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Attacking cancer biomarker clusters from all sides

- bispecific, bivalent and polyspecific affinity proteins towards HER family receptors

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Title (English)

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Abstract

Potential cancer therapy drugs target and inhibit the phosphorylation of the human epidermal growth factor receptor (HER) family. Due to signalling crosstalk between the receptors drugs targeting two or more receptors can improve the inhibition. Affibodies are targeting affinity proteins that have been derived from the immunoglobulin-binding staphylococcal protein A. An ABD - derived affinity protein (ADAPT) has a HER target region as well as an albumin binding domain (ABD) binding human serum albumin in the bloodstream enabling longer in-vivo half-life. The two types of affinity proteins were used to produce constructs, bispecific, bivalent and polyspecific, for cancer biomarker receptors, HER1, HER2, HER3 as well as VEGFR-2 and IGF1R. The resulting affinity was measured with SPR, and it gave the lowest K_D in the range of 1^{-10} M.

Keywords

Cancer therapy drugs, HER family, ADAPT, SPR

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Attacking cancer biomarker clusters from all sides

- bispecific, bivalent and polyspecific small protein binders towards HER-family receptors

Populärvetenskaplig sammanfattning

Katarina Ivermark

Det har visats att en grupp av receptorer den så kallade human epidermal growth factor receptor-familjen, HER familjen, är biomarkörer för cancersjukdomar. HER familjen har fyra medlemmar. HER1, HER2, HER3 och HER4, och dessa kan vara uttryckta på cancerceller i huvud, nacke, bröst och äggstockar. Receptorerna har tyrosinkinas aktivitet som leder till fosforylering och kan öka celltillväxten och bilda tumörer. HER2 överuttrycks ofta på bröstcancerceller och cancerterapi som inhiberar dess fosforylering har utvecklats och testats. HER3 kan vid inbindning av den naturliga liganden heregulin heterodimerisera d. v. s. bilda ett complex med ytterligare en receptor i HER familjen, och framkalla fosforylering som driver tillväxten av cancertumörer. Att använda en cancermedicin som binder flera receptorer i HER familjen kan därmed ge ett bättre resultat än en terapi som enbart binder en utav dem.

Antikroppars funktion som specifikt bindande molekyler till antigener i immunförsvaret har imiterats för att producera molekyler med hög specifik bindning, hög affinitet, till specifika målmolekyler. Affibody molekyler är ett sådant exempel på ett affinitetsprotein och har producerats med HER receptorer som målmolekyler. Affibody molekyler är uppbyggda från protein A uttryckt i organismen *Staphylococcus aureus*, de består av tre sammankopplade helixar och är mer än 20 gånger så små som stora antikroppar. Den mindre storleken för affibody molekyler medför en lättare penetration genom cellerna i kroppen. Ett problem som uppstår är att affibody molekyler inte kan stanna i kroppen tillräcklig länge, på grund av sin ringa storlek, de kan lätt forslas ut ur kroppen via njurarna. Detta kan lösas genom att länka affibodys till albumin bindande domäner, ABDs. Dessa binder humant serum albumin, HSA, vilket är närvarande i blodet och behåller därmed affibody molekylerna i blodströmmen. En ytterligare utveckling har varit att kombinera dessa specifikt bindande egenskaper, affinitet för specifika målmolekyler, med en ABD vilket ger ett **A**BD-**d**eriverat **a**ffinitets **p**ro**t**ein, en ADAPT molekyl.

I detta examensarbete länkades affibody- och ADAPT molekyler samman för att producera protein som binder biomarkörer för cancer, tre HER receptorer samt ytterligare två cancerrelaterade receptorer; VEGFR-2 och IGF1R. Detta för att öka affiniteten och motverka resistensen som kan uppstå om endast en receptor binds åt gången. Bivalenta protein vilka binder två av samma receptormedlem i detta fall HER2 alternativt HER3 producerades. Bispecifika protein vilka var tänkta att binda både HER2 och HER3 samtidigt producerades också. Slutligen producerades polyspecifika protein som var tänkta att binda så många cancerrelaterade receptorer som möjligt. Dessa hade subdomäner med bindningsegenskaper för HSA, HER1, HER2, HER3, VEGFR-2 samt IGF1R. En polyspecifik hexamer producerades vilken hade sex stycken bindande domäner och en polyspecifik oktamer vilken hade åtta stycken bindande domäner. Alla dessa proteins affinitet testades sedan med en optisk biosensor och de resulterande värdena evaluerades för att avgöra molekylernas förmåga att binda cancerrelaterade receptorer.

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Civilingenjörsprogrammet i Molekylär bioteknik

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Abbreviations

HSA - Human Serum Albumin

ABD - Albumin Binding Domain

ADAPT – ABD-Derived Affinity Protein

SPR – Surface Plasmon Resonance

MS - Mass Spectrometry

BCA - Bicinchoninic acid assay

HPLC – High Performance Liquid Chromatography

1. Introduction

The human epidermal growth factor receptor (HER) family consist of four receptors with tyrosine kinase activity. The phosphorylation signalling of HER family receptors are present in various cancer types such as head, neck, breast and ovarian cancer (Salomon et al., 1995). One of the receptors, HER2 is overexpressed on breast cancer cells (Slamon et al., 1987). There are already anti-cancer drugs on the market, targeting and inhibiting the phosphorylation of the HER2 receptor (Sergina et al., 2007), however their effect might be diminished due to crosstalk signalling between different members of the HER family. A bispecific drug targeting two or more receptors is a possible solution to the resistance and has been tested in earlier studies (Kontermann, 2012). The receptor HER3 as opposed to HER2 does not have a tyrosine kinase unit that is independently functional (Guy et al., 1994). The binding to HER3 by the natural ligand heregulin (Burden and Yarden, 1997) will induce heterodimerisation and form a HER2-HER3 complex. This unit is oncogenic (Fig. 1) and produces downstream phosphorylation (Holbro et al., 2003; Sergina et al., 2007).

In addition to the HER family there are other receptors that are cancer biomarkers. The vascular endothelial growth factor receptor 2 (VEGFR-2) is present in tumor angiogenesis and has been related to different types of cancers (Yan et al., 2015). The insulin-like growth factor type 1 receptor (IGF1R) has an abnormal regulation in breast cancer (Kang et al., 2015).

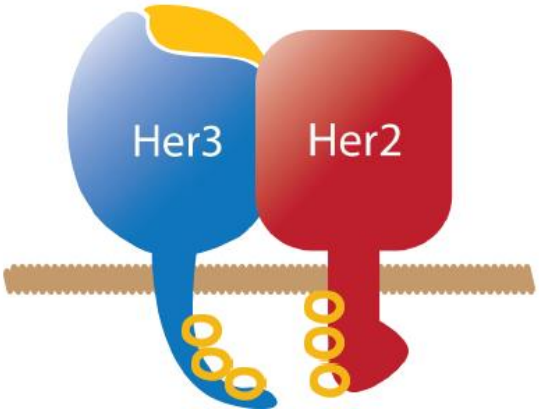

Figure 1. Crosstalk between the membrane proteins HER2 and HER3 when the ligand heregulin binds HER3 resulting into downstream phosphorylation.

Cancer receptor targeting and therapy can be achieved by designing affinity proteins with affinity for a cancer receptor. The general model, an antibody (immunoglobulin) with high specificity, can be imitated by generating affinity proteins using non-immunoglobulin based protein scaffolds (Löfblom et al., 2011). The affibody is one example of an affinity protein which has been developed into binding HER receptors. The affibody consists of a single three-helix domain with 13 randomized amino acids to achieve affinity for the target (Fig. 2A). Affibodies binding HER2 with 22 pM affinity and HER3 with 0.7 nM affinity have been selected through phage display and staphylococcal surface display (Kronqvist et al., 2011; Orlova et al., 2006). The affibody is derived from the B-domain of the immunoglobulin-binding region of protein A in *Staphylococcus aureus* (Uhlén et al., 1984). It has a molecular weight of 6.5 kDa (Löfblom et al., 2010) and is significantly smaller than a large immunoglobulin molecule of 150 kDa (Holliger and Hudson, 2005). The small size of the affibody enables easier penetration through a cancer tumor (Göstring et al., 2012).

A disadvantage of the affibodys size is that it can be eliminated fast through the kidneys (Nilvebrant et al., 2014), this may be solved by linkage between the affibody and an three-helix albumin binding domain (ABD) binding human serum albumin (HSA) in the blood stream (Jonsson et al., 2008;

Malm et al., 2014) (Fig. 2B). It is also possible to combine the HSA-binding features of an ABD with the targeting features of an affibody into one single three-helix domain with HER targeting on one side of the backbone and HSA binding on the other side. The molecule is an ABD-derived affinity protein, an ADAPT molecule, derived from the streptococcal protein G and has a similar size to an affibody with the molecular weight of 5 kDa (Nilvebrant et al., 2011, 2014) (Fig. 2C).

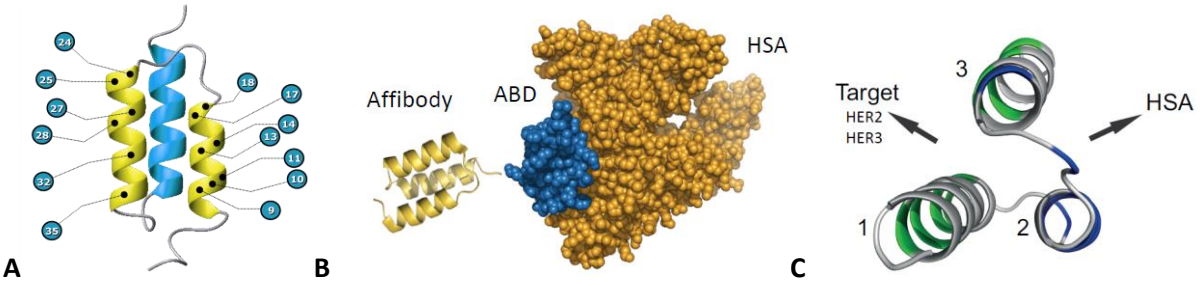

Figure 2. (**A**) Affibody with 13 randomized amino acids to increase the affinity for the target. (**B**) Affibody linked to ABD binding HSA. (**C**) ADAPT molecule with and ABD engineered to bind the target HER2 or HER3 on one side and HSA on the other side of the backbone.

Another difference between the antibody and the affibody or ADAPT molecule is the ability of the antibody to bind two targets. The antibody binds two epitopes which are the recognition sites on the target antigen. The resulting accumulated affinity strength from both binding sites will give a functional affinity, called avidity. This avidity has been imitated with engineering, in the study of Malm et al. (2014). Avidity with affibody constructs, binding HER targets, were achieved by linking ABD and two affibodies, binding HER2 and HER3. The measured affinity was 0.6 nM for HER2 and 0.6 nM for HER3 for a ZHER3-ABD-ZHER2 construct. For the opposite ZHER3-ABD-ZHER3 orientation the affinity was 0.7 nM for HER2 and 0.8 nM for HER3. Furthermore the constructs ability to inhibit HER receptor phosphorylation was examined. The study suggested that the orientation of the construct of the HER2 and HER3 may affect the downstream phosphorylation, it could be agonistic (favoured) or antagonistic (disfavoured). The ZHER3-ZHER2 was antagonistic according to the study, which was the preferred effect.

In the master thesis project, affibodies (referred to as Z) and ADAPT molecules were linked together in order to produce constructs targeting two or more cancer biomarkers, HER1, HER2, HER3, VEGFR-2 and IGF1R. The constructs had bispecificity, bivalency or polyspecificity. The bispecific construct had specificity for two HER receptor members, HER2 and HER3, and the ability to bind two targets simultaneously. The bivalent construct had specificity for one HER receptor member, either HER2 or HER3 and the ability to bind to two of this receptor simultaneously. The polyspecific constructs had specificity for five different receptors including HER1, HER2, HER3, VEGFR-2 and IGF1R. Due to the results in previous studies where the orientation seemed to influence the phosphorylation different orientation with the affibody and ADAPT was produced for these targets. Bispecific constructs targeting HER2 and HER3 (Fig. 3A) were produced in two orientations by PCR cloning. The linker used was a single G_4S flexible linker. Bivalent constructs targeting HER2 and HER3 were produced in two orentations (Fig. 3B, 3C). Different linker lengths were tested for the constructs bivalent for the HER2 receptor since this is the receptor mostly characterized as cancer related. The linker had three different lengths; G_4S, $(G_4S)_2$ and $(G_4S)_3$. The constructs targeting two receptors had a molecular weight of around 13 kDa which was half the size of the constructs produced in the study by Malm et al. (2014) which had a molecular weight of approximately 28 kDa. Polyspecific constructs were expressed in order to bind as many targets as possible. Their targets were HER1 (EGFR), HER2, HER3 and HSA as well

as the receptors VEGFR-2 and IGF1R. Two polyspecific constructs were expressed; one polyspecific hexamer consisting of six protein domains and one polyspecific octamer consisting of eight protein domains. The polyspecific hexamer (Fig. 4A) had ADAPT molecules targeting HER2 and HER3. It also had affibodies targeting HER1, two epitopes of VEGFR-2 and IGF1R. The polyspecific octamer (Fig. 4B) had the same subdomains with the addition of two affibodies targeting HER2 and HER3. The polyspecific hexamer had a molecular weight of 39 kDa and the polyspecific octamer a molecular weight of 53 kDa. All the produced construct, their targets, specificity, linker length and molecular weight are listed in Table 1.

Gel electrophoresis and Sanger sequencing were used to confirm the correct DNA sequence of the construct. After the protein expression Mass spectrometry (MS) and SDS-PAGE was used to validate the correct molecular weight for the protein constructs. The affinity was measured with Surface Plasmon Resonance (SPR). The majority of the constructs had an affinity between 10 and 0.1 nM which was close to the affinity previously achieved by Malm et al. (2014).

Table 1. All the produced construct, their target receptor, specificity, linker and molecular weight.

Construct name	Target receptor	Specificity	Linker	Molecular weight [kDa]
ADAPTHER3-ZHER2	HSA, HER2, HER3	Bispecific	G_4S	12.7
ZHER2-ADAPTHER3	HSA, HER2, HER3	Bispecific	G_4S	12.7
ADAPTHER3-ZHER3	HSA, HER3	Bivalent	G_4S	12.7
ZHER3-ADAPTHER3	HSA, HER3	Bivalent	G_4S	12.7
ADAPTHER2-1-ZHER2	HSA, HER2	Bivalent	G_4S	12.8
ZHER2-1-ADAPTHER2	HSA, HER2	Bivalent	G_4S	12.8
ADAPTHER2-2-ZHER2	HSA, HER2	Bivalent	$(G_4S)_2$	13.1
ZHER2-2-ADAPTHER2	HSA, HER2	Bivalent	$(G_4S)_2$	13.1
ADAPTHER2-3-ZHER2	HSA, HER2	Bivalent	$(G_4S)_3$	13.4
ZHER2-3-ADAPTHER2	HSA, HER2	Bivalent	$(G_4S)_3$	13.4
Polyspecific hexamer	HSA, HER1, HER2, HER3, VEGFR-2, IGF1R	Polyspecific	G_4S	38.7
Polyspecific octamer	HSA, HER1, HER2, HER3, VEGFR-2, IGF1R	Polyspecific	G_4S	52.7

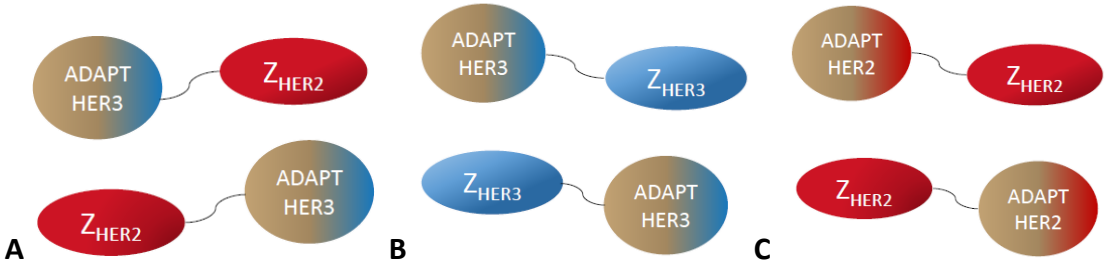

Figure 3. Constructs with two protein domains linked with a G_4S flexible linker. (**A**) Bispecific constructs targeting HER2 and HER3 with ADAPT for HER3 and an affibody for HER2. (**B**) Bivalent constructs targeting HER3 with ADAPT and affibody for HER3. (**C**) Bivalent constructs targeting HER2 with ADAPT and affibody for HER2.

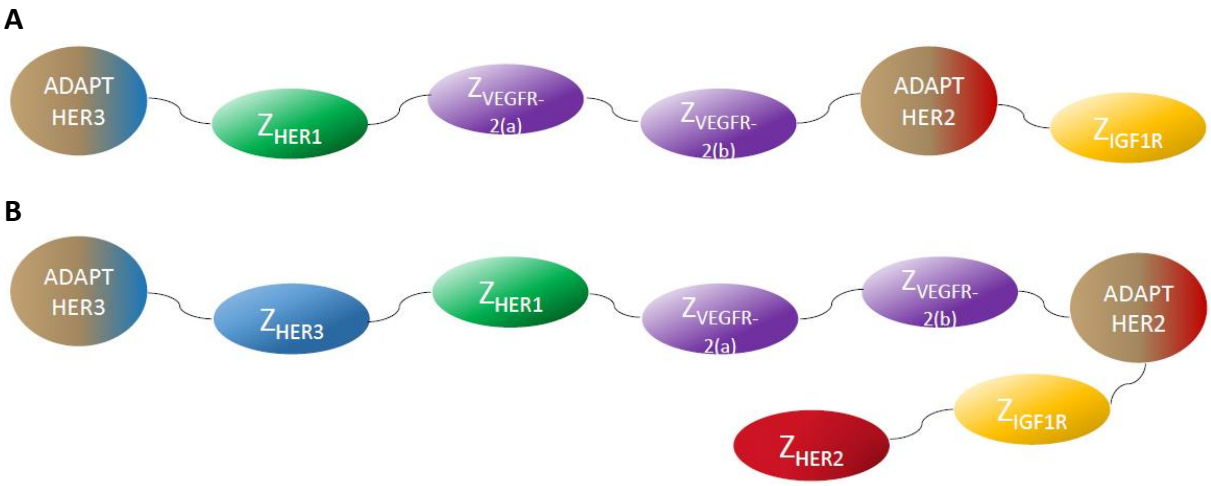

Figure 4. Polyspecific constructs. (**A**) Polyspecific hexamer with ADAPT molecules targeting HER3 and HER2 and affibodies for HER1, two epitopes of VEGFR-2 and IGF1R. (**B**) Polyspecific octamer with ADAPT molecules targeting HER2 and HER3 and affibodies for HER2, HER3, EGFR, two epitopes of VEGFR-2, and IGF1R.

2. Materials and Methods

2.1. Cloning bispecific constructs for HER2 and HER3 and bivalent constructs for HER3

Bispecific and bivalent molecules were engineered using monovalent binders and linked by PCR cloning. The constructs contained subdomains for ADAPTHER3, ZHER2, and ZHER3, a G_4S-linker, the outer restriction sites NcoI and AscI, and a cysteine in the C-terminal (Fig. 5). The outer primers were flanking the restriction sites NcoI and AscI and the inner primers created an overlap for the G_4S linker (Table S1).

The first step of the cloning concerned the amplification of each subdomain, ADAPTHER3, ZHER2 and ZHER3 according to their orientation in the construct. For the PCR New England Biolabs® Phusion® HF Reaction Buffer or New England Biolabs® Phusion® GC Reaction Buffer was used, depending on the GC content of the primers, and Phusion® DNA polymerase (Table S2). A test cloning for each subdomain was used to establish the appropriate annealing temperature in order to obtain the correct PCR product (Table S9). The correct size was confirmed by gel electrophoresis using 1 % Agarose gels (2g Standard Agarose – Type LE BioNordika dissolved in 200 ml TAE Buffer) with GelRed™ Nucleic Acid Gel stain x 10.000 in water BIOTIUM, Thermo Scientific DNA Loading Dye and GeneRuler™ DNA Ladder Mix. Thereafter the product was amplified by making 5 PCR reactions for each subdomain with the same PCR protocol and PCR program. The 5 PCR products were pooled and purified with QIAGEN QIAquick PCR Purification Kit. In the case of unspecific products visible on the Agarose gel the PCR product was run through a 1 % GTG gel (2 g SeaKem®GTG® Agarose Lonza, 200 mL 1 x TAE Buffer), the correct product was cut out with a scalpel in UV-light and purified with QIAGEN QIAquick® Gel Extraction Kit.

The cloning gave the subdomains ADAPTHER3-N, ZHER2-N, ZHER3-N for the N-terminal position of the construct and ADAPTHER3-C, ZHER2-C, ZHER3-C for the C-terminal position of the construct. The constructs were built in the second step using the amplified and purified PCR Product of each subdomain as template and the outer primers flanking the restriction sites in a Phusion PCR (Table S3). The ends of the cloned constructs were cleaved off in a digestion at the restriction sites NcoI and AscI with New England Biolabs® Cutsmart™ Buffer and the enzymes AscI and NcoI from Thermo Scientific (Table S4).

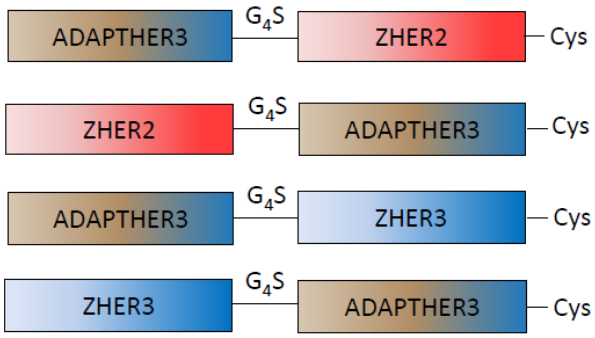

Figure 5. Bispecific constructs for HER3 and HER2 in two orientations; ADAPTHER3-ZHER2, ZHER2-ADAPTHER3 and bivalent constructs for HER3 in two orientations; ADAPTHER3-ZHER3, ZHER3-ADAPTHER3. The constructs have a G_4S-linker and a cysteine in the C-terminal.

2.2. Vector

The vector for protein expression was pHisDummy with a T7 promoter inducible by IPTG and a kanamycin resistance gene. The vector initially had a nonsense Dummy insert with a His-tag in the C-terminal. The vector that was used had the Dummy insert cleaved off and instead a Z wildtype binding IGG. The plasmid vector was expressed in Top10 cells. The insert was removed in a digestion at the restriction sites NcoI and AscI in New England Biolabs® Cutsmart™ Buffer and the enzymes AscI and NcoI from Thermo Scientific (Table S5). The digest was incubated on heat-block at 37 °C for 3 hours. Dephosphorylation to prevent religation was performed with New England Biolabs® Antarctic Phos Reaction buffer and New England Biolabs® Antarctic Phosphatase (Table S6) and incubated on heat-block at 37 °C for 1 hour. Gel extraction was used to isolate the vector from the Dummy insert with a 1 % GTG-gel (2 g SeaKem®GTG® Agarose Lonza, 200 mL 1 x TAE Buffer), the fragment was cut out in UV-light and purified with QIAGEN QIAquick Gel extraction Kit.

2.3. Ligation

Ligation between the vector and purified PCR fragment of the constructs was performed with New England Biolabs® Buffer for T4 DNA ligase with 10 mM ATP and New England Biolabs® T4 DNA Ligase (Table S7). A negative control with no insert was included for each ligation and transformation. A test-ligation for the vector with another insert confirmed that the pHisDummy vector was working properly. The test-ligation was then transformed into Top10 cells and the amount of cell growth was noticed.

2.4. Presynthesized bivalent constructs for HER2 and polyspecific constructs

Construct bivalent for HER2 were ordered for synthesis as well as polyspecific constructs binding HSA, HER1, HER2, HER3, IGF1R and VEGFR-2. The bivalent constructs for HER2 had one ADAPT molecule and one affibody molecule linked together with G_4S linkers in different lengths and orientation and had a cysteine in the C-terminal. They were called ADAPTHER2-1-ZHER2, ADAPTHER2-2-ZHER2, ADAPTHER2-3-ZHER2, ZHER2-1-ADAPTHER2, ZHER2-2-ADAPTHER2 and ZHER2-3-ADAPTHER2 (Fig. 6). The polyspecific constructs had ADAPT and affibodies linked with single G_4S linkers, a cysteine in the C-terminal. They had either 6 binding regions in a polyspecific hexamer and or 8 binding regions in a polyspecific octamer (Fig. 7). The constructs came in pET26b(+) vectors with a T7 promoter and a kanamycin resistance gene.

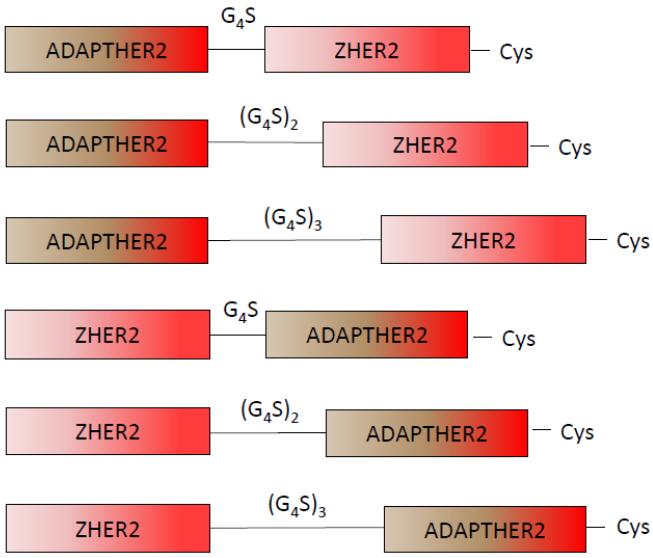

Figure 6. Bivalent constructs for HER2 and HSA in two orientations with 3 different linker lengths G_4S, $(G_4S)_2$ and $(G_4S)_3$. From the top; ADAPTHER2-1-ZHER2, ADAPTHER2-2-ZHER2, ADAPTHER2-3-ZHER2, ZHER2-1-ADAPTHER2, ZHER2-2-ADAPTHER2 and ZHER2-3-ADAPTHER2.

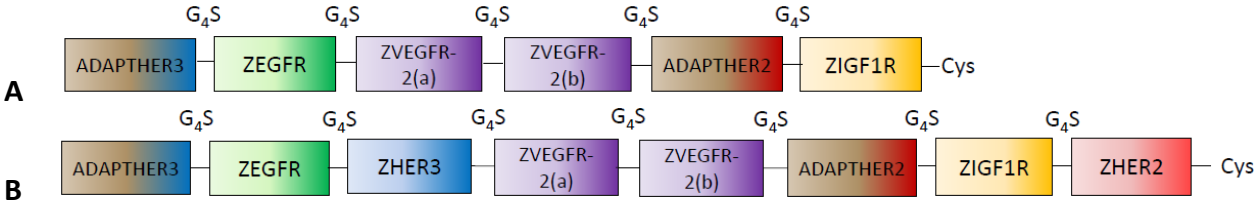

Figure 7. Polyspecific constructs for HER1, HER2, HER3 and two epitopes of VEGFR-2 and ZIGF1R. (**A**) Polyspecific hexamer. (**B**) Polyspecific octamer.

2.5. Transformation in Top 10 cells

Heat shock transformation into chemically competent Top 10 *Escherichia coli* cells were performed for each correct construct. A mixture of 8 µl ligation and 2 µl 5 x KCM was incubated on ice for 2-5 min. Thereafter 10 µl chemically competent *E. coli* cells were added to the mixture and incubated on ice for 20 min. The cells were heat-shocked at 42 °C for 40 seconds, 200 µl TSB media was added to the samples and the cells were incubated on a rotamixer at 37 °C for 30-60 min. The cell suspensions were thereafter plated on Agar kanamycin plates. The plated cells were grown overnight at 37 °C.

2.6. Colony PCR and Sanger sequencing

Colony PCR was used to confirm that the construct had the correct size. Colonies from the transformation into Top 10 cells were picked for screening in colony PCR. The PCR was performed using Thermo Scientific F-511 10 x Optimized DynAzyme Buffer and Thermo Scientific DynAzyme II DNA Polymerase (Table S7, S10). Sequencing was used to confirm correct colonies for the constructs. The preparation for Sanger sequencing concerned a PCR (Table S8, S11), followed by ethanol precipitation. In the ethanol precipitation 25 µl cold 95 % ethanol mixed with 1 µl 3 M NaAc pH 5 was added to each well from the PCR. The plate was closed, vortexed, the samples were spun down and put in the freezer for at least 15 minutes. The samples were centrifuged at 4600 rpm for 30 minutes. The liquid was then

eliminated by putting the plate upside down, discarding the liquid in the sink and hitting the plate upside down on paper. After addition of 150 µl 70 % ethanol the plate was centrifuged at 4600 rpm for 15 minutes. The liquid was eliminated by discarding it in the zink and the plate was kept upside down and spun on paper. The samples were then let airdry in a dark environment and thereafter resuspended in 20 µl Staq. The samples were sequenced in AME Bioscience 3730xl DNA Analyzer. The sequences were then analysed by Geneious software.

2.7. Inoculation Top 10 cells

Inoculation of the correct colonies in 10 ml TSB media with 1% kanamycin in a 100 ml sterile E-flask was grown overnight on shaking at 37 °C. The culture were miniprepped by QIAGEN QIAquick Miniprep Kit.

2.8. Protein expression

Heat shock transformation of the plasmids containing the constructs into chemically competent BL21*DE3 cells was performed for protein expression. A mixture of 1 µl ligation, 7 µl Staq and 2 µl 5 x KCM was incubated on ice for 2-5 minutes. Thereafter 10 µl chemically competent *E. coli* cells were added to the mixture and incubated on ice for 20 min. The cells were heat-shocked at 42 °C for 40 seconds and 200 µl TSB media was added to the sample. The cell suspension was incubated on rotamixer, ELMI Sky Line Intelli-mixer, in 37 °C for 30-60 min. The suspension was thereafter plated on Agar kanamycin plates. The plated cells were grown overnight at 37 °C.

Inoculation into 10 ml culture with 10 ml TSB+Y media with 500 µg/ml kanamycin in a 100 ml sterile E-flask for protein expression was grown overnight on shaking at 37 °C. The next day a culture of 1 l with 1 l TSB+Y media, 500 µg/ml kanamycin and 1 ml of the 10 ml culture added were grown on shaking at 37 °C. OD_{600} for *E. coli* was measured continuously until it had reached 0.8 - 1.5. Induction was started adding 100 µl 0.5 M IPTG and incubating the culture in 25 °C on shaking overnight. Glycerol stock was made mixing 200 µl Glycerol with 800 µl IPTG-induced sample and stored at -80°C. The cultures were put in GS3-tubes and centrifuged at 4628 rpm for 8 min. The supernatant was poured off and the pellet was put in a -20 °C freezer.

The cells were lysed by sonication. The pellet was resuspended by adding 10 ml 1 x TST buffer and using vortex. Dilution was then made to 20 ml with 1 x TST Buffer. The solution was transferred to a SS34-tube and put on in a beaker with ice. The beaker with the open tube was placed in the sonicator, a High Intensity Liquid Processor, so that the Microtip reached inside the tube, about 1 cm above the bottom of the tube. Sonication with at on pulse 1.0 second, off pulse 1.0 second, 40 % force was performed for 1.5-3 minutes. The sample was thereafter centrifugated to eliminate cell debris at 10000 g for 20 minutes. The sample was filtered with a 0.45 µm-filter into a Falcon tube, 25 µl was saved for SDS-PAGE analysis.

2.9. Anti-ABD purification

A test purification with an Anti-ABD column revealed if the ADAPT molecule had an ABD region binding Anti-ABD. A 1.7 ml Anti-ABD column containing 1 x TST + 20 % ethanol was used. Two test lysates containing ADAPT6 were tested, GC-SS-HE3-DEADANS-ADAPT6 and GC-VDANS-ADAPT6. The column was pre-equilibrated with 7 column volumes (CV) 1 x TST. Thereafter 3 ml sample was loaded onto the column. The flow-through was saved to see if the sample bound. The column was washed with 5 CV 1 x TST followed by 10 CV 5 mM NH_4Ac pH 5.5. Fractions were collected by eluation with 3 CV 0.1 M HAc into 0.5 ml fractions in Eppendorf tubes. Re-equilibration with 7 CV of 1 x TST and cleaning-in-place with 3 CV 0.5 M NaOH followed by 5 CV 1 x TST to re-equilibrate the matrix in between the two purification runs. The OD_{280} for protein content was measured for each fraction to

determine the fractions containing protein. The measurement gave no protein peaks which is why HSA columns binding HSA was used instead for the purification, see below.

2.10. HSA purification

A HSA column was used to purify the expressed protein. Buffers of TST pH 8.0, 0.5 M HAc pH 2.8 and 5 mM NH_4Ac pH 5.5 was prepared and filtered with a 0.45 µM filter. The column was firstly pulsed with 30 ml Milli-Q water, 30 ml 0.5M HAc and 60 ml TST. For the purification 75 ml TST was applied followed by the 20 ml lysate sample. An additional 75 ml TST was applied, then 50 ml 5 mM NH_4Ac. Fractions was collected by eluation in 12 fractions of 1 ml with 0.5 M HAc. The column was again pulsed with 30 ml 0.5 M HAc, 30 ml 5 mM NH_4Ac, 30 ml TST, 30 ml TST + 20 % EtOH. The OD_{280} for protein content was measured for each fraction to determine the fractions containing protein. The 0.5 M HAc fractions with the highest OD_{280} were freeze dried over night with SCANVAC Cool Safe. The dried protein was diluted in PBS to 100 µM. The sample with correct size according to the gel were pooled to create a protein batch for each construct.

2.11. SDS-PAGE

SDS-PAGE was used to confirm the purification and correct size of the fractions with higher protein content according to the OD_{280} measurement. To prepare the samples for the denaturation 5 -10 µl protein of each sample was mixed it with a 1:1 solution of beta-mercaptoethanol and glycerol. The samples were boiled for at least 5 minutes at 95 °C. Using Mini-PROTEAN® TGX™ Precast Gels, Any kD™, 10-well comb, 30 µl/well BIO-RAD and Mini-PROTEAN®Tetra System BIO-RAD. The gel was run at 150 V for 35 minutes. The gel was washed with deionized water until excess dye was removed from the gel. The gel was stained with GelCode®Blue Stain Reagent and left on a shaking for 45 minutes. The gel was then washed with deionized water for 60 minutes on shaking. The water was changed and left for another 60 minutes which after the gel was put on shaking over night with fresh deionized water. All the 12 expressed and HSA-purified constructs was visualized by a gel with Novex by life technologies NuPAGE 4-12% Bis-Tris Gel 1.0 mm X 15 well and a XCell Sure Lock TM Electrophoresis Cell.

2.12. Bicinchoninic acid assay

The concentration of protein in solution of the pooled batch was determined with Bicinchoninic acid assay (BCA). The protein fraction concentration of µg/ml was calculated and adjusted to be within the range of the reference concentrations. The protein batch was diluted to create replicates. The assay was performed in a Greiner bio one REF 655161 PS – Microplate, Sterile, 96 w Flat Bottom well plate were 25 µl sample was mixed with a 200 µl solution of Pierce® BCA Protein Assay Reagent A and Pierce® BCA Protein Assay Reagent B in a 51:1 ratio. The mixtures were incubated for 30 minutes at room temperature. The resulting color change was then measured with Sunrise TECAN and analysed with Magellan 4 software. A standard curve was created and the concentration of protein in solution were calculated from the equation of the trendline from the standard curve.

2.13. Liquid chromatography mass spectrometry

The protein samples were prepared for Liquid Chromatography Mass Spectrometry (LC-MS) by taking 50 ng/µl of each sample and adding it into a solution of 5 % acetonitrile and 0.1 % Formic Acid to a total volume of 50 µl in a 1.5 ml Eppendorftube. The samples were sent in for LC-MS to obtain data. The data showed a molecular weight of 300 Da more than expected for three constructs. Therefore the protein samples were also measured by MS under reduced conditions to prevent the cysteine in the C-terminal from reacting forming disulphide bridges with unwanted fragments giving

this excess molecular weight. A solution of 0.1 M Sigma-Aldrich DL-Dithiothreitol (DTT) was prepared and added to a final concentration of 20 mM. The samples were incubation on a 40 °C heating block for 1 hour. The buffer was changed with a GE Healthcare NAP-5 columns to PBS. The protein concentration was measured with Nanodrop. Thereafter 5 % acetonitrile and 0.1 % Formic Acid was mixed with the reduced protein sample to a total volume of 50 µl in a 1.5 ml Eppendorftube and sent for LC-MS measurement.

2.14. High performance liquid chromatography

To analyse the purity of the cloned protein samples, ADAPTHER3-ZHER2, ZHER2-ADAPTHER3 and ZHER3-ADAPTHER3, high performance liquid chromatography (HPLC) was used. The ADAPTHER3-ZHER3 protein solution did not have the correct molecular weight according to the MS results and was therefore not analysed by HPLC. Agilent Technologies HPLC with a 4.6 x 150 mm column and the software Agilent Chem Station for LC and LC/MS systems was used for the analysis. To prepare the sample 50 µg protein sample diluted in 1 x PBS was mixed with 20 % Acetonitrile + 0.1 % Trifluoroacetic acid (TFA), 80 % H_2O + 0.1 % TFA to a volume of 250 µl to use the same buffers as the system. The system contained initially Isopropanol. To prepare it for the run, 100 % Acetonitrile + 0.1 TFA with flow rate 0.3 ml/min was added for 10 minutes. Thereafter 20 % Acetonitrile + 0.1 TFA, 80 % H_2O + 0.1 TFA was added at a flow rate 0.3-1 ml/min for 10 minutes. For the run 180-190 µl sample was injected and a gradient of 20-50 % Acetonitrile + 0.1 % TFA in H_2O + 0.1 % TFA was set to run in the system. The absorbance was measured at 220 and 280 nm. The Column Thermostat was 30.0 °C. A threshold/slope was used for collect and the threshold was 30. Max pressure was 220 bar.

2.15. Biotinylation

Biotinylation was performed on all constructs to obtain only monomers preventing the cysteine to build dimers. The proteins were reduced by adding 0.1 M Sigma-Aldrich DL-Dithiothreitol (DTT) added to a final concentration of 20 mM. The samples were incubation on a 40 °C heating block for 1 hour. The buffer was changed with a NAP-5 columns to PBS. Thereafter Sigma-Aldrich Biotin-maleimide was added, 5 times the molar mass of the construct. The samples were incubated in room temperature for 1h 30 minutes. The proteins was stored in the 4 °C fridge overnight. The buffer was changed to PBS with GE Healthcare PD-10 columns.

2.16. Surface plasmon resonance

In this work Bio-rad Laboratories ProteOn XPR36 System was used to measure the affinity. The system design had six parallel flow paths. The flow paths could in vertical direction be used to immobilize the target protein on six parallel protein surface lanes. The six flow paths was then rotated to horizontal position to enable injection of six parallel analyte solutions over the surfaces. The design enabled so called “one-shot” kinetics where 6 x 6 interactions could be measured by only one injection. The measured kinetic data was then visualized in a sensorgram (Fig. 8) where the response RU (pg protein/mm^2) was measured over time. Two phases can be seen in a sensorgram, one association phase where the association rate constant k_a was measured in 1/M·s and dissociation phase where the dissociation rate constant k_d was measured in 1/s. The affinity could be described with the k_d/k_a ratio, the unit K_D in mol/L. A low K_D meant a low dissociation rate k_d divided by a high association rate k_a, hence a strong interaction.

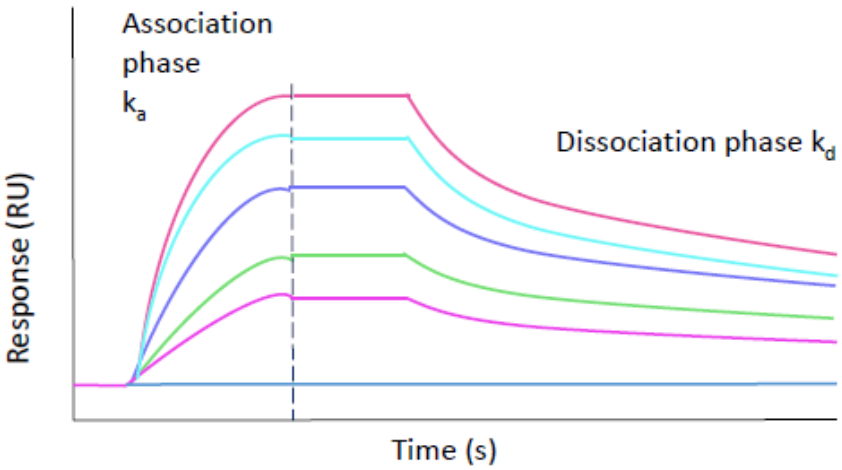

Figure 8. An illustration of a sensorgram with an injection of six analyte concentrations.

The affinity of the constructs to HSA, HER2, HER3, VEGFR-2, HER1 and IGF1R was measured. Two GLC- chips were used for the SPR measurements. On the first chip three proteins HSA, HER2, HER3 were immobilized by amine coupling, each to a separate surface. The concentration of protein was 5 µg/ml, the flow rate was 30 µl/min, the contact time 300 seconds and the volume 150 µl. First the chip surface was activated by adding NHS and EDC, the ligand to be immobilized was thereafter added and deactivation was performed by adding ethanolamine. The immobilization level which was the RU max when the ligand had been flown over the chip was noted for each immobilization. A surface test was made with the controls ABD* for HSA, ADAPT6 for HER2, ABDHER3 mat-1 for HSA and HER3. The surfaces was tested by flowing over a solution of 50 nM and 100 nM of the controls over the immobilized protein surfaces. The flow rate was 50 µl/min, the contact time was 250 seconds, the volume was 208 µl and the dissociation time was 600 seconds. The running buffer was PBST (PBS, 0.005% (v/v) Tween 20).

The analyte samples were diluted in PBST. In between the analyte runs regeneration was performed by adding 10 mM NaOH to wash off the bound analyte. After the test had confirmed that binding to the surface the cloned constructs ADAPTHER3-ZHER2, ZHER2-ADAPTHER3, ADAPTHER3-ZHER3, ZHER3-ADAPTHER3 were flown over the surfaces. The flow rate was 50 µl/min, the contact time was 250 seconds, the volume was 208 µl and the dissociation time was 3000 seconds. The constructs were flown in a dilution series with 100, 50, 25, 12.5, 6.25, 0 nM protein construct. On the second chip all six proteins HSA, HER1, HER2, HER3, VEGFR-2 and IGF1R were immobilized to a separate surface. The concentration of protein was 5 µg/ml, the flow rate was 30 µl/min, the contact time was 300 seconds and the volume was 150 µl. The controls were ABD* for HSA, ADAPT6 for HER2, ABDHER3 mat-1 for HER3 and HSA, Link2 for VEGFR-2, His6-Z02377 for HER1 and IGF1 for IGF1R. All 12 constructs were flown over the immobilized protein surfaces.

3. Results

3.1 Cloning

During the first cloning step (Fig. S1) the ADAPTHER3-N of 179 bp and ADAPTHER3-C of 189 bp subdomains were amplified at an annealing temperature of 55 °C. The rest of the fragments did not show the correct size which were 197 bp for ZHER2-N, 205 bp for ZHER2-C, 197 bp for ZHER3-N and 205 bp for ZHER3-C. Cloning with new longer primers (Table S1) was tried with an annealing temperature of 50 °C and 55 °C giving correct fragments for ZHER2-N and ZHER2-C which were gel

extracted to isolate them from the larger unspecific products. The primers also gave correct products for ZHER3-N and ZHER3-C in low concentrations (Fig. S2, S3). The six correct products were purified (Fig. S4).

The overlap extension PCR for the complete constructs was optimized using 100 ng of each template, a PCR reaction mix of 100 µl and an annealing temperature of 55 °C (Table S3). The expected size was 366 bp for ADAPTHER3-ZHER2 and ADAPTHER3-ZHER3 and 368 bp for ZHER2-ADAPTHER3 and ZHER3-ADAPTHER3. The resulting PCR products had the correct size for ADAPTHER3-ZHER2, ZHER2-ADAPTHER3 and ADAPTHER3-ZHER3 (Fig. S5) as well as unspecific smaller fragment produced for ADAPTHER3-ZHER3 and ZHER3-ADAPTHER3. The correct larger fragment for ADAPTHER3-ZHER3 for the PCR with annealing temperature of 55 °C was gel extracted.

The vector was cleaved and compared with the cleaved vector showing a smaller size for the cleaved vector signifying it was correctly digested and could be used for the ligation and transformation (Fig. S6). The colony PCR of clones from the cloned constructs ADAPTHER3-ZHER2, ZHER2-ADAPTHER3 and ADAPTHER3-ZHER3 showed a correct expected size of 485 bp (Fig. S7). Sequencing of all the clones showed correct DNA sequence for ADAPTHER3-ZHER2 and ZHER2-ADAPTHER3. Sequencing showed a correct ADAPTHER3-ZHER3 amino acid sequence, the template used for ZHER3 (Z8699 PCR product) had a different DNA sequence than initially intended but the same amino acid sequence. The ADAPTHER3-ZHER3 construct was used for the PCR of the ZHER3-ADAPTHER3 construct. The subdomain of ZHER3-N was amplified using the correct colony PCR product of ADAPTHER3-ZHER3. It produced the correct subdomain for ZHER3-N of 197 bp (Fig. S8A). The overlap extension PCR thereafter obtained the correct product for ZHER3-ADAPTHER3 of 368 bp (Fig. S7B). The colony PCR gave correct size for the construct on gel (Fig. S9) and the sequencing proved correct clones for the construct ZHER3-ADAPTHER3.

3.2. Protein expression

Transformation into BL21*DE3 cells and culturing of the cloned construct ADAPTHER3-ZHER2, ZHER2-ADAPTHER3, ADAPTHER3-ZHER3 and ZHER3-ADAPTHER3 as well as bivalent constructs for HER2 and the polyspecific hexamer and polyspecific octamer was performed.

3.3. Anti-ABD purification

The testing for Anti-ABD purification gave negative results. The absorbance measurement at 280 nm from the fractions of the anti-ABD purified GC-SS-HE3-DEAVDANS-ADAPT6 and GC-VDANS-ADAPT6 gave no clear peaks (Fig. 9, 10). According to the OD_{280} measurement the constructs with ADAPT molecules did not bind to the column.

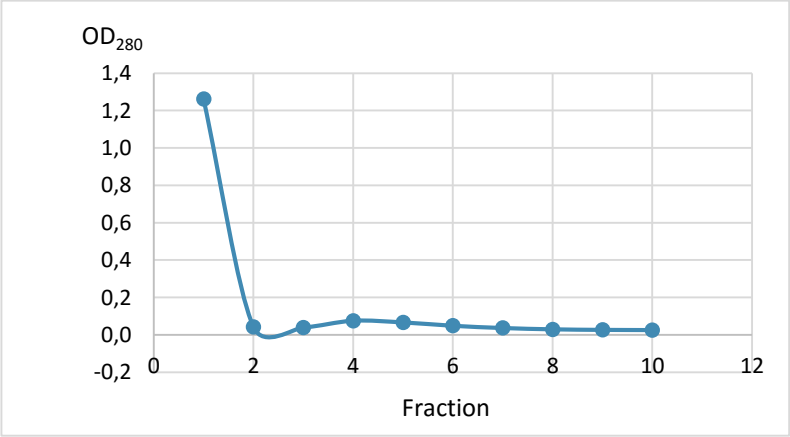

Figure 9. Plot for the absorbance measurement at OD_{280} for the protein content resulting after Anti-ABD purification of the ADAPT molecule GC-VDANS-ADAPT6.

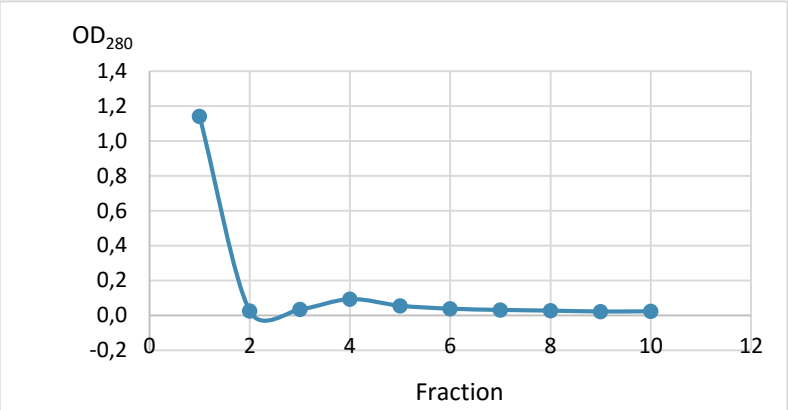

Figure 10. Plot for the absorbance measurement at OD_{280} for the protein content resulting after Anti-ABD purification of the ADAPT molecule GC-SS-HE3-DEAVDANS-ADAPT6.

3.4. HSA purification

The absorbance measurement at 280 nm gave clear peaks for all the constructs indicating a purified protein (example Fig. 11).

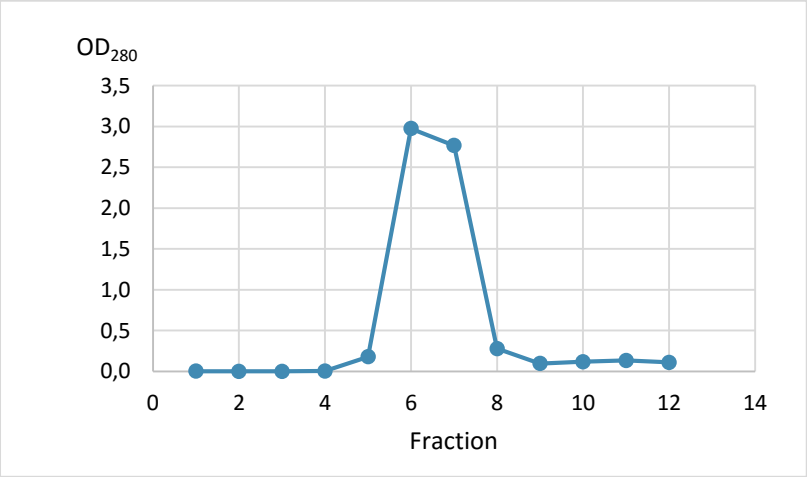

Figure 11. Plot for the absorbance measurement of protein content of ADAPTHER3-ZHER2 at OD_{280}.

3.5. SDS-PAGE

The gel for the fractions of each construct showed pure samples except for ZHER2-ADAPTHER3 which had visible contaminations. All the 12 construct were later visualized in a Novex gel (Fig. 12). It appeared that the 12 constructs have the correct molecular weight. The molecular weight was expected to be 12.8 kDa for ADAPTHER2-1-ZHER2 and ZHER2-1-ADAPTHER2, 13.1 kDa for ADAPTHER2-2-ZHER2 and ZHER2-2-ADAPTHER2, 13.4 kDa for ADAPTHER2-3-ZHER2 and ZHER2-3-ADAPTHER2, 38.7 kDa for the polyspecific hexamer and 52.7 kDa for the polyspecific octamer, 12.7 kDa for ADAPTHER3-ZHER2, ZHER2-ADAPTHER3, ADAPTHER3-ZHER3 and ZHER3-ADAPTHER3. There are visible dimers for example for the polyspecific hexamer and polyspecific octamer.

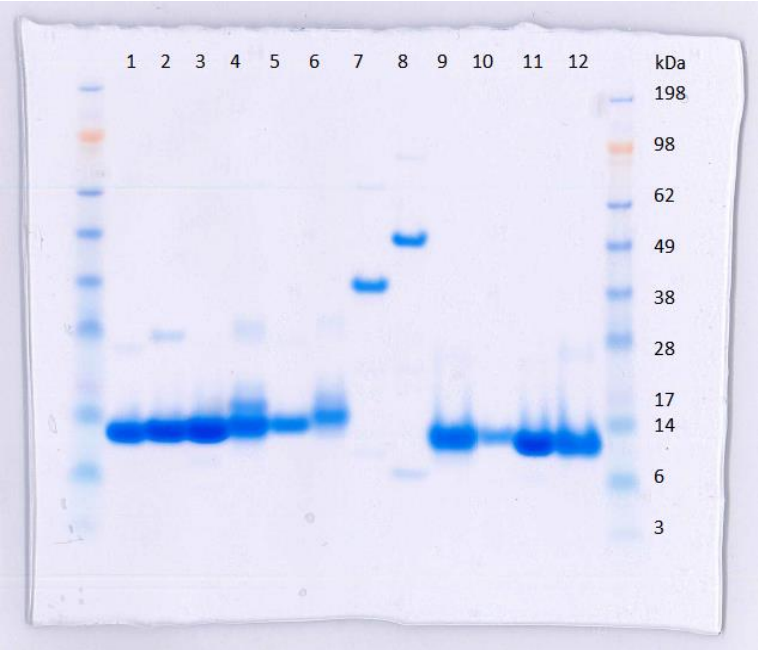

Figure 12. SDS-PAGE gel of all 12 purified constructs. SeaBlue®Plus 2 Ladder. (**1**) ADAPTHER2-1-ZHER2, expected size 12.8 kDa. (**2**) ZHER2-1-ADAPTHER2, expected size 12.8 kDa. (**3**) ADAPTHER2-2-ZHER2, expected size 13.1 kDa. (**4**) ZHER2-2-ADAPTHER2, expected size 13.1 kDa. (**5**) ADAPTHER2-3-ZHER2, expected size 13.4 kDa. (**6**) ZHER2-3-ADAPTHER2, expected size 13.4 kDa. (**7**) Polyspecific hexamer, expected size 38.7 kDa. (**8**) Polyspecific octamer, expected size 52.7 kDa. (**9**) ADAPTHER3-ZHER2, expected size 12.7 kDa. (**10**) ZHER2-ADAPTHER3, expected 12.7 kDa. (**11**) ADAPTHER3-ZHER3, expected size 12.7 kDa. (**12**) ZHER3-ADAPTHER3 expected size 12.7 kDa, SeaBlue®Plus 2 Ladder.

3.6. Mass spectrometry

The first MS measurement gave a size of about 300 Da more than estimated for ADAPTHER3-ZHER2, ZHER2-ADAPTHER3 and ZHER3-ADAPTHER3. Furthermore ADAPTHER3-ZHER3 and ZHER3-ADAPTHER3 also had peaks for at 7391 Da which was 5280 Da under the expected molecular weight. Measurements under reduced conditions gave the correct size for ADAPTHER3-ZHER2, ZHER2-ADAPTHER3 and ZHER3-ADAPTHER3. In addition ZHER2-ADAPTHER3 had several smaller peaks suggesting impurities. ADAPTHER3-ZHER3 and ZHER3-ADAPTHER3 still had peaks at 7391 Da. The peaks for ADAPTHER2-1-ZHER2, ZHER2-1-ADAPTHER2, ADAPTHER2-2-ZHER2, ZHER2-2-ADAPTHER2, ADAPTHER2-3-ZHER2, ZHER2-3-ADAPTHER2, the polyspecific hexamer and the polyspecific octamer gave with a measurement under reduced conditions the correct size. After biotinylation MS measurement of proteins gave correct size for the cloned constructs and ADAPTHER2-1-ZHER2 and ZHER2-2-ADAPTHER2. ADAPTHER2-2-ZHER2, ZHER2-2-ADAPTHER2, ADAPTHER2-3-ZHER2, ZHER2-3-ADAPTHER2, the polyspecific hexamer and polyspecific octamer had lots of background. Even after the concentration the the polyspecific hexamer and polyspecific octamer had too much background to distinguish peaks for the correct molecular weight.

3.7. High performance liquid chromatography

The analytical measurement of ADAPTHER3-ZHER2, ZHER2-ADAPTHER3 and ZHER3-ADAPTHER3 with HPLC gave a gradient curve of 20-50 % Acetonitrile + 0.1 % TFA in H_2O + 0.1 % TFA as where expected. ADAPTHER3-ZHER2 (Fig. 13) had two large peaks possibly monomer and dimer of the construct. ZHER2-ADAPTHER3 (Fig. 14) had 3 larger peaks and several small peaks, this constructs was purified with HPLC. ZHER3-ADAPTHER3 had one large peak (Fig. 15) implying that the protein solution was free from impurities.

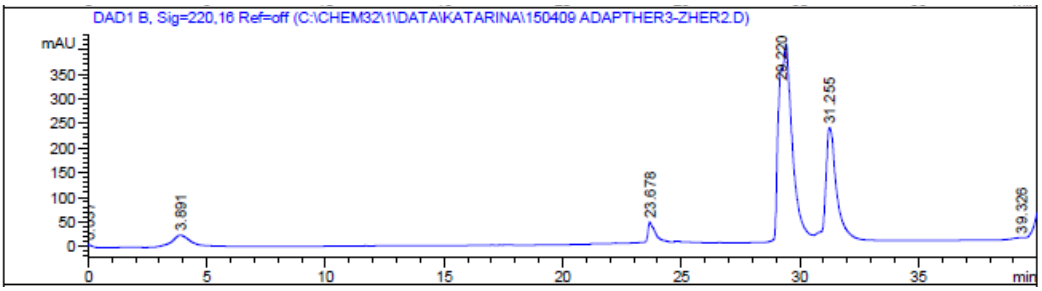

Figure 13. HPLC resulting plot from ADAPTHER3-ZHER2.

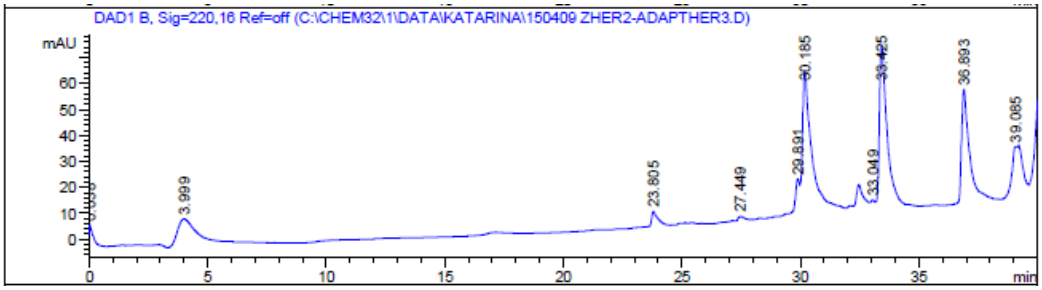

Figure 14. HPLC resulting plot from ZHER2-ADAPTHER3.

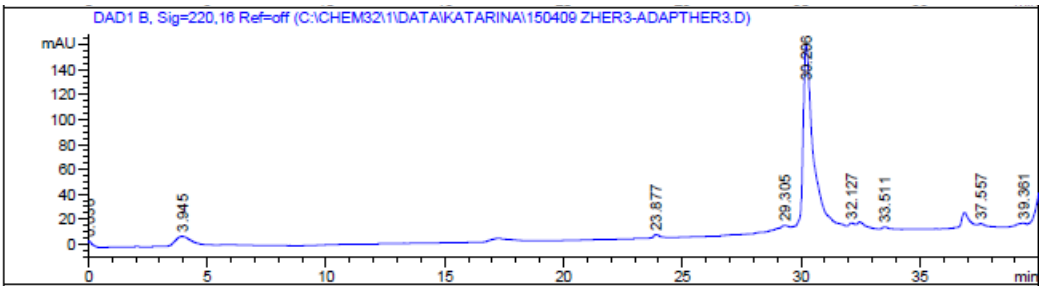

Figure 15. HPLC resulting plot from ZHER3-ADAPTHER3.

3.8. Surface plasmon resonance

Replicate measurements were made. Two GLC-chips were used for the SPR. On the first chip the target receptors HSA, HER2-His and HER3-His were immobilized. The level of immobilisation was 1800 RU for HSA, 2800 RU for HER2-His and 1200 RU for HER3-His. The surface test result confirmed that surface with HSA immobilized bound ABD* and ABDHER3 mat-1, the surface with HER2 immobilised bound ADAPT6 and the surface with HER3 immobilised bound ABDHER3 mat-1. On the second chip all the receptors were immobilized. The level of immobilisation was 400 and an additional 1000 RU for HSA, 1500 RU for HER2-His, 500 RU and an additional 1800 RU for HER3-His, 1800 RU for VEGFR-2, 800 RU for His-EGFR and 400 RU for IGF1R. The bivalent and bispecific construct bound to the surface with HSA, HER2 and HER3 which confirmed that the surfaces had receptors immobilized to them. A surface test confirmed that VEGFR-2 was working properly but not HER1 and IGF1R. The polyspecific constructs did not manage to bind VEGFR-2 even though the control worked.

Data for all the twelve constructs were obtained and sensorgrams were produced (Fig. S30-S40). For the cloned constructs, bispecific for HER2 and HER3 and bivalent for HER3, three measurements received results with data that could be evaluated. The KD values for the three measurements with the bispecific constructs for HER2 and HER3 and the bivalent constructs for HER3 was summarized and compared in three graphs (Fig. S41-S46). The first measurements were taken on the GLC-chip with the immobilized receptors for HSA, HER2 and HER3. The second and third measurements were taken on the second GLC-chip with all the target receptors immobilized. For the target HSA the relationship

between the constructs looked similar with a slight variation for ZHER2-ADAPTHER3 and ZHER3-ADAPTHER3 (Fig. S41). For the target HER2 the relationship was similar (Fig. S42). For the target HER3 the relationship differed for ZHER2-ADAPTHER3 and ZHER3-ADAPTHER3 (Fig. S43). The bivalent constructs for HER2 did not receive good enough results to look at replicates, neither did the polyspecific octamer nor the polyspecific hexamer.

The KD values for all the twelve constructs regarding HSA, HER2 and HER3 was summarized and compared (Table 2). For HSA and the bispecific construct the ZHER3-ZHER2 orientation with a KD of 3.7 nM seemed to have a slightly better affinity than the ZHER2-ZHER3 orientation with a KD of 4.5 nM. For the bivalent construct for HER3 the highest affinity for HSA was obtained for ADAPTHER3-ZHER3 with the lowest KD of 0.82 nM. The ZHER3-ADAPTHER3 orientation got 2.6 nM for HSA. For the bivalent constructs for HER2 the affinity for HSA was very similar for all the constructs, between 0.94 and 1.6 nM. The lowest KD was obtained for ADAPTHER2-2-ZHER2 with 0.94 nM for HSA. The polyspecific hexamer and octamer had higher KD values for HSA than the smaller constructs with a KD of 12 nM and 6.7 nM respectively.

For the HER2 receptor the ZHER3-ZHER2 orientation with a KD of 5.6 nM gave also in this case a lower KD than the ZHER2-ZHER3 orientation with a KD value of 12 nM. For the bivalent constructs for HER2 the lowest KD value of 1.1 nM for HER2 was obtained for ADAPTHER2-1-ZHER2. The other bivalent construct for HER2 had KD values between 1.2 and 2.6 nM. The polyspecific hexamer got a KD of 3.5 nM for HER2 and the polyspecific octamer had a KD of 0.13 µM for HER2.

For HER3 the KD was very similar for the bispecific constructs, 3.3 nM for ADAPTHER3-ZHER2 and 3.1 nM for ZHER2-ADAPTHER3. The bivalent molecules for HER3 have a lower KD with only a slight difference, 0.26 nM for ADAPTHER3-ZHER3 and 0.13 nM for ZHER3-ADAPTHER3.

Table 2. Kinetic parameter values k_a, k_d, KD from the SPR measurements for the targets HSA, HER2 and HER3 for the twelve expressed constructs.

Construct	Target	k_a (1/M·s)	k_d (1/s)	KD (M)
ADAPTHER3-ZHER2	HSA	8,4E+04	3,1E-04	3,7E-09
	HER2	3,6E+04	2,0E-04	5,6E-09
	HER3	1,3E+05	4,3E-04	3,3E-09
ZHER2-ADAPTHER3	HSA	7,0E+04	3,1E-04	4,5E-09
	HER2	3,9E+04	4,8E-04	1,2E-08
	HER3	1,3E+05	4,0E-04	3,1E-09
ADAPTHER3-ZHER3	HSA	1,2E+05	9,4E-05	8,2E-10
	HER3	2,3E+05	5,9E-05	2,6E-10
ZHER3-ADAPTHER3	HSA	1,3E+05	3,3E-04	2,6E-09
	HER3	4,4E+05	5,7E-05	1,3E-10
ADAPTHER2-1-ZHER2	HSA	1,4E+05	1,3E-04	9,5E-10
	HER2	5,3E+04	6,1E-05	1,1E-09
ZHER2-1-ADAPTHER2	HSA	2,3E+05	2,4E-04	1,1E-09
	HER2	3,9E+04	8,2E-05	2,1E-09
ADAPTHER2-2-ZHER2	HSA	1,7E+05	1,6E-04	9,4E-10
	HER2	2,2E+04	5,6E-05	2,6E-09
ZHER2-2-ADAPTHER2	HSA	1,7E+05	2,5E-04	1,5E-09
	HER2	6,4E+04	1,1E-04	1,7E-09
ADAPTHER2-3-ZHER2	HSA	1,3E+05	1,9E-04	1,6E-09
	HER2	1,2E+05	1,8E-04	1,5E-09
ZHER2-3-ADAPTHER2	HSA	1,6E+05	2,3E-04	1,4E-09
	HER2	6,6E+04	9,8E-05	1,5E-09
Polyspecific hexamer	HSA	1,6E+04	2,0E-04	1,2E-08
	HER2	3,7E+04	1,3E-04	3,5E-09
Polyspecific octamer	HSA	3,7E+04	2,5E-04	6,7E-09
	HER2	2,0E+00	2,5E-05	1,3E-05
	HER3	1,2E+04	7,9E-05	6,7E-09

4. Discussion

The goal with the master thesis project was to create new molecules targeting cancer biomarkers, and furthermore analyse the molecules' binding skills. The cancer related receptor included three members of the HER family, HER1, HER2 and HER3, and two additional cancer related receptors; IGF1R and VEGFR-2. The constructs were to have affinity for HSA to increase the *in vivo* half-life. Affinity analysis was to be performed by the use of SPR.

During the cloning there were problems retrieving the subdomain for the ZHER3. Sequencing showed that it worked using a different ZHER3 sequence than was initially intended. This ZHER3 was used for both the N- and C-terminal domains.

The MS showed 300 Da larger proteins than expected which is why MS under reduced conditions was made. This gave more accurate results indicating that some extra amino acids were attached to the cysteine of the proteins in the C-terminal. However results for ADAPTHER3-ZHER3 showed only about half the size of the expected molecular weight suggesting truncation of the protein during the expression. Reculturing of this construct was made which solved the problem.

The Anti-ABD purification did not give any peaks, indicating that the ADAPTHER3 domain did not bind to the column and therefore had no albumin binding domain. In contrast the HSA purification gave clear peaks which demonstrates that the ADAPT domain bound HSA as expected. This HSA purification was used with all the constructs. One problem that occurred after the purification was the inability of the purified protein fractions of the polyspecific hexamer and polyspecific octamer to be dissolved in PBS. A possible explanation is the multiple domain design enabling easy attachment between the domains which was hard to break once attached. In order to try to solve this problem, the buffer was changed with desalting columns after the purification instead of freeze dry.

The SDS-PAGE gel showed dimers for the polyspecific hexamer and polyspecific octamer that could potentially have had an impact on the SPR measurements for the constructs. With the biotinylation the dimers were hopefully eliminated. A gel to verify this would have been very useful but the time was limited.

For the MS there were results that indicated carry over between the measurements, i.e. samples residing in the column and affecting the subsequent results. This made it more difficult to determine if the samples were pure or not. The MS for constructs after the biotinylation did not give exact results and sometimes the background made it hard to evaluate the results. The MS results suggested that some of the samples were not correctly biotinylated. This would give the conclusion that the SPR results might not have been entirely correct.

For the SPR there were some examples where the data was unstable and the curve fitting was not perfect. For ZHER3-ADAPTHER3 the analyte binding reached saturation. For the polyspecific hexamer the data was very fluctuating, it was only possible to see an interaction for HSA and HER2. For ZHER2-1-ADAPTHER2 air peaks seemed to affect the data. The required time for running all the analyte samples caused evaporation for the samples at the end of the run. Air peaks occurred and the samples had to be rerun and the order of the analytes changed. The SPR results from the repetitive measurements for the constructs targeting HER2 and HER3 bispecificly and HER3 bivalently showed similar results suggesting a reliability for the SPR measurements. The orientation of the HER2 and HER3 did not seem to affect the affinity significantly, there was a slightly lower KD for the HER3-HER2 orientation for HSA and HER2, but no significant difference for HER3. The KD for the bivalent construct compared to the bispecific construct was lower for HER2 and 10-fold lower for HER3, which could be explained by their potential avidity. The level of immobilisation for HER1 and IGF1R was relatively low which could explain why their controls did not bind and why the protein surfaces could not be used for the analysis.

5. Conclusion

The master thesis project shows that it was possible to produce constructs bispecific, bivalent and polyspecific for HER2 and HER3 receptors with ADAPT and affibodies. It was possible to reach a similar affinity to the study by Malm et al. (2014) with 1^{-9} M affinity for HER2 and 1^{-10} M affinity for HER3. Furthermore the construct bound HSA as in the study by Malm et al. (2014) with 1^{-10} M affinity for HSA. The constructs had a molecular weight of 13 kDa which was half the size of the constructs produced in the study by Malm et al. (2014) which had a molecular weight of approximately 28 kDa. The orientation of the constructs did not seem to affect the affinity. The avidity of the bivalent construct seemed to give a slightly better affinity than for the bispecific constructs. It was also possible to produce polyspecific constructs in the shape of a polyspecific hexamer and a polyspecific octamer.

Additional affinity measurements are needed to determine the affinity for the target receptors of the polyspecific hexamer and polyspecific octamer. It would also be interesting to grow cancer cells and test binding and inhibition of the construct on receptors occurring naturally on cancer cells. There were plans for a cell binding assay to test the binding of the constructs to the receptors, a cell phosphorylation-inhibition assay to test the amount of phosphorylation when the constructs were binding to their target, and a cell proliferation assay to test the resulting cell growth of the cancer cells. It would be interesting to check the phosphorylation inhibition affect during interaction between construct and receptor on cancer cells as was performed in earlier studies and compare the agonistic and antagonistic affect for the different orientations.

A complete analysis of the HER binding constructs could potentially give rise to new and improved HER targeting biomarkers and cancer drugs in a long-term perspective, drugs that have good affinity for their target and have potential of decreasing phosphorylation that affects the tumor growth.

It is important to discuss the advantages and disadvantages for affibodies versus antibodies in order to deliberate the affibody's potential as immunotherapeutic cancer drug. Affibodies has the advantage of being smaller and easy to produce. However they are not a natural component of the human immune system. Antibodies already have two binding sites, hence they are bivalent, and the function of their Fc region is important for the immune response and provides and additional possibility for engineering. Affibodies gives an alternative option to antibodies, they focus solely on the targeting and inhibiting of a target molecule. Future testing and engineering can improve their potential as cancer drugs.

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I would like to thank the advisors Tarek Bass and Mikael Åstrand for their earlier work and their support and guidance through the entire Master thesis work. My thanks is also sent to John Löfblom and Stefan Ståhl for their support of the Master thesis project at KTH. I am also very grateful for the valuable questions, comment and support I received from my scientific reviewer Frank Roche and from my opponent Stina Åhag.

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Appendix A

Table S1. Primers

Name	*Binding protein*	*Region*
AgeneralfwdNcoI	ADAPTHER3	N-terminal
AHER3revG4S	ADAPTHER3	N-terminal
AHER3fwdG4S	ADAPTHER3	C-terminal
AHER3revcys	ADAPTHER3	C-terminal
ZHER2fwdlong	ZHER2	N-terminal
ZHER2revG4S	ZHER2	N-terminal
ZHER2fwdG4Slong	ZHER2	C-terminal
ZHER2revCyslong	ZHER2	C-terminal
ZHER3fwdlong	ZHER3	N-terminal
ZHER3revG4Slong	ZHER3	N-terminal
ZHER3G4Slong	ZHER3	C-terminal
ZHER3revCyslong	ZHER3	C-terminal

Table S2. Subdomain PCR

10 µl	New England Biolabs® 5x Phusion® HF/GC Reaction Buffer
5 µl	Chase nucleotidemix
1 µl	Forward primer 5 ρmol
1 µl	Reverse primer 5 ρmol
1 µl	template
0.5 µl	Phusion® DNA polymerase
31.5 µl	Staq
50 µl	Total

Table S3. Construct PCR 100 ng template

20 µl	New England Biolabs® 5x Phusion® HF/GC Reaction Buffer
10 µl	Chase nucleotidemix
12 µl	Forward primer 5 ρmol
12 µl	Reverse primer 5 ρmol
100 ng	N-terminal template
100 ng	C-terminal template
0.5 µl	Phusion® DNA polymerase
	Staq
100 µl	Total

Table S4. Digestion

10 µl	New England Biolabs® Cutsmart™ 10 x Buffer
1 µl	Thermo Scientific AscI 10 U/ µl
1 µl	Thermo Scientific NcoI HF 10 U/ µl
	DNA
	Staq
100 µl	Total

Table S5. Dephosphorylation

	New England Biolabs® 10 x Antarctic Phos Reaction Buffer
	Digested DNA
1 µl	New England Biolabs® Antarctic phosphatase
50 µl	Total

Table S6. Ligations

1 µl	Digested plasmid vector
1-3 µl	Digested fragment
2 µl	Biolabs® 10 x Buffer for T4 DNA ligase with 10 mM ATP
1 µl	New England Biolabs® T4 DNA Ligase
	Staq
20 µl	Total

Table S7. Colony PCR

10 µl	Thermo Scientific F-511 10 x Optimized DynAzyme Buffer
5 µl	Chase nucleotidemix
1 µl	Forward primer 5 ρmol
1 µl	Reverse primer 5 ρmol
0.5 µl	Thermo Scientific DynAzyme II DNA Polymerase
37.5 µl	Staq
50 µl	Total

Table S8. Sanger PCR

7 µl	1x Cs-buffert
0.5 µl	BigDye terminator mix
1 µl	Forward primer 5 ρmol
1 µl	Template

PCR Programs

Table S9. Phusion PCR

Step	Temperature	Time	Cycles
Initiation	94 °C	5 min	1
Denaturation	94 °C	30 s	30
Annealing	30-55 °C	30 s	
Extension	72 °C	1.5 min	
Termination	72 °C	10 min	1
	4 °C	∞	-

Table S10. Colony PCR

Step	Temperature	Time	Cycles
Initiation	94 °C	5 min	1
Denaturation	94 °C	30 s	30
Annealing	60 °C	30 s	
Extension	72 °C	1.5 min	
Termination	72 °C	10 min	1
	4 °C	∞	-

Table S11. Sanger PCR

Step	Temperature	Time	Cycles
Denaturation	96 °C	10 s	30
Annealing	50 °C	15 s	
Extension	60 °C	4 min	
	4 °C	∞	-

Appendix B

PCR gels

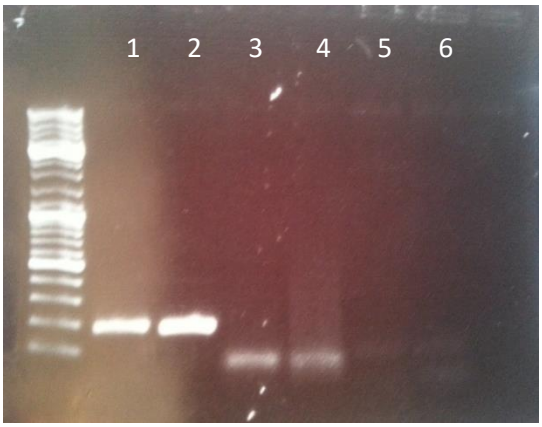


Figure S1. Cloning of subdomains with an annealing temperature of 55 °C. (**1**) ADAPTHER3-C, (**2**) ADAPTHER3-N, (**3**) ZHER2-C, (4) ZHER3-C, (**5**) ZHER2-N and (**6**) ZHER3-N. Correct products were obtained for ADAPTHER3-C with 189 bp and ADAPTHER3-N with 179 bp. Correct products were not obtained for ZHER2-C with 205 bp, ZHER2-N with 197 bp, ZHER3-C with 205 bp and ZHER3-N with 197 bp.

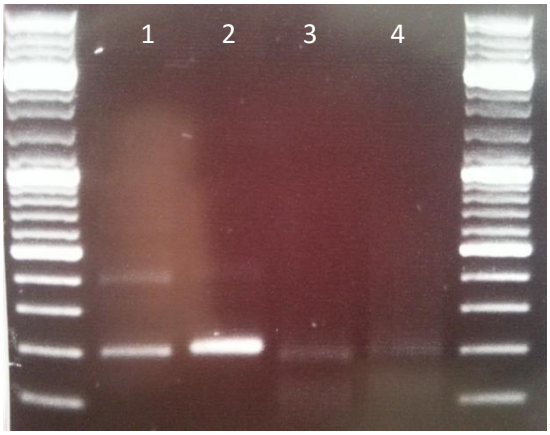


Figure S2. Cloning of subdomains with new longer primers seen in and annealing temperature of 50 °C. (**1**) ZHER2-N, (**2**) ZHER2-C, (**3**) ZHER3-N, (**4**) ZHER3-C. ZHER2-C with 205 bp and ZHER2-N with 197 bp has multiple products and correct products. ZHER3-N with 197 bp and ZHER3-C with 205 bp has corrects products in low concentrations.

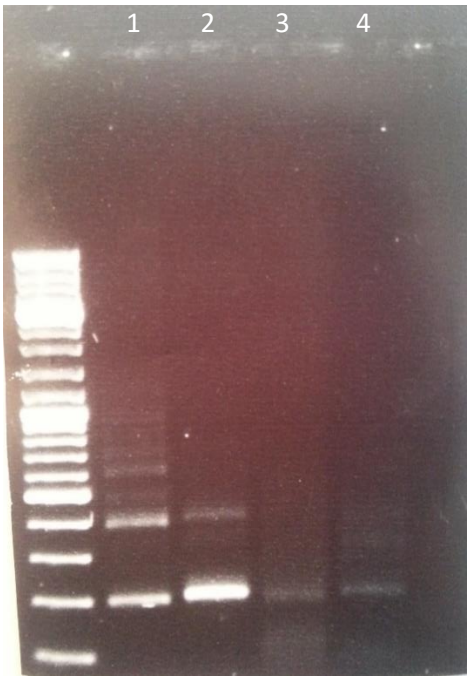


Figure S3. Cloning of subdomains (1) ZHER2-N, (2) ZHER2-C, (3) ZHER3-N, (4) ZHER3-C with new longer primers and annealing temperature of 55 °C. ZHER2-N with 197 bp and ZHER2-C with 205 bp has multiple products and correct products. ZHER3-N with 197 bp and ZHER3-C with 205 bp also has correct products in low concentrations.

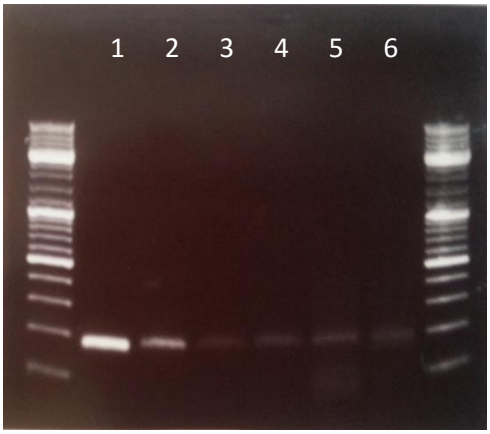


Figure S4. Purified products from the cloning. (1) ADAPTHER3-N, (2) ADAPTHER3-C, (3) ZHER2-N, (4) ZHER2-C, (5) ZHER3-N, (6) ZHER3-C ZHER2-C is 205 bp, ZHER2-N is 197 bp, ZHER3-C is 205 bp, ZHER3-N is 197 bp. All clones have the correct products.

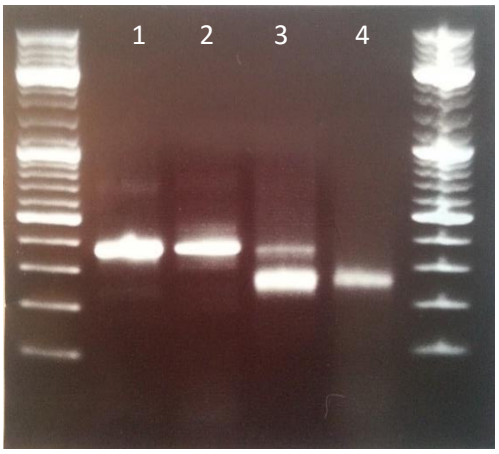


Figure S5. Cloning of complete constructs (1) ADAPTHER3-ZHER2, (2) ZHER2-ADAPTHER3, (3) ADAPTHER3-ZHER3 and (4) ZHER3-ADAPTHER3 with 100 ng of each subdomain template with annealing temperature of 55 °C. The expected size was 366 bp for ADAPTHER3-ZHER2, 368 bp for ZHER2-ADAPTHER3, 366 bp for ADAPTHER3-ZHER3 and 368 bp for ZHER3-ADAPTHER3. Correct products were obtained for ADAPTHER3-ZHER2, ZHER2-ADAPTHER3 and ADAPTHER3-ZHER3. ZHER3-ADAPTHER3 had a too short product.

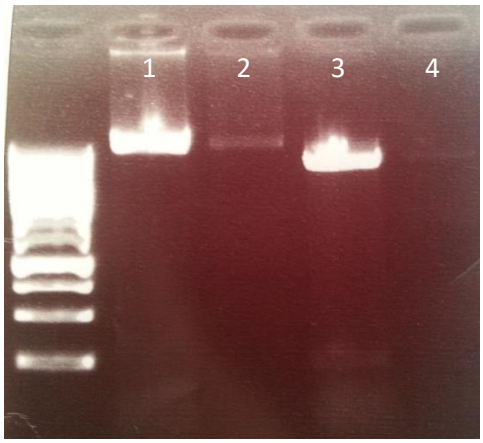


Figure S6. HisDummy vector with Z wildtype binding IGG inserted diluted (1) 1:10 and (2) 1:100 compared with the cleaved vector without insert diluted (3) 1:10 and (4) 1:100.

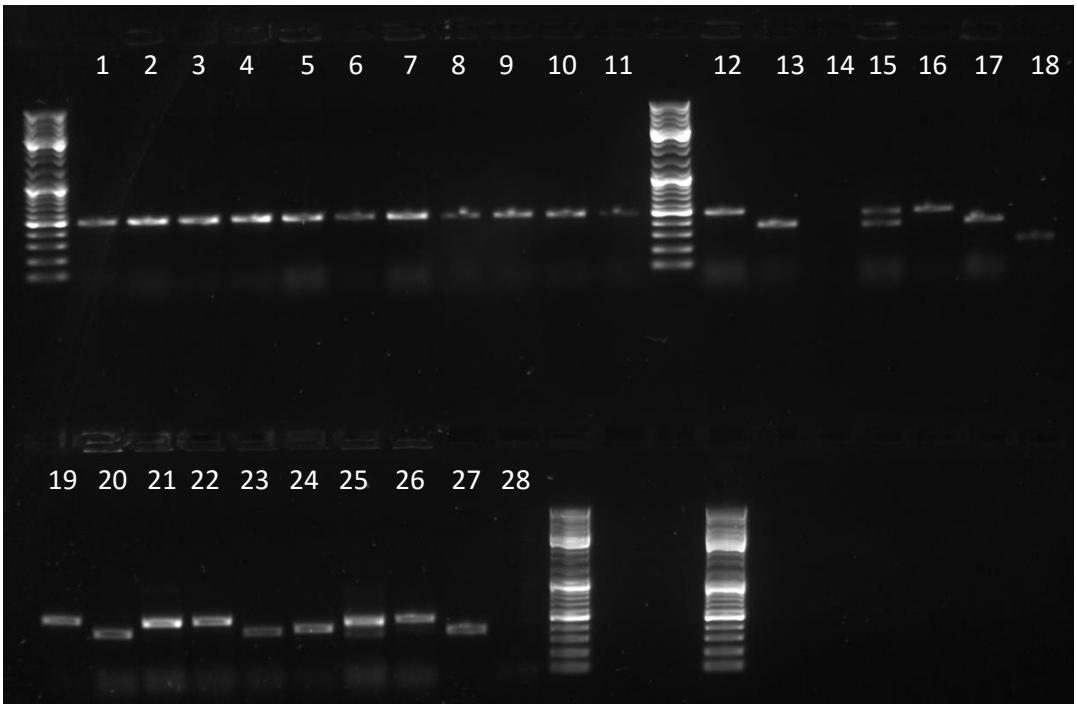


Figure S7. Colony PCR on (1-10) ADAPTHER3-ZHER2, (11) ZHER2-ADAPTHER3, (12-26) ADAPTHER3-ZHER3. Correct insert was 485 bp. The positive control (27) was the cleaved HisDummy vector of 460 bp. The negative control (28) of the PCR mix did not have contaminations.

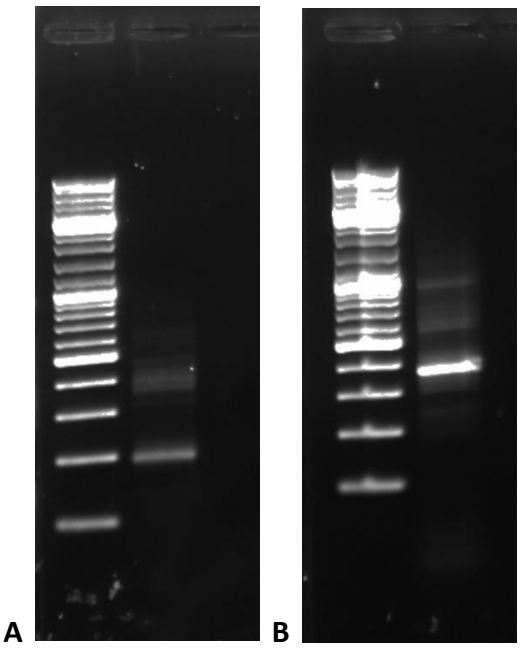


Figure S8. (**A**) Recloning of ZHER3-N with the colony PCR product of ADAPTHER3-ZHER3 as a template. It gave the correct product of 197 bp and also a low concentration of larger unspecific product. (**B**) Cloning of ZHER3-ADAPTHER3 with the obtained ZHER3-N as subdomain template and the previously cloned ADAPTHER3-C giving the correct product of 368 bp and also a low concentration of larger unspecific products.

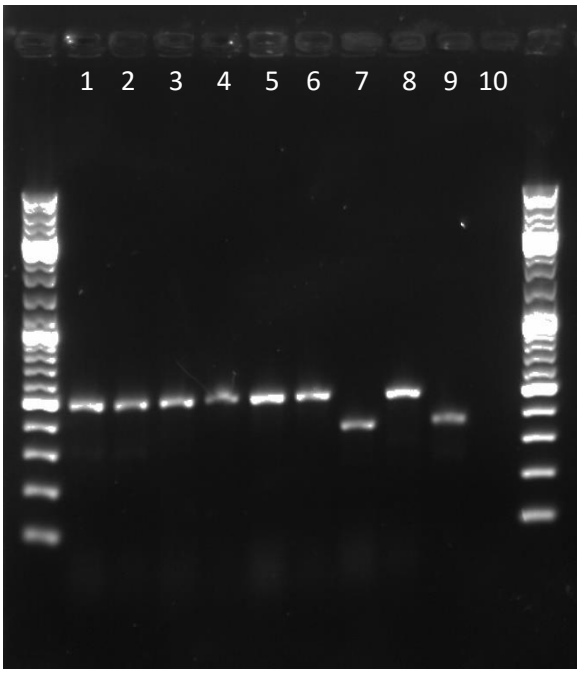


Figure S9. Colony PCR of (1-8) ZHER3-ADAPTHER3. The correct size of 485 bp was obtained. The positive control (9) was the cleaved HisDummy vector of 460 bp. The negative control (10) of the PCR mix did not have contaminations.

SPR Sensorgrams

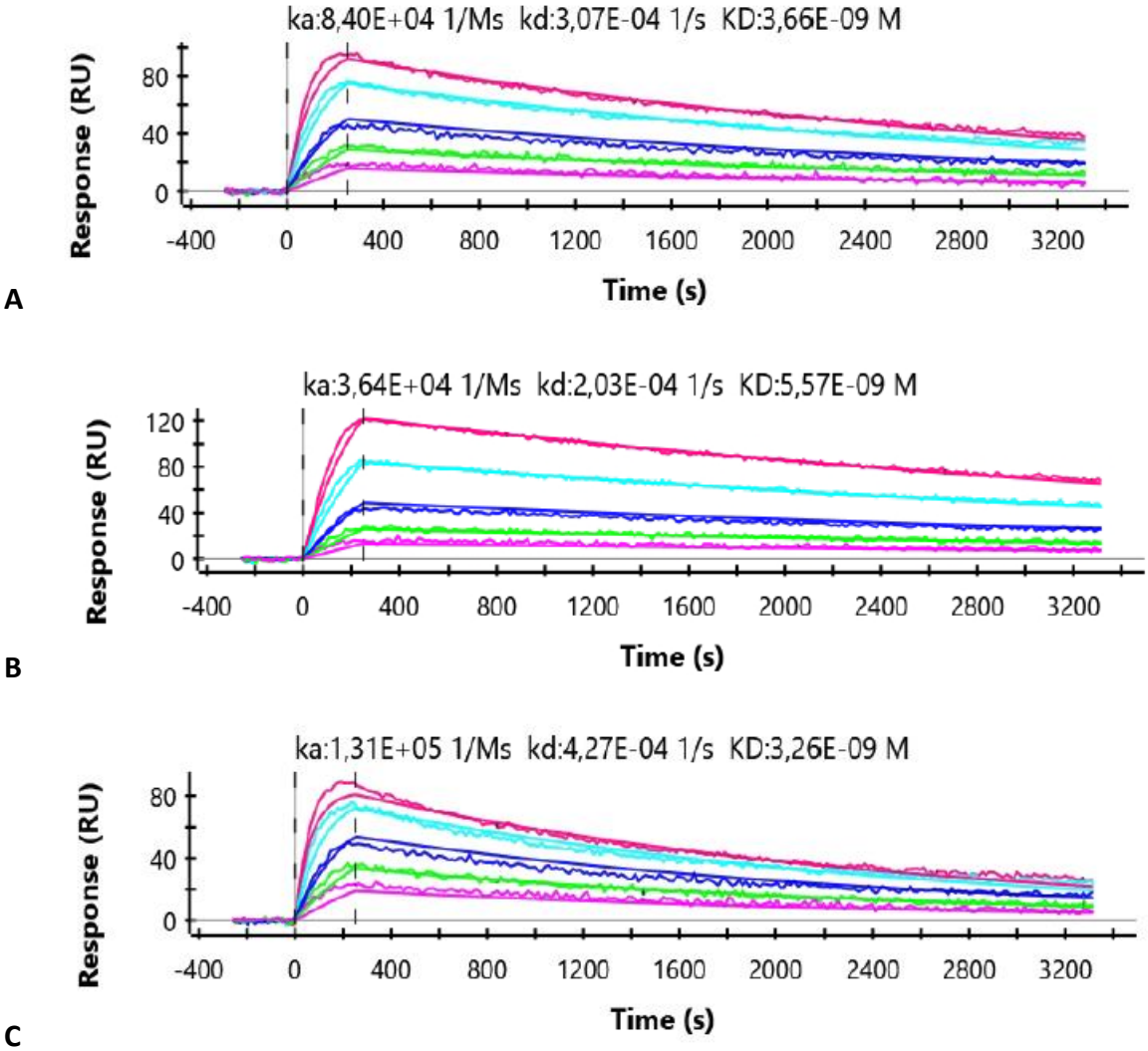
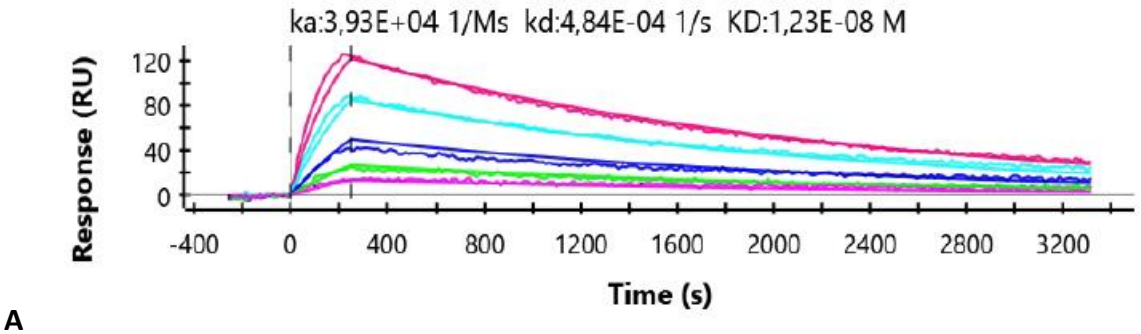
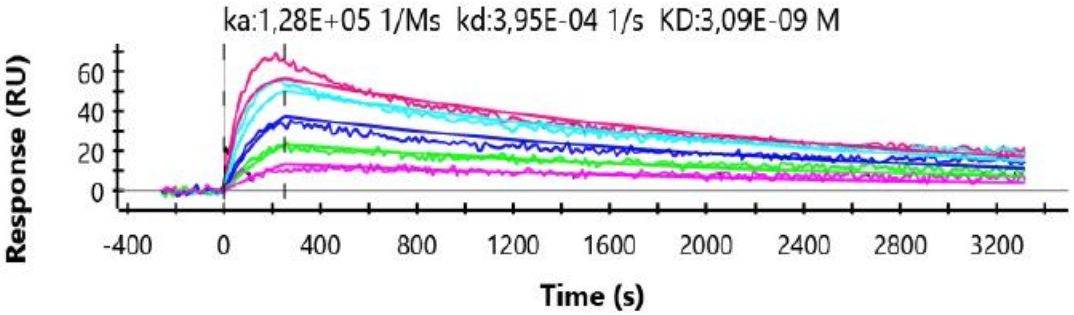


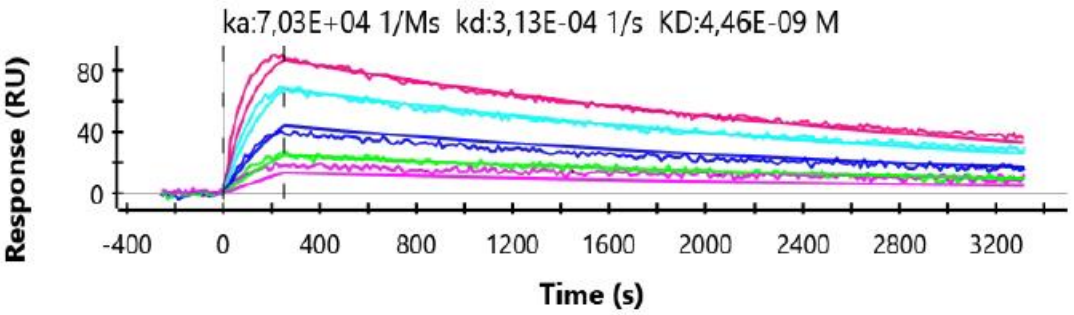
Figure S30. Sensorgrams for ADAPTHER3-ZHER2 flowing over a surface of immobilized (**A**) HSA, (**B**) HER2, (**C**) HER3.



A

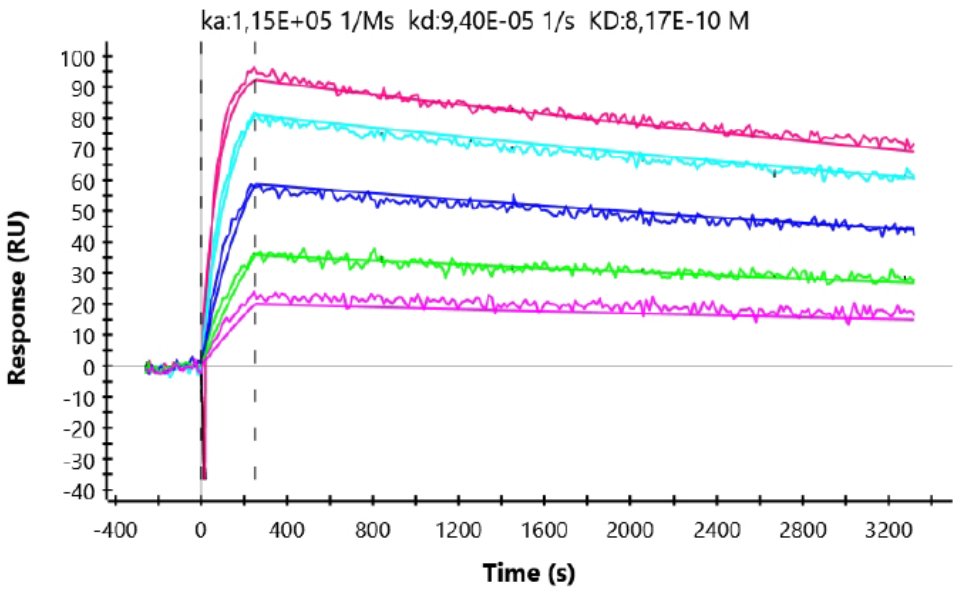


B

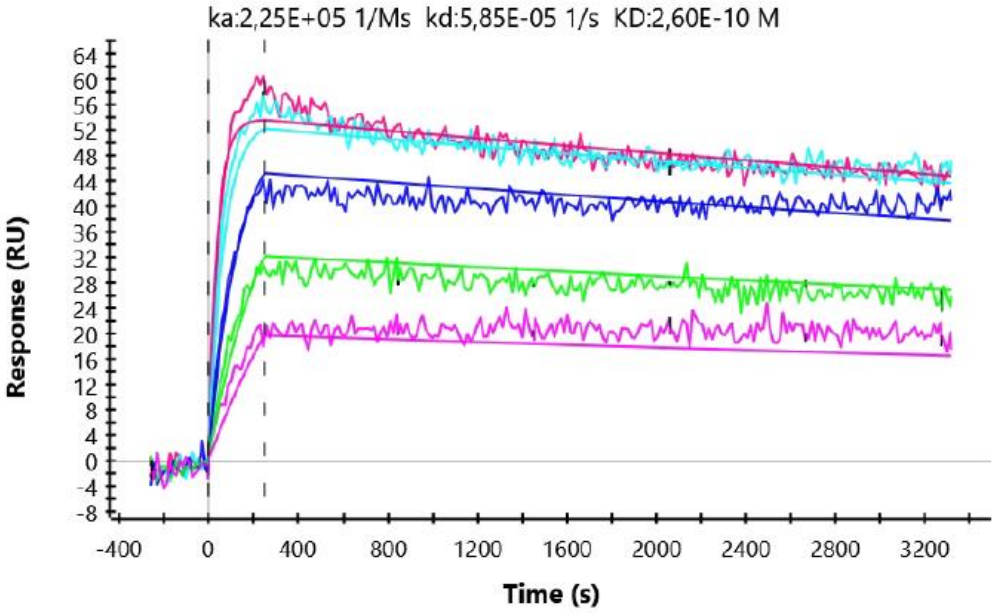


C

Figure S31. Sensorgrams for ZHER2-ADAPTHER3 flowing over a surface of immobilized (**A**) HSA, (**B**) HER2, (**C**) HER3.

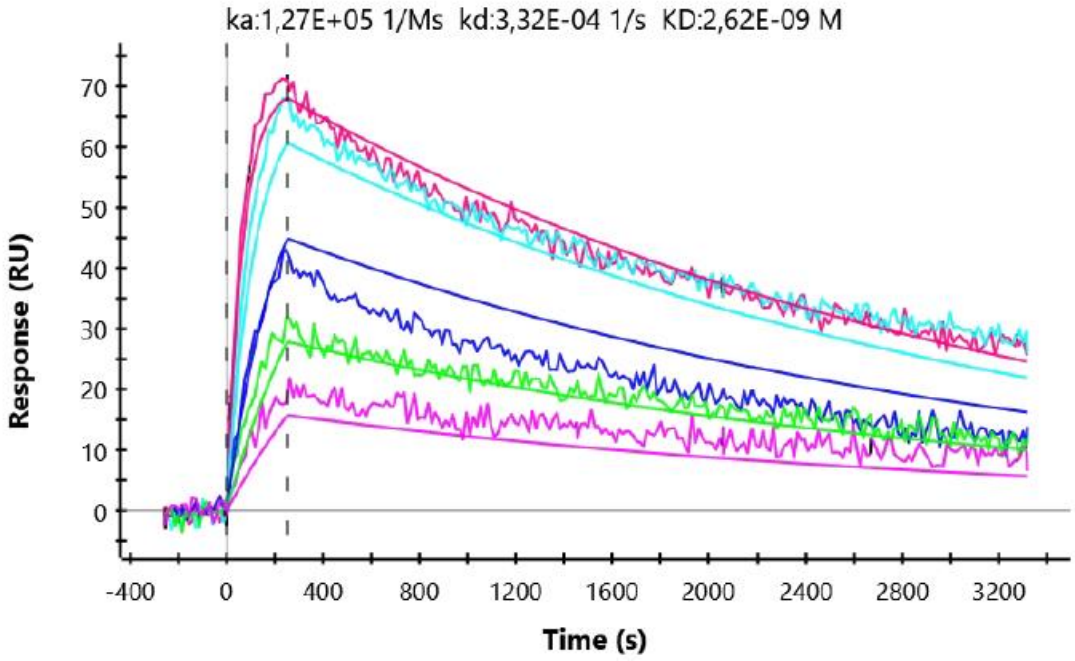


A

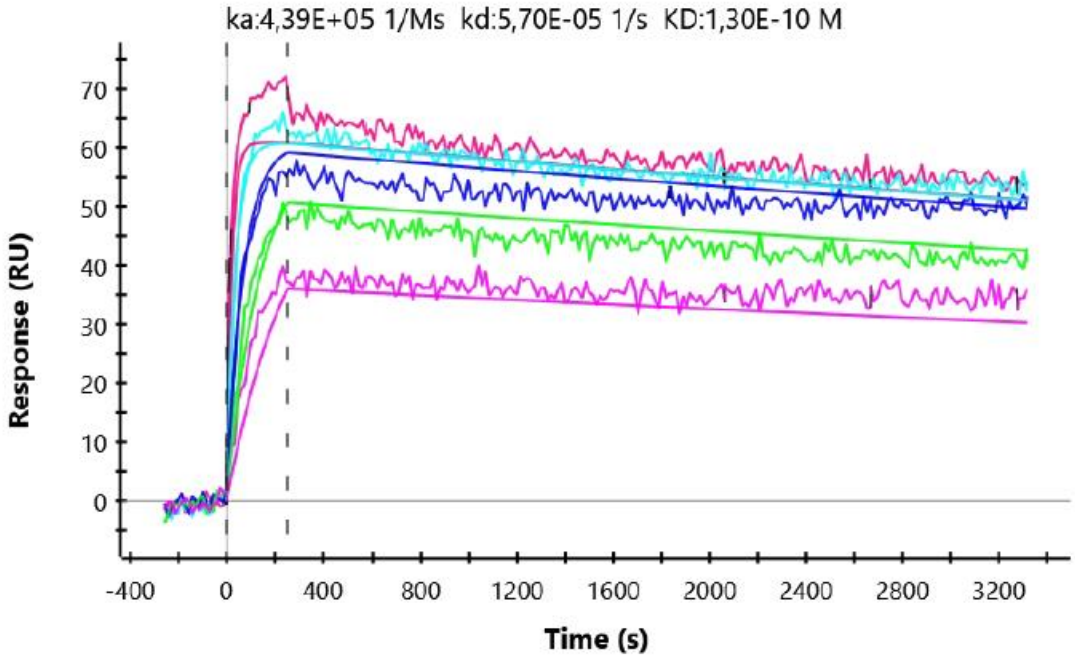


B

Figure S32. Sensorgrams for ADAPTHER3- ZHER2 flowing over a surface of immobilized (**A**) HSA, (**B**) HER3.

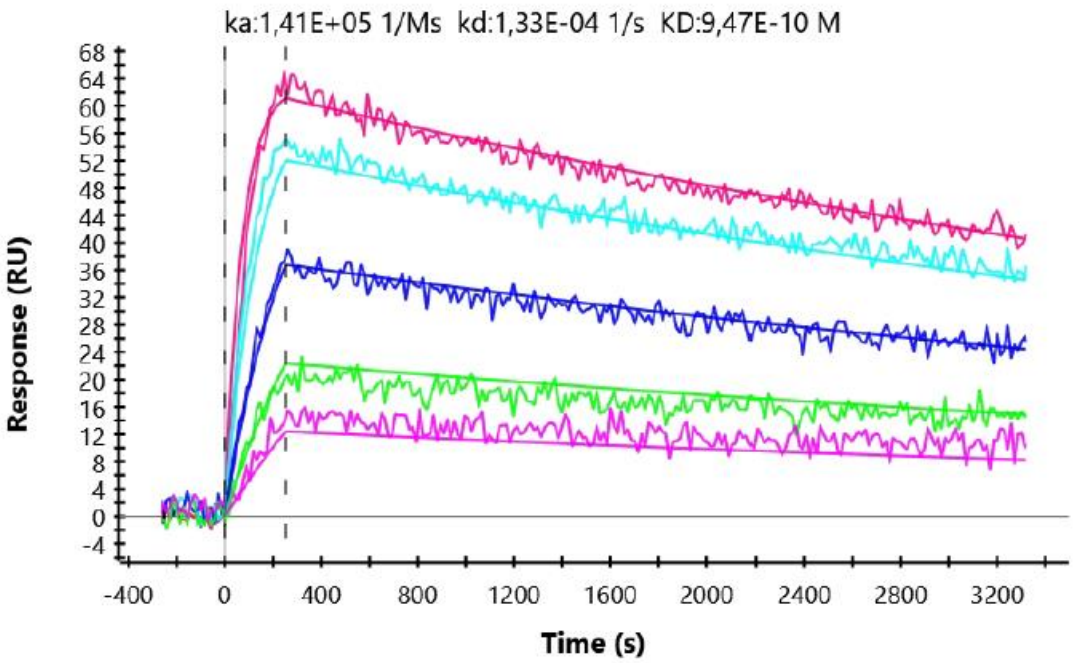


A

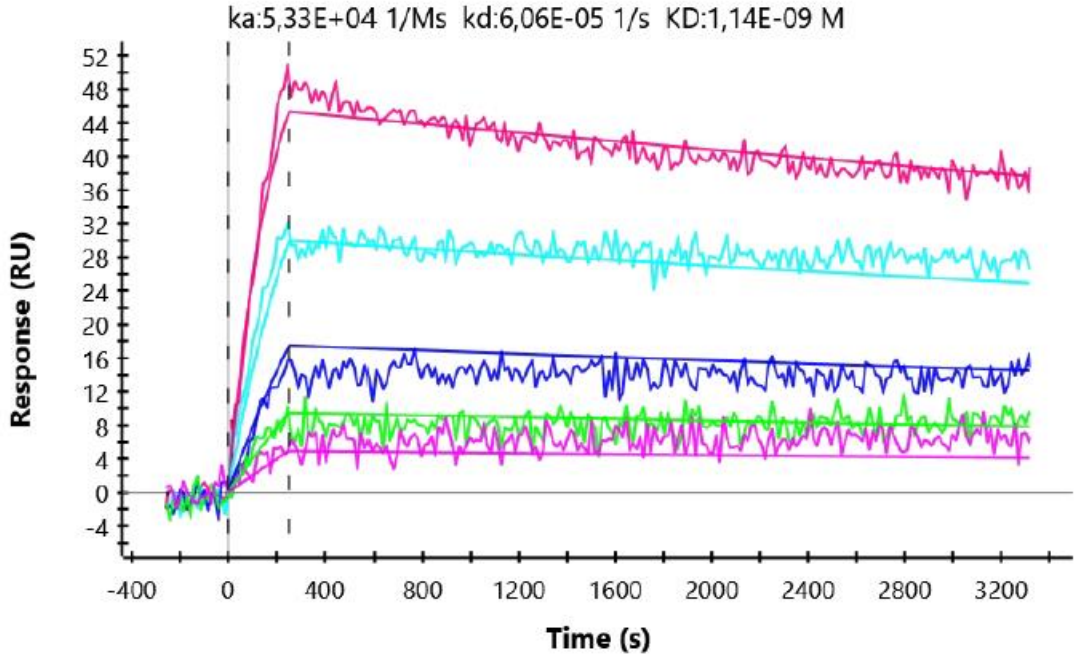


B

Figure S33. Sensorgrams for ZHER3-ADAPTHER3 flowing over a surface of immobilized (**A**) HSA, (**B**) HER3.



A



B

Figure S34. Sensorgrams for ADAPHER2-1-ZHER2 flowing over a surface of immobilized (**A**) HSA, (**B**) HER2.

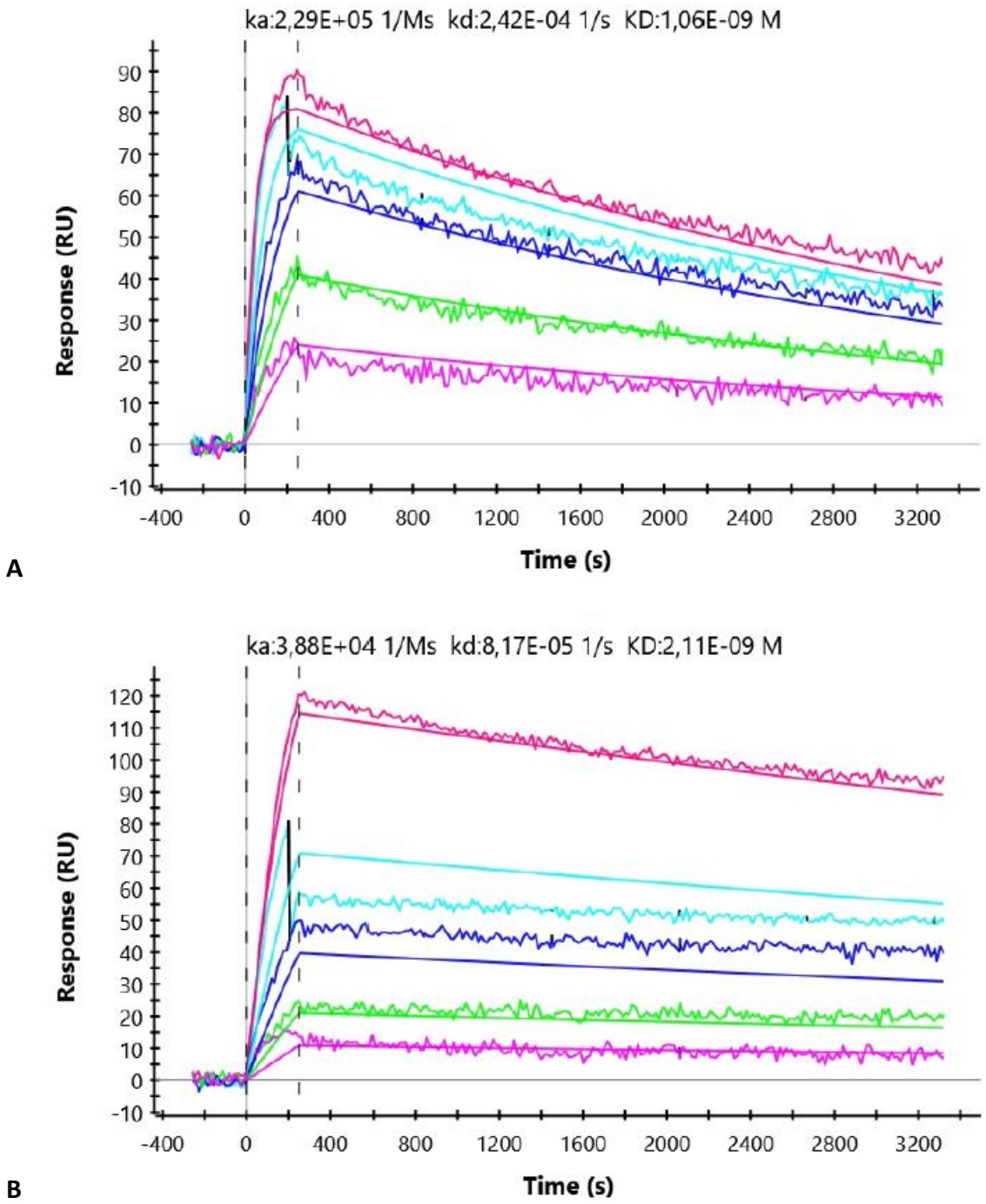
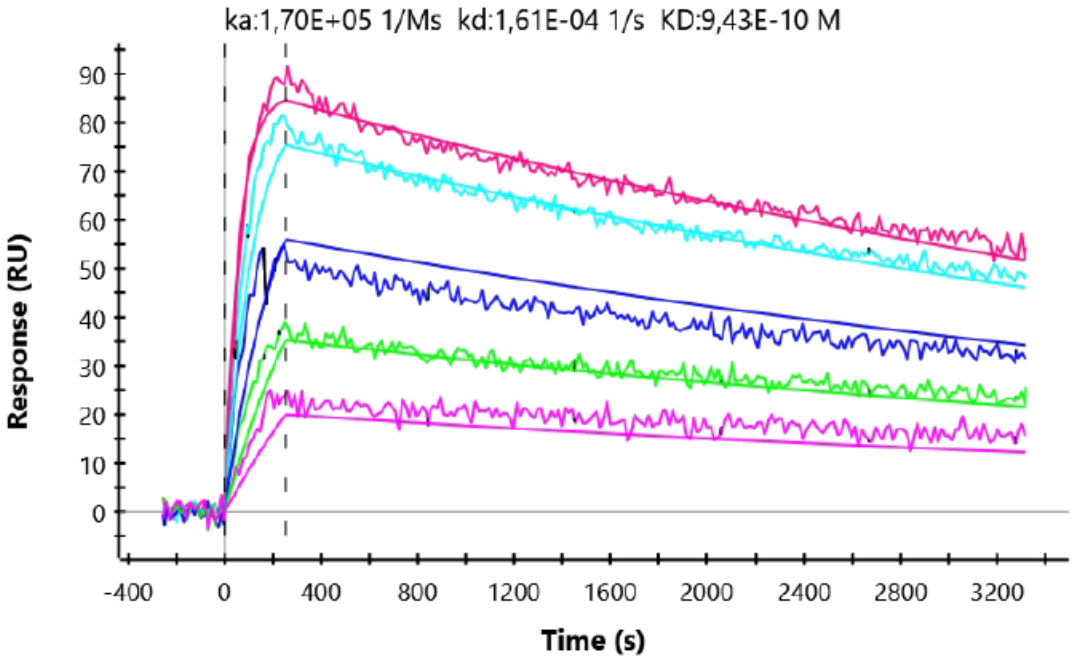
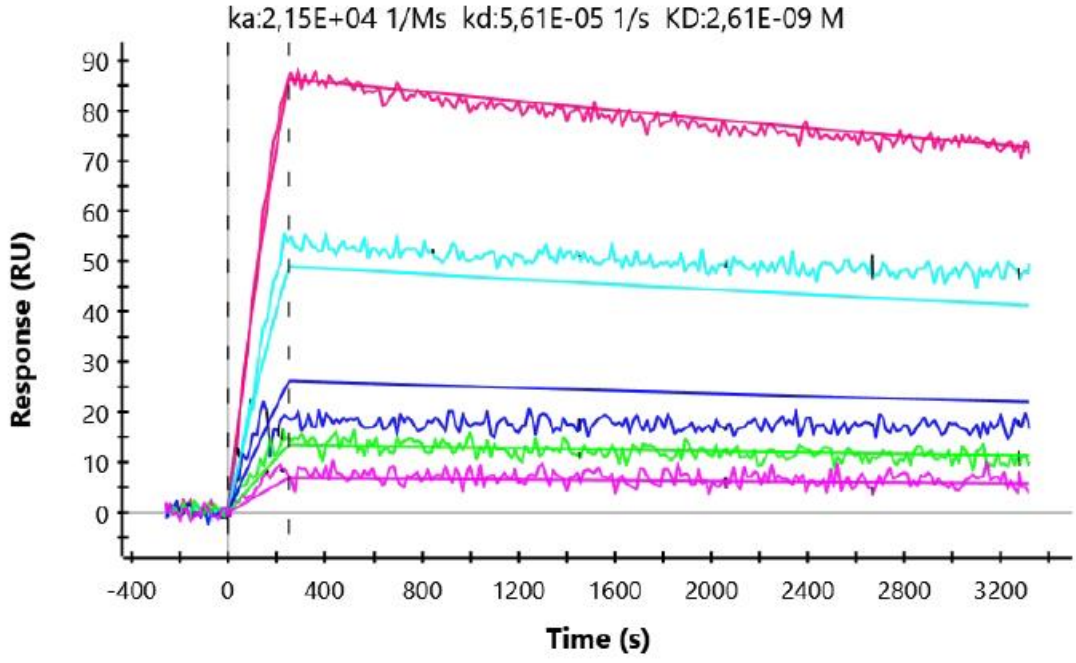


Figure S35. Sensorgrams for ZHER2-1-ADAPTHER2 flowing over a surface of immobilized (**A**) HSA, (**B**) HER2.

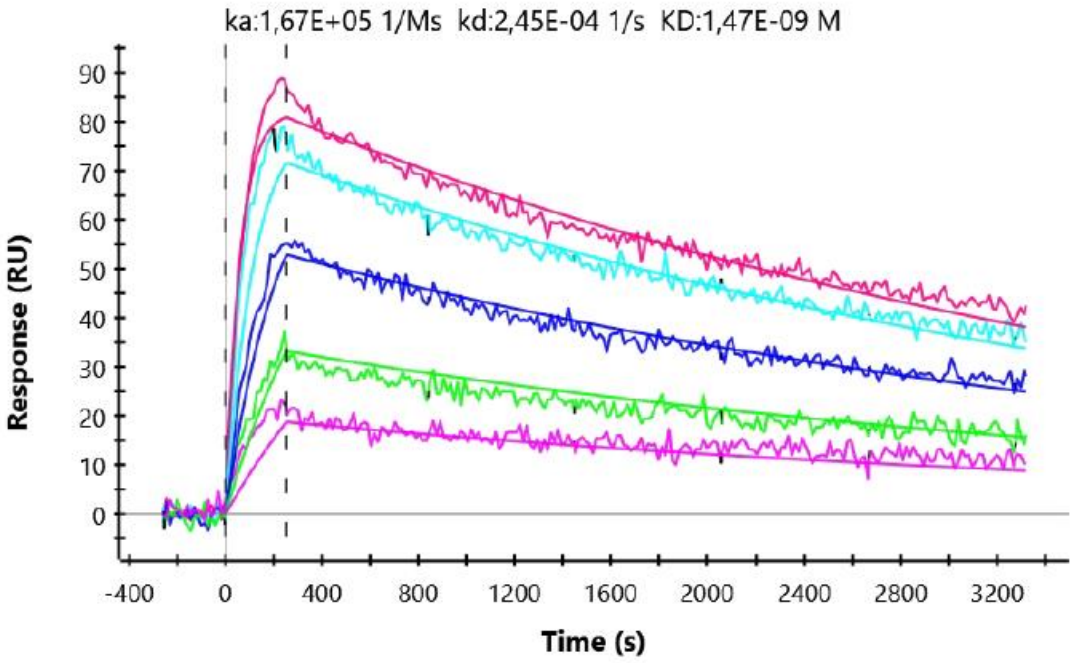


A

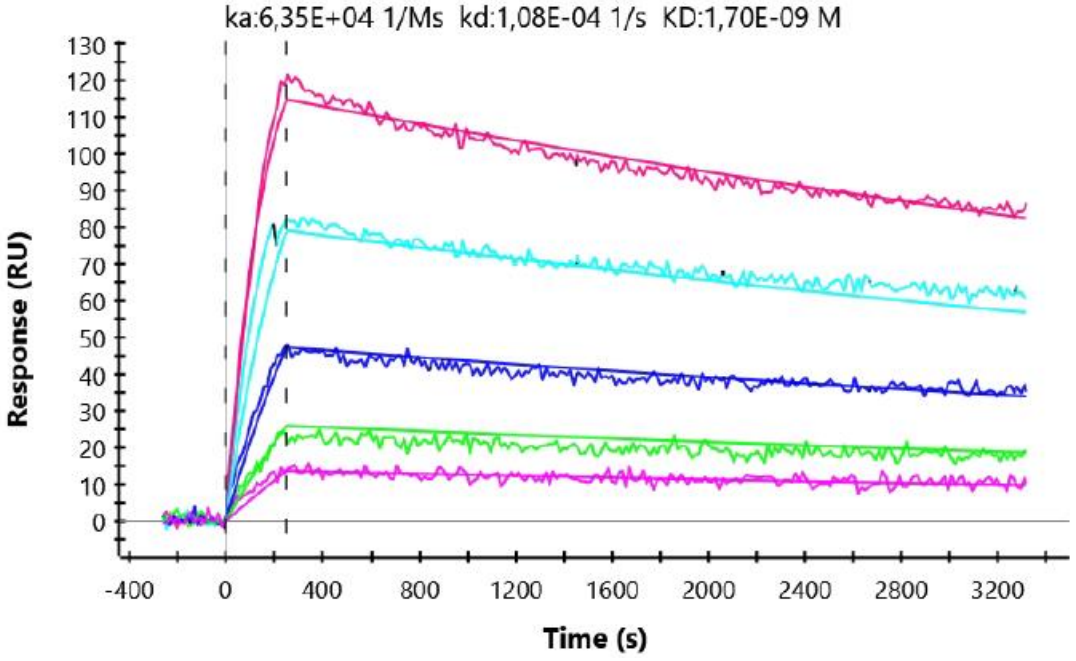


B

Figure S36. Sensorgrams for ADAPTHER2-2-ZHER2 flowing over a surface of immobilized (**A**) HSA, (**B**) HER2.

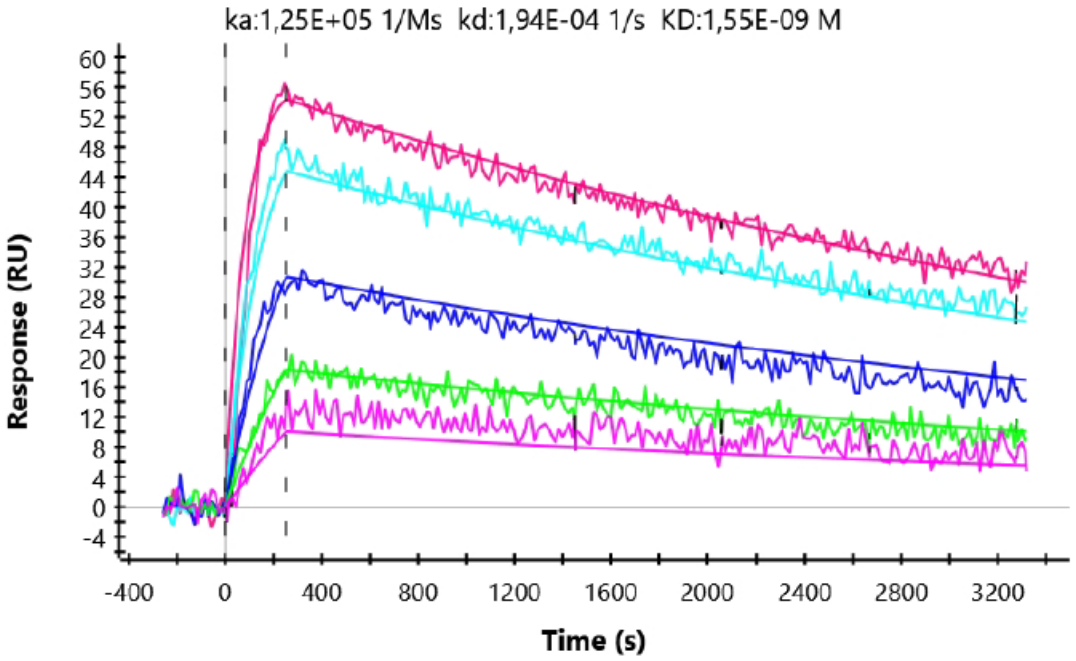


A

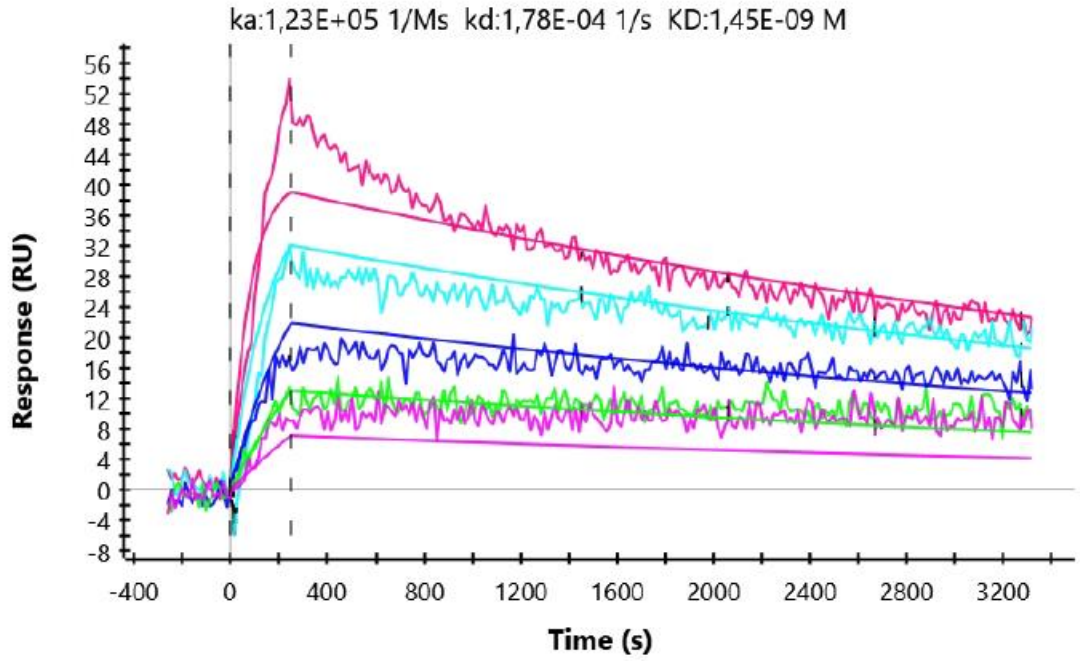


B

Figure S37. Sensorgrams for ZHER2-2-ADAPTHER2 flowing over a surface of immobilized (**A**) HSA, (**B**) HER2.

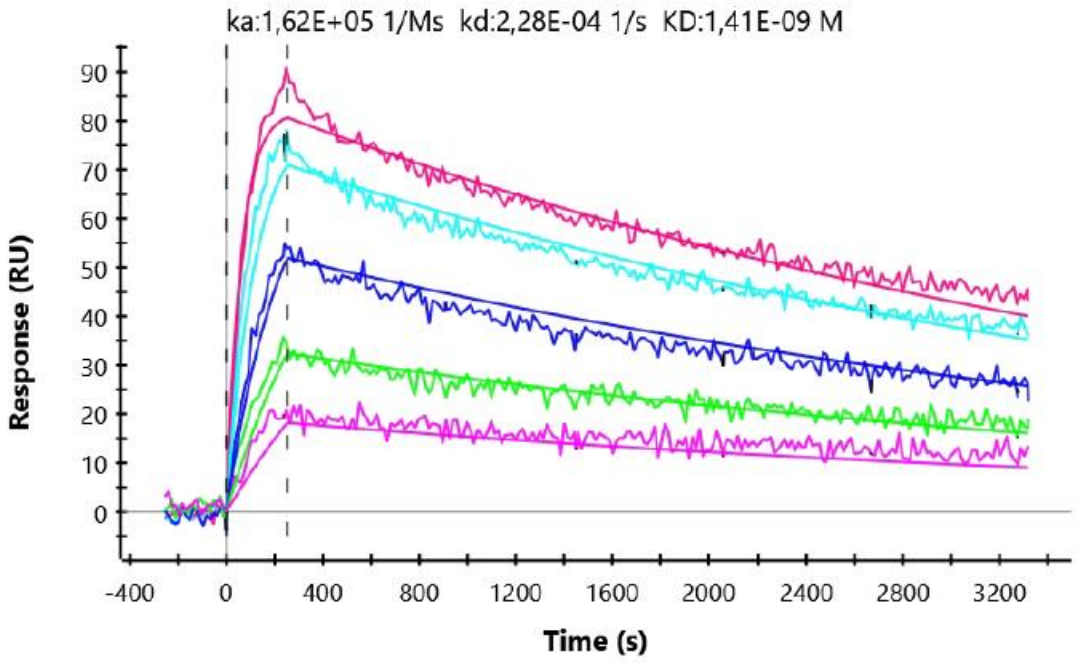


A

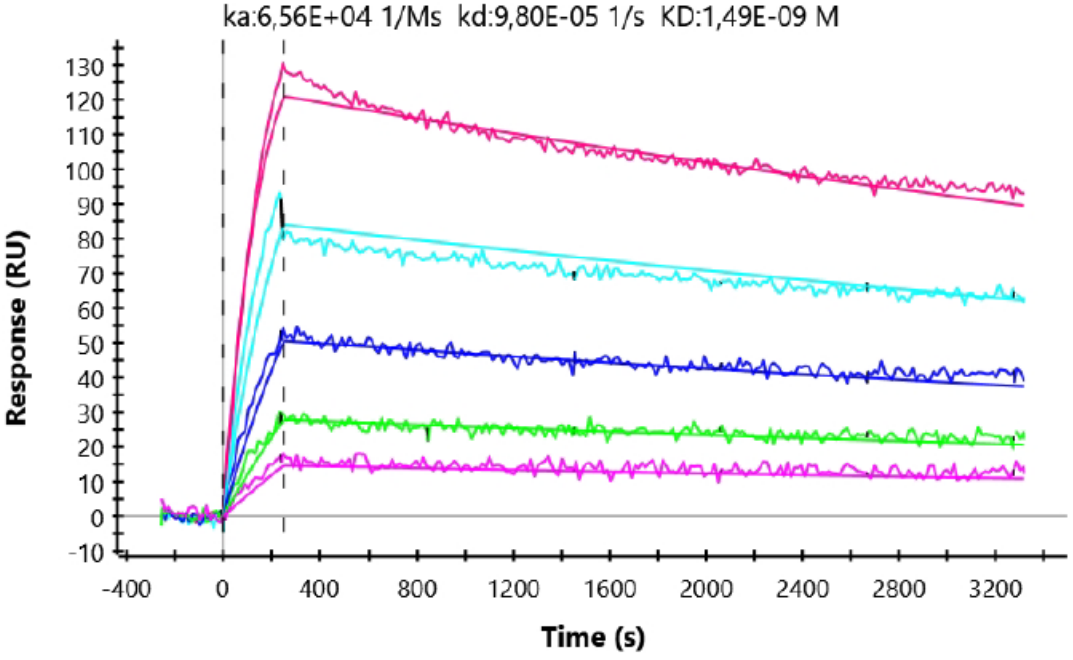


B

Figure S38. Sensorgrams for ADADPHER2-3-ZHER2 over a surface of immobilized (**A**) HSA, (**B**) HER2.

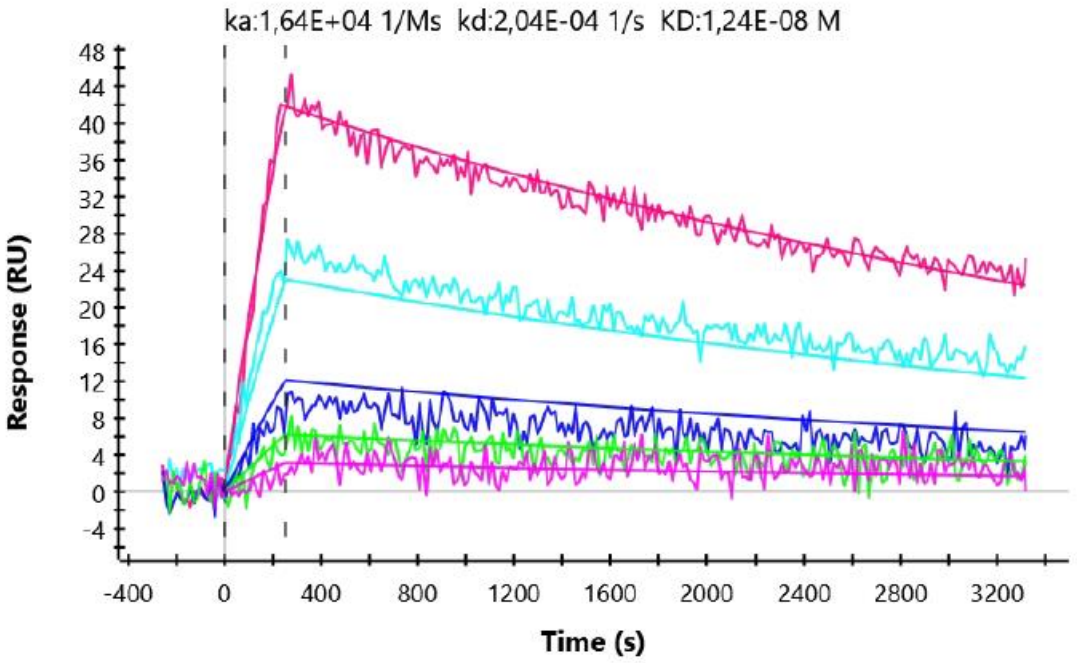


A

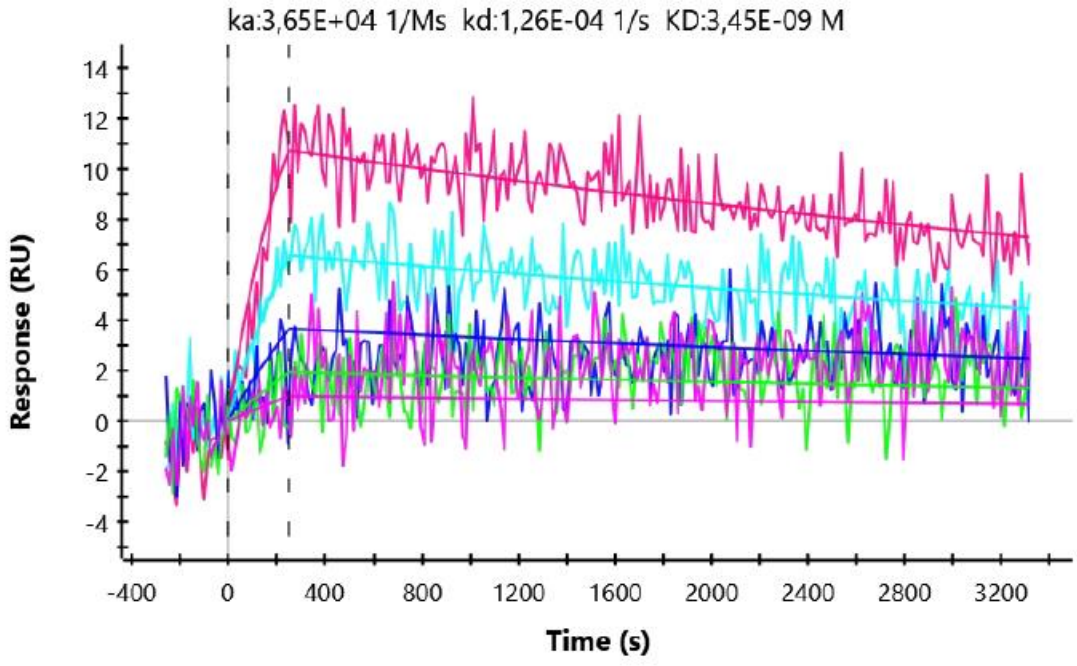


B

Figure S39. Sensorgrams for ZHER2-3-ADAPTHER2 over a surface of immobilized (**A**) HSA, (**B**) HER2.



A



B

Figure S40. Sensorgrams for the polyspecific hexamer over a surface of immobilized (**A**) HSA, (**B**) HER2.

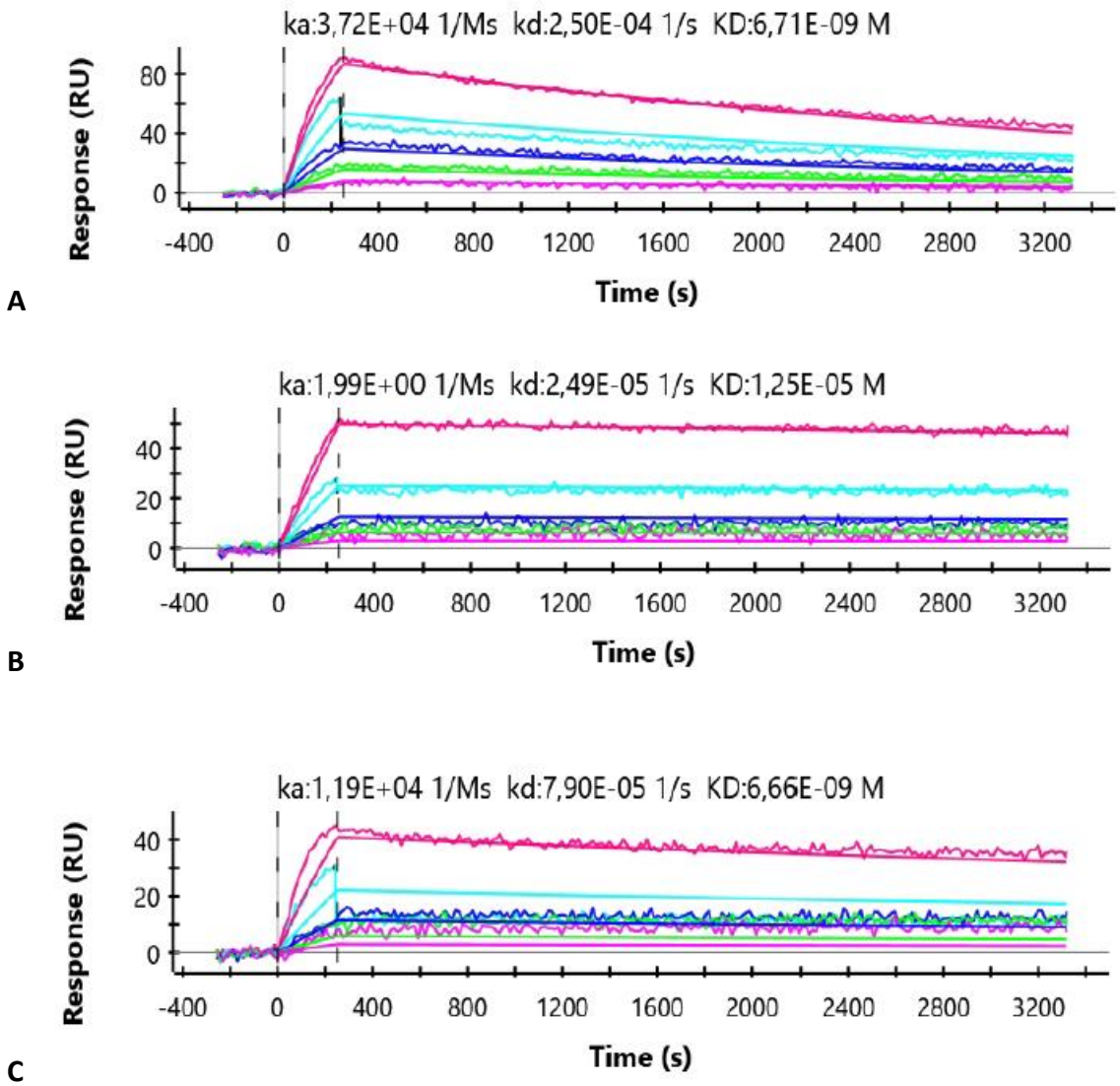


Figure S40. Sensorgrams for the polyspecific octamer over a surface of immobilized (**A**) HSA, (**B**) HER2, (**C**) HER3.

SPR diagrams

Figure S41. Comparison of the three measured KD values for HSA for ADAPTHER3-ZHER2, ZHER2-ADAPTHER3, ADAPTHER3-ZHER3, ZHER3-ADAPTHER3. Two GLC-chips were used. Measurement 1 was taken on the first chip, measurement 2 and 3 on the second chip.

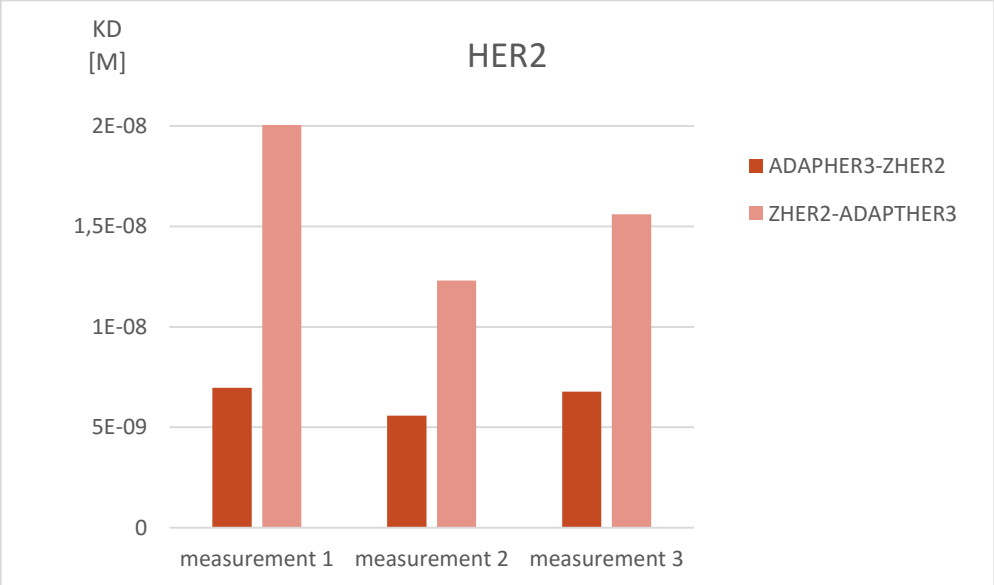


Figure S42. Comparison of the three measured KD values for HER2 for ADAPTHER3-ZHER2, ZHER2-ADAPTHER3. Two GLC-chips were used. Measurement 1 was taken on the first chip, measurement 2 and 3 on the second chip.

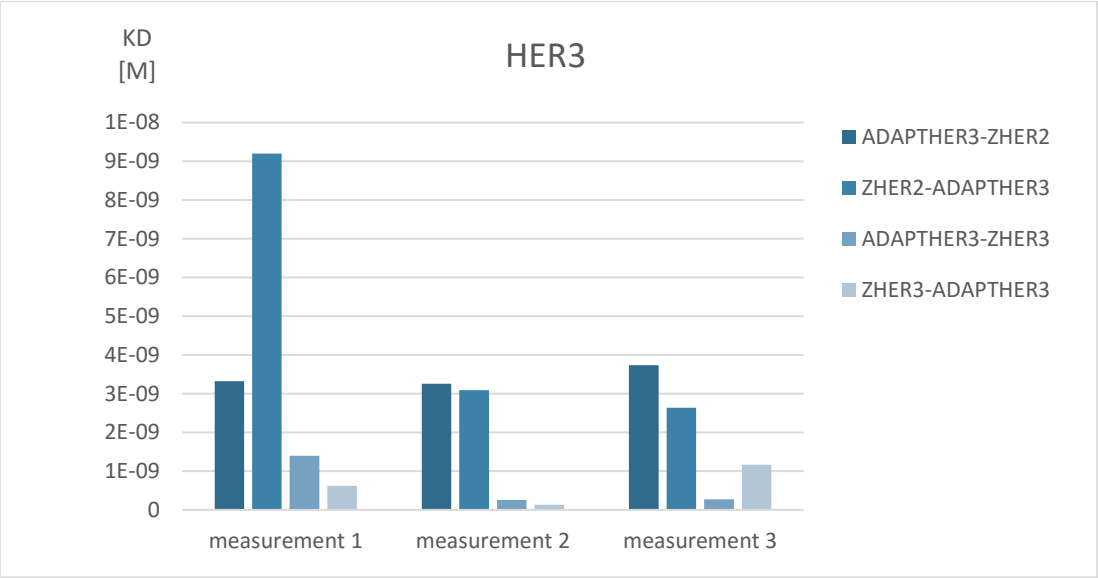


Figure S43. Comparison of the three measured KD values for HER3 for ADAPTHER3-ZHER2, ZHER2-ADAPTHER3, ADAPTHER3-ZHER3, ZHER3-ADAPTHER3. Two GLC-chips were used. Measurement 1 was taken on the first chip, measurement 2 and 3 on the second chip.