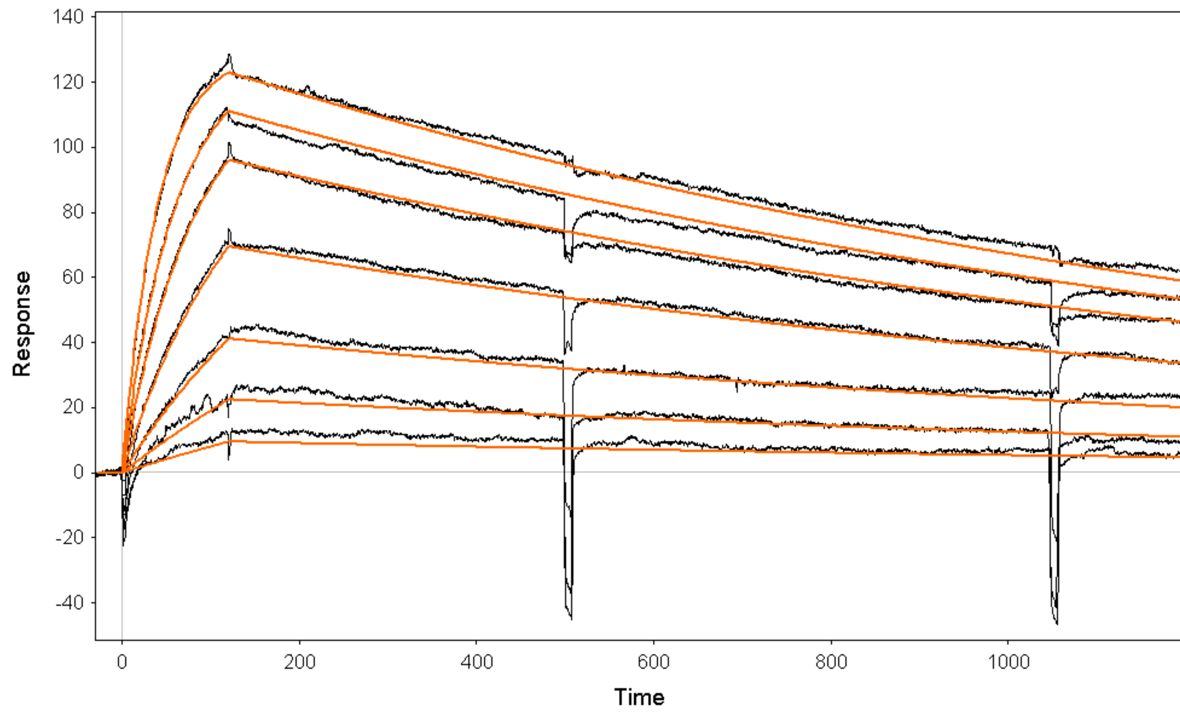
n) I_11



$K_D = 137 \pm 1$ nM $k_{on} = (48.6 \pm 0.3) \times 10^4\ M^{-1}s^{-1}$ $k_{off} = (66.5 \pm 0.5) \times 10^{-3}s^{-1}$

DARPin concentrations [nM]: 10, 25, 50, 75, 100, 150, 250

o) I_19



$K_D = 2.07 \pm 0.01$ nM $k_{on} = (33.7 \pm 0.3) \times 10^4\ M^{-1}s^{-1}$ $k_{off} = (0.695 \pm 0.004) \times 10^{-3} s^{-1}$

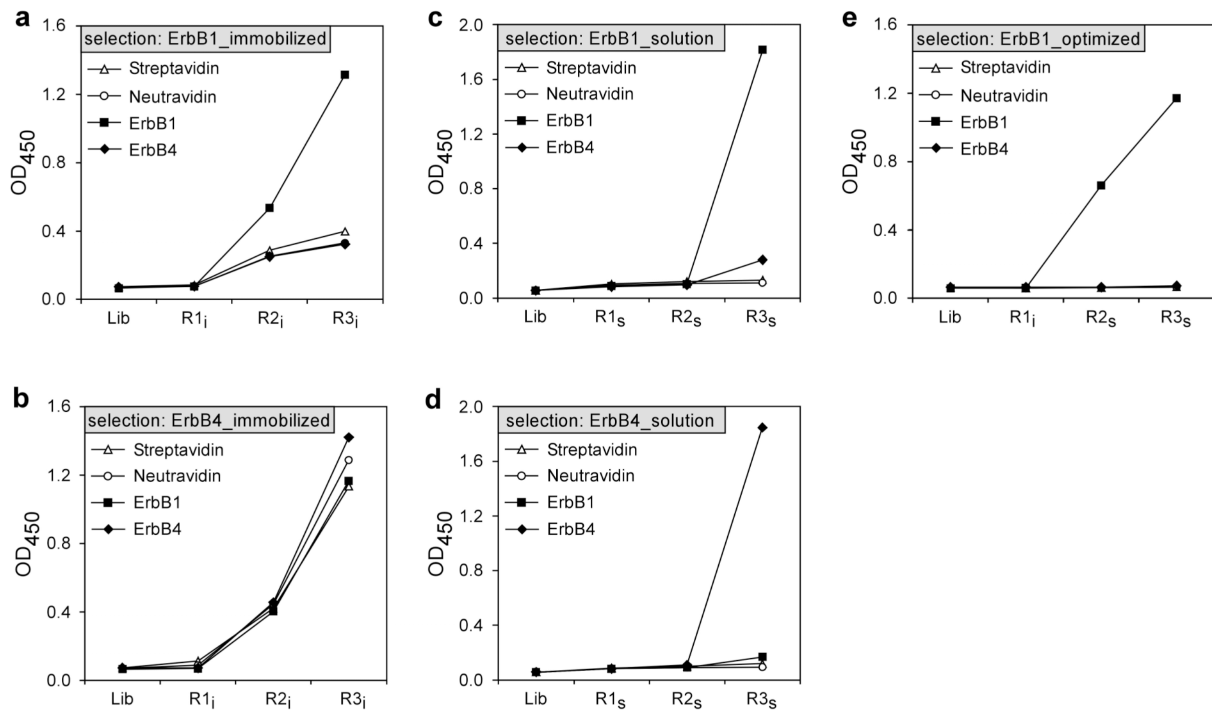
DARPin concentrations [nM]: 2, 5, 10, 20, 35, 50, 75

Supplementary Figure 3. Sequences of DARPins selected by phage display. The amino acid sequences of the different DARPins are shown in the alignment, grouped according to their target. The designed sequences for the N-Cap, the C-Cap and the internal consensus[1] repeat modules are given above the selected sequences (x represents a randomized potential interaction residue, where any amino acid was allowed except Cys, Gly or Pro; z represents a randomized framework residue where the three amino acids Asn, His or Tyr were allowed). In the alignment, only differences compared to the designed sequence are highlighted and residues that had been randomized in the original design are boxed. The names of the clones and their length are given on the left side of the respective sequence. Names beginning with *I* represent huIgG binders, those beginning with *T* represent TNFα binders, those beginning with *E* represent EGF-R (ErbB1), those beginning with *B* represent ErbB4 binders, those beginning with *9* represent HER2_509 (ErbB2_509) binders and those beginning with *H* represent HER2_631 (ErbB2_631) binders. Note that in this sequence representation, the N-terminal MRGSHHHHHH-tag has been removed.

References

1. Binz, H. K., Stumpp, M. T., Forrer, P., Amstutz, P. & Plückthun, A. (2003). Designing repeat proteins: Well-expressed, soluble and stable proteins from combinatorial libraries of consensus ankyrin repeat proteins. *J. Mol. Biol.* **332**, 489-503.

Clone	NxC	N-Cap	1. repeat	2. repeat	3. repeat	4. repeat	5. repeat	C-Cap
		DLGKKLLEAARAGQDDEVRILMANGADVNA	xDxxGxTPLHLAAxxGHLEIVEVLLKzGADVNA	xDxxGxTPLHLAAxxGHLEIVEVLLKzGADVNA	xDxxGxTPLHLAAxxGHLEIVEVLLKzGADVNA	xDxxGxTPLHLAAxxGHLEIVEVLLKzGADVNA	xDxxGxTPLHLAAxxGHLEIVEVLLKzGADVNA	QDKFGKTAFDISIDNGNEDLAEILQ
E_01	N3C	....	D.TW.W.......YQ...........N......	Y.YI.W.......D............N......	S.YI.D.......HN...........H......			....
E_67	N3C	....	T.ND.N.....S.WI...........H......	D.LL.M.......DT...........Y......	R.TR.K.......RD...........HD.....			....
E_68	N3C	....	F.YW.M.......DN...........H......	S.NF.F.......FY...........H......	F.MW.N.......QN...........N......			....
E_69	N4C	....	D.NA.R.......NF...........N......	KGHHCN.......WA...........Y......	D.DE.Y.......DI.D.........Y......	W.MY.R.......SA...........Y......		....
9_16	N3C	....	H.FH.L.......GM...........N......	V.TD.I.L.....YY...........H......	H.YA.S.......NT...........N......			....
9_26	N3C	....	K.FY.I.......AY...........H......	H.WN.W.......KY...........H......	I.NA.K.......AH...........Y......			....
9_29	N3C	....	H.FY.I.......NF...........H......	F.Y.DN.......DA...........Y......	S.RD.H.......RE...........N......			....
H_14	N3C	..................C...........	T.IH.H.......AM...........N......	N.WR.F.......LN...........N......	T.TA.N.......WF...........N......			....
B4_01	N4C	..................H...........	V.WM.D.......FY...........N......	K.TW.D.......LL.R.........H......	I.MR.T.......PA...........Y......	D.VH.N.......MS...........Y......		....
B4_02	N3C	....	K.NA.K.A.....VW...........N......	Y.AS.Y.L.....RM...........Y......	R.RF.S.......WH...........H......			....
B4_07	N3C	....	R.VF.W.......VD...........Y......	R.VA.R.......SF...........Y......	V.YT.T.......WH...........H......			....
B4_33	N3C	....	E.AT.F.......VW...........N......	N.QY.Y.......RM...........H......	I.VL.T.......WH...........N......			....
B4_45	N3C	....	R.DG.T.......NH...........Y......	N.RY.Y.T.....RH...........H......	F.NT.Q.......WH...........Y......			....
B4_50	N4C	....	H.RY.V.......YF...........N......	D.HD.Y.......DK...........H......	D.SM.N.......RH...........H......	N.FM.S.......WS...........H......		....
B4_58	N5C	.........T....................	F.SN.I.......FF...........N......	H.SY.S.......NR...........Y......	F.ST.Q.......SQ...........Y......	S.RM.F.......YT...........N......	K.FV.W.......YR...........H......	....
I_01	N3C	....	N.IS.Y.......YV..Q........N......	D.TW.D.......LF...........Y......	H.RF.F.......SS...........H......			....
I_02	N2C	....	R.MS.Y.......HM...........N......	K.NW.D.......IF...........N......				....
I_07	N2C	....	S.KS.Y.......HI...........H......	H.SW.D.......TF...........H......				....
I_11	N3C	....	I.TI.L.......HD...........N......	A.NW.I.......RR...........H......	D.VQ.N.....T.HH...........H......			....
I_13	N2C	....	F.MS.Y.......YD...........N......	N.LW.D.......TR...........Y......				....
I_19	N3C	....	D.NK.D.......SF...........N......	D.YF.D.......WS...........Y......	Q.QR.F.......IA...........Y......			....
T_01	N3C	............D.................	N.IW.I.......IF.......F...N......	S.FS.F.......YK...........H......	N.AT.T.......KK...........N......			....
T_02	N3C	........V.....................	A.HQSF.....Y.IF...........N......	S.WH.N.......WI...........Y......	T.HS.S.......TL...........Y......			....
T_07	N3C	....	Y.WK.L.......IF.......SAM.N......	I.FS.R.......LI...........Y......	H.SA.S.......TK...........Y......			....
T_08	N3C	....	W.FL.LI..R...AF...........N......	K.TY.I.......MN...........N......	L.NT.S.......NY...........H......			....
T_09	N3C	............S.................	N.FQ.I.......IF...........N......	Y.QM.M.......WT...........H......	D.TH.A.......HT...........Y......			....
T_16	N3C	.....P........................	Y.IV.I.......IF...........N......	Y.MQVN.......WL...........N......	E.SY.N.......DK...........N......			....
T_25	N3C	....	D.RR.IP......IF...........H......	H.MQ.R.......YT...........Y......	I.FT.H.......FR...........H......			....
T_27	N3C	.....P........................	Y.RH.L.....V.IF...........H......	I.II.Y.......WS...........N......	S.VT.S.......DK...........Y......			....
T_37	N3C	....	H.KR.I.......IT....M......H......	V.IQ.R.......WI...........Y......	M.DF.E.......RT...........H......			....
T_40	N3C	............S.................	N.RV.F.......MF....L......N......	I.FQ.K.......QL...........Y......	L.AR.I.......IH..P........Y......			....



Supplementary Figure 4. Selection approaches on ErbB1 and ErbB4. The enrichments for the selections performed on (a) immobilized ErbB1, (b) immobilized ErbB4, (c) ErbB1 in solution, (d) ErbB4 in solution and (e) ErbB1 by using the optimized selection protocol were analyzed by phage ELISA. Equivalent amounts of the initial library (Lib) and pools of amplified phage particles from each selection round (R1, R2 and R3) were tested for binding to immobilized streptavidin, neutravidin, immobilized ErbB1 and ErbB4. For each sample applied the signal of bound phage particles, detected with an anti-M13 antibody, is shown. For each round the subscript denotes if the selection was performed on immobilized target protein (i) or on target protein in solution (s).