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Supplemental Material to:

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Monoclonal antibody therapeutics with up to five specificities: Functional enhancement through fusion of target-specific peptides

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Supplemental Information: Methods, Tables and Figures

Table S1. FACS analysis of receptor expression

Cell lines	Mean Fluorescence Intensity			
	EGFR	ErbB2	ErbB3	IGF-1R
MCF-7	249	661	3289	9681
MCF-7 ^{ErbB2}	284	6750	2770	6971
BxPC-3	4441	548	1344	2185
SK-BR-3	1008	16786	2142	466

MCF-7^{ErbB2} cells were generated by transfecting MCF-7 cells (American Type Culture Collection, Manassas, VA) with the construct, pcDNA3.1/Zeo+:huErbB2, using FuGENE6 transfection reagent (Roche, Indianapolis, IN, USA) according to the manufacturer's instructions. After three weeks of selection in 250 μg/ml zeocin (Invitrogen), the clone 7C76 (MCF-7^{ErbB2}) was identified based on ErbB2 expression levels. MCF-7^{ErbB2} along with the parental MCF-7, SK-BR-3 and BxPC-3 cells were assessed for cell surface expression of EGFR, ErbB2, ErbB3 and IGF1R using commercial antibodies from Becton Dickinson. Briefly, adherent cells were detached from flasks using 0.05% Trypsin-EDTA solution (Invitrogen), washed with FACS buffer (PBS with 0.2% BSA and 0.1% NaN_3) and resuspended in FACS buffer at $0.15x10^6$ cells per 200 μl. Staining with directly conjugated primary antibodies was performed at 4 °C for 30 minutes. Cells were washed once with FACS buffer, resuspended in 250 μl FACS buffer, and analyzed on a FACSCanto flow-cytometer with FACS Diva software (Becton Dickinson). Directly matched directly conjugated mouse IgG antibodies (Becton Dickinson) were used as isotype controls to determine instrument settings. Data are presented as mean fluorescence intensity (MFI). Debris and dead cells were gated out on the FSC/SSC dot plot based on the size and granularity.

Table S2. Summary of SPR data for Ang2 binding zybodies, MBP fusions, and scaffold antibody

	Ang2						ErbB2					
	Average			Standard Error			Average			Standard Error		
	K_D (nM)	k_a (1/Ms)	k_d (1/s)	K_D (nM)	k_a (1/Ms)	k_d (1/s)	K_D (nM)	k_a (1/Ms)	k_d (1/s)	K_D (nM)	k_a (1/Ms)	k_d (1/s)
TRA-*a1*L	3.50	2.36 E+05	8.32 E-04	0.34	9.74 E+03	1.08 E-04	1.73	4.51 E+04	7.78 E-05	0.35	1.12 E+03	1.54 E-05
TRA-*a1*H	1.26	4.89 E+05	6.28 E-04	0.21	2.38 E+04	1.29 E-04	1.29	4.51 E+04	5.79 E-05	0.28	1.35 E+03	1.07 E-05
***a1*L-TRA**	1.82	6.53 E+05	1.20 E-03	0.19	3.19 E+04	1.73 E-04	2.49	3.56 E+04	8.87 E-05	0.09	4.70 E+02	2.48 E-06
***a1*H-TRA**	1.29	6.19 E+05	8.13 E-04	0.19	3.81 E+04	1.49 E-04	1.76	4.32 E+04	7.42 E-05	0.02	3.67 E+02	4.08 E-08
MBP-*a1*	22.20	7.46 E+04	1.66 E-03	0.08	3.47 E+03	8.57 E-05	nd	nd	nd	nd	nd	nd
TRA	nd	nd	nd	nd	nd	nd	2.23	5.99 E+04	1.21 E-04	0.55	1.14 E+04	1.40 E-05

For ErbB2 measurements, anti-Fc antibody (Sigma) was amine coupled to a CM5 sensor chip as per the standard procedure described in Biacore 3000 manual, followed by capture of Zybodies at 1 μg/ml. ErbB2 (domains I-IV) was then injected for 4 min at concentrations ranging from 2.4 nM to 200 nM. A dissociation time of 6 minutes was included. The surface was regenerated using 10 mM glycine (pH 2.0) for 60 seconds. Data was fit to 1:1 Langmuir binding model. For Ang2 binding, an anti-HIS antibody (Abcam) was amine-coupled to a CM5 sensor chip as per the standard procedure described in Biacore manual, followed by capture of Ang2 (R&D Systems) at 1 μg/ml. Zybodies or an MBP-*a1* fusion were injected at concentrations ranging from 0.8 nM to 66 nM over the Ang-2 surface for 3 minutes. A dissociation time of 6 minutes was included. The surface was regenerated using 10 mM glycine (pH 2.0) for 60 seconds. Data was fit to 1:1 Langmuir binding model. Values represent averages of three independent measurements. nd= not determined.

Figure S1

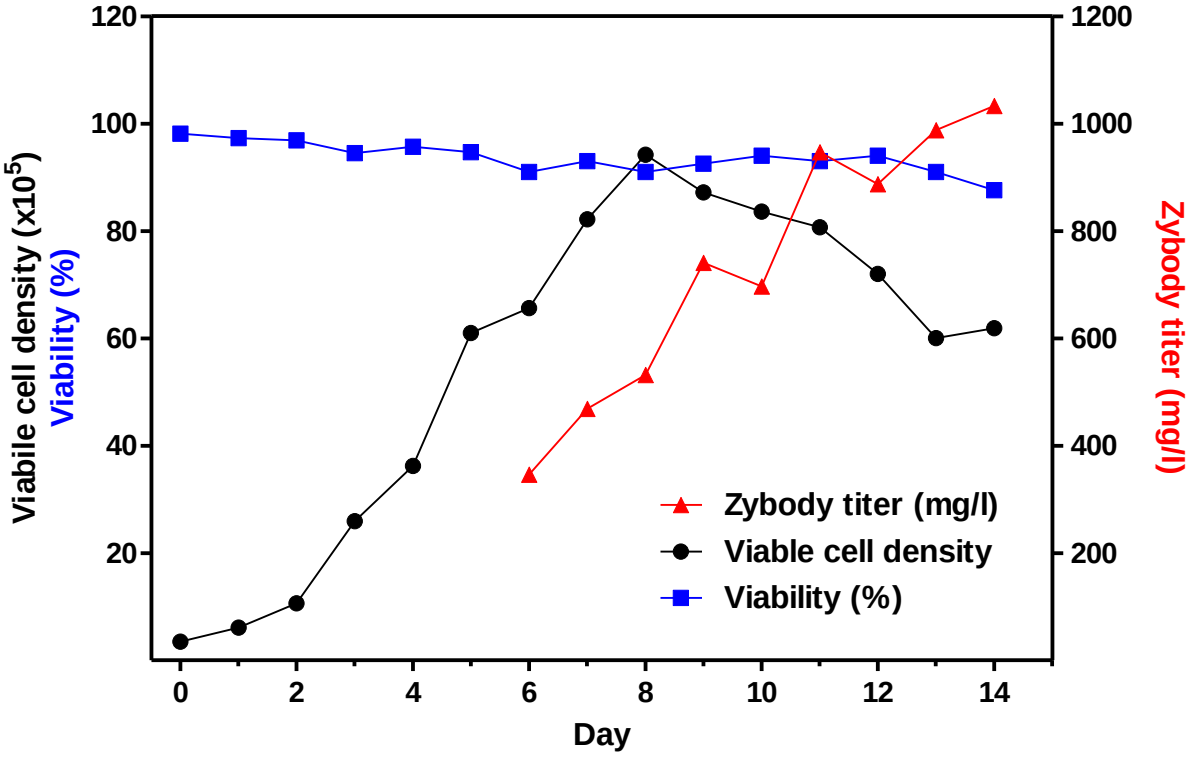

Figure S1. *Stable expression of a zybody in CHO cell line.*
A CHO cell line, stably expressing the ErbB2/Ang2-targeting, bi-specific zybody, TRA-*a2*H, was established at Selexis SA and used for bioreactor production studies in a 10-liter disposable stirred tank equipped with Rockwell/Allen Bradley compact Logix controller (Xcellerex). The reactor was inoculated with a target seeding density of $0.3x10^6$ cells/ml and then followed a fourteen day, fed batch process. VCD, lactate, glucose, pH and CO_2 were measured using a NOVA Bioprofile. Osmolality was measured using Auto Osmometer (Advanced Instruments). Supernatant samples were sterile filtered and stored at 2–8 °C for further titer and product quality analyses. The zybody titer was measured by ELISA using anti-human Fc (Abcam) for capture and anti-human κ HRP (Abcam) for detection. Viable cell density reached a maximum of 9.4 x 10^6 cell/ml on day 8, and total cell viability remained above 90% throughout the entire run. By day 14, the titer of TRA-*a2*H had exceeded 1.0 g/l, at which point the culture was harvested.

Figure S2

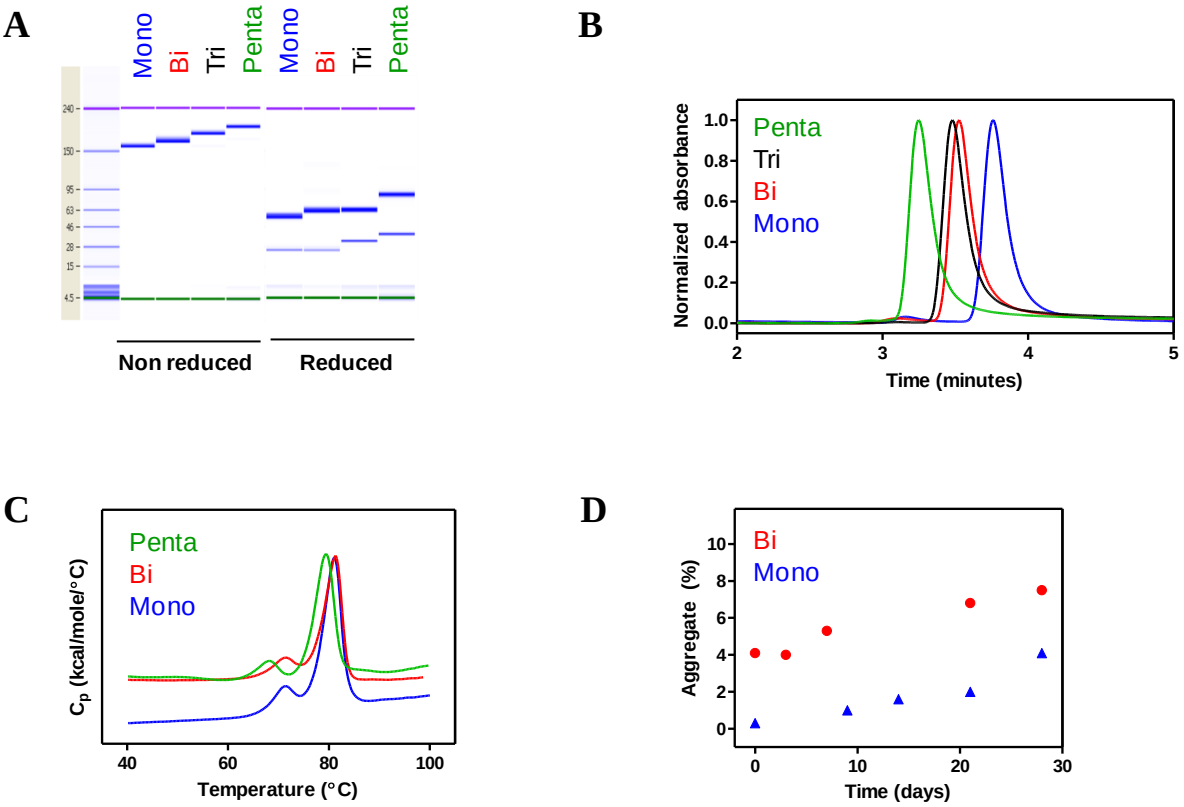

Figure S2. *Analytical assessment of multi-specific zybodies compared to mono-specific scaffold antibody.* Representative penta-, tri-, and bi-specific zybodies, along with the parental antibody scaffold, trastuzumab, were produced from transiently transfected HEK293 cells and were purified over a single column of Protein A resin. (**A**) The mono-specific trastuzumab scaffold (Mono), along with TRA-*i1*H (Bi), TRA-*i1*-H-*e1*L (Tri) and *a2*H-*n1*L-TRA-*i1*H-*e1*L (Penta) were analyzed by capillary electrophoresis (Agilent 2100 Bioanalyzer) under reducing and non-reducing conditions. (**B**) Analytical size-exclusion chromatograms of the same proteins described in **A**. Normalized absorbance is the ratio of absorbance at 280 nm to the maximum absorbance of entire run. (**C**) Differential scanning calorimetry thermograms were generated for trastuzumab and the same bi- and penta-specific zybodies indicated in **A**. (**D**) A stability study compared the stability of the bi-specific zybody, TRA-*a1*H (Bi) to that of trastuzumab (Mono). Samples were diluted to 1 mg/ml in PBS and stored at 50 °C for up to 4 weeks. Samples were analyzed by SEC at weekly intervals. Although the initial levels of aggregation differed between the pharmaceutical grade trastuzumab and the zybody, the rates of aggregation over time were similar.

Figure S3

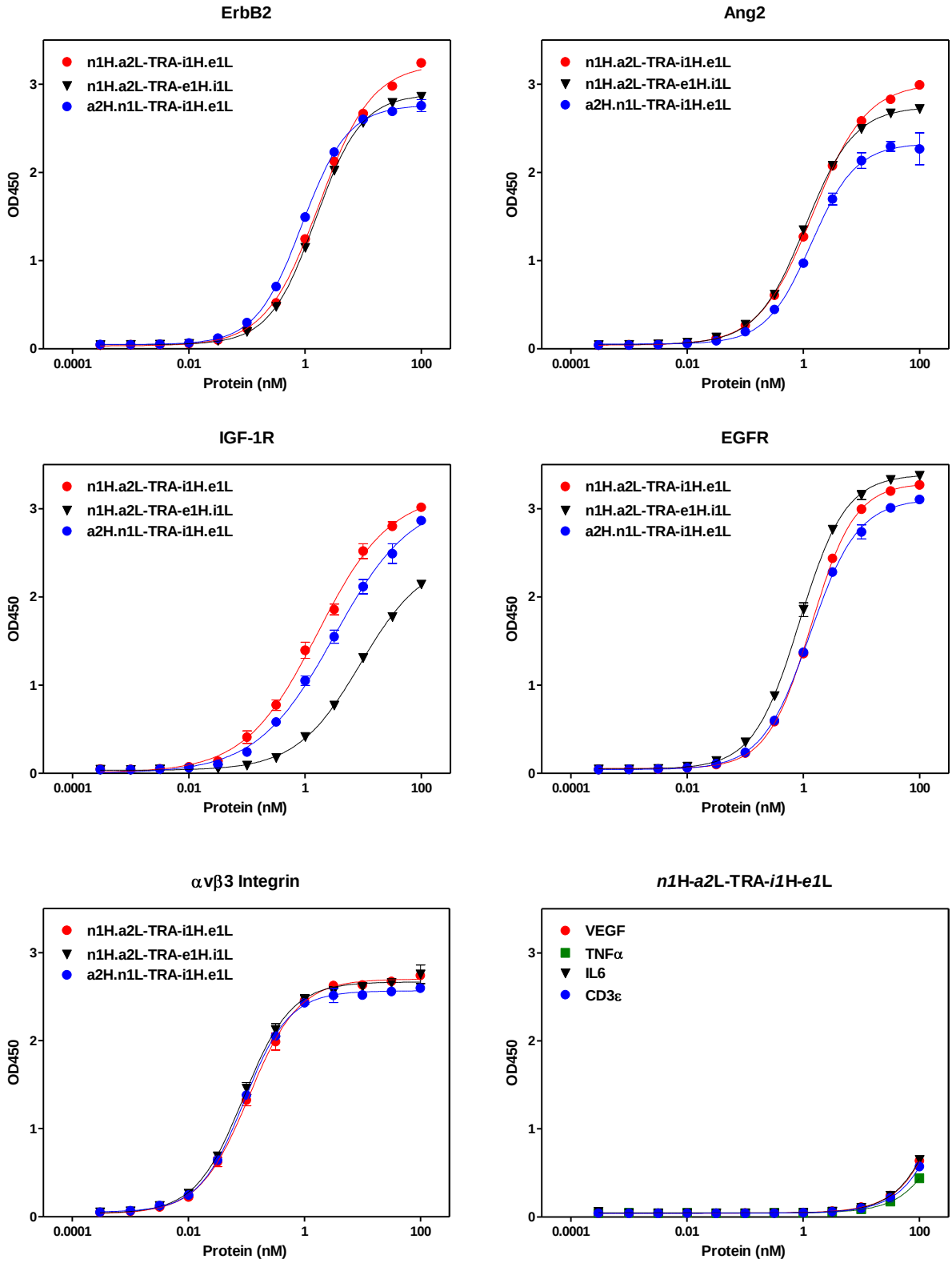
ErbB2
Ang2
IGF-1R
EGFR
αvβ3 Integrin
n1H-a2L-TRA-i1H-e1L
n1H.a2L-TRA-i1H.e1L
n1H.a2L-TRA-e1H.i1L
a2H.n1L-TRA-i1H.e1L
VEGF
TNFα
IL6
CD3ε
OD450
Protein (nM)
0.0001
0.01
1
100
0
1
2
3

Figure S3. *Direct binding of multi-specific zybodies to various targets and non-specific proteins.*
ELISA was used to characterize binding of multi-specific zybodies to ErbB2-Fc, Ang2, IGF-1R, EGFR, αvβ3 integrin, VEGF, TNFα, IL6 and CD3ε. Microtiter plates were coated with target protein: 30 ng/ml Ang2 (R&D Systems), 30 ng/ml ErbB2-Fc (R&D Systems), 500 ng/ml IGF-1R (R&D Systems), 500 ng/ml EGFR (R&D Systems), 500 ng/ml αvβ3 integrin (R&D Systems), VEGF 500 ng/ml (R&D Systems), TNFα 500 ng/ml (Peprotech), IL6 500 ng/ml (Peprotech) or CD3ε 500 ng/ml (Sino Biological) at 4 ℃ overnight. After blocking (ELISA Blocker, Thermo Scientific) and washing (PBS, 0.1% Tween 20), serially diluted zybody was added to wells and incubated for 2 hours at room temperature. Plates were washed again, followed by addition of anti-human κ HRP and incubation for 1 hr at room temperature. After a final wash step, 3,3',5,5'-tetramethylbenzidine reagent (KPL, Inc.) was added and absorbance was measured at 450 nm using a plate reader (SpectraMax).

Figure S4

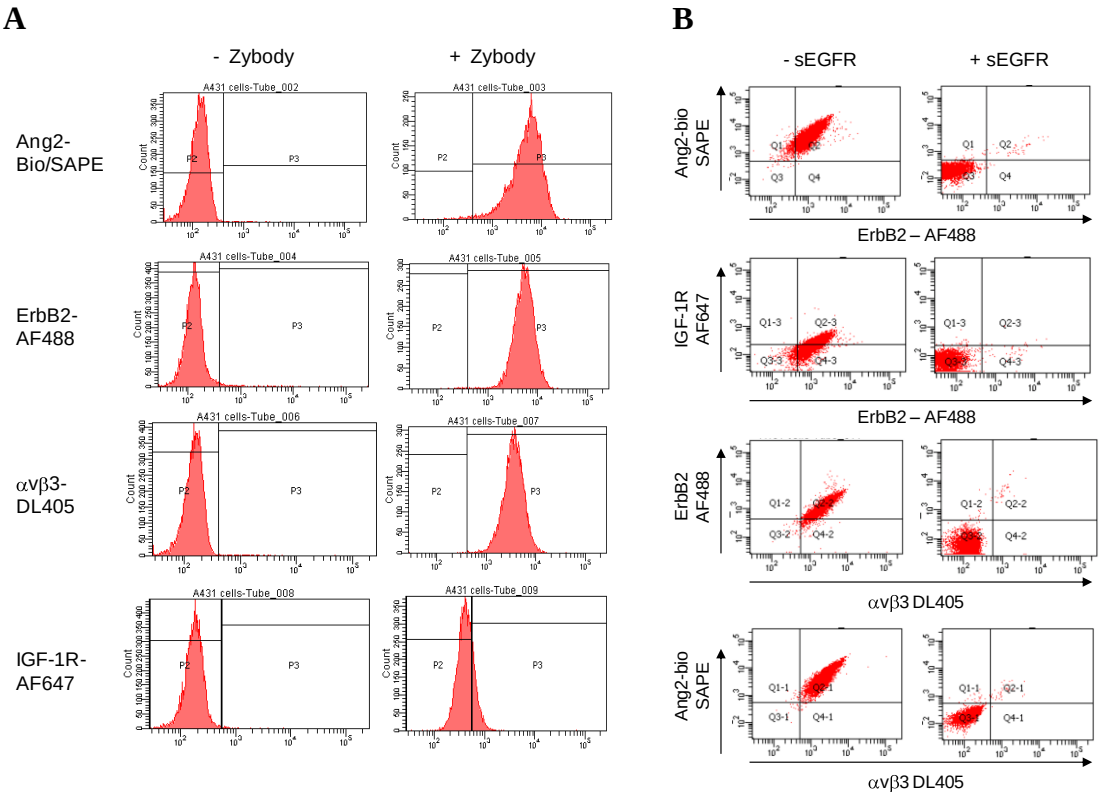

Figure S4. Pentaspecific zybody binds to EGFR on A431 cells and four additional soluble targets. A431 cells (10^6) in 100ul of FACS buffer (PBS, 0.1% BSA, 0.1% sodium azide) received either 53 nM of the penta-specific zybody, *a2*H-*n1*L-TRA-*e1*H-*i1*L, or zybody (53 nM) plus sEGFR (158 nM). After incubation for 20 minutes and washing in FACSBuffer, cells received; Ang-2 biotin (18 nM), ErbB2-AF488 (58 nM), αvβ3 DL405 (52 nM), and IGF-1R AF647 (49 nM). After incubation for 20 minutes and washing, cells received streptavidin-PE. After an additional 15 minute incubation and wash, cells were resuspend cells in 200 μl of FACS buffer and analyzed on a FACSCanto flow-cytometer with FACS Diva software (Becton Dickinson). (**A)** Single parameter histograms indicating increased binding for each of the four soluble targets occur in the presence of penta-specific zybody. (**B**) Two-parameter analysis is performed in a pair-wise fashion, indicating simultaneously binding of soluble targets to the cell surface. Penta-specific zybody binding to A431 cells is mediated through EGFR, as binding is inhibited with soluble EGFR.