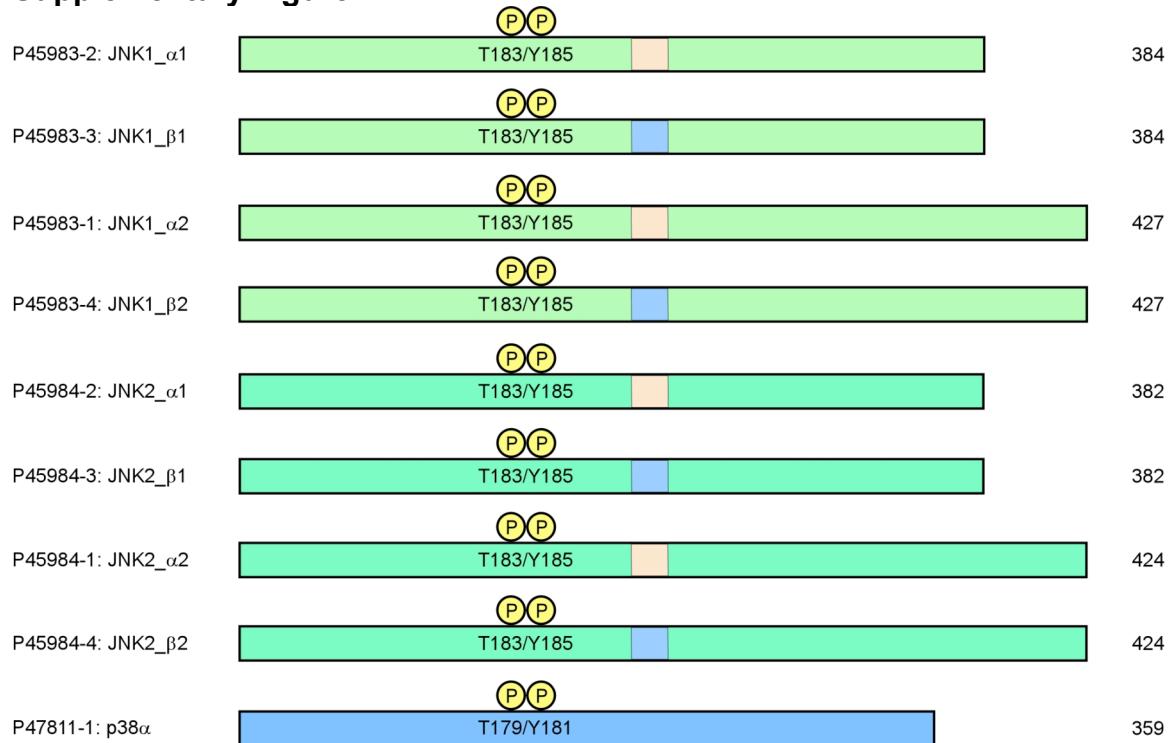


**Supplementary Figure 1.** Illustration and sequence alignment of different mitogen activated protein kinases. **A.)** Schematic representation of the different isoforms of human c-Jun N-terminal kinase 1 (green boxes), of the different isoforms of human c-Jun N-terminal kinase 2 (cyan boxes) and of the mouse p38 $\alpha$  (blue box). SwissProt accession numbers and the names are depicted on the left, the total number of amino acids is indicated on the right. Phosphothreonine and phosphotyrosine residues are indicated in the figure at the respective positions. The  $\alpha$  and  $\beta$  isoforms of JNK1 and JNK2 differ by a distinct sequence region indicated by the differently colored boxes (alternative exon usage). The isoforms  $\alpha 1$  and  $\beta 1$  differ from the isoforms  $\alpha 2$  and  $\beta 2$  by length as indicated by longer boxes. **B.)** Amino acid sequence alignment of the different isoforms of human c-Jun N-terminal kinase 1 with the different isoforms of human c-Jun N-terminal kinase 2 and the mouse p38 $\alpha$ . The SwissProt accession number is indicated on the left, followed by the name and the amino acid sequence. For orientation, the sequence alignment is numbered and the sequences are represented in blocks of 10 amino acids. A consensus sequence is given below the kinase sequences.

### Supplementary Figure 1A.



## Supplementary Figure 1B.

Swissprot	Name	Amino acid position/sequence				
Accession		1				
P45983-2: JNK1_a1	MSRSKRDNNF YSVEIGDSTF TVLKRYQNLK PIGSGAQGIV CAAVDAILER NVAIKKLSRP FQNQTHAKRA YRELVLMKCV NHKNIIGLLN VFTPQKSLEE	51				
P45983-1: JNK1_a2	MSRSKRDNNF YSVEIGDSTF TVLKRYQNLK PIGSGAQGIV CAAVDAILER NVAIKKLSRP FQNQTHAKRA YRELVLMKCV NHKNIIGLLN VFTPQKSLEE		FQDVYIVMEL	MDANLCQVIQ	.MELDHERMS	YLYQMLCGI KHLHSAGIIH
P45983-3: JNK1_b1	MSRSKRDNNF YSVEIGDSTF TVLKRYQNLK PIGSGAQGIV CAAVDAILER NVAIKKLSRP FQNQTHAKRA YRELVLMKCV NHKNIIGLLN VFTPQKSLEE		FQDVYIVMEL	MDANLCQVIQ	.MELDHERMS	YLYQMLCGI KHLHSAGIIH
P45983-4: JNK1_b2	MSRSKRDNNF YSVEIGDSTF TVLKRYQNLK PIGSGAQGIV CAAVDAILER NVAIKKLSRP FQNQTHAKRA YRELVLMKCV NHKNIIGLLN VFTPQKSLEE		FQDVYIVMEL	MDANLCQVIQ	.MELDHERMS	YLYQMLCGI KHLHSAGIIH
P45984-2: JNK2_a1	MSDSKCDSQF YSVQVADSTF TVLKRYQQQLK PIGSGAQGIV CAAFDTVLGI SAVAKKLSRP FQNQTHAKRA YRELVLLKCV NHKNIISLLN VFTPQKTL		E FQDVYIVMEL	MDANLCQVIH	.MELDHERMS	YLYQMLCGI KHLHSAGIIH
P45984-1: JNK2_a2	MSDSKCDSQF YSVQVADSTF TVLKRYQQQLK PIGSGAQGIV CAAFDTVLGI SAVAKKLSRP FQNQTHAKRA YRELVLLKCV NHKNIISLLN VFTPQKTL		E FQDVYIVMEL	MDANLCQVIH	.MELDHERMS	YLYQMLCGI KHLHSAGIIH
P45984-3: JNK2_b1	MSDSKCDSQF YSVQVADSTF TVLKRYQQQLK PIGSGAQGIV CAAFDTVLGI SAVAKKLSRP FQNQTHAKRA YRELVLLKCV NHKNIISLLN VFTPQKTL		E FQDVYIVMEL	MDANLCQVIH	.MELDHERMS	YLYQMLCGI KHLHSAGIIH
P45984-4: JNK2_b2	MSDSKCDSQF YSVQVADSTF TVLKRYQQQLK PIGSGAQGIV CAAFDTVLGI SAVAKKLSRP FQNQTHAKRA YRELVLLKCV NHKNIISLLN VFTPQKTL		E FQDVYIVMEL	MDANLCQVIH	.MELDHERMS	YLYQMLCGI KHLHSAGIIH
P47811-1: p38alpha	~~~SQERTPFF YRQELENKNTI EWVERYQNLS PVGSGAYGSV CAAFDTKTGH RAVAVKKLSRP FQSIIHAKRT YRELRLKKHM KHENVIGLLD VFTPARSLEE		FNDVYLVTHL MGADLNNIVK CQKLTDDHVQ FLIYQILRGL KYIHSADIH			
Consensus	MS-SK-D-- FYS---DSTF TVLKRYQ-LK PIGSGAQGIV CAAFD--L--	-VAVKKLSRP FQNQTHAKRA YRELVL-KCV NHKNII--LLN VFTPQK-LEE	FQDVY-VMEL	MDANLCQVI-	-MELDHERMS	YLYQMLCGI KHLHSAGIIH
Accession		150				
P45983-2: JNK1_a1	RDLKPNSIVV KSDCTLKLDF GLARTAGTS FMMTPYVVTR YYRAPEVILG .MGYKENVDL WSVGCI	201				
P45983-1: JNK1_a2	RDLKPNSIVV KSDCTLKLDF GLARTAGTS FMMTPYVVTR YYRAPEVILG .MGYKENVDL WSVGCI		M GYKENVDL FPEF MKKL.QPTVR TYVENRPKYA GYSFEKLF	PD VLFPADSEHN	KLKASQARDL	
P45983-3: JNK1_b1	RDLKPNSIVV KSDCTLKLDF GLARTAGTS FMMTPYVVTR YYRAPEVILG .MGYKENVDI WSVGCI		M GYKENVDI FPEF MKKL.QPTVR TYVENRPKYA GYSFEKLF	PD VLFPADSEHN	KLKASQARDL	
P45983-4: JNK1_b2	RDLKPNSIVV KSDCTLKLDF GLARTAGTS FMMTPYVVTR YYRAPEVILG .MGYKENVDI WSVGCI		M GYKENVDI FPEF MKKL.QPTVR TYVENRPKYA GYSFEKLF	PD VLFPADSEHN	KLKASQARDL	
P45984-2: JNK2_a1	RDLKPNSIVV KSDCTLKLDF GLARTACTN FMMTPYVVTR YYRAPEVILG .MGYKENVDI WSVGCI		M GYKENVDI FPEF MKKL.QPTVR TYVENRPKYA GYSFEKLF	PD VLFPADSEHN	KLKASQARDL	
P45984-1: JNK2_a2	RDLKPNSIVV KSDCTLKLDF GLARTACTN FMMTPYVVTR YYRAPEVILG .MGYKENVDI WSVGCI		M GYKENVDI FPEF MKKL.QPTVR TYVENRPKYA GYSFEKLF	PD VLFPADSEHN	KLKASQARDL	
P45984-3: JNK2_b1	RDLKPNSIVV KSDCTLKLDF GLARTACTN FMMTPYVVTR YYRAPEVILG .MGYKENVDI WSVGCI		M GYKENVDI FPEF MKKL.QPTVR TYVENRPKYA GYSFEKLF	PD VLFPADSEHN	KLKASQARDL	
P45984-4: JNK2_b2	RDLKPNSIVV KSDCTLKLDF GLARTACTN FMMTPYVVTR YYRAPEVILG .MGYKENVDI WSVGCI		M GYKENVDI FPEF MKKL.QPTVR TYVENRPKYA GYSFEKLF	PD VLFPADSEHN	KLKASQARDL	
P47811-1: p38alpha	RDLKPNSIVV NEDCELKIDF GLARH.. TD DEMTGYVATR WYRAPEIMLN WMHYNQVTDI WSVGCI		M GYKENVDI MAEL LTGRTLFPGT DHIDQLKLIL RLVGTGAE	L LKKISSESAR NYIQSLAQM P KMNFANVF.. .IGAN PL...AVDL		
Consensus	RDLKPNSIVV KSDCTLKLDF GLARTA-T- FMMTPYVVTR YYRAPEVILG -MGYKENVDI WSVGCI		GEM V--VLFPG- D-IDQWNKVI EQLGTP--EF MKKL.QPTVR -YVENRPKY- G--FE-LFPD --FP--SE-- K-K-SQARDL			
Accession		300				
P45983-2: JNK1_a1	LSKMLVIDAS KRISVDEALQ HPYINVWYDP SEAEAPPKPKI PDQLDEREH TIEEWKELIY KEV.....	301				
P45983-1: JNK1_a2	LSKMLVIDAS KRISVDEALQ HPYINVWYDP SEAEAPPKPKI PDQLDEREH TIEEWKELIY KEV.....		MDLEERTKNG VIRGQPSPL. AQVQQ~~~~~	~~~~~	~~~~~	
P45983-3: JNK1_b1	LSKMLVIDAS KRISVDEALQ HPYINVWYDP SEAEAPPKPKI PDQLDEREH TIEEWKELIY KEV.....		MDLEERTKNG VIRGQPSPL. AQVQQ~~~~~	~~~~~	~~~~~	
P45983-4: JNK1_b2	LSKMLVIDAS KRISVDEALQ HPYINVWYDP SEAEAPPKPKI PDQLDEREH TIEEWKELIY KEV.....		MDLEERTKNG VIRGQPSPL. AQVQQ~~~~~	~~~~~	~~~~~	
P45984-2: JNK2_a1	LSKMLVIDDP KRISVDEALR HPYITWYDP AEEAAPPQPI YDAQLEEREH AIEEWKELIY KEV.....		MDWEERSKNG VVKDQFS.. A AQMQQ~~~~~	~~~~~	~~~~~	
P45984-1: JNK2_a2	LSKMLVIDDP KRISVDEALR HPYITWYDP AEEAAPPQPI YDAQLEEREH AIEEWKELIY KEV.....		MDWEERSKNG VVKDQFS.. A AQMQQ~~~~~	~~~~~	~~~~~	
P45984-3: JNK2_b1	LSKMLVIDDP KRISVDEALR HPYITWYDP AEEAAPPQPI YDAQLEEREH AIEEWKELIY KEV.....		MDWEERSKNG VVKDQFS.. A AQMQQ~~~~~	~~~~~	~~~~~	
P45984-4: JNK2_b2	LSKMLVIDDP KRISVDEALR HPYITWYDP AEEAAPPQPI YDAQLEEREH AIEEWKELIY KEV.....		MDWEERSKNG VVKDQFS.. A AQMQQ~~~~~	~~~~~	~~~~~	
P47811-1: p38alpha	LEKMLVLDSKRITAAQALA HAYFAQYHDP DDEPVADP.. YDQSFESRDL LIDEWKSLTY DEVISFVPPP LDQEEMES~		~ ~~~~~	~~~~~	~~~~~	
Consensus	LSKMLVID-- KRISVDEAL- HPYI-WYDP -EAEAPPPI -D-QL-EREH -IEEWKELIY KEV-----		MD-EER-KNG V---QPS-- A-V-----			
Accession		437				

**Supplementary Figure 2.** Sequences of DARPins selected for JNK2, JNK1 or p38 binding. The amino acid sequences of the different DARPins are shown in three alignments, grouped with respect to their target. Note that in this representation only the amino acid sequences of the DARPin domains are shown, but N- or C-terminal sequence extensions, which were present due to the selection system used, are not shown. The designed sequence for the N3C library is 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). The residue numbers are given above the designed sequence for orientation. The target name and the name of the DARPins are given on the left side of the respective sequence. If several constructs were identical, only one sequence is listed and the names of the identical sequences are given in addition (i.e. for JNK2: pB4=pC4=pD4, pF6=pG6, pF7=pA12=rD12, pE8=pC6; for JNK1: p3=p7, p1=p8=p10=p11). Sequences that had recombined from N3C to N2C are shown at the top of each alignment group.

## Supplementary Figure 2.

## Binder Sequences JNK2alpha2:

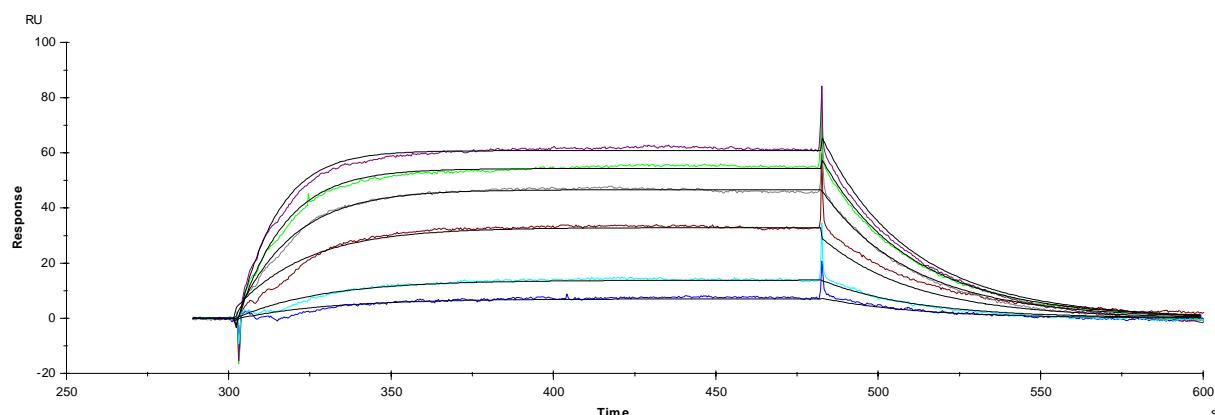
### Binder Sequences JNK1:

### Binder Sequences p38alpha:

## Affinity of selected DARPin

SPR was measured using a BIACore 3000 instrument (BIACore, Uppsala, Sweden). All measurements were done in HBS buffer (20 mM HEPES pH 7.4, 150 mM NaCl, 10 mM MgCl<sub>2</sub>, 1 mM DTT, 0.005% Tween 20) at a flow rate of 50 µl/min. Biotinylated pD\_JNK2α2 fusion protein was immobilized (600 RU) on a SA chip (BIACore). For the determination of kinetic data, the interactions were measured as follows: five minutes initial buffer flow, followed by a three-minute injection of DARPin in varying concentrations (2 nM to 200 nM) and a final off-rate measurement of 45 minutes with buffer flow. Clone pB4 was measured at higher concentrations (50 nM to 1 µM) using a shorter dissociation time (15 min). The signal of an uncoated reference cell was always subtracted from the sensorgrams. The kinetic data of the interaction were evaluated with a global fit using BIAevaluation 3.1 (BIACore). A representative set of curves with the corresponding fits are shown below:

**pB4**



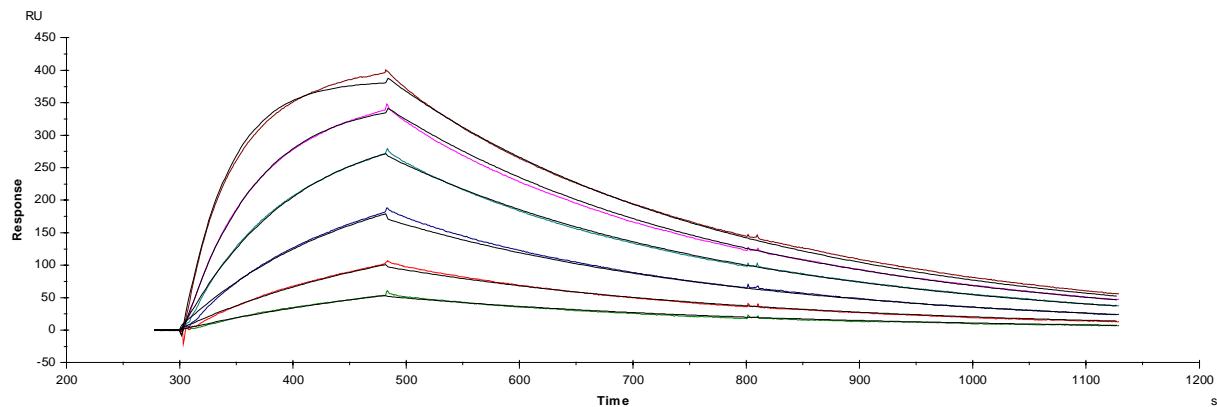
$$k_{\text{on}} = 5.8 \cdot 10^4 \text{ M}^{-1} \text{ s}^{-1}$$

$$k_{\text{off}} = 0.0422 \text{ s}^{-1}$$

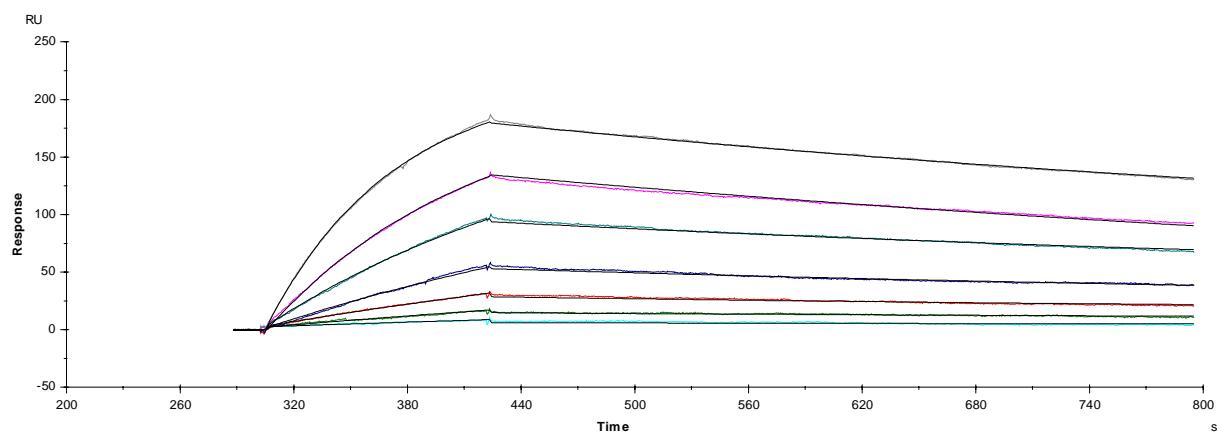
$$K_D = 7.27 \cdot 10^{-7} \text{ M}$$

DARPin concentrations [M]:  $5 \cdot 10^{-8}$ ,  $1 \cdot 10^{-7}$ ,  $2.5 \cdot 10^{-7}$ ,  $5 \cdot 10^{-7}$ ,  $7.5 \cdot 10^{-7}$ ,  $1 \cdot 10^{-6}$

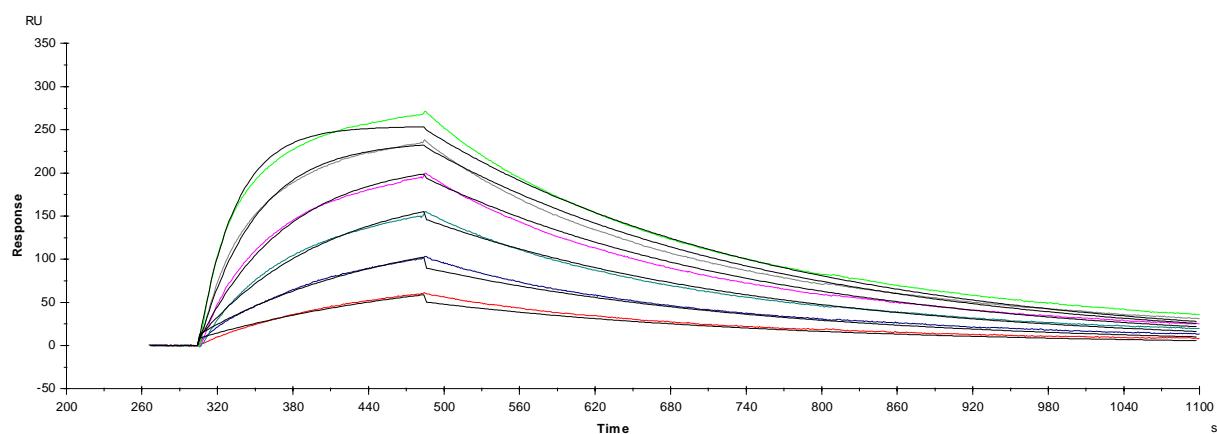
### pG7



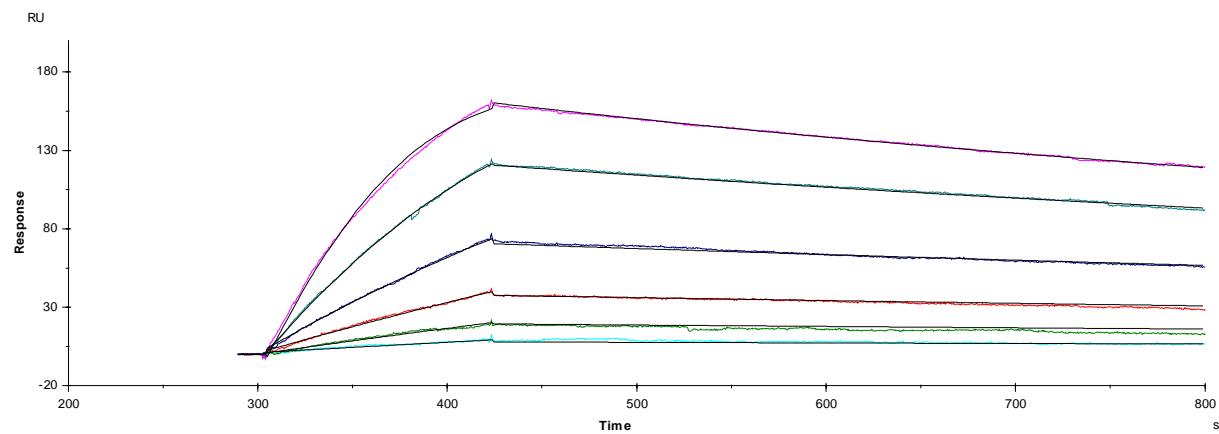
### pB8



### pB11



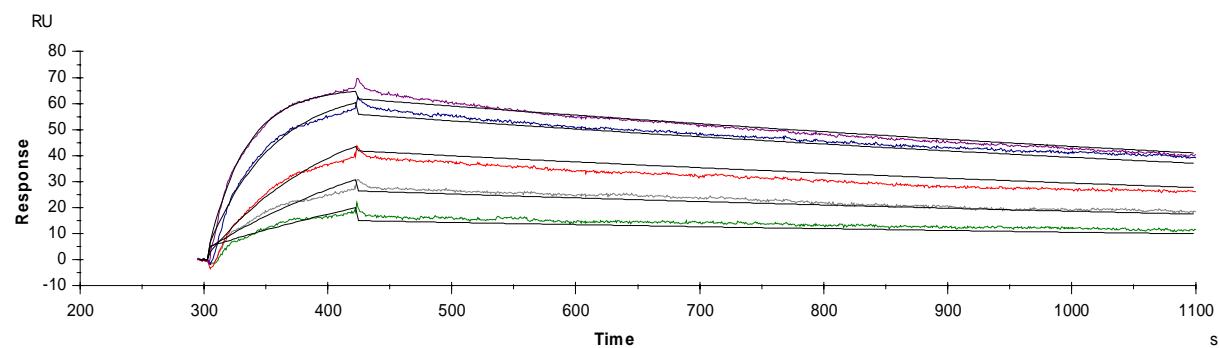
## pE11



$$k_{\text{on}} = 3.99 \cdot 10^5 \text{ M}^{-1} \text{ s}^{-1} \quad k_{\text{off}} = 1.01 \cdot 10^{-3} \text{ s}^{-1} \quad K_D = 2.53 \cdot 10^{-9} \text{ M}$$

DARPin concentrations [M]:  $2 \cdot 10^{-9}$ ,  $5 \cdot 10^{-9}$ ,  $1 \cdot 10^{-8}$ ,  $2 \cdot 10^{-8}$ ,  $4 \cdot 10^{-8}$ ,  $7 \cdot 10^{-8}$

## pD12



$$k_{\text{on}} = 4.65 \cdot 10^5 \text{ M}^{-1} \text{ s}^{-1} \quad k_{\text{off}} = 6.09 \cdot 10^{-4} \text{ s}^{-1} \quad K_D = 1.31 \cdot 10^{-9} \text{ M}$$

DARPin concentrations [M]:  $5 \cdot 10^{-9}$ ,  $1 \cdot 10^{-8}$ ,  $2 \cdot 10^{-8}$ ,  $4 \cdot 10^{-8}$ ,  $7 \cdot 10^{-8}$