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(71) Demandeur/Applicant:
GENMAB A/S, DK

(72) Inventeurs/Inventors:
NEIJSEEN, JOOST J., NL;
MEESTERS, JOYCE I., NL;
DE GOEIJ, BART, NL;
LABRIJN, ARAN FRANK, NL;
PARREN, PAUL, NL;
SCHUURMAN, JANINE, NL

(74) Agent: BORDEN LADNER GERVAIS LLP

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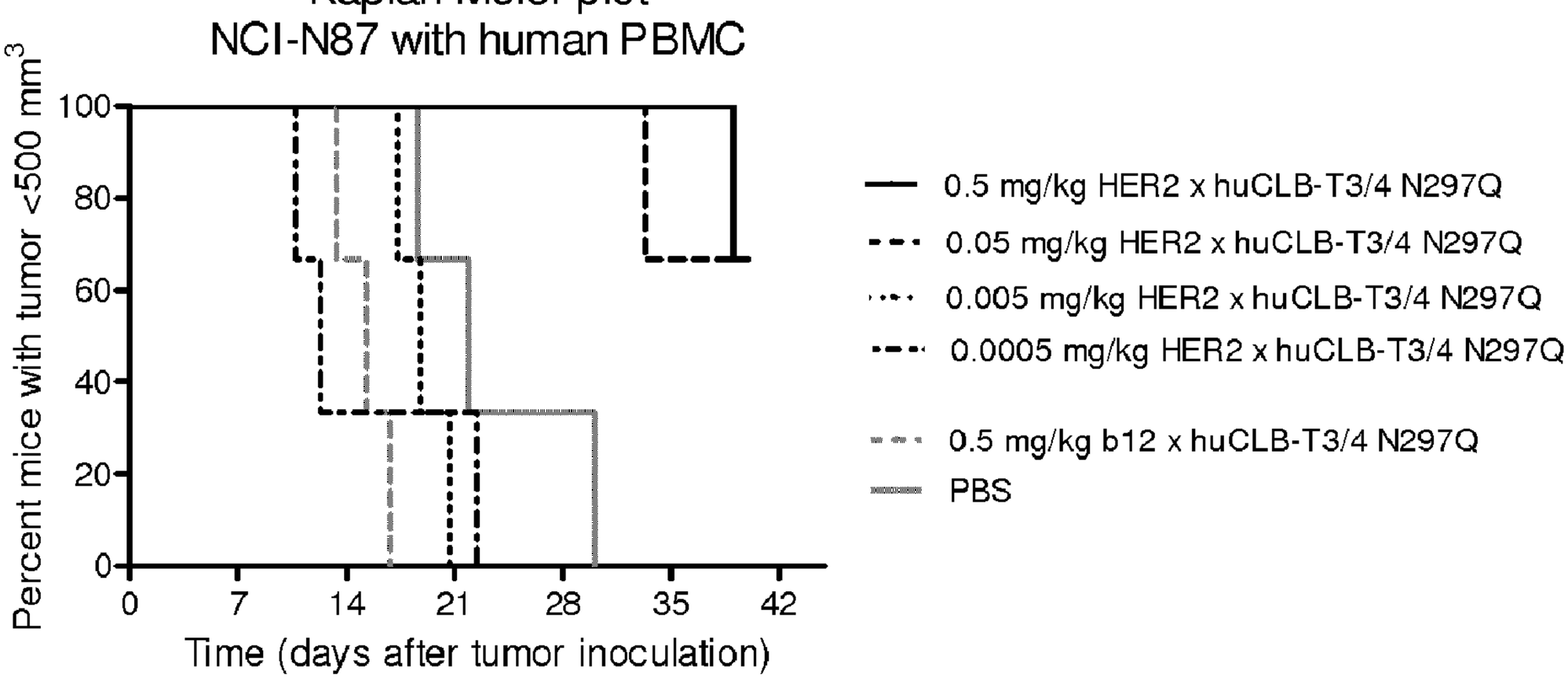
Figure 33B



(57) Abrégé/Abstract:
Bispecific antibodies which comprise one antigen-binding region binding to an epitope of human epidermal growth factor receptor 2 (HER2) and one antigen-binding region binding to human CD3, and related antibody-based compositions and molecules, are disclosed. Pharmaceutical compositions comprising the antibodies and methods for preparing and using the antibodies are also disclosed.

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(71) Applicant *(for all designated States except US)*: **GENMAB A/S** [DK/DK]; Bredgade 34, DK-1260 Copenhagen K (DK).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **NEIJSEEN, Joost, J.** [NL/NL]; c/o Genmab B.V., Yalelaan 60, NL-3584 CM Utrecht (NL). **MEESTERS, Joyce, I.** [NL/NL]; c/o Genmab B.V., Yalelaan 60, NL-3584 CM Utrecht (NL). **DE GOEIJ, Bart** [NL/NL]; c/o Genmab B.V., Yalelaan 60, NL-3584 CM Utrecht (NL). **LABRIJN, Aran, Frank** [NL/NL]; c/o Genmab B.V., Yalelaan 60, NL-3584 CM Utrecht (NL). **PARREN, Paul** [NL/NL]; c/o Genmab B.V., Yalelaan 60, NL-3584 CM Utrecht (NL). **SCHUURMAN, Janine** [NL/NL]; c/o Genmab B.V., Yalelaan 60, NL-3584 CM Utrecht (NL).

(74) Common Representative: **GENMAB A/S**; Bredgade 34, DK-1260 Copenhagen K (DK).

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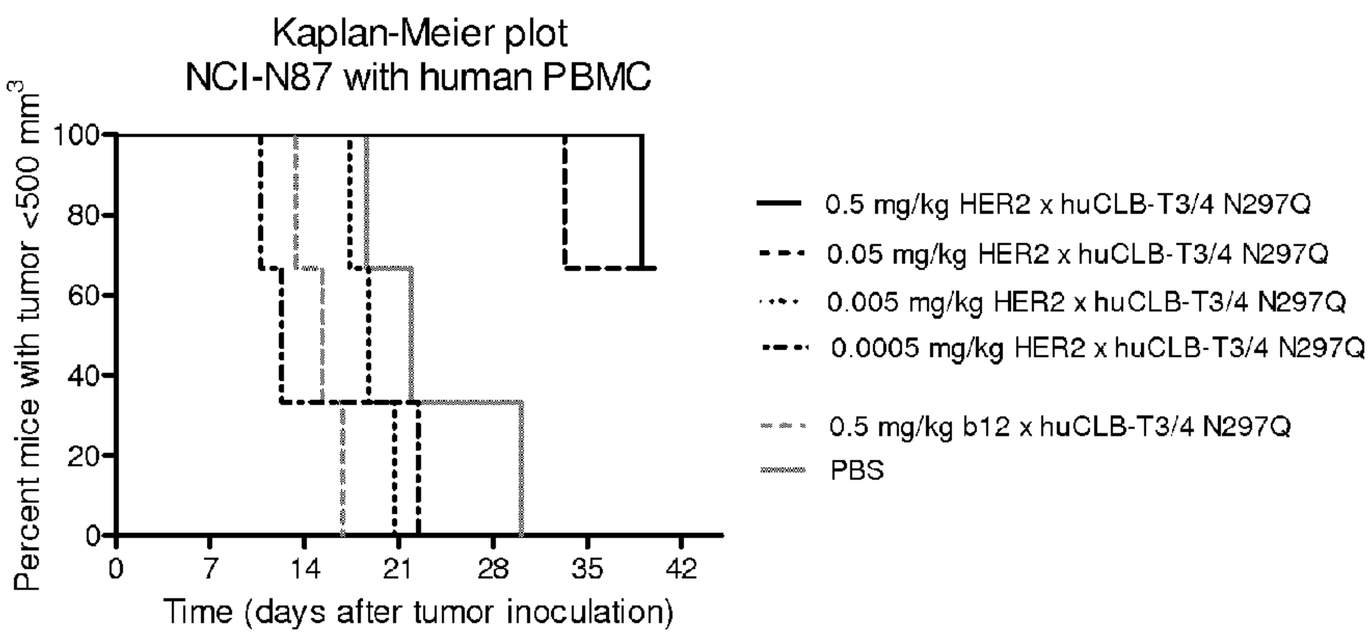
(54) **Title**: BISPECIFIC ANTIBODIES AGAINST HER2 AND CD3

Figure 33B



(57) **Abstract**: Bispecific antibodies which comprise one antigen-binding region binding to an epitope of human epidermal growth factor receptor 2 (HER2) and one antigen-binding region binding to human CD3, and related antibody-based compositions and molecules, are disclosed. Pharmaceutical compositions comprising the antibodies and methods for preparing and using the antibodies are also disclosed.

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BISPECIFIC ANTIBODIES AGAINST HER2 AND CD3

FIELD OF THE INVENTION

The present invention relates to bispecific antibodies directed to human epidermal growth factor receptor 2 (HER2) and cluster determinant 3 (CD3) and to uses of such antibodies, in particular their use in the treatment of cancer.

BACKGROUND OF THE INVENTION

HER2 is a 185-kDa cell surface receptor tyrosine kinase and member of the epidermal growth factor receptor (EGFR) family that comprises four distinct receptors: EGFR/ErbB-1, HER2/ErbB-2, HER3/ErbB-3, and HER4/ErbB-4. Both homo- and heterodimers are formed by the four members of the EGFR family, with HER2 being the preferred and most potent dimerization partner for other ErbB receptors (Graus-Porta *et al.*, Embo J 1997;16:1647-1655; Tao *et al.*, J Cell Sci 2008;121:3207-3217). HER2 can be activated by overexpression or by heterodimerization with other ErbBs that can be activated by ligand binding (Riese and Stern, Bioessays 1998;20:41-48). For HER2, no ligand has been identified. HER2 activation leads to receptor phosphorylation, which triggers a cascade of downstream signals through multiple signaling pathways, such as MAPK, phosphoinositol 3-kinase/AKT, JAK/STAT and PKC, which ultimately results in the regulation of multiple cellular functions, such as growth, survival and differentiation (Huang *et al.*, Expert Opin Biol Ther 2009;9:97-110).

Much of the attention on HER2 in tumors has been focused on its role in breast cancer, in which HER2 overexpression is reported in approximately 20% of the cases and is correlated with poor prognosis (Reese *et al.*, Stem Cells 1997;15:1-8; Andrechek *et al.*, Proc Natl Acad Sci U S A 2000;97:3444-3449; and Slamon *et al.*, Science 1987;235:177-182). Besides breast cancer, HER2 expression has also been associated with other human carcinoma types, including prostate cancer, non-small cell lung cancer, bladder cancer, ovarian cancer, gastric cancer, colon cancer, esophageal cancer and squamous cell carcinoma of the head & neck (Garcia de Palazzo *et al.*, Int J Biol Markers 1993;8:233-239; Ross *et al.*, Oncologist 2003;8:307-325; Osman *et al.*, J Urol 2005;174:2174-2177; Kapitanovic *et al.*, Gastroenterology 1997;112:1103-1113; Turken *et al.*, Neoplasma 2003;50:257-261; and Oshima *et al.*, Int J Biol Markers 2001;16:250-254).

Trastuzumab (Herceptin®) is a recombinant, humanized monoclonal antibody directed against domain IV of the HER2 protein, thereby blocking ligand-independent HER2 homodimerization, and to a lesser extend heterodimerization of HER2 with other family members in cells with high HER2 overexpression (Cho *et al.*, Nature 2003;421:756-760 and

Wehrman *et al.*, Proc Natl Acad Sci U S A 2006;103:19063-19068). In cells with modest HER2 expressing levels, trastuzumab was found to inhibit the formation of HER2/EGFR heterodimers (Wehrman *et al.*, (2006), *supra*; Schmitz *et al.*, Exp Cell Res 2009;315:659-670). Trastuzumab mediates antibody-dependent cellular cytotoxicity (ADCC) and prevents ectodomain shedding, which would otherwise result in the formation of a truncated constitutively active protein in HER2 overexpressing cells. Also inhibition of both *in vitro* and *in vivo* proliferation of tumor cells expressing high levels of HER2 has been reported for trastuzumab (reviewed in Nahta and Esteva, Oncogene 2007;26:3637-3643). Herceptin® has been approved both for first-line and adjuvant treatment of HER2 overexpressing metastatic breast cancer, either in combination with chemotherapy, or as a single agent following one or more chemotherapy regimens. Trastuzumab has been found to be effective only in 20-50% of HER2 overexpressing breast tumor patients and many of the initial responders show relapse after a few months (Dinh *et al.*, Clin Adv Hematol Oncol 2007;5:707-717).

Pertuzumab (Omnitarg™) is another humanized monoclonal antibody. It is directed against domain II of the HER2 protein, resulting in inhibition of ligand-induced heterodimerization (*i.e.*, HER2 dimerizing with another member of the ErbB family to which a ligand has bound); a mechanism reported to not strictly require high HER2 expression levels (Franklin *et al.*, Cancer Cell 2004;5:317-328.). Although pertuzumab also mediates ADCC, the main mechanism of action of pertuzumab relies on its dimerization blockade (Hughes *et al.*, Mol Cancer Ther 2009;8:1885-1892). Moreover, pertuzumab was found to enhance EGFR internalization and downregulation by inhibiting the formation of EGFR/HER2 heterodimers, which otherwise tethers EGFR at the plasma membrane (Hughes *et al.*, 2009, *supra*). This correlates with the observation that EGFR homodimers internalize more efficient than EGFR/HER2 dimers (Pedersen *et al.*, Mol Cancer Res 2009;7:275-284. The complementary mechanisms of action of pertuzumab and trastuzumab reportedly results in enhanced anti-tumor effects and efficacy when combined in patients who progressed during prior trastuzumab therapy (Baselga *et al.*, J Clin Oncol 2010;28:1138-1144), and a phase III trial to evaluate this antibody combination together with Docetaxel in previously untreated HER2-positive metastatic breast cancer is underway.

An alternative approach to improve targeted antibody therapy is by delivering cytotoxic cells or drugs specifically to the antigen-expressing cancer cells. This concept of using T-cell for efficient killing of tumor cells has been described already in 1985 (Stearz at al. Nature 1985, 314:628-631). For example, the so-called trifunctional antibodies are bispecific antibodies, targeting with one arm the antigen on the tumor cell and with the other arm for instance CD3 on T cells, and provide Fc receptor binding by the Fc region.

Upon binding, a complex of T cells, tumor cells and effector cells that bind the antibody Fc domain is formed, leading to killing of the tumor cells (Muller and Kontermann, BioDrugs 2010;24:89-98.). Ertumaxomab is one such trifunctional antibody against HER2 and CD3, which induces cytotoxicity in cell lines with low HER2 expression and which is in Phase II clinical development in metastatic breast cancer (Jones *et al.*, Lancet Oncol 2009;10:1179-1187 and Kiewe *et al.*, Clin Cancer Res 2006;12:3085-3091).

Alternatively, a complex of T cells and tumor cells are formed, leading to killing of the tumor cells (Muller and Kontermann, BioDrugs 2010;24:89-98, Baeuerle and Reinhardt 2009, Cancer Research 96: 4941) by an dual targeting antibody fragment (*e.g.* dual targeting single chain antibodies). Blinatumomab (Bargou *et al*, Science 2008, 321:974-976) is a single chain antibody construct named BiTE which induces cytotoxicity by targeting CD19 and CD3. Other antibody fragment based T-cell engaging bispecifics have been described (Moore *et al*. 2011, Blood 117:4542-4551, Baeuerle et el. Current opinion in Molecular Therapeutics 2009, 11:22-30).

The complex mechanisms regulating the function of HER2 warrant further research on new and optimized therapeutic strategies against this proto-oncogene. Accordingly, there remains a need for effective and safe products for treating HER2-related diseases, such as cancer.

SUMMARY OF THE INVENTION

It is an object of the present invention to provide novel effective bispecific antibodies comprising a first antigen-binding region derived from a HER2 antibody and a second region having a binding specificity for CD3, for medical use. Typically, the second region is an antigen-binding region derived from a CD3 antibody, optionally a known CD3 antibody.

As shown herein, the novel bispecific HER2xCD3 antibodies are capable of dose-dependent killing of HER2-expressing cells in *in vitro* cytoxicity assays, effectively prevent tumor growth *in vivo*, and/or have other advantages over monospecific HER2 or CD3 antibodies. In one aspect, the monospecific HER2 antibodies from which the HER2-binding region is derived exhibit HER2 binding characteristics or variable region sequences that differ from HER2 antibodies described in the art.

In preferred embodiments, the bispecific HER2xCD3 antibodies of the invention are prepared from HER2 antibodies that are fully human or humanized, bind to novel epitopes, and/or have favorable properties for therapeutic use in human patients. Each Fab-arm of the bispecific antibodies may further include an Fc-region, optionally comprising modifications promoting the formation of the bispecific antibody, modifications affecting Fc-mediated effector functions, and/or other features described herein.

These and other aspects of the invention are described in further detail below.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: Alignment of HER2 HuMab heavy chain variable region (VH) sequences with germline (reference) sequences (A-O). In each VH sequence, the amino acids that differ from those of the germline (reference) at specific positions are highlighted. Consensus VH sequences are shown, where "X" indicates positions at which alternative amino acids (selected from those aligned at each position) are possible. The CDR1, CDR2, and CDR3 sequences are underlined in each VH sequence. The consensus CDR sequences are further defined in Table 4.

Figure 2: Alignment of HuMab light chain variable region (VL) sequences with germline (reference) sequences (panels A-H). In each VL sequence, the amino acids that differ from those of the germline (reference) at specific positions are highlighted. In, *e.g.*, Figure 2A, all VL sequences derived from the same V-segment (IgKV1-12-01), but the closest J-segment differed between antibodies. Consensus VL sequences are shown, where "X" indicates positions at which alternative amino acids (selected from those aligned at the indicated position) are possible. The CDR1, CDR2, and CDR3 sequences are underlined in each VL sequence. The consensus CDR sequences are further defined in Table 4.

Figure 3: Binding curves of HER2 antibodies to (A, B, E) high (AU565) and (C, D, F) low (A431) HER2 expressing cell lines, determined as described in Example 12. Data shown are mean fluorescence intensities (MFI) of one representative experiment for each cell line. The EC_{50} values indicate the apparent affinities.

Figure 4: Binding of HER2 antibodies to HER2 expressed on monkey Rhesus epithelial cells. Data shown are mean fluorescence intensities (MFI) of one experiment, described in Example 13.

Figure 5: Chromium-release (ADCC) assay of HER2 antibodies, showing PBMC-mediated lysis of ^{51}Cr-labeled SK-BR-3 cells after incubation with HER2 antibody. Values depicted are the mean maximum percentages ^{51}Cr-release ± the standard deviation from one representative *in vitro* ADCC experiment with SK-BR-3 cells. See Example 15 for details.

Figure 6: Effect of HER2 antibodies on the proliferation of AU565 cells, as compared to untreated cells (set to 100%). Data shown are percentages proliferation of AU565 cells compared to untreated cells measured in three independent experiments ± the standard deviation. * Significant ($P<0.05$). See Example 16 for details.

Figure 7: Percentage of viable MCF7 cells stimulated with Heregulin-β1 and treated with the indicated HER2 antibodies, relative to cells stimulated with Heregulin-β1 only. As a control, the percentage proliferation of unstimulated cells is shown (none). Data was obtained from three independent experiments ± the stdev. * Significant inhibition of Heregulin-β1-induced proliferation ($P<0.05$). See Example 17 for details.

Figure 8: ADC assay, showing killing of AU565 cells (A, B) or A431 cells (C, D) via anti-kappa-ETA'-conjugated HER2 antibodies. (A, B) Data shown are fluorescence intensities (FI) of one representative experiment with AU565 cells treated with non-conjugated and anti-kappa-ETA'-conjugated HER2 antibodies. (C, D) Data shown are mean fluorescence intensities (MFI) of one representative experiment with A431 cells treated with non-conjugated and anti-kappa-ETA'-conjugated HER2 antibodies. See Example 18 for details.

Figure 9: Binding of bispecific HER2 x CD3 antibodies to Jurkat cells. All generated bispecific antibodies show binding to Jurkat, albeit with a lower apparent affinity than the monospecific parental antibodies (nomenclature = CD3 clone x HER2 clone).

Figure 10: (A) Dose-dependent simultaneous binding of HER2xCD3 antibodies (HER2 169 x huCLB-T3/4) to labeled AU565 cells (CFSE – Y-axis) and Jurkat cells (PKH26 – X-asis), thereby creating doublets of interconnected cells as shown by the double-positive cells in FACS dot plot (Q2). (B) Representative examples of FACS experiments showing the double positive events in Q2 (dotted line) representing the cells simultanously bound via the bispecific HER2 x CD antibody.

Figure 11: Dose dependent killing of AU565 cells by bispecific HER2 x CD3 antibodies. Bispecific antibodies were generated from 4 different CD3 antibodies combined with two different HER2 antibodies (169 and 153) or control antibody IgG1 b12. (A) huOKT3, (B) HUM291, (C) YTH12.5 and (D) huCLB-T3/4. See Example 21 for details.

Figure 12: Antibody induced downmodulation of HER2. Relative percentage of HER2 expressed in AU565 cell lysate after 3 days incubation with 10 µg/mL antibody. The amount of HER2 was quantified using a HER2-specific capture ELISA and plotted as a percentage relative to untreated cells. Data shown are mean of three experiments ± standard deviation.

Figure 13: Colocalization analysis of HER2 antibodies (FITC) with lysosomal marker LAMP1 (Cy5). FITC pixel intensity overlapping with Cy5 for various monospecific HER2 antibodies. FITC pixel intensity in LAMP1/Cy5 positive pixels of three different images is plotted for each antibody. Group 3 antibodies 098 and 153 show higher FITC pixel intensities in the LAMP1/Cy5 positive compartments compared to antibodies 025 and pertuzumab from Group 2 and 169 and Herceptin® from Group 1.

Figure 14: HER2 antibody binding to CHO-S cells transfected with different HER2 ECD construct analyzed by means of flow cytometry. Hu-HER2 = fully human HER2, Hu-HER2-ch(I) CR1 = hu-HER2 with chicken domain I, Hu-HER2-ch(II) = hu-HER2 with chicken domain II, hu-HER2-ch(III) = hu-HER2 with chicken domain III and Hu-HER2-ch(IV) = hu-HER2 with chicken domain IV. Data shown are mean fluorescence intensities (MFI) of one representative antibody, TH1014-153. See Example 24 for details.

Figure 15: *In vivo* effect of HER2-HuMabs in the NCI-N87 human gastric carcinoma xenograft model in female CB.17 severe combined immunodeficiency (SCID) mice. Data shown are mean tumorsize ± S.E.M. per group (n = 10 mice per group) (A, C) and survival (B, D). See Example 25 for details.

Figure 16: *In vivo* effect of HER2 HuMabs in BT-474 breast tumor xenografts in Balb/C nude mice. Data shown are mean tumorsize ± S.E.M. per group (n = 8 mice per group) (A) and survival (B). See Example 26 for details.

Figure 17: Non-specific Fc-mediated killing in a cytotoxic assay with PBMCs can be further reduced using antibodies with a modified Fc region (LFLEDANQPS), whereas non-glycosylation via N297Q alone does not completely remove this activity. These mutations do no compromise the specific killing activity of bispecific HER2xCD3 antibody. See Example 27 for details.

Figure 18: Location of HER2 epitope has a strong effect on the efficacy of the HER2xCD3 antibodies as shown by comparison studies of three mAbs combined with the same anti-CD3 antibody (huCLB-T3/4) in cytoxicity assays with either T-cells (A) or PBMCs (B) as effector cells.

Figure 19: T cell cytotoxicity assay using target cell lines with various HER2 expression levels. Shown is the percentage of viable cells after three days incubation with T cells in the presence of HER2xCD3 bispecific antibody. The efficacy positively correlated with the expression levels, as the cells with the highest expression were killed at the lowest antibody concentrations.

Figure 20: CD69 expression of T cells co-cultured with AU565 tumor cells in the presence of bispecific HER2 x CD3 antibody and monospecific controls.

Figure 21: CD69 expression of T cells in PBMC pool treated with different Fc variants of DuoBody HER2 169 x huCLBT3/4 in the absence of tumor cells.

Figure 22: Cytokine profile resulting from incubation of PBMCs or T-cells with DuoBody huCLB T3/4-Q x HER2-169-Q (CD3-Q/169Q) antibodies and HER2 positive tumor cells.

Figure 23: GM-CSF production as a measure for T cell activation by bispecific HER2 x CD3 antibodies and the contribution of non-specific Fc mediated activation.

Figure 24: Evaluation of the *in vivo* efficacy of HER2 x CD3 bispecific mAb in a subcutaneous xenograft model with HER2 expressing tumor cell line and human PBMCs. In (A), tumor development (mean & SEM) in mice with NCI-N87 S.C. xenografts and S.C. human PBMCs treated with bispecific HER2 x CD3 antibodies is shown. Three dosing schedules were being compared, and the lowest dose appeared to be most effective. In (B) the percentage surviving mice (with tumor sizes smaller then 500 mm^3) is shown in a Kaplan-Meier plot.

Figure 25: Comparison between triple mutant (ITL), double mutants (IT, IL, TL) and single mutant (L) human IgG1-2F8 in the generation of bispecific antibodies by Fab-arm exchange with human IgG4-7D8. The generation of bispecific antibodies after 2-MEA-induced *in vitro* Fab-arm exchange between the human IgG1-2F8 triple and double mutants and wild type IgG4-7D8 with a CPSC hinge (A) or mutant IgG4-7D8-CPPC with a stabilized hinge (B), or the single mutant IgG1-2F8-F405L and IgG4-7D8 with a wild type CPSC or stabilized CPPC hinge (C), was determined by an ELISA. A concentration series (total antibody) of 0-20 µg/mL or 0-10 µg/mL was analyzed in the ELISA for the experiments including the double and single mutants, respectively. Combinations with the double mutants IgG1-2F8-IL and –TL result in bispecific EGFR/CD20 binding similar as the triple mutant IgG1-ITL. Combinations with the IgG1-2F8-IT do not result in a bispecific product. Combinations with the single mutant IgG1-2F8-F405L result in bispecific EGFR/CD20 binding.

Figure 26: 2-MEA-induced Fab-arm exchange between IgG1-2F8-ITL and IgG1-7D8-K409X mutants. The generation of bispecific antibodies after 2-MEA-induced *in vitro* Fab-arm exchange between IgG1-2F8-ITL and the indicated IgG1-7D8-K409X mutants was determined by an ELISA. (A) A concentration series (total antibody) of 0-20 µg/mL was analyzed. The positive control is a purified batch of bispecific antibody, derived from IgG1-2F8-ITL x IgG4-7D8-CPPC. (B) The exchange is presented as bispecific binding at 20 µg/mL relative to the positive control (black bar). Dark grey bars represents the bispecific binding between the IgG4 control (IgG4-7D8 x IgG4-2F8), the negative control (IgG1-2F8 x IgG1-7D8-K409R) and between IgG1-2F8-ITL and IgG4-7D8-CPPC. Light grey bars represent results from simultaneously performed Fab-arm-exchange reactions between the indicated IgG1-7D8-K409X mutants and IgG1-2F8-ITL.

Figure 27: 2-MEA-induced Fab-arm-exchange between IgG1-2F8-F405X mutants and IgG1-7D8-K409R. The generation of bispecific antibodies after 2-MEA-induced *in vitro* Fab-arm-exchange between the indicated IgG1-2F8-F405X mutants and IgG1-7D8-K409R was determined by an ELISA. (A) A concentration series (total antibody) of 0-20 µg/mL was analyzed in the ELISA. The positive control is a purified batch of bispecific antibody, derived from IgG1-2F8-F405L x IgG1-7D8-K409R. (B) The exchange is presented as bispecific binding at 20 µg/mL antibody concentration relative to the positive control (black bar). Dark grey bars represents the bispecific binding between the IgG4 control (IgG4-7D8 x IgG4-2F8) and the negative control (IgG1-2F8 x IgG1-7D8-K409R). Light grey bars represent results from simultaneously performed Fab-arm-exchange reactions between the indicatedIgG1-2F8-F405X mutants and IgG1-7D8-K409R or controls.

Figure 28: 2-MEA-induced Fab-arm-exchange between IgG1-2F8-Y407X mutants and IgG1-7D8-K409R. The generation of bispecific antibodies after 2-MEA-induced *in vitro* Fab-

arm-exchange between the indicated IgG1-2F8-Y407X mutants and IgG1-7D8-K409R was determined by an ELISA. **(A)** A concentration series (total antibody) of 0-20 µg/mL was analyzed in the ELISA. The positive control is a purified batch of bispecific antibody, derived from IgG1-2F8-F405L x IgG1-7D8-K409R. **(B)** The exchange is presented as bispecific binding at 20 µg/mL antibody concentration relative to the positive control (black bar). Dark grey bars represents the bispecific binding between the IgG4 control (IgG4-7D8 x IgG4-2F8) and the negative control (IgG1-2F8 x IgG1-7D8-K409R). Light grey bars represent results from simultaneously performed Fab-arm-exchange reactions between the indicated IgG1-2F8-Y407X mutants and IgG1-7D8-K409R or controls.

Figure 29: Generation of bispecific antibodies after 2-MEA-induced *in vitro* Fab-arm exchange between the indicated IgG1-2F8-L368X mutants and IgG1-7D8-K409R was determined by an ELISA using a concentration series (total antibody) of 0-20 µg/mL (A). The positive control is a purified batch of bispecific antibody, derived from IgG1-2F8-F405L x IgG1-7D8-K409R. (B) Bispecific binding at 20 µg/mL relative to the positive control (black bar). Dark grey bars represents the bispecific binding between the IgG4 control (IgG4-7D8 x IgG4-2F8) and the negative control (IgG1-2F8 x IgG1-7D8-K409R). Light grey bars represent results from simultaneously performed Fab-arm-exchange reactions between the indicated IgG1-2F8-L368X mutants and IgG1-7D8-K409R.

Figure 30: Generation of bispecific antibodies after 2-MEA-induced *in vitro* Fab-arm exchange between the indicated IgG1-2F8-K370X mutants and IgG1-7D8-K409R was determined by an ELISA using a concentration series (total antibody) of 0-20 µg/mL (A). The positive control is a purified batch of bispecific antibody, derived from IgG1-2F8-F405L x IgG1-7D8-K409R. (B) Bispecific binding at 20 µg/mL relative to the positive control (black bar). Dark grey bars represents the bispecific binding between the IgG4 control (IgG4-7D8 x IgG4-2F8) and the negative control (IgG1-2F8 x IgG1-7D8-K409R). Light grey bars represent results from simultaneously performed Fab-arm-exchange reactions between the indicated IgG1-2F8-D370X mutants and IgG1-7D8-K409R.

Figure 31: Generation of bispecific antibodies after 2-MEA-induced *in vitro* Fab-arm exchange between the indicated IgG1-2F8-D399X mutants and IgG1-7D8-K409R was determined by an ELISA using a concentration series (total antibody) of 0-20 µg/mL (A). (B) Bispecific binding at 20 µg/mL antibody concentration relative to the positive control (black bar). Dark grey bars represents the bispecific binding between the IgG4 control (IgG4-7D8 x IgG4-2F8) and the negative control (IgG1-2F8 x IgG1-7D8-K409R). Light grey bars represent results from simultaneously performed Fab-arm-exchange reactions between the indicated IgG1-2F8-D399X mutants and IgG1-7D8-K409R.

Figure 32: Generation of bispecific antibodies after 2-MEA-induced *in vitro* Fab-arm exchange between the indicated IgG1-2F8-T366X mutants and IgG1-7D8-K409R was determined by an ELISA using a concentration series (total antibody) of 0-20 µg/mL (A). (B) The bispecific binding at 20 µg/mL antibody concentration relative to the positive control (black bar). Dark grey bars represents the bispecific binding between the IgG4 control (IgG4-7D8 x IgG4-2F8) and the negative control (IgG1-2F8 x IgG1-7D8-K409R). Light grey bars represent results from simultaneously performed Fab-arm-exchange reactions between the indicated IgG1-2F8-T366X mutants and IgG1-7D8-K409R.

Figure 33: Evaluation of the *in vivo* efficacy of HER2 x CD3 bispecific mAb in a subcutaneous xenograft model with an HER2 expressing tumor cell line and human PBMCs. In **(A)**, tumor development (mean & SEM) in mice with NCI-N87 s.c. xenografts and s.c. human PBMCs treated with bispecific HER2 x CD3 antibodies is shown. Different dosing schedules were being compared, and 0.05 mg/kg and 0.5 mg/kg appeared to be effective. In **(B)**, the percentage mice with tumor sizes smaller then 500 mm^3 is shown in a Kaplan-Meier plot.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

The term "HER2" (also known as ErbB-2, NEU, HER-2, and CD340), when used herein, refers to human epidermal growth factor receptor 2 (SwissProt P04626) and includes any variants, isoforms and species homologs of HER2 which are naturally expressed by cells, including tumor cells, or are expressed on cells transfected with the HER2 gene or cDNA. Species homologs include rhesus monkey HER2 (macaca mulatta; Genbank accession No. GI:109114897).

The term "CD3" refers to the human CD3 protein complex, which is composed of six distinct chains (a CD3γ chain (SwissProt P09693), a CD3δ chain (SwissProt P04234), two CD3ε chains (SwissProt P07766), and one CD3 zeta chain homodimer (SwissProt P20963) (ε γ: ε δ:ζζ), and which is associated with the T cell receptor α and β chain. The term includes any CD3 variants, isoforms and species homologs which are naturally expressed by cells, including T cells, or are expressed on cells transfected with genes or cDNA encoding the aforementioned chains.

The term "immunoglobulin" refers to a class of structurally related glycoproteins consisting of two pairs of polypeptide chains, one pair of light (L) low molecular weight chains and one pair of heavy (H) chains, all four inter-connected by disulfide bonds. The structure of immunoglobulins has been well characterized. See for instance Fundamental Immunology Ch. 7 (Paul, W., ed., 2nd ed. Raven Press, N.Y. (1989)). Briefly, each heavy

chain typically is comprised of a heavy chain variable region (abbreviated herein as V_H or VH) and a heavy chain constant region. The heavy chain constant region typically is comprised of three domains, C_H1, C_H2, and C_H3. Each light chain typically is comprised of a light chain variable region (abbreviated herein as V_L or VL) and a light chain constant region. The light chain constant region typically is comprised of one domain, C_L. The V_H and V_L regions may be further subdivided into regions of hypervariability (or hypervariable regions which may be hypervariable in sequence and/or form of structurally defined loops), also termed complementarity determining regions (CDRs), interspersed with regions that are more conserved, termed framework regions (FRs). Each V_H and V_L is typically composed of three CDRs and four FRs, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4 (see also Chothia and Lesk J. Mol. Biol. 196, 901-917 (1987)). Unless otherwise stated or contradicted by context, CDR sequences herein are identified according to IMGT rules (Brochet X., Nucl Acids Res. 2008;36:W503-508 and Lefranc MP., Nucleic Acids Research 1999;27:209-212; see also internet http address imgt.cines.fr/IMGT_vquest/vquest?livret=0&Option=humanIg. However, the numbering of amino acid residues in an antibody sequence can also be performed by the method described in Kabat *et al.*, Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991) (phrases such as "variable domain residue numbering as in Kabat", "Kabat position" or "according to Kabat" herein refer to this numbering system). Particularly, for numbering of amino acids in the constant region, the EU index numbering system (Kabat *et al*, supra), can be used. The Kabat numbering of residues may be determined for a given antibody as described in Kabat *et al.,* supra.

In the present invention reference to amino acid positions is, unless contradicted by the context, according to the EU-index as described in Kabat et al., Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991).

The term "antibody" (Ab) in the context of the present invention refers to an immunoglobulin molecule, a fragment of an immunoglobulin molecule, or a derivative of either thereof, which has the ability to specifically bind to an antigen under typical physiological conditions with a half life of significant periods of time, such as at least about 30 minutes, at least about 45 minutes, at least about one hour, at least about two hours, at least about four hours, at least about 8 hours, at least about 12 hours, about 24 hours or more, about 48 hours or more, about 3, 4, 5, 6, 7 or more days, etc., or any other relevant functionally-defined period (such as a time sufficient to induce, promote, enhance, and/or modulate a physiological response associated with antibody binding to the antigen and/or

time sufficient for the antibody to recruit an effector activity). The variable regions of the heavy and light chains of the immunoglobulin molecule contain a binding domain that interacts with an antigen. The constant regions of the antibodies (Abs) may mediate the binding of the immunoglobulin to host tissues or factors, including various cells of the immune system (such as effector cells) and components of the complement system such as C1q, the first component in the classical pathway of complement activation. A HER2 antibody may also be a multispecific antibody, such as a bispecific antibody, diabody, or similar molecule (see for instance PNAS USA 90(14), 6444-8 (1993) for a description of diabodies). Indeed, bispecific antibodies, diabodies, and the like, provided by the present invention may bind any suitable target in addition to a portion of HER2. As indicated above, the term antibody herein, unless otherwise stated or clearly contradicted by context, includes fragments of an antibody that are antigen-binding fragments, *i.e.*, retain the ability to specifically bind to the antigen. It has been shown that the antigen-binding function of an antibody may be performed by fragments of a full-length antibody. Examples of antigen-binding fragments encompassed within the term "antibody" include (i) a Fab' or Fab fragment, a monovalent fragment consisting of the V_L, V_H, C_L and C_H1 domains, or a monovalent antibody as described in WO2007059782 (Genmab); (ii) $F(ab')_2$ fragments, bivalent fragments comprising two Fab fragments linked by a disulfide bridge at the hinge region; (iii) a Fd fragment consisting essentially of the V_H and C_H1 domains; (iv) a Fv fragment consisting essentially of the V_L and V_H domains of a single arm of an antibody, (v) a dAb fragment (Ward *et al.*, Nature 341, 544-546 (1989)), which consists essentially of a V_H domain and also called domain antibodies (Holt *et al*; Trends Biotechnol. 2003 Nov;21(11):484-90); (vi) camelid or nanobodies (Revets *et al*; Expert Opin Biol Ther. 2005 Jan;5(1):111-24) and (vii) an isolated complementarity determining region (CDR). Furthermore, although the two domains of the Fv fragment, V_L and V_H, are coded for by separate genes, they may be joined, using recombinant methods, by a synthetic linker that enables them to be made as a single protein chain in which the V_L and V_H regions pair to form monovalent molecules (known as single chain antibodies or single chain Fv (scFv), see for instance Bird *et al.*, Science 242, 423-426 (1988) and Huston *et al.*, PNAS USA 85, 5879-5883 (1988)). Such single chain antibodies are encompassed within the term antibody unless otherwise noted or clearly indicated by context. Although such fragments are generally included within the meaning of antibody, they collectively and each independently are unique features of the present invention, exhibiting different biological properties and utility. These and other useful antibody fragments in the context of the present invention, as well as bispecific formats of such fragments, are discussed further herein. It also should be understood that the term antibody, unless specified otherwise, also includes polyclonal

antibodies, monoclonal antibodies (mAbs), antibody-like polypeptides, such as chimeric antibodies and humanized antibodies, and antibody fragments retaining the ability to specifically bind to the antigen (antigen-binding fragments) provided by any known technique, such as enzymatic cleavage, peptide synthesis, and recombinant techniques. An antibody as generated can possess any isotype.

The term "bispecific antibody" is in the context of the present invention to be understood as an antibody having two different antigen-binding regions defined by different antibody sequences. This can be understood as different target binding but includes as well binding to different epitopes in one target.

The term "bispecific antibody" is in the context of the present invention to be understood as an antibody with two different antigen-binding regions (based on sequence information). This can mean different target binding but includes as well binding to different epitopes in one target.

When used herein, unless contradicted by context, the term "Fab-arm" or "arm" refers to one heavy chain-light chain pair.

When used herein, unless contradicted by context, the term "Fc region" refers to an antibody region comprising at least a hinge region, a CH2 domain, and a CH3 domain.

As used herein, "isotype" refers to the immunoglobulin class (for instance IgG1, IgG2, IgG3, IgG4, IgD, IgA, IgE, or IgM) that is encoded by heavy chain constant region genes.

The term "monovalent antibody" means in the context of the present invention that an antibody molecule is capable of binding a single molecule of the antigen, and thus is not able of antigen crosslinking.

An "antibody deficient in effector function" or an "effector-function-deficient antibody" refers to an antibody which has a significantly reduced or no ability to activate one or more effector mechanisms, such as complement activation or Fc receptor binding. Thus, effector-function deficient antibodies have significantly reduced or no ability to mediate antibody-dependent cell-mediated cytotoxicity (ADCC) and/or complement-dependent cytotoxicity (CDC). An example of such an antibody is IgG4. Another example is the introduction of mutations in Fc-region which can strongly reduce the interaction with complement proteins and Fc-receptors. See, for example, Bolt S *et al*., Eur J Immunol 1993, 23:403-411; Oganesyan, Acta Crys. 2008, D64, 700–704; and Shields *et al*., JBC 2001, 276: 6591–6604.

A "HER2 antibody" or "anti-HER2 antibody" is an antibody as described above, which binds specifically to the antigen HER2.

A "HER2xCD3 antibody" or "anti-HER2xCD3 antibody" is a multispecific antibody, optionally a bispecific antibody, which comprises two different antigen-binding regions, one of which binds specifically to the antigen HER2 and one of which binds specifically to CD3.

The term "human antibody", as used herein, is intended to include antibodies having variable and constant regions derived from human germline immunoglobulin sequences. The human antibodies of the invention may include amino acid residues not encoded by human germline immunoglobulin sequences (*e.g.*, mutations introduced by random or site-specific mutagenesis *in vitro* or by somatic mutation *in vivo*). However, the term "human antibody", as used herein, is not intended to include antibodies in which CDR sequences derived from the germline of another mammalian species, such as a mouse, have been grafted onto human framework sequences.

As used herein, a human antibody is "derived from" a particular germline sequence if the antibody is obtained from a system using human immunoglobulin sequences, for instance by immunizing a transgenic mouse carrying human immunoglobulin genes or by screening a human immunoglobulin gene library, and wherein the selected human antibody is at least 90%, such as at least 95%, for instance at least 96%, such as at least 97%, for instance at least 98%, or such as at least 99% identical in amino acid sequence to the amino acid sequence encoded by the germline immunoglobulin gene. Typically, outside the heavy chain CDR3, a human antibody derived from a particular human germline sequence will display no more than 20 amino acid differences, *e.g.* no more than 10 amino acid differences, such as no more than 9, 8, 7, 6 or 5, for instance no more than 4, 3, 2, or 1 amino acid difference from the amino acid sequence encoded by the germline immunoglobulin gene.

When used herein, the term "heavy chain antibody" or "heavy-chain antibody" refers to an antibody which consists only of two heavy chains and lacks the two light chains usually found in antibodies. Heavy chain antibodies, which naturally occur in *e.g.* camelids, can bind antigens despite their lack of VL domains.

In a preferred embodiment, the antibody of the invention is isolated. An "isolated antibody," as used herein, is intended to refer to an antibody which is substantially free of other antibodies having different antigenic specificities (for instance an isolated antibody that specifically binds to HER2 is substantially free of antibodies that specifically bind antigens other than HER2). An isolated antibody that specifically binds to an epitope, isoform or variant of HER2 may, however, have cross-reactivity to other related antigens, for instance from other species (such as HER2 species homologs). Moreover, an isolated antibody may be substantially free of other cellular material and/or chemicals. In one

embodiment of the present invention, two or more "isolated" monoclonal antibodies having different antigen-binding specificities are combined in a well-defined composition.

When used herein in the context of two or more antibodies, the term "competes with" or "cross-competes with" indicates that the two or more antibodies compete for binding to HER2, *e.g.* compete for HER2 binding in the assay described in Example 14. An antibody "blocks" or "cross-blocks" one or more other antibodies from binding to HER2 if the antibody competes with the one or more other antibodies 25% or more, with 25%-74% representing "partial block" and 75%-100% representing "full block", preferably as determined using the assay of Example 14. For some pairs of antibodies, competition or blocking in the assay of the Examples is only observed when one antibody is coated on the plate and the other is used to compete, and not vice versa. Unless otherwise defined or negated by context, the terms "competes with", "cross-competes with", "blocks" or "cross-blocks" when used herein is also intended to cover such pairs of antibodies.

The term "epitope" means a protein determinant capable of specific binding to an antibody. Epitopes usually consist of surface groupings of molecules such as amino acids or sugar side chains and usually have specific three dimensional structural characteristics, as well as specific charge characteristics. Conformational and nonconformational epitopes are distinguished in that the binding to the former but not the latter is lost in the presence of denaturing solvents. The epitope may comprise amino acid residues directly involved in the binding and other amino acid residues, which are not directly involved in the binding, such as amino acid residues which are effectively blocked or covered by the specifically antigen binding peptide (in other words, the amino acid residue is within the footprint of the specifically antigen binding peptide).

The term "monoclonal antibody" as used herein refers to a preparation of antibody molecules of single molecular composition. A monoclonal antibody composition displays a single binding specificity and affinity for a particular epitope. Accordingly, the term "human monoclonal antibody" refers to antibodies displaying a single binding specificity which have variable and constant regions derived from human germline immunoglobulin sequences. The human monoclonal antibodies may be generated by a hybridoma which includes a B cell obtained from a transgenic or transchromosomal nonhuman animal, such as a transgenic mouse, having a genome comprising a human heavy chain transgene and a light chain transgene, fused to an immortalized cell.

As used herein, the term "binding" in the context of the binding of an antibody to a predetermined antigen or epitope typically is a binding with an affinity corresponding to a K_D of about 10^{-7} M or less, such as about 10^{-8} M or less, such as about 10^{-9} M or less, about 10^{-10} M or less, or about 10^{-11} M or even less when determined by for instance surface

plasmon resonance (SPR) technology in a BIAcore 3000 instrument using the antigen as the ligand and the antibody as the analyte, and binds to the predetermined antigen with an affinity corresponding to a K_D that is at least ten-fold lower, such as at least 100 fold lower, for instance at least 1,000 fold lower, such as at least 10,000 fold lower, for instance at least 100,000 fold lower than its affinity for binding to a non-specific antigen (*e.g.*, BSA, casein) other than the predetermined antigen or a closely-related antigen. The amount with which the affinity is lower is dependent on the K_D of the antibody, so that when the K_D of the antibody is very low (that is, the antibody is highly specific), then the amount with which the affinity for the antigen is lower than the affinity for a non-specific antigen may be at least 10,000 fold.

The term "k_d" (sec^{-1}), as used herein, refers to the dissociation rate constant of a particular antibody-antigen interaction. Said value is also referred to as the k_{off} value.

The term "k_a" ($M^{-1} \times sec^{-1}$), as used herein, refers to the association rate constant of a particular antibody-antigen interaction.

The term "K_D" (M), as used herein, refers to the dissociation equilibrium constant of a particular antibody-antigen interaction.

The term "K_A" (M^{-1}), as used herein, refers to the association equilibrium constant of a particular antibody-antigen interaction and is obtained by dividing the k_a by the k_d.

When used herein the term "heterodimeric interaction between the first and second CH3 regions" refers to the interaction between the first CH3 region and the second CH3 region in a first-CH3/second-CH3 heterodimeric protein.

When used herein the term "homodimeric interactions of the first and second CH3 regions" refers to the interaction between a first CH3 region and another first CH3 region in a first-CH3/first-CH3 homodimeric protein and the interaction between a second CH3 region and another second CH3 region in a second-CH3/second-CH3 homodimeric protein.

The term "reducing conditions" or "reducing environment" refers to a condition or an environment in which a substrate, here a cysteine residue in the hinge region of an antibody, is more likely to become reduced than oxidized.

As used herein, the term "inhibits proliferation" (*e.g.* referring to cells, such as tumor cells) is intended to include any substantial decrease in the cell proliferation when contacted with a HER2 antibody as compared to the proliferation of the same cells not in contact with a HER2 antibody, *e.g.*, the inhibition of proliferation of a cell culture by at least about 10%, at least about 20% or at least about 30%, or at least as much as a reference antibody such as trastuzumab, *e.g.*, as determined by an assay in the Examples, *e.g.* Example 16.

As used herein, the term "promotes proliferation" (*e.g.* referring to cells, such as tumor cells) is intended to include any substantial increase in the cell proliferation when

contacted with a HER2 antibody as compared to the proliferation of the same cells not in contact with a HER2 antibody, *e.g.*, the promotion of proliferation of a cell culture by at least about 10%, at least about 20% or at least about 30%, or at least as much as a reference antibody as F5, *e.g.*, as determined by an assay in the Examples.

As used herein, the term "internalization", when used in the context of a HER2 antibody includes any mechanism by which the antibody is internalized into a HER2-expressing cell from the cell-surface and/or from surrounding medium, *e.g.*, via endocytosis. The internalization of an antibody can be evaluated using a direct assay measuring the amount of internalized antibody (such as, *e.g.*, the fab-CypHer5E assay described in Example 19), or an indirect assay where the effect of an internalized antibody-toxin conjugate is measured (such as, *e.g.*, the anti-kappa-ETA´assay of Example 18).

The present invention also provides antibodies comprising functional variants of the V_L region, V_H region, or one or more CDRs of the antibodies of the examples. A functional variant of a V_L, V_H, or CDR used in the context of a HER2 antibody still allows the antibody to retain at least a substantial proportion (at least about 50%, 60%, 70%, 80%, 90%, 95% or more) of the affinity/avidity and/or the specificity/selectivity of the parent antibody and in some cases such a HER2 antibody may be associated with greater affinity, selectivity and/or specificity than the parent antibody.

Such functional variants typically retain significant sequence identity to the parent antibody. The percent identity between two sequences is a function of the number of identical positions shared by the sequences (*i.e.*, % homology = # of identical positions/total # of positions x 100), taking into account the number of gaps, and the length of each gap, which need to be introduced for optimal alignment of the two sequences. The percent identity between two nucleotide or amino acid sequences may *e.g.* be determined using the algorithm of E. Meyers and W. Miller, Comput. Appl. Biosci 4, 11-17 (1988) which has been incorporated into the ALIGN program (version 2.0), using a PAM120 weight residue table, a gap length penalty of 12 and a gap penalty of 4. In addition, the percent identity between two amino acid sequences may be determined using the Needleman and Wunsch, J. Mol. Biol. 48, 444-453 (1970) algorithm.

Exemplary variants include those which differ from a parent antibody VH and/or VL sequence shown in Figures 1 and 2 at one or more "variant" amino acid positions, denoted "X" in the corresponding consensus sequence. Preferred variants are those in which the new amino acid is selected from those at the corresponding position in one of the aligned sequences in Figure 1 or 2 (for details on CDR sequence variants, see Table 4). Alternatively or additionally, the sequence of VH, VL or CDR variants may differ from the sequence of the VH, VL or CDR of the parent antibody sequences mainly by conservative

substitutions; for instance at least 10, such as at least 9, 8, 7, 6, 5, 4, 3, 2 or 1 of the substitutions in the variant are conservative amino acid residue replacements.

In the context of the present invention, conservative substitutions may be defined by substitutions within the classes of amino acids reflected in the following table:

Amino acid residue classes for conservative substitutions

Acidic Residues	Asp (D) and Glu (E)
Basic Residues	Lys (K), Arg (R), and His (H)
Hydrophilic Uncharged Residues	Ser (S), Thr (T), Asn (N), and Gln (Q)
Aliphatic Uncharged Residues	Gly (G), Ala (A), Val (V), Leu (L), and Ile (I)
Non-polar Uncharged Residues	Cys (C), Met (M), and Pro (P)
Aromatic Residues	Phe (F), Tyr (Y), and Trp (W)

In the context of the present invention the following notations are, unless otherwise indicated, used to describe a mutation; i) substitution of an amino acid in a given position is written as *e.g.* K405R which means a substitution of a Lysine in position 405 with an Arginine; and ii) for specific variants the specific three or one letter codes are used, including the codes Xaa and X to indicate any amino acid residue. Thus, the substitution of Arginine for Lysine in position 405 is designated as: K405R, or the substitution of any amino acid residue for Lysine in position 405 is designated as K405X. In case of deletion of Lysine in position 405 it is indicated by K405*.

The term "recombinant host cell" (or simply "host cell"), as used herein, is intended to refer to a cell into which an expression vector has been introduced, *e.g.* an expression vector encoding an antibody of the invention. Recombinant host cells include, for example, transfectomas, such as CHO cells, HEK293 cells, NS/0 cells, and lymphocytic cells.

The term “transgenic non-human animal” refers to a non-human animal having a genome comprising one or more human heavy and/or light chain transgenes or transchromosomes (either integrated or non-integrated into the animal’s natural genomic DNA) and which is capable of expressing fully human antibodies. For example, a transgenic mouse can have a human light chain transgene and either a human heavy chain transgene or human heavy chain transchromosome, such that the mouse produces human HER2 antibodies when immunized with HER2 antigen and/or cells expressing HER2. The human heavy chain transgene may be integrated into the chromosomal DNA of the mouse, as is the case for transgenic mice, for instance HuMAb® mice, such as HCo7, HCo12, or HCo17

mice, or the human heavy chain transgene may be maintained extrachromosomally, as is the case for transchromosomal KM mice as described in WO02/43478. Similar mice, having a larger human Ab gene repertoire, include HCo7 and HCo20 (see *e.g.* WO2009097006). Such transgenic and transchromosomal mice (collectively referred to herein as "transgenic mice") are capable of producing multiple isotypes of human monoclonal antibodies to a given antigen (such as IgG, IgA, IgM, IgD and/or IgE) by undergoing V-D-J recombination and isotype switching. Transgenic, nonhuman animal can also be used for production of antibodies against a specific antigen by introducing genes encoding such specific antibody, for example by operatively linking the genes to a gene which is expressed in the milk of the animal.

"Treatment" refers to the administration of an effective amount of a therapeutically active compound of the present invention with the purpose of easing, ameliorating, arresting or eradicating (curing) symptoms or disease states.

An "effective amount" or "therapeutically effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve a desired therapeutic result. A therapeutically effective amount of a HER2 antibody may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of the HER2 antibody to elicit a desired response in the individual. A therapeutically effective amount is also one in which any toxic or detrimental effects of the antibody or antibody portion are outweighed by the therapeutically beneficial effects.

An "anti-idiotypic" antibody is an antibody which recognizes unique determinants generally associated with the antigen-binding site of an antibody.

Further aspects and embodiments of the invention

As described above, the invention relates to a bispecific antibody comprising two different antigen-binding regions, one which has a binding specificity for HER2 and one which has a binding specificity for CD3.

In one aspect, the invention relates to a bispecific molecule comprising a first antigen binding region from a HER2 antibody described herein and a second antigen binding region from a CD3 antibody described herein.

In one embodiment, the HER2 antigen-binding region is from a HER2 antibody which cross-blocks or binds to the same epitope as a reference antibody from cross-block group 1, described herein. In a specific embodiment, the bispecific antibody comprises an antigen-binding region from an antibody of cross-block group 1, as described herein.

In one embodiment, the HER2 antigen-binding region is from a HER2 antibody which cross-blocks or binds to the same epitope as a reference antibody from cross-block group 2,

described herein. In a specific embodiment, the bispecific antibody comprises an antigen-binding region from an antibody of cross-block group 2, as described herein.

In one embodiment, the HER2 antigen-binding region is from a HER2 antibody which cross-blocks or binds to the same epitope as a reference antibody from cross-block group 3, described herein. In a specific embodiment, the bispecific antibody comprises an antigen-binding region from an antibody of cross-block group 3, as described herein.

In one embodiment, the HER2 antigen-binding region is from a HER2 antibody which cross-blocks or binds to the same epitope as a reference antibody from cross-block group 4, described herein. In a specific embodiment, the bispecific antibody comprises an antigen-binding region from an antibody of cross-block group 4, as described herein.

In a particular embodiment, the bispecific antibody of any one of the preceding embodiments comprises an antigen-binding region which cross-blocks or binds to the same epitope as a reference CD3 antibody comprising the VH and VL regions of CD3 antibody YTH12.5, HUM291 (also known as visilizumab), huOKT3-C114S-gLC (related to teplizumab), all known in the art, or comprising the VH and VL regions of CD3 antibody huCLB-T3/4, which represents a humanized variant of CLB-T3/4. Further details on these CD3 antibodies are provided in Example 21. In another particular embodiment, the bispecific antibody of any one of the preceding embodiments comprises an antigen-binding region from an antibody selected from YTH12.5, HUM291 huOKT3-C114S-gLC and huCLB-T3/4.

Thus, the bispecifc antibody of the present invention may comprise a first antigen-binding region and a second antigen-binding region, which first antigen-binding region binds an epitope on human epidermal growth factor receptor 2 (HER2) and which second antigen-binding region binds an epitope on human CD3.

Antibodies and antigen-binding regions

The bispecific antibody of the present invention comprises two different antigen-binding regions which bind HER2 and CD3, respectively. Furthermore, as described below one method of producing a bispecific antibody of the present invention is based on incubating a first HER2 antibody and a second CD3 antibody under reducing conditions.

Antigen-binding regions binding to HER2 antibodies of the present invention may belong to any of cross-block groups 1, 2, 3 and 4. In the following examples of such antigen-binding regions bind to HER2 belonging to cross-block groups 1, 2, 3, and 4 are given, and reference to "antigen-binding region" in this context is intended to include both an antigen-binding region of a bispecific antibody of the present invention, *e.g.* a first antigen-binding region, and first HER2 antibody.

In a further or alternative embodiment of the present invention, the bispecific antibody comprises an antigen-binding region of one or more of the human antibodies of cross-blocks 1, 2, 3, or 4, which blocks the binding to HER2.

In a further or alternative embodiment of the present invention, the bispecific antibody comprises an antigen-binding region which blocks the binding to the same epitope on soluble HER2 as one or more of the human antibodies of cross-blocks 1, 2, 3, or 4.

In a further or alternative embodiment of the present invention, the bispecific antibody comprises an antigen-binding region which binds to the same epitope on HER2 as one or more of the human antibodies of cross-blocks 1, 2, 3, or 4.

Thus, the bispecific antibody of the present invention may comprise a first antigen-binding region and a second antigen-binding region, which first and second antigen-binding regions bind different epitopes, and wherein the first antigen-binding region binds an epitope on human epidermal growth factor receptor 2 (HER2).

The first antigen-binding region of the bispecific antibody of the present invention may be an antigen-binding region from any of cross-block groups 1, 2, 3, and 4.

Cross-block group 1

In one aspect, the bispecific antibody of the invention comprises an antigen-binding region which blocks the binding to HER2, *e.g.* soluble HER2, or binds to the same epitope on HER2 as one or more of the human antibodies of cross-block group 1 described herein.

In one embodiment, the antigen-binding region cross-blocks the binding to soluble HER2 of trastuzumab, when determined as described in Example 14.

In one embodiment, the antigen-binding region blocks the binding to HER2, *e.g.* soluble HER2, or binds to the same epitope as a reference antibody comprising a VH region comprising the sequence of SEQ ID NO:1 and a VL region comprising the sequence of SEQ ID NO:5 (**169**).

In one embodiment, the antigen-binding region blocks the binding to HER2, *e.g.* soluble HER2, or binds to the same epitope as a reference antibody comprising a VH region comprising the sequence of SEQ ID NO:8 and a VL region comprising the sequence of SEQ ID NO:12 (**050**).

In one embodiment, the antigen-binding region blocks the binding to HER2, *e.g.* soluble HER2, or binds to the same epitope as a reference antibody comprising a VH region comprising the sequence of SEQ ID NO:15 and a VL region comprising the sequence of SEQ ID NO:19 (**084**).

In one embodiment, the antigen-binding region blocks the binding to HER2, *e.g.* soluble HER2, or binds to the same epitope as a reference antibody comprising VH and VL regions selected from the group consisting of:

a) a VH region comprising the sequence of SEQ ID NO:77 and a VL region comprising the sequence of SEQ ID NO:78 (**049**);
b) a VH region comprising the sequence of SEQ ID NO:79 and a VL region comprising the sequence of SEQ ID NO:80 (**051**);
c) a VH region comprising the sequence of SEQ ID NO:81 and a VL region comprising the sequence of SEQ ID NO:82 (**055**);
d) a VH region comprising the sequence of SEQ ID NO:83 and a VL region comprising the sequence of SEQ ID NO:84 (**123**);
e) a VH region comprising the sequence of SEQ ID NO:85 and a VL region comprising the sequence of SEQ ID NO:86 (**161**); and
f) a VH region comprising the sequence of SEQ ID NO:87 and a VL region comprising the sequence of SEQ ID NO:88 (**124**).

In another additional or alternative aspect of the bispecific antibody of the invention, one antigen-binding region binds to HER2 and comprises a VH CDR3, VH region and/or VL region sequence similar or identical to such a sequence of an antibody described herein.

In one embodiment, the antigen-binding region comprises a VH CDR3 region having a sequence selected from the group consisting of

SEQ ID NO:11 (**050, 049, 051, 055**), optionally wherein the VH region is derived from the IgHV3-21-1 germline sequence;

SEQ ID No:130, such as the sequence of SEQ ID NO:18 (**084**), optionally wherein the VH region is derived from the IgHV1-69-04 germline sequence;

SEQ ID NO:133 (**169, 123, 161, 124**), such as the sequence of SEQ ID NO:4 (**169**), optionally wherein the VH region is derived from the IgHV1-18-1 germline sequence; or

In one embodiment, the antigen-binding region comprises a VH CDR3 region of one of antibodies 123, 161, or 124, as shown in Figure 1, optionally wherein the VH region is derived from an IgHV1-18-1 germline.

In one embodiment, the bispecific antibody or the first antigen-binding region comprises a VH region selected from the group consisting of

a) a VH region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:9, 127 and 11, such as the CDR1, CDR2 and CDR3 sequences of SEQ ID NOS: 9, 10 and 11 (**050**); optionally where the VH region is derived from an IgHV3-23-1 germline;
b) a VH region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:128, 129 and 130, such the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:16, 17 and 18, respectively (**084**), optionally where the VH region is derived from an IgHV1-69-04 germline; and
c) a VH region comprising the CDR1, CDR2, and CDR3 sequences of SEQ ID NOs:131, 132, and 133, such as the CDR1, CDR2, and CDR3 sequences of SEQ ID NOs: 2, 3 and 4 (**169**), respectively, optionally where the VH region is derived from an IgHV1-18-1 germline.

In one embodiment, the bispecific antibody or the first antigen-binding region comprises a VH region selected from the preceding embodiments (a) or (b) and a VL region comprising the CDR1, CDR2, and CDR3 sequences of SEQ ID NO:13, XAS (wherein X is A or V), and SEQ ID No:155, respectively, such as a CDR1 sequence selected from SEQ ID Nos: 13 or 20, a CDR2 which is AAS or VAS, and a CDR3 sequence selected from SEQ ID NOs:14 and 21 (**050**, **084**); respectively, optionally where the VL region is derived from an IgKV1-12-01 germline.

In one embodiment, the bispecific antibody or the first antigen-binding region comprises a VH region which is the preceding embodiment (c) and a VL region comprising the CDR1, CDR2, and CDR3 sequences of SEQ ID NO:6, DXS (wherein X=A or T), and SEQ ID NO:156 (**169**), respectively, optionally wherein the VL region is derived from IgKV3-11-01.

In one embodiment, the bispecific antibody or the first antigen-binding region comprises a VH region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:2, 3 and 4, respectively; and, optionally, a VL region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:6, DAS, and SEQ ID NO:7, respectively (**169**).

In one embodiment, the bispecific antibody or the first antigen-binding region comprises a VH region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:9, 10 and 11, respectively; and a VL region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:13, AAS, and SEQ ID NO:14, respectively (**050**).

In one embodiment, the bispecific antibody or the first antigen-binding region comprises a VH region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:16,

17 and 18, respectively; and a VL region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:20, VAS, and SEQ ID NO:21, respectively (**084**).

In separate embodiments, the bispecific antibody or the first antigen-binding region comprises:

a) a VH region comprising the sequence of SEQ ID NO:1 and, optionally, a VL region comprising the sequence of SEQ ID NO:5 (**169**);
b) a VH region comprising the sequence of SEQ ID NO:8 and, preferably, a VL region comprising the sequence of SEQ ID NO:12 (**050**);
c) a VH region comprising the sequence of SEQ ID NO:15 and, preferably, a VL region comprising the sequence of SEQ ID NO:19 (**084**);
d) a VH region comprising the sequence of SEQ ID NO:77 and, preferably, a VL region comprising the sequence of SEQ ID NO:78 (**049**);
e) a VH region comprising the sequence of SEQ ID NO:79 and, preferably, a VL region comprising the sequence of SEQ ID NO:80 (**051**);
f) a VH region comprising the sequence of SEQ ID NO:81 and, preferably, a VL region comprising the sequence of SEQ ID NO:82 (**055**);
g) a VH region comprising the sequence of SEQ ID NO:83 and, preferably, a VL region comprising the sequence of SEQ ID NO:84 (**123**);
h) a VH region comprising the sequence of SEQ ID NO:85 and, preferably, a VL region comprising the sequence of SEQ ID NO:86 (**161**);
i) a VH region comprising the sequence of SEQ ID NO:87 and, preferably, a VL region comprising the sequence of SEQ ID NO:88 (**124**); and/or
j) a variant of any of said antibodies, wherein said variant preferably has at most 1, 2 or 3 amino-acid modifications, more preferably amino-acid substitutions, such as conservative amino acid substitutions and substitutions where the new amino acid is one at the same position in an aligned sequence in Figures 1 or 2, particularly at positions indicated by "X" in the corresponding consensus sequence.

Cross-block group 2

In one aspect of the antibody of the invention, the bispecific antibody comprises an antigen-binding region which blocks the binding to HER2, *e.g.* soluble HER2, or binds to the same epitope on HER2 as one or more of the human antibodies of cross-block group 2 described herein.

In one embodiment, the antigen-binding region cross-blocks the binding to soluble HER2 of pertuzumab, when determined as described in Example 14.

In one embodiment, the antigen-binding region blocks the binding to HER2, *e.g.* soluble HER2, or binds to the same epitope as a reference antibody comprising a VH region comprising the sequence of SEQ ID NO:22 and a VL region comprising the sequence of SEQ ID NO:26 (**025**).

In one embodiment, the antigen-binding region blocks the binding to HER2, *e.g.* soluble HER2, or binds to the same epitope as a reference antibody comprising a VH region comprising the sequence of SEQ ID NO:29 and a VL region comprising the sequence of SEQ ID NO:32 (**091**).

In one embodiment, the antigen-binding region blocks the binding to HER2, *e.g.* soluble HER2, or binds to the same epitope as a reference antibody comprising a VH region comprising the sequence of SEQ ID NO:35 and a VL region comprising the sequence of SEQ ID NO:39 (**129**).

In one embodiment, the antigen-binding region blocks the binding to HER2, *e.g.* soluble HER2, or binds to the same epitope as a reference antibody comprising VH and VL regions selected from the group consisting of:

a) a VH region comprising the sequence of SEQ ID NO:89 and a VL region comprising the sequence of SEQ ID NO:90 (**001**);
b) a VH region comprising the sequence of SEQ ID NO:91 and a VL region comprising the sequence of SEQ ID NO:92 (**143**);
c) a VH region comprising the sequence of SEQ ID NO:93 and a VL region comprising the sequence of SEQ ID NO:94 (**019**);
d) a VH region comprising the sequence of SEQ ID NO:95 and a VL region comprising the sequence of SEQ ID NO:96 (**021**);
e) a VH region comprising the sequence of SEQ ID NO:97 and a VL region comprising the sequence of SEQ ID NO:98 (**027**);
f) a VH region comprising the sequence of SEQ ID NO:99 and a VL region comprising the sequence of SEQ ID NO:100 (**032**)
g) a VH region comprising the sequence of SEQ ID NO:101 and a VL region comprising the sequence of SEQ ID NO:102 (**035**);
h) a VH region comprising the sequence of SEQ ID NO:103 and a VL region comprising the sequence of SEQ ID NO:104 (**036**);
i) a VH region comprising the sequence of SEQ ID NO:105 and a VL region comprising the sequence of SEQ ID NO:106 (**054**); and
j) a VH region comprising the sequence of SEQ ID NO:107 and a VL region comprising the sequence of SEQ ID NO:108 (**094**).

In another additional or alternative aspect of the bispecific antibody of the invention, the bispecific antibody or the first antigen-binding region comprises a VH CDR3, VH region and/or VL region sequence similar or identical to a sequence of the novel antibodies described herein.

In one embodiment, the bispecific antibody or the first antigen-binding region comprises a VH CDR3 region having a sequence selected from the group consisting of SEQ ID NO:136, such as the sequence of SEQ ID NO:25 (**025**), optionally wherein the VH region is derived from the IgHV4-34-1 germline sequence;

SEQ ID NO:139, such as the sequence of SEQ ID NO:31 (**091**), optionally wherein the VH region is derived from the IgHV4-34-01 germline sequence; and

SEQ ID NO:142, such as the sequence of SEQ ID NO:38 (**129**), optionally wherein the VH region is derived from the IgHV3-30-01 germline sequence.

In one embodiment, the bispecific antibody or the first antigen-binding region comprises a VH CDR3 region of one of antibodies 001, 143, 019, 021, 027, 032, 035, 036, 054 or 094 as shown in Figure 1, optionally wherein the VH region is derived from an IgHV4-34-1 germline.

In one embodiment, the bispecific antibody or the first antigen-binding region comprises a VH region selected from the group consisting of

a) a VH region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:134, 135 and 136, such as the CDR1, CDR2 and CDR3 sequences of SEQ ID NOS: 23, 24 and 25 (**025**); optionally where the VH region is derived from an IgHV4-34-1 germline;
b) a VH region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:137, 138 and 139, such the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:30, 163, and 31, respectively (**091**), optionally where the VH region is derived from an IgHV4-34-01 germline; and
c) a VH region comprising the CDR1, CDR2, and CDR3 sequences of SEQ ID NOs:140, 141 and 142, such as the CDR1, CDR2, and CDR3 sequences of SEQ ID NOs: 36, 37 and 38 (**129**), respectively, optionally where the VH region is derived from an IgHV3-30-01 germline.

In one embodiment, the bispecific antibody or the first antigen-binding region comprises a VH region selected from the preceding embodiment (a) and a VL region comprising the CDR1, CDR2, and CDR3 sequences of SEQ ID NO:157, AAS, and SEQ ID No:164, respectively, such as the CDR1, CDR2, and CDR3 sequences of SEQ ID Nos:27, AAS, and

SEQ ID NO:28 (**025**); respectively, optionally where the VL region is derived from an IgKV1D-16-01 germline.

In one embodiment, the bispecific antibody or the first antigen-binding region comprises a VH region selected from the preceding embodiment (b) and a VL region comprising the CDR1, CDR2, and CDR3 sequences of SEQ ID NO:33, AX_1X_2 (wherein X_1 is A or T, preferably A; and X_2 is S or F, preferably S), and SEQ ID No:158, respectively, such as the CDR1, CDR2 and CDR3 sequences of SEQ ID Nos:33, AAS, and SEQ ID NO:34 (**091**); respectively, optionally where the VL region is derived from an IgKV1D-16-01 germline.

In one embodiment, the bispecific antibody or the first antigen-binding region comprises a VH region which is the preceding embodiment (c) and a VL region comprising the CDR1, CDR2, and CDR3 sequences of SEQ ID NO:40, DAS and SEQ ID NO:41 (**129**), respectively, optionally wherein the VL region is derived from IgKV3-11-01.

In one embodiment, the bispecific antibody or the first antigen-binding region comprises a VH region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:23, 24 and 25, respectively; and, optionally, a VL region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:27, AAS, and SEQ ID NO:28, respectively (**025**).

In one embodiment, the bispecific antibody comprises a VH region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:30, 163 and 31, respectively; and a VL region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:33, AAS, and SEQ ID NO:34, respectively (**091**).

In one embodiment, the bispecific antibody or the first antigen-binding region comprises a VH region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:36, 37 and 38, respectively; and a VL region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:40, DAS, and SEQ ID NO:41, respectively (**129**).

In separate embodiments, the bispecific antibody or the first antigen-binding region comprises:

a) a VH region comprising the sequence of SEQ ID NO:22 and, optionally, a VL region comprising the sequence of SEQ ID NO:26 (**025**);
b) a VH region comprising the sequence of SEQ ID NO:29 and, preferably, a VL region comprising the sequence of SEQ ID NO:32 (**091**);
c) a VH region comprising the sequence of SEQ ID NO:35 and, preferably, a VL region comprising the sequence of SEQ ID NO:39 (**129**);
d) a VH region comprising the sequence of SEQ ID NO:89 and, preferably, a VL region comprising the sequence of SEQ ID NO:90 (**001**);
e) a VH region comprising the sequence of SEQ ID NO:91 and, preferably, a VL region comprising the sequence of SEQ ID NO:92 (**143**);

f) a VH region comprising the sequence of SEQ ID NO:93 and, preferably, a VL region comprising the sequence of SEQ ID NO:94 (**019**);
g) a VH region comprising the sequence of SEQ ID NO:95 and, preferably, a VL region comprising the sequence of SEQ ID NO:96 (**021**);
h) a VH region comprising the sequence of SEQ ID NO:97 and, preferably, a VL region comprising the sequence of SEQ ID NO:98 (**027**);
i) a VH region comprising the sequence of SEQ ID NO:99 and, preferably, a VL region comprising the sequence of SEQ ID NO:100 (**032**);
j) a VH region comprising the sequence of SEQ ID NO:101 and, preferably, a VL region comprising the sequence of SEQ ID NO:102 (**035**);
k) a VH region comprising the sequence of SEQ ID NO:103 and, preferably, a VL region comprising the sequence of SEQ ID NO:104 (**036**);
l) a VH region comprising the sequence of SEQ ID NO:105 and, preferably, a VL region comprising the sequence of SEQ ID NO:106 (**054**);
m) a VH region comprising the sequence of SEQ ID NO:106 and, preferably, a VL region comprising the sequence of SEQ ID NO:108 (**094**); and/or
n) a variant of any of said antibodies, wherein said variant preferably has at most 1, 2 or 3 amino-acid modifications, more preferably amino-acid substitutions, such as conservative amino acid substitutions and substitutions where the new amino acid is one at the same position in an aligned sequence in Figures 1 or 2, particularly at positions indicated by "X" in the corresponding consensus sequence.

Cross-block group 3

In one aspect of the bispecific antibody of the invention, the bispecific antibody comprises an antigen-binding region which blocks the binding to HER2, *e.g.* soluble HER2, or binds to the same epitope on HER2 as one or more of the human antibodies of cross-block group 3 described herein.

In one embodiment, the antigen-binding region cross-blocks the binding to soluble HER2 of F5 and/or C1, when determined as described in Example 14.

In one embodiment, the antigen-binding region blocks the binding to HER2, *e.g.* soluble HER2, or binds to the same epitope as a reference antibody comprising a VH region comprising the sequence of SEQ ID NO:46 and a VL region comprising the sequence of SEQ ID NO:49 (**127**).

In one embodiment, the antigen-binding region blocks the binding to HER2, *e.g.* soluble HER2, or binds to the same epitope as a reference antibody comprising a VH region

comprising the sequence of SEQ ID NO:49 and a VL region comprising the sequence of SEQ ID NO:53 (**159**).

In one embodiment, the antigen-binding region blocks the binding to HER2, *e.g.* soluble HER2, or binds to the same epitope as a reference antibody comprising a VH region comprising the sequence of SEQ ID NO:56 and a VL region comprising the sequence of SEQ ID NO:60 (**098**).

In one embodiment, the antigen-binding region blocks the binding to HER2, *e.g.* soluble HER2, or binds to the same epitope as a reference antibody comprising a VH region comprising the sequence of SEQ ID NO:63 and a VL region comprising the sequence of SEQ ID NO:67 (**153**).

In one embodiment, the antigen-binding region blocks the binding to HER2, *e.g.* soluble HER2, or binds to the same epitope as a reference antibody comprising a VH region comprising the sequence of SEQ ID NO:70 and a VL region comprising the sequence of SEQ ID NO:74 (**132**).

In one embodiment, the antigen-binding region blocks the binding to HER2, *e.g.* soluble HER2, or binds to the same epitope as a reference antibody comprising VH and VL regions selected from the group consisting of:

a) a VH region comprising the sequence of SEQ ID NO:109 and a VL region comprising the sequence of SEQ ID NO:110 (**105**);
b) a VH region comprising the sequence of SEQ ID NO:111 and a VL region comprising the sequence of SEQ ID NO:112 (**100**);
c) a VH region comprising the sequence of SEQ ID NO:113 and a VL region comprising the sequence of SEQ ID NO:114 (**125**);
d) a VH region comprising the sequence of SEQ ID NO:115 and a VL region comprising the sequence of SEQ ID NO:116 (**162**);
e) a VH region comprising the sequence of SEQ ID NO:117 and a VL region comprising the sequence of SEQ ID NO:118 (**033**);
f) a VH region comprising the sequence of SEQ ID NO:119 and a VL region comprising the sequence of SEQ ID NO:120 (**160**)
g) a VH region comprising the sequence of SEQ ID NO:121 and a VL region comprising the sequence of SEQ ID NO:122 (**166**);
h) a VH region comprising the sequence of SEQ ID NO:123 and a VL region comprising the sequence of SEQ ID NO:124 (**152**); and
i) a VH region comprising the sequence of SEQ ID NO:125 and a VL region comprising the sequence of SEQ ID NO:126 (**167**).

In another additional or alternative aspect of the bispecific antibody of the invention, the bispecific antibody or the first antigen-binding region comprises a VH CDR3, VH region and/or VL region sequence similar or identical to a sequence of the novel antibodies described herein.

In one embodiment, the bispecific antibody or the first antigen-binding region comprises a VH CDR3 region having a sequence selected from the group consisting of SEQ ID NO:148, such as the sequence of SEQ ID NO:48 (**127**), optionally wherein the VH region is derived from the IgHV5-51-01 germline sequence;

SEQ ID NO:52 (**159**), optionally wherein the VH region is derived from the IgHV5-51-01 germline sequence;

SEQ ID NO:145, such as the sequence of SEQ ID NO:59 (**098**), optionally wherein the VH region is derived from the IgHV3-23-01 germline sequence;

SEQ ID NO:154, such as the sequence of SEQ ID NO:66 (**153**), optionally wherein the VH region is derived from the IgHV3-30-03-01 germline sequence; and

SEQ ID NO:151, such as the sequence of SEQ ID NO:73 (**132**), optionally wherein the VH region is derived from the IgHV1-18-01 germline sequence.

In one embodiment, the bispecific antibody or the first antigen-binding region comprises a VH CDR3 region of one of antibodies 105, 100, 125 or 162 as shown in Figure 1, optionally wherein the VH region is derived from an IgHV3-23-1 germline.

In one embodiment, the bispecific antibody or the first antigen-binding region comprises a VH CDR3 region of one of antibodies 033, 160, 166, 152 or 167 as shown in Figure 1, optionally wherein the VH region is derived from an IgHV3-30-3-01 germline.

In one embodiment, the bispecific antibody or the first antigen-binding region comprises a VH region selected from the group consisting of

a) a VH region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:146, 147 and 148, such as the CDR1, CDR2 and CDR3 sequences of SEQ ID NOS: 43, 44 and 45 (**127**); optionally where the VH region is derived from an IgHV5-51-01 germline;

b) a VH region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:149, 51 and 52, such as the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:50, 51 and 52, respectively (**159**), optionally where the VH region is derived from an IgHV5-51-01 germline;

c) a VH region comprising the CDR1, CDR2, and CDR3 sequences of SEQ ID NOs:143, 144 and 145, such as the CDR1, CDR2, and CDR3 sequences of SEQ ID NOs: 57, 58 and 59 (**098**), respectively, optionally where the VH region is derived from an IgHV3-23-01 germline;

d) a VH region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:152, 153 and 154, such as the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:64, 65 and 66, respectively (**153**), optionally where the VH region is derived from an IgHV3-30-03-01 germline; and

e) a VH region comprising the CDR1, CDR2, and CDR3 sequences of SEQ ID NOs:71, 150 and 151, such as the CDR1, CDR2, and CDR3 sequences of SEQ ID NOs: 71, 72 and 73 (**132**), respectively, optionally where the VH region is derived from an IgHV1-18-01 germline.

In one embodiment, the bispecific antibody or the first antigen-binding region comprises a VH region selected from the preceding embodiment (a) and a VL region comprising the CDR1, CDR2, and CDR3 sequences of SEQ ID NO:47, AAS and SEQ ID NO:48, respectively (**127**); respectively, optionally where the VL region is derived from an IgKV1D-8-01 germline.

In one embodiment, the bispecific antibody or the first antigen-binding region comprises a VH region selected from the preceding embodiment (b) and a VL region comprising the CDR1, CDR2, and CDR3 sequences of SEQ ID NO:54, AAS, and SEQ ID No:55 (**159**); respectively, optionally where the VL region is derived from an IgKV1D-16-01 germline.

In one embodiment, the bispecific antibody or the first antigen-binding region comprises a VH region which is the preceding embodiment (c) and a VL region comprising the CDR1, CDR2, and CDR3 sequences of SEQ ID NO:159, AAS and SEQ ID NO:160, respectively, such as the VL CDR1, CDR2 and CDR3 sequences of SEQ ID NOS: 61, AAS and SEQ ID NO:62 (**098**), optionally wherein the VL region is derived from IgKV1D-16-01.

In one embodiment, the bispecific antibody or the first antigen-binding region comprises a VH region which is the preceding embodiment (d) and a VL region comprising the CDR1, CDR2, and CDR3 sequences of SEQ ID NO:161, XAS (wherein X=D or A, preferably D), and SEQ ID NO:162 (**153**), respectively, such as the VL CDR sequences of SEQ ID NO:68, DAS, and 69, optionally wherein the VL region is derived from IgKV1D-16-01.

In one embodiment, the bispecific antibody or the first antigen-binding region comprises a VH region which is the preceding embodiment (e) and a VL region comprising the CDR1, CDR2, and CDR3 sequences of SEQ ID NO:75, DAS and SEQ ID NO:76 (**132**), respectively, optionally wherein the VL region is derived from IgKV3-11-01.

In one embodiment, the bisspecific antibody or the first antigen-binding region comprises a VH region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:43, 44 and 45, respectively; and a VL region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:47, AAS, and SEQ ID NO:48, respectively (**127**).

In one embodiment, the bispecific antibody or the first antigen-binding region comprises a VH region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:50, 51 and 52, respectively; and a VL region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:54, AAS, and SEQ ID NO:55, respectively (**159**).

In one embodiment, the bispecific antibody or the first antigen-binding region comprises a VH region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:57, 58 and 59, respectively; and a VL region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:60, AAS, and SEQ ID NO:61, respectively (**098**).

In one embodiment, the bispecific antibody or the first antigen-binding region comprises a VH region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:64, 65 and 66, respectively; and, optionally, a VL region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:68, DAS, and SEQ ID NO:69, respectively (**153**).

In one embodiment, the bispecific antibody or the first antigen-binding region comprises a VH region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:71, 72 and 73, respectively; and a VL region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:75, DAS, and SEQ ID NO:76, respectively (**132**).

In separate embodiments, the bispecific antibody or the first antigen-binding region comprises:

a) a VH region comprising the sequence of SEQ ID NO:46 and, preferably, a VL region comprising the sequence of SEQ ID NO:49 (**127**);
b) a VH region comprising the sequence of SEQ ID NO:49 and, preferably, a VL region comprising the sequence of SEQ ID NO:53 (**159**);
c) a VH region comprising the sequence of SEQ ID NO:56 and, preferably, a VL region comprising the sequence of SEQ ID NO:60 (**098**);
d) a VH region comprising the sequence of SEQ ID NO:63 an, optionally, a VL region comprising the sequence of SEQ ID NO:67 (**153**);
e) a VH region comprising the sequence of SEQ ID NO:70 and, preferably, a VL region comprising the sequence of SEQ ID NO:74 (**132**);
f) a VH region comprising the sequence of SEQ ID NO:109 and, preferably, a VL region comprising the sequence of SEQ ID NO:110 (**105**);

g) a VH region comprising the sequence of SEQ ID NO:111 and, preferably, a VL region comprising the sequence of SEQ ID NO:112 (**100**);
h) a VH region comprising the sequence of SEQ ID NO:113 and, preferably, a VL region comprising the sequence of SEQ ID NO:114 (**125**);
i) a VH region comprising the sequence of SEQ ID NO:115 and, preferably, a VL region comprising the sequence of SEQ ID NO:116 (**162**);
j) a VH region comprising the sequence of SEQ ID NO:117 and, preferably, a VL region comprising the sequence of SEQ ID NO:118 (**033**);
k) a VH region comprising the sequence of SEQ ID NO:119 and, preferably, a VL region comprising the sequence of SEQ ID NO:120 (**160**)
l) a VH region comprising the sequence of SEQ ID NO:121 and, preferably, a VL region comprising the sequence of SEQ ID NO:122 (**166**);
m) a VH region comprising the sequence of SEQ ID NO:123 and, preferably, a VL region comprising the sequence of SEQ ID NO:124 (**152**);
o) a VH region comprising the sequence of SEQ ID NO:125 and, preferably, a VL region comprising the sequence of SEQ ID NO:126 (**167**); and/or
p) a variant of any of said antibodies, wherein said variant preferably has at most 1, 2 or 3 amino-acid modifications, more preferably amino-acid substitutions, such as conservative amino acid substitutions and substitutions where the new amino acid is one at the same position in an aligned sequence in Figures 1 or 2, particularly at positions indicated by "X" in the corresponding consensus sequence.

Cross-block group 4

In one aspect of the bispecific antibody of the invention, the bispecific antibody comprises an antigen-binding region which binds HER2 but which does not block the binding to soluble HER2 of a second antibody, optionally in immobilized form, comprising the VH and VL sequences of any of trastuzumab, pertuzumab, F5, and C1, when determined as described in Example 14.

In an additional or alternative aspect of the antibody of the invention, the antigen-binding region blocks or cross-blocks the binding to HER2 of one or more of the human antibodies of cross-block group 4.

In one embodiment, the antigen-binding region blocks the binding to HER2 of a reference antibody, optionally immobilized, wherein the reference antibody comprises a VH region comprising the sequence of SEQ ID NO:165 and a VL region comprising the sequence of SEQ ID NO:169 (**005**), preferably wherein the antibody is fully blocking when determined as described in Example 14.

In one embodiment, the antigen-binding region blocks the binding to HER2 of a reference antibody, optionally immobilized, wherein the reference antibody comprises a VH region comprising the sequence of SEQ ID NO:172 and a VL region comprising the sequence of SEQ ID NO:176 (**006**), preferably wherein the antibody is fully-blocking when determined as described in Example 14.

In one embodiment, the antigen-binding region blocks the binding to HER2 of a reference antibody, optionally immobilized, wherein the reference antibody comprises a VH region comprising the sequence of SEQ ID NO:179 and a VL region comprising the sequence of SEQ ID NO:183 (**059**), preferably wherein the antibody is fully-blocking when determined as described in Example 14.

In one embodiment, the antigen-binding region blocks the binding to HER2 of a reference antibody, optionally immobilized, wherein the reference antibody comprises a VH region comprising the sequence of SEQ ID NO:186 and a VL region comprising the sequence of SEQ ID NO:190 (**060**), preferably wherein the antibody is fully-blocking when determined as described in Example 14.

In one embodiment, the antigen-binding region blocks the binding to HER2 of a reference antibody, optionally immobilized, wherein the reference antibody comprises a VH region comprising the sequence of SEQ ID NO:193 and a VL region comprising the sequence of SEQ ID NO:197 (**106**), preferably wherein the antibody is fully-blocking when determined as described in Example 14.

In one embodiment, the antigen-binding region blocks the binding to HER2 of a reference antibody, optionally immobilized, wherein the reference antibody comprises a VH region comprising the sequence of SEQ ID NO:200 and a VL region comprising the sequence of SEQ ID NO:204 (**111**), preferably wherein the antibody is fully-blocking when determined as described in Example 14.

In separate and specific embodiments, the antigen-binding region blocks the binding of two, three, four, five, or six reference antibodies of the preceding embodiment, such as, *e.g.*, antibodies 005 and 111, antibodies 005 and 006; antibodies 059 and 106; antibodies 006 and 059; antibodies 059, 106, 005 and 060; antibodies 006, 59, 060, and 111; or antibodies 059, 106, 005, 060, 111 and 006.

In one embodiment, the antibody, when immobilized, competes for binding to soluble HER2 with all antibodies defined in the preceding embodiment for 25% or more, preferably 50% or more, when determined as described in Example 14.

In one aspect of the antibody of the invention, the antibody binds the same epitope on HER2 as one or more of the novel human antibodies described herein.

In one embodiment, the antigen-binding region binds the same epitope as an antibody comprising a VH region comprising the sequence of SEQ ID NO:165 and, optionally, a VL region comprising the sequence of SEQ ID NO:169 (**005**).

In one embodiment, the antigen-binding region binds the same epitope as an antibody comprising a VH region comprising the sequence of SEQ ID NO:172 and a VL region comprising the sequence of SEQ ID NO:176 (**006**).

In one embodiment, the antigen-binding region binds the same epitope as an antibody comprising a VH region comprising the sequence of SEQ ID NO:179 and a VL region comprising the sequence of SEQ ID NO:183 (**059**).

In one embodiment, the antigen-binding region binds the same epitope as an antibody comprising a VH region comprising the sequence of SEQ ID NO:186 and a VL region comprising the sequence of SEQ ID NO:190 (**060**).

In one embodiment, the antigen-binding region binds the same epitope as an antibody comprising a VH region comprising the sequence of SEQ ID NO:193 and a VL region comprising the sequence of SEQ ID NO:197 (**106**).

In one embodiment, the antigen-binding region binds the same epitope as an antibody comprising a VH region comprising the sequence of SEQ ID NO:200 and a VL region comprising the sequence of SEQ ID NO:204 (**111**).

In one embodiment, the antigen-binding region binds to the same epitope as at least one antibody selected from the group consisting of:

a) an antibody comprising a VH region comprising the sequence of SEQ ID NO:207 and a VL region comprising the sequence of SEQ ID NO:208 (**041**)

b) an antibody comprising a VH region comprising the sequence of SEQ ID NO:209 and a VL region comprising the sequence of SEQ ID NO:210 (**150**), and

c) an antibody comprising a VH region comprising the sequence of SEQ ID NO:211 and a VL region comprising the sequence of SEQ ID NO:212 (**067**);

d) an antibody comprising a VH region comprising the sequence of SEQ ID NO:213 and a VL region comprising the sequence of SEQ ID NO:214 (**072**);

e) an antibody comprising a VH region comprising the sequence of SEQ ID NO:215 and a VL region comprising the sequence of SEQ ID NO:216 (**163**);

f) an antibody comprising a VH region comprising the sequence of SEQ ID NO:217 and a VL region comprising the sequence of SEQ ID NO:218 (**093**);

g) an antibody comprising a VH region comprising the sequence of SEQ ID NO:219 and a VL region comprising the sequence of SEQ ID NO:220 (**044**).

In another additional or alternative aspect of the bispecific antibody of the invention, the bispecific antibody or the first antigen-binding region comprises a VH CDR3, VH region and/or VL region sequence similar or identical to a sequence of the HER2 antibodies described herein.

In one embodiment, the bispecific antibody or the first antigen-binding region comprises a VH CDR3 region having an amino acid sequence selected from the group consisting of

SEQ ID No:223, such as the sequence of SEQ ID No:168, 189, 196 (**005, 060, 106**), optionally wherein the VH region is derived from the IgHV5-51-1 germline;

SEQ ID No:226, such as the sequence of SEQ ID NO:175 (**006**), optionally wherein the VH region is derived from the IgHV3-23-1 germline sequence;

SEQ ID NO:229, such as the sequence of SEQ ID NO:182 (**059**), optionally wherein the VH region is derived from the IgHV1-18-1 germline sequence; or

SEQ ID NO:231, such as the sequence of SEQ ID NO:203 (**111**), optionally wherein the VH region is derived from the IgHV1-69-4 germline sequence.

In one embodiment, the bispecific antibody or the first antigen-binding region comprises a VH CDR3 region comprising the amino acid sequence of SEQ ID NO: 223, wherein X1=Q, H, or L; X2 = R, A, T, or K; X3=G; X4=D; X5=R or none; X6=G or none; X7=Y or F; X8=Y or D; X9=Y, F, or H; X10=Y, D, S, F, or N; X11=M or L; and X12=V or I; preferably, wherein X1=Q, X2=R or A; X5=X6=none; X7=Y or F; X8=Y; X9=F; X10=Y; and X12=V. In a particular embodiment the antibody comprises a VH CDR3 region comprising the amino acid sequence of SEQ ID NO: 223, wherein X1=Q, X2=R or A; X3=G; X4=D, X5=X6=none; X7=Y or F; X8=Y; X9=F; X10=Y; and X12=V.

In one embodiment the antibody or the first antigen-binding region comprises a VH CDR3 region comprising the amino acid sequence of SEQ ID NO:223, wherein X1=Q, X2=K; X3=G; X4=D, X5=X6=none; X7=F; X8=Y; X9=X10=F; X11=L; and X12=V; or wherein X1=Q, X2=A; X3=G; X4=D, X5=X6=none; X7=X8=Y; X9=Y; X10=N; X11=M; and X12=V; or wherein X1=Q, X2=K; X3=G; X4=D, X5=X6=none; X7=X8=Y; X9=H; X10=Y; X11=L; and X12=V; or wherein X1=Q, X2=K; X3=G; X4=D, X5=X6=none; X7=Y; X8=Y; X9=F; X10=N; X11=L; and X12=V; or wherein X1=Q, X2=R; X3=G; X4=D, X5=X6=none; X7=Y; X8=Y; X9=F; X10=N; X11=L; and X12=V; or wherein X1=Q, X2=R; X3=G; X4=D, X5=X6=none; X7=Y; X8=Y; X9=X10=F; X11=L; and X12=I; or wherein X1=Q, X2=A; X3=G; X4=D, X5=X6=none; X7=X8=Y; X9=Y; X10=N; X11=M; and X12=V.

In one embodiment, the bispecific antibody or the first antigen-binding region comprises a VH CDR3 region of one of antibodies 041, 150, 067, 072, 163, or 093, as

shown in Figure 1, optionally wherein the VH region is derived from an IgHV5-51-1 germline.

In one embodiment, the bispecific antibody or the first antigen-binding region comprises a VH region selected from the group consisting of

a) a VH region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:221, 222 and 223, such as
   a. a CDR1 sequence selected from SEQ ID NOs:166, 187, and 194; a CDR2 sequence selected from 167, 188, and 195; and a CDR3 sequence selected from 168, 189, and 196 (**005, 060, 106**),
   b. the CDR1, CDR2, and CDR3 sequences of SEQ ID NOs:166, 167 and 168, respectively (**005**),
   c. the CDR1, CDR2, and CDR3 sequences of SEQ ID NOs:187, 188 and 189, respectively (**060**),
   d. the CDR1, CDR2, and CDR3 sequences of SEQ ID NOs:196, 197 and 198, respectively (**106**),

   optionally where the VH region is derived from an IgHV5-51-1 germline;

b) a VH region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:224, 225 and 226, such the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:173, 174, and 175, respectively (**006**), optionally where the VH region is derived from an IgHV3-23-1 germline; and

c) a VH region comprising the CDR1, CDR2, and CDR3 sequences of SEQ ID NOs:227, 228, and 229, such as the CDR1, CDR2, and CDR3 sequences of SEQ ID NOs: 180, 181 and 182 (**059**), respectively, optionally where the VH region is derived from an IgHV1-18-1 germline; and

d) a VH region comprising the CDR1, CDR2, and CDR3 sequences of SEQ ID NOs:230, 202 and 231, such as the CDR1, CDR2, and CDR3 sequences of SEQ ID NOs: 201, 202 and 203 (**111**), respectively, optionally where the VH region is derived from an IgHV1-69-4 germline.

In one embodiment, the bispecific antibody or the first antigen-binding region comprises a VH region selected from the preceding embodiments (a), (c) or (d) and a VL region comprising the CDR1, CDR2, and CDR3 sequences of SEQ ID NO:232, GAS, and SEQ ID No:233, respectively, such as a CDR1 sequence selected from SEQ ID Nos: 170, 184, 191, 198 and 205, a CDR2 which is GAS, and a CDR3 sequence selected from 171, 85, 192, 199 and 206 (**005**, **059**, **060**, **106**, **111**); respectively, optionally where the VL region is derived from an IgKV3-20-01 germline.

In one embodiment, the bispecific antibody or the first antigen-binding region comprises a VH region which is the preceding embodiment (b) and a VL region comprising the CDR1, CDR2, and CDR3 sequences of SEQ ID NO:177, DAS, and SEQ ID NO:178 (**006**), respectively, optionally where the VL region is derived from IgKV3-11-01.

In one embodiment, the bispecific antibody or the first antigen-binding region comprises a VH region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:166, 167 and 168, respectively; and, optionally, a VL region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:170, GAS, and SEQ ID NO:171, respectively (**005**).

In one embodiment, the bispecific antibody or the first antigen-binding region comprises a VH region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:173, 174 and 175, respectively; and a VL region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:177, DAS, and SEQ ID NO:178, respectively (**006**).

In one embodiment, the bispecific antibody or the first antigen-binding region comprises a VH region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:180, 181 and 182, respectively; and a VL region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:184, GAS, and SEQ ID NO:185, respectively (**059**).

In one embodiment, the bispecific antibody or the first antigen-binding region comprises a VH region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:187, 188 and 189, respectively; and a VL region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:191, GAS, and SEQ ID NO:192, respectively (**060**).

In one embodiment, the bispecific antibody or the first antigen-binding region comprises a VH region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:194, 195 and 196, respectively; and a VL region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:198, GAS, and SEQ ID NO:199, respectively (**106**).

In one embodiment, the bispecific antibody or the first antigen-binding region comprises a VH region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:201, 202 and 203, respectively; and a VL region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:205, GAS, and SEQ ID NO:206, respectively (**111**).

In one embodiment, the bispecific antibody or the first antigen-binding region comprises a VH region comprising the CDR1 sequence of SEQ ID NO:221, wherein X1=S; X2=T and X3=S; the CDR2 sequence of SEQ ID NO:226, wherein X1=Y and X2=H and the CDR3 sequence of SEQ ID NO:227, wherein X1=Q, X2=K; X3=G; X4=D, X5=X6=none; X7=F; X8=Y; X9=X10=F; X11=L; and X12=V; and a VL region comprising the CDR1 sequence of SEQ ID NO:232, wherein X1=X2=S; the CDR2 sequence GAS; and the CDR3 sequence of SEQ ID NO: 233, wherein X1=Q, X2=S, X3=X4=none and X5=L (**041**).

In one embodiment, the bispecific antibody or the first antigen-binding region comprises a VH region comprising the CDR1 sequence of SEQ ID NO:221, wherein X1=S; X2=T and X3=S; the CDR2 sequence of SEQ ID NO:222, wherein X1=Y and X2=H, and the CDR3 sequence of SEQ ID NO:223, wherein X1=Q, X2=A; X3=G; X4=D, X5=X6=none; X7=X8=Y; X9=Y; X10=N; X11=M; and X12=V; and a VL region comprising the CDR1 sequence of SEQ ID NO:232, wherein X1=X2=S; the CDR2 sequence GAS; and the CDR3 sequence of SEQ ID NO: 233, wherein X1=Q, X2=S, X3=X4=none and X5=L (**150**).

In one embodiment, the bispecific antibody or the first antigen-binding region comprises a VH region comprising the CDR1 sequence of SEQ ID NO:221, wherein X1=S; X2=T and X3=S; the CDR2 sequence of SEQ ID NO:222, wherein X1=Y and X2=D, and the CDR3 sequence of SEQ ID NO:223, X1=Q, X2=K; X3=G; X4=D, X5=X6=none; X7=X8=Y; X9=H; X10=Y; X11=L; and X12=V; and a VL region comprising the CDR1 sequence of SEQ ID NO:232, wherein X1=X2=S; the CDR2 sequence GAS; and the CDR3 sequence of SEQ ID NO: 233, wherein X1=Q, X2=S, X3=P, X4=R and X5=L (**067**).

In one embodiment, the bispecific antibody or the first antigen-binding region comprises a VH region comprising the CDR1 sequence of SEQ ID NO:221, wherein X1=S; X2=T and X3=S; the CDR2 sequence of SEQ ID NO:222, wherein X1=Y and X2=D, and the CDR3 sequence of SEQ ID NO:223, wherein X1=Q, X2=K; X3=G; X4=D, X5=X6=none; X7=Y; X8=Y; X9=F; X10=N; X11=L; and X12=V; and a VL region comprising the CDR1 sequence of SEQ ID NO:232, wherein X1=X2=S; the CDR2 sequence GAS; and the CDR3 sequence of SEQ ID NO: 233, wherein X1=Q, X2=S, X3=P, X4=R and X5=L (**072**).

In one embodiment, the bispecific antibody or the first antigen-binding region comprises a VH region comprising the CDR1 sequence of SEQ ID NO:221, wherein X1=R; X2=I and X3=S; the CDR2 sequence of SEQ ID NO:222, wherein X1=Y and X2=D, and the CDR3 sequence of SEQ ID NO:223, wherein X1=Q, X2=R; X3=G; X4=D, X5=X6=none; X7=Y; X8=Y; X9=F; X10=N; X11=L; and X12=V; and a VL region comprising the CDR1 sequence of SEQ ID NO:232, wherein X1=X2=S; the CDR2 sequence GAS; and the CDR3 sequence of SEQ ID NO: 233, wherein X1=Q, X2=S, X3=X4=none and X5=L (**163**).

In one embodiment, the bispecific antibody or the first antigen-binding region comprises a VH region comprising the CDR1 sequence of SEQ ID NO:221, wherein X1=S; X2=T and X3=S; the CDR2 sequence of SEQ ID NO:222, wherein X1=Y and X2=D, and the CDR3 sequence of SEQ ID NO:223, wherein X1=Q, X2=R; X3=G; X4=D, X5=X6=none; X7=Y; X8=Y; X9=X10=F; X11=L; and X12=I; and a VL region comprising the CDR1 sequence of SEQ ID NO:232, wherein X1=X2=S; the CDR2 sequence GAS; and the CDR3 sequence of SEQ ID NO: 233, wherein X1=Q, X2=S, X3=X4=none and X5=L (**093**).

In one embodiment, the bispecific antibody or the first antigen-binding region comprises a VH region comprising the CDR1 sequence of SEQ ID NO:221, wherein X1=R; X2=S and X3=S; the CDR2 sequence of SEQ ID NO:222, wherein X1=F and X2=D, and the CDR3 sequence of SEQ ID NO:223, wherein X1=Q, X2=A; X3=G; X4=D, X5=X6=none; X7=X8=Y; X9=Y; X10=N; X11=M; and X12=V; and a VL region comprising the CDR1 sequence of SEQ ID NO:232, wherein X1=X2=S; the CDR2 sequence GAS; and the CDR3 sequence of SEQ ID NO: 233, wherein X1=Q, X2=S, X3=X4=none and X5=L (**044**).

In separate embodiments, the bispecific antibody or the first antigen-binding region comprises:

a) a VH region comprising the sequence of SEQ ID NO:165 and, optionally, a VL region comprising the sequence of SEQ ID NO:169 (**005**)
b) a VH region comprising the sequence of SEQ ID NO:172 and, preferably, a VL region comprising the sequence of SEQ ID NO:176 (**006**)
c) a VH region comprising the sequence of SEQ ID NO:179 and, preferably, a VL region comprising the sequence of SEQ ID NO:183 (**059**)
d) a VH region comprising the sequence of SEQ ID NO:186 and, preferably, a VL region comprising the sequence of SEQ ID NO:190 (**060**)
e) a VH region comprising the sequence of SEQ ID NO:193 and, preferably, a VL region comprising the sequence of SEQ ID NO:197 (**106**)
f) a VH region comprising the sequence of SEQ ID NO:200 and, preferably, a VL region comprising the sequence of SEQ ID NO:204 (**111**)
g) a VH region comprising the sequence of SEQ ID NO:297 and, preferably, a VL region comprising the sequence of SEQ ID NO:208 (**041**)
h) a VH region comprising the sequence of SEQ ID NO:209 and, preferably, a VL region comprising the sequence of SEQ ID NO:210 (**150**),
i) a VH region comprising the sequence of SEQ ID NO:211 and, preferably, a VL region comprising the sequence of SEQ ID NO:212 (**067**),
j) a VH region comprising the sequence of SEQ ID NO:213 and, preferably, a VL region comprising the sequence of SEQ ID NO:214 (**072**),
k) a VH region comprising the sequence of SEQ ID NO:215 and, preferably, a VL region comprising the sequence of SEQ ID NO:216 (**163**),
l) a VH region comprising the sequence of SEQ ID NO:217 and, preferably, a VL region comprising the sequence of SEQ ID NO:218 (**093**),
m) a VH region comprising the sequence of SEQ ID NO:219 and, preferably, a VL region comprising the sequence of SEQ ID NO:220 (**044**), and/or

n) a variant of any of said antibodies, wherein said variant preferably has at most 1,2 or 3 amino-acid modifications, more preferably amino-acid substitutions, such as conservative amino acid substitutions and substitutions where the new amino acid is one at the same position in an aligned sequence in Figures 1 or 2, particularly at positions indicated by "X" in the corresponding consensus sequence.

Functional properties of antigen-binding regions or HER2 antibodies of cross-block groups 1, 2, 3, and 4

In another aspect of the antibody of the invention, the bispecific antibody comprises an antigen-binding region from a HER2 antibody which binds to the same HER2 epitope as one or more of the novel Group 1, 2, 3 or 4 antibodies described herein, preferably when determined as described in Example 14; and is further characterized by one or more properties determined as described in Examples 12, 13, 15, 16, 17, 18 and 19.

Thus the first antigen-binding region of the bispecific antibody of the present invention may be same as one of the following HER2 antibodies. The first HER2 antibody of the present invention may have one or more of the following characteristics.

In one embodiment, the HER2 antibody has a lower EC_{50} value (half maximal effective concentration) than trastuzumab in binding to A431 cells, preferably an EC_{50} value lower than 0.80 µg/ml, 0.50 µg/ml, or 0.30 µg/ml, when determined as described in Example 12, and preferably binds the same epitope as at least one reference antibody comprising the VH and VL regions selected from the group consisting of

a) a VH region comprising the sequence of SEQ ID NO:1 and a VL region comprising the sequence of SEQ ID NO:5 (**169**);
b) a VH region comprising the sequence of SEQ ID NO:15 and a VL region comprising the sequence of SEQ ID NO:19 (**084**);
c) a VH region comprising the sequence of SEQ ID NO:22 and a VL region comprising the sequence of SEQ ID NO:26 (**025**);
d) a VH region comprising the sequence of SEQ ID NO:29 and a VL region comprising the sequence of SEQ ID NO:32 (**091**);
e) a VH region comprising the sequence of SEQ ID NO:46 and a VL region comprising the sequence of SEQ ID NO:49 (**127**);
f) a VH region comprising the sequence of SEQ ID NO:49 and a VL region comprising the sequence of SEQ ID NO:53 (**159**);
g) a VH region comprising the sequence of SEQ ID NO:56 and a VL region comprising the sequence of SEQ ID NO:60 (**098**);

h) a VH region comprising the sequence of SEQ ID NO:63 and a VL region comprising the sequence of SEQ ID NO:67 (**153**);
i) a VH region comprising the sequence of SEQ ID NO:70 and a VL region comprising the sequence of SEQ ID NO:74 (**132**)
j) a VH region comprising the sequence of SEQ ID NO:1 and a VL region comprising the sequence of SEQ ID NO:5 (**005**);
k) a VH region comprising the sequence of SEQ ID NO:8 and a VL region comprising the sequence of SEQ ID NO:11 (**006**); and
l) a VH region comprising the sequence of SEQ ID NO:15 and a VL region comprising the sequence of SEQ ID NO:19 (**059**).

In an additional or alternative embodiment, the HER2 antibody or the first antigen-binding region specifically binds HER2-positive Rhesus monkey epithelial cells, when determined as described in Example 13, and preferably binds the same epitope as at least one reference antibody comprising the VH and VL regions selected from the group consisting of the VH and VL regions of any of antibodies 169, 050, 084, 025, 091, 129, 127, 159, 098, 153 132, 005, 006, 059, 060, 106 and 111.

In an additional or alternative embodiment, the anti-HER2 antibody or the first antigen-binding region efficiently induces ADCC (antibody-dependent cell-mediated cytotoxicity), preferably achieving a specific ^{51}Cr-release of at least 30%, more preferably at least 40%, when determined as described in Example 15, and preferably binds the same epitope as at least one reference antibody comprising the VH and VL regions selected from the group consisting of:

a) a VH region comprising the sequence of SEQ ID NO:1 and a VL region comprising the sequence of SEQ ID NO:5 (**169**);
b) a VH region comprising the sequence of SEQ ID NO:8 and a VL region comprising the sequence of SEQ ID NO:12 (**050**);
c) a VH region comprising the sequence of SEQ ID NO:15 and a VL region comprising the sequence of SEQ ID NO:19 (**084**);
d) a VH region comprising the sequence of SEQ ID NO:22 and a VL region comprising the sequence of SEQ ID NO:26 (**025**);
e) a VH region comprising the sequence of SEQ ID NO:29 and a VL region comprising the sequence of SEQ ID NO:32 (**091**);
f) a VH region comprising the sequence of SEQ ID NO:35 and a VL region comprising the sequence of SEQ ID NO:39 (**129**); and

g) a VH region comprising the sequence of SEQ ID NO:63 an, preferably, a VL region comprising the sequence of SEQ ID NO:67 (**153**).

In an additional or alternative embodiment, the anti-HER2 antibody or the first antigen-binding region specifically binds HER2-expressing AU565 cells but promotes ligand-independent proliferation of the cells less than any of F5 and C1 when determined as described in Example 16, and preferably binds the same epitope as at least one reference antibody comprising the VH and VL regions selected from the group consisting of

a) a VH region comprising the sequence of SEQ ID NO:1 and a VL region comprising the sequence of SEQ ID NO:5 (**169**);
b) a VH region comprising the sequence of SEQ ID NO:8 and a VL region comprising the sequence of SEQ ID NO:12 (**050**);
c) a VH region comprising the sequence of SEQ ID NO:15 and a VL region comprising the sequence of SEQ ID NO:19 (**084**);
d) a VH region comprising the sequence of SEQ ID NO:22 and a VL region comprising the sequence of SEQ ID NO:26 (**025**);
e) a VH region comprising the sequence of SEQ ID NO:29 and a VL region comprising the sequence of SEQ ID NO:32 (**091**);
f) a VH region comprising the sequence of SEQ ID NO:35 and a VL region comprising the sequence of SEQ ID NO:39 (**129**);
g) a VH region comprising the sequence of SEQ ID NO:46 and a VL region comprising the sequence of SEQ ID NO:49 (**127**);
h) a VH region comprising the sequence of SEQ ID NO:49 and a VL region comprising the sequence of SEQ ID NO:53 (**159**);
i) a VH region comprising the sequence of SEQ ID NO:56 and a VL region comprising the sequence of SEQ ID NO:60 (**098**);
j) a VH region comprising the sequence of SEQ ID NO:63 and a VL region comprising the sequence of SEQ ID NO:67 (**153**);
k) a VH region comprising the sequence of SEQ ID NO:70 and a VL region comprising the sequence of SEQ ID NO:74 (**132**)
l) a VH region comprising the sequence of SEQ ID NO:1 and a VL region comprising the sequence of SEQ ID NO:5 (**005**); and
m) a VH region comprising the sequence of SEQ ID NO:22 and a VL region comprising the sequence of SEQ ID NO:26 (**060**).

In an additional or alternative embodiment, the anti-HER2 antibody or the /first antigen-binding region specifically binds HER2-expressing AU565 cells and inhibits ligand-independent proliferation of the cells, preferably inhibiting proliferation by at least 20%, more preferably at least 25%, when determined as described in Example 16, and preferably binds the same epitope as at least one reference antibody comprising the VH and VL regions selected from the group consisting of:

a) a VH region comprising the sequence of SEQ ID NO:1 and a VL region comprising the sequence of SEQ ID NO:5 (**169**); and
b) a VH region comprising the sequence of SEQ ID NO:8 and a VL region comprising the sequence of SEQ ID NO:12 (**050**).

In an additional or alternative embodiment, the anti-HER2 antibody specifically binds HER2-expressing AU565 cells but has no significant effect on, or does not promote, ligand-induced proliferation of the cells, preferably inhibiting proliferation by no more than 25%, more preferably by no more than 15%, when determined as described in Example 17, and binds the same epitope as at least one reference antibody comprising the VH and VL regions selected from the group consisting of:

a) a VH region comprising the sequence of SEQ ID NO:1 and a VL region comprising the sequence of SEQ ID NO:5 (**169**);
b) a VH region comprising the sequence of SEQ ID NO:8 and a VL region comprising the sequence of SEQ ID NO:12 (**050**);
c) a VH region comprising the sequence of SEQ ID NO:15 and a VL region comprising the sequence of SEQ ID NO:19 (**084**); and
d) a VH region comprising the sequence of SEQ ID NO:56 and a VL region comprising the sequence of SEQ ID NO:60 (**098**).

In an additional or alternative embodiment, the anti-HER2 antibody specifically binds HER2-expressing MCF-7 cells and inhibits ligand-induced proliferation, *e.g.* it may completely inhibit the ligand-induced effect or inhibit the total proliferation by 50%, *e.g.* 60% or 70% or 80%, of the cells when determined as described in Example 17, and binds the same epitope as at least one reference antibody comprising the VH and VL regions selected from the group consisting of:

a) a VH region comprising the sequence of SEQ ID NO:22 and a VL region comprising the sequence of SEQ ID NO:26 (**025**);
b) a VH region comprising the sequence of SEQ ID NO:29 and a VL region comprising the sequence of SEQ ID NO:32 (**091**);

c) a VH region comprising the sequence of SEQ ID NO:35 and a VL region comprising the sequence of SEQ ID NO:39 (**129**); and
d) a VH region comprising the sequence of SEQ ID NO:63 and, preferably, a VL region comprising the sequence of SEQ ID NO:67 (**153**).

In an additional or alternative embodiment, the first anti-HER2 antibody is internalized by tumor cells expressing HER2, such as AU565 cells, to a higher degree than trastuzumab and pertuzumab, preferably more than twice or three times the amount of internalized trastuzumab, preferably when determined according to Example 18, and binds to the same epitope as an antibody comprising VH and VL regions selected from the group consisting of:

a) a VH region comprising the sequence of SEQ ID NO:46 and a VL region comprising the sequence of SEQ ID NO:49 (**127**);
b) a VH region comprising the sequence of SEQ ID NO:49 and a VL region comprising the sequence of SEQ ID NO:53 (**159**);
c) a VH region comprising the sequence of SEQ ID NO:56 and a VL region comprising the sequence of SEQ ID NO:60 (**098**);
d) a VH region comprising the sequence of SEQ ID NO:63 and a VL region comprising the sequence of SEQ ID NO:67 (**153**); and
e) a VH region comprising the sequence of SEQ ID NO:70 and a VL region comprising the sequence of SEQ ID NO:74 (**132**).

Preferably, the antibody binds to the same epitope as an antibody comprising VH and VL regions selected from

a) a VH region comprising the sequence of SEQ ID NO:46 and a VL region comprising the sequence of SEQ ID NO:49 (**127**) and
b) a VH region comprising the sequence of SEQ ID NO:56 and a VL region comprising the sequence of SEQ ID NO:60 (**098**).

In a further embodiment, the antibody binds to Domain II or IV of HER2, preferably wherein the antibody does not significantly promote proliferation of HER2 expressing cells, and is more efficiently internalized, or is internalized to a higher degree, than trastuzumab or pertuzumab into HER2-expressing tumor cells, preferably when determined as described in the Examples, *e.g.* examples 16 and 19, respectively.

In a further embodiment the antibody enhanced HER2 downmodulation more than trastuzumab, *e.g.* the antibody enhanced HER2 downmodulation by more 30%, such as more than 40% or more than 50% when determined as described in Example 22, preferably

wherein the antibody binds to the same epitope as an antibody of cross-block group 3 of the present invention, *e.g.* an antibody binding to the same epitope as an antibody comprising VH and VL regions selected from the group consisting of:

a) a VH region comprising the sequence of SEQ ID NO:56 and a VL region comprising the sequence of SEQ ID NO:60 (**098**);
b) a VH region comprising the sequence of SEQ ID NO:63 and a VL region comprising the sequence of SEQ ID NO:67 (**153**).

In another or alternative embodiment the antibody decreased tumour growth and improved survival *in vivo* more than trastuzumab, when determined as described in Example 25, preferably wherein the antibody binds to the same epitope as an antibody of cross-block 1 or cross-block 2 of the present invention, *e.g.* an antibody binding to the same epitope as an antibody comprising VH and VL regions selected from the group consisting of:

a) a VH region comprising the sequence of SEQ ID NO:1 and a VL region comprising the sequence of SEQ ID NO:5 (**169**);
b) a VH region comprising the sequence of SEQ ID NO:15 and a VL region comprising the sequence of SEQ ID NO:19 (**084**); and
c) a VH region comprising the sequence of SEQ ID NO:29 and a VL region comprising the sequence of SEQ ID NO:32 (**091**).

In another or alternative embodiment the antibody decreased tumour growth and improved survival *in vivo* more than trastuzumab, when determined as described in Example 26, preferably wherein the antibody binds to the same epitope as an antibody of cross-block 2 or cross-block 3 of the present invention, *e.g.* an antibody binding to the same epitope as an antibody comprising VH and VL regions selected from the group consisting of:

a) a VH region comprising the sequence of SEQ ID NO:22 and a VL region comprising the sequence of SEQ ID NO:26 (**025**);
b) a VH region comprising the sequence of SEQ ID NO:29 and a VL region comprising the sequence of SEQ ID NO:32 (**091**);
c) a VH region comprising the sequence of SEQ ID NO:35 and a VL region comprising the sequence of SEQ ID NO:39 (**129**); and
d) a VH region comprising the sequence of SEQ ID NO:63 and a VL region comprising the sequence of SEQ ID NO:67 (**153**).

More particularly, wherein the antibody binds to the same epitope as an antibody comprising VH and VL regions selected from the group consisting of:

a) a VH region comprising the sequence of SEQ ID NO:22 and a VL region comprising the sequence of SEQ ID NO:26 (**025**); and
b) a VH region comprising the sequence of SEQ ID NO:29 and a VL region comprising the sequence of SEQ ID NO:32 (**091**).

In one embodiment, the conjugated antibody kills at least 60%, preferably at least 70% AU565 cells or A431 cells, when determined as described in Example 18, and cross-blocks at least one antibody selected from

a) an antibody comprising a VH region comprising the sequence of SEQ ID NO:1 and a VL region comprising the sequence of SEQ ID NO:5 (**005**)
b) an antibody comprising a VH region comprising the sequence of SEQ ID NO:22 and a VL region comprising the sequence of SEQ ID NO:26 (**060**)
c) an antibody comprising a VH region comprising the sequence of SEQ ID NO:15 and a VL region comprising the sequence of SEQ ID NO:19 (**059**), and
d) an antibody comprising a VH region comprising the sequence of SEQ ID NO:36 and a VL region comprising the sequence of SEQ ID NO:40 (**111**).

In separate and specific embodiments, the antibody of the preceding embodiment fully cross-blocks, preferably bind to the same epitope as, antibody 005, 060, 059, 111, or a combination thereof.

In one embodiment, the antibody of the preceding embodiment kills at least 80% of A431 cells when determined as described in Example 18, and cross-blocks at least one antibody selected from

a) an antibody comprising a VH region comprising the sequence of SEQ ID NO:1 and a VL region comprising the sequence of SEQ ID NO:5 (**005**), and
b) an antibody comprising a VH region comprising the sequence of SEQ ID NO:22 and a VL region comprising the sequence of SEQ ID NO:26 (**060**).

In separate and specific embodiments, the antibody of the preceding embodiment fully cross-blocks, preferably bind to the same epitope as, antibody 005, 060, or a combination thereof.

In an additional or alternative embodiment, the antibody is internalized by tumor cells expressing HER2, such as AU565 cells, more than trastuzumab is, preferably more than twice or three times the amount of internalized trastuzumab, preferably when determined according to Example 19, and cross-blocks at least one antibody selected from the group consisting of:

a) an antibody comprising a VH region comprising the sequence of SEQ ID NO:1 and a VL region comprising the sequence of SEQ ID NO:5 (**005**)
b) an antibody comprising a VH region comprising the sequence of SEQ ID NO:8 and a VL region comprising the sequence of SEQ ID NO:11 (**006**)
c) an antibody comprising a VH region comprising the sequence of SEQ ID NO:15 and a VL region comprising the sequence of SEQ ID NO:19 (**059**)
d) an antibody comprising a VH region comprising the sequence of SEQ ID NO:22 and a VL region comprising the sequence of SEQ ID NO:26 (**060**)
e) an antibody comprising a VH region comprising the sequence of SEQ ID NO:29 and a VL region comprising the sequence of SEQ ID NO:33 (**106**)
f) an antibody comprising a VH region comprising the sequence of SEQ ID NO:36 and a VL region comprising the sequence of SEQ ID NO:40 (**111**).

In separate and specific embodiments, the antibody of the preceding embodiment fully cross-blocks, preferably bind to the same epitope as, antibody 005, 006, 059, 060, 106, 111, or a combination thereof.

Bispecific antibodies

In one embodiment, the antibody is a bispecific antibody, comprising (i) a first antigen-binding region of a HER2 antibody as defined herein, *e.g.* an antibody of cross-block 1, 2, 3 or 4, and (ii) a second antibody comprising an antigen-binding region of an antibody which binds to CD3.

First antigen-binding region

In one embodiment the first antigen-binding region comprises a VH region comprising a CDR3 sequence of an antibody of cross-block 1, 2, 3 or 4 as defined herein, such as SEQ ID NO: 4, 25, 66 or 168 (**169**, **025**,**153**, or **005**). In a particular embodiment, the first antigen-binding region comprises a VH region comprising a CDR3 sequence of SEQ ID NO: 4 (**169**).

In one embodiment the first antigen-binding region comprises a VH region comprising CDR1, CDR2 and CDR3 sequences of an antibody of cross-block 1, 2, 3 or 4 as defined herein, such as CDR1, CDR2, and CDR3 sequences SEQ ID NOs: 2, 3 and 4 (**169**), or CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:23, 24 and 25 (**025**), or CDR1, CDR2 and CDR3 sequences of SEQ ID NOs: 64, 65 and 66 (**153**), or CDR1, CDR2 CDR3 sequence of SEQ ID NOs: 166, 167 and 168 (**005**). In a particular embodiment, the first antigen-binding region comprises a VH region comprising CDR1, CDR2 and CDR3 sequences of CDR1, CDR2, and CDR3 sequences SEQ ID NOs: 2, 3 and 4 (**169**).

In a further or alternative embodiment the first antigen-binding region comprises a VH region comprising a CDR3 sequence of an antibody of cross-block 1, 2, 3 or 4 as defined herein, such as CDR3 sequence an antibody of cross-block 1 of SEQ ID NO: 11 (**050**), or SEQ ID NO: 18 (**084**); or a CDR3 sequence of an antibody of cross-block 2 of SEQ ID NO: 31 (**091**), or SEQ ID NO: 38 (**129**), or a CDR3 sequence of an antibody of cross-block 3 of SEQ ID NO: 45 (**127**), or SEQ ID NO:52 (**159**), or SEQ ID NO:59 (**098**), or SEQ ID NO:73 (**132**), or a CDR3 sequence of an antibody of cross-block 4 of SEQ ID NO:175 (**006**), SEQ ID NO: 182 (**059**), SEQ ID NO:189 (**060**), SEQ ID NO:196 (**106**), or SEQ ID NO:203 (**111**).

In one embodiment the first antigen-binding region comprises a VH region comprising CDR1, CDR2 and CDR3 sequences of an antibody of cross-block 1, 2 or 3 as defined herein, such as CDR1, CDR2, and CDR3 sequences SEQ ID NOs: 2, 3 and 4 (**169**), or CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:23, 24 and 25 (**025**), or CDR1, CDR2 and CDR3 sequences of SEQ ID NOs: 64, 65 and 66 (**153**), or CDR1, CDR2 CDR3 sequence of SEQ ID NOs: 166, 167 and 168 (**005**).

In one embodiment the first antigen-binding region comprises a VH region comprising CDR1, CDR2 and CDR3 sequences of an antibody of cross-block 1, 2, 3 or 4 as defined herein, and a VL region comprising CDR1, CDR2 and CDR3 sequences of an antibody of cross-block 1, 2, 3 or 4 as defined herein.

In a further or alternative embodiment the first antigen-binding region comprises a VH region comprising CDR1, CDR2 and CDR3 sequences of an antibody of cross-block 1, 2, 3 or 4 as defined herein, such as CDR1, CDR2, and CDR3 sequences of an antibody of cross-block 1 of SEQ ID NOs: 9, 10 and 11 (**050**), or SEQ ID NOs: 16, 17 and 18 (**084**); or CDR1, CDR2, and CDR3 sequences of an antibody of cross-block 2 of SEQ ID NOs: 30, 163 and 31 (**091**), or SEQ ID NOs: 36, 37 and 38 (**129**), or CDR1, CDR2, and CDR3 sequences of an antibody of cross-block 3 SEQ ID NOs: 43, 44 and 45 (**127**), or SEQ ID NOs:50, 51 and 52 (**159**), or SEQ ID NOs:57, 58 and 59 (**098**), or SEQ ID NOs:71, 72 and 73 (**132**), or CDR1, CDR2 and CDR3 sequences of an antibody of cross-block 4 such as SEQ ID NOS: 173, 174,_and 175 (**006**), SEQ ID NOS: 180, 181 , and 182 (**059**), SEQ ID NOS:187, 188, and 189 (**060**), SEQ ID NOS:194, 195, and 196 (**106**), or SEQ ID NOS:201, 202, and 203 (**111**).

In one embodiment the first antigen-binding region comprises a VH region and a VL region selected from the group consisting of:

a) a VH region comprising the CDR1, CDR2, and CDR3 sequences of SEQ ID NOs: 2, 3 and 4; and a VL region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID: 6, DAS and SEQ ID NO:7, respectively (**169**);
b) a VH region comprising the CDR1, CDR2, and CDR3 sequences of SEQ ID NOs: 23, 24 and 25; and a VL region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NO: 27, AAS and SEQ ID NO:28, respectively (**025**);
c) a VH region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:64, 65 and 66; and a VL region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NO: 68, DAS and SEQ ID NO:69 (**153**); and
d) a VH region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:166, 167 and 168; and a VL region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NO: 170, GAS and SEQ ID NO:171 (**005**).

In a particular embodiment, the VH region comprises the CDR1, CDR2, and CDR3 sequences of SEQ ID NOs: 2, 3 and 4 the VL region comprises the CDR1, CDR2 and CDR3 sequences of SEQ ID: 6, DAS and SEQ ID NO:7, respectively (**169**).

In a further or alternative embodiment the first antigen-binding region comprises a VH region and a VL region selected from the group consisting of:

a) a VH region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:9, 127 and 11, such as the CDR1, CDR2 and CDR3 sequences of SEQ ID NOS: 9, 10 and 11 (**050**); optionally where the VH region is derived from an IgHV3-23-1 germline;
b) a VH region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:128, 129 and 130, such the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:16, 17 and 18, respectively (**084**), optionally where the VH region is derived from an IgHV1-69-04 germline; and
c) a VH region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:137, 138 and 139, such the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:30, 163, and 31, respectively (**091**), optionally where the VH region is derived from an IgHV4-34-01 germline; and
d) a VH region comprising the CDR1, CDR2, and CDR3 sequences of SEQ ID NOs:140, 141 and 142, such as the CDR1, CDR2, and CDR3 sequences of SEQ ID NOs: 36, 37 and 38 (**129**), respectively, optionally where the VH region is derived from an IgHV3-30-01 germline.
e) a VH region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:146, 147 and 148, such as the CDR1, CDR2 and CDR3 sequences of SEQ

ID NOS: 43, 44 and 45 (**127**); optionally where the VH region is derived from an IgHV5-51-01 germline;

f) a VH region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:149, 51 and 52, such as the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:50, 51 and 52, respectively (**159**), optionally where the VH region is derived from an IgHV5-51-01 germline;

g) a VH region comprising the CDR1, CDR2, and CDR3 sequences of SEQ ID NOs:143, 144 and 145, such as the CDR1, CDR2, and CDR3 sequences of SEQ ID NOs: 57, 58 and 59 (**098**), respectively, optionally where the VH region is derived from an IgHV3-23-01 germline;

h) a VH region comprising the CDR1, CDR2, and CDR3 sequences of SEQ ID NOs:71, 150 and 151, such as the CDR1, CDR2, and CDR3 sequences of SEQ ID NOs: 71, 72 and 73 (**132**), respectively, optionally where the VH region is derived from an IgHV1-18-01 germline;

i) a VH region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:221, 222 and 223, such as the CDR1, CDR2, and CDR3 sequences of SEQ ID NOs:187, 188 and 189, respectively (**060**), optionally where the VH region is derived from an IgHV5-51-1 germline;

j) A VH region comprising the CDR1, CDR2, and CDR3 sequences of SEQ ID NOs:194, 195 and 196, respectively (**106**), optionally where the VH region is derived from an IgHV5-51-1 germline;

k) a VH region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:224, 225 and 226, such the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:173, 174, and 175, respectively (**006**), optionally where the VH region is derived from an IgHV3-23-1 germline;

l) a VH region comprising the CDR1, CDR2, and CDR3 sequences of SEQ ID NOs:227, 228, and 229, such as the CDR1, CDR2, and CDR3 sequences of SEQ ID NOs: 180, 181 and 182 (**059**), respectively, optionally where the VH region is derived from an IgHV1-18-1 germline; and

m) a VH region comprising the CDR1, CDR2, and CDR3 sequences of SEQ ID NOs:230, 202 and 231, such as the CDR1, CDR2, and CDR3 sequences of SEQ ID NOs: 201, 202 and 203 (**111**), respectively, optionally where the VH region is derived from an IgHV1-69-4 germline.

<u>Second antigen-binding region</u>

In any one of the preceding embodiments, the second antigen-binding region can be derived from a CD3 antibody.

In one embodiment, the second antigen-binding region is derived from a CD3 antibody comprising the VH CDR3 sequence of SEQ ID NO: 244 (**huCLB-T3/4**).

In a further embodiment, the second antigen-binding region is derived from a CD3 antibody comprising the VL CDR3 sequence of SEQ ID NO: 246 (**huCLB-T3/4**).

In further embodiment, the second antigen-binding region is derived from a CD3 antibody is an antibody comprising a VH region selected from the group consisting of:

a) a VH region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOS:242, 243 and 244, respectively (**huCLB-T3/4**);
b) a VH region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NO:234 (**YTH12.5**);
c) a VH region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NO:238 (**huOKT3-C114S-gLC**); and
d) a VH region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NO:236 (**HUM291**).

In a further embodiment, the second antigen-binding region is derived from a CD3 antibody comprising a VH region comprising the VH CDR1, CDR2 and CDR3 sequences of SEQ ID NO:234 and a VL region comprising the VLCDR1, CDR2 and CDR3 sequences of SEQ ID NO:235 (**YTH12.5**).

In a specific embodiment, the second antigen-binding region is derived from a CD3 antibody comprising a VH region comprising the sequence of SEQ ID NO: 234 (**YTH12.5**) and VL region comprising the sequence of SEQ ID NO:235 (**YTH12.5**).

In one embodiment, the second antigen-binding region is derived from a CD3 antibody comprising a VH region comprising the VH CDR1, CDR2 and CDR3 sequences of SEQ ID NOS:242, 243 and 244, respectively, and, optionally, a VL region comprising the VL CDR1, CDR2 and CDR3 sequences of SEQ ID NOS:245, DTS and 246, respectively (**huCLB-T3/4**).

In a specific embodiment, the second antigen-binding region is derived from a CD3 antibody comprising a VH region comprising the sequence of SEQ ID NO: 240 (**huCLB-T3/4**) and, optionally, VL region comprising the sequence of SEQ ID NO:241 (**huCLB-T3/4**).

In one embodiment, the second antigen-binding region is derived from a CD3 antibody comprising a VH region comprising the VH CDR1, CDR2 and CDR3 sequences of SEQ ID NO:238 and a VL region comprising the VL CDR1, CDR2 and CDR3 sequences of SEQ ID NO:239 (**huOKT3-C114S-gLC**).

In a specific embodiment, the second antigen-binding region is derived from a CD3 antibody comprising a VH region comprising the sequence of SEQ ID NO: 238 and VL region comprising the sequence of SEQ ID NO:239 (**huOKT3-C114S-gLC**).

In one embodiment, the second antigen-binding region is derived from a CD3 antibody comprising a VH region comprising the VH CDR1, CDR2 and CDR3 sequences of SEQ ID NO:236 and a VL region comprising the VL CDR1, CDR2 and CDR3 sequences of SEQ ID NO:237 (**HUM291**).

In a specific embodiment, the second antigen-binding region is derived from a CD3 antibody comprising a VH region comprising the sequence of SEQ ID NO:236 and VL region comprising the sequence of SEQ ID NO:237 (**HUM291**).

Specific combinations of bispecific antibodies

One embodiment of the present invention relates to a bispecific antibody having a second antigen-binding region comprising a VH region comprising a CDR3 sequence of a CD3 antibody according to SEQ ID NO: 244 (**huCLB-T3/4**), and a first antigen-binding region comprising a VH region comprising a CDR3 sequence of a HER2 antibody of cross-block 1, 2, 3, or 4 as defined herein, such as SEQ ID NOs: 4, 25, 66, or 168 (**169**, **025**, **153**, or **005**).

Another embodiment of the present invention relates to a bispecific antibody having a second antigen-binding region comprising a VH region comprising a CDR3 sequence of a CD3 antibody according to SEQ ID NO: 244 (**huCLB-T3/4**) and a VL region comprising a CDR3 sequence of a CD3 antibody according to SEQ ID NO: 246 (**huCLB-T3/4**), and a first antigen-binding region comprising a VH region comprising a CDR3 sequence of a HER2 antibody of cross-block 1, 2, 3, or 4 as defined herein, such as SEQ ID NOs: 4, 25, 66, or 168 (**169**, **025**, **153**, or **005**) and a VL region comprising a CDR3 sequence of a HER2 antibody of cross-block 1, 2, 3, or 4 as defined herein, such as SEQ ID NOs: 7, 28, 69, or 171 (**169**, **025**, **153**, or **005).**

Another embodiment of the present invention relates to a bispecific antibody having a second antigen-binding region comprising a VH region comprising CDR1, CDR2, and CDR3 sequences of a CD3 antibody according to SEQ ID NOs: 242, 243, and 244 (**huCLB-T3/4**), and a first antigen-binding region comprises a VH region comprising CDR1, CDR2, and CDR3 sequences of a HER2 antibody of cross-block 1, 2, 3, or 4 as defined herein, such as SEQ ID NOs: 2, 3, and 4 (**169**), SEQ ID NOs: 23, 24, and 25 (**025**), SEQ ID NOs: 64, 65, and 66 (**153**), or SEQ ID NOs: 166, 167, and 168 (**005**).

Another embodiment of the present invention relates to a bispecific antibody having a second antigen-binding region comprising a VH region comprising CDR1, CDR2, and CDR3

sequences of a CD3 antibody according to SEQ ID NOs: 242, 243, and 244 (**huCLB-T3/4**) and a VL region comprising CDR1, CDR2, and CDR3 sequences of a CD3 antibody according to SEQ ID NOs: 245, DTS, and 246 (**huCLB-T3/4**), and a first antigen-binding region comprising a VH region comprising CDR1, CDR2, and CDR3 sequences of a HER2 antibody of cross-block 1, 2, 3, or 4 as defined herein and a VL region comprising CDR1, CDR2, and CDR3 sequences of a HER2 antibody of cross-block 1, 2, 3, or 4 as defined herein, such as SEQ ID NOs: 2, 3, 4, 6, DAS, and 7 (**169**), SEQ ID NOs: 23, 24, 25, 27, AAS, and 28 (**025**), SEQ ID NOs: 64, 65, 66, 68, DAS, and 69 (**153**), or SEQ ID NOs: 166, 167, 168, 170, GAS, and 171 (**005**).

Another embodiment of the present invention relates to a bispecific antibody having a second antigen-binding region comprising a VH region of a CD3 antibody according to SEQ ID NO: 240 (**huCLB-T3/4**), and a first antigen-binding region comprising a VH region of a HER2 antibody of cross-block 1, 2, 3, or 4 as defined herein, such as SEQ ID NOs: 1, 22, 63, or 165 (**169**, **025**, **153**, or **005**).

Another embodiment of the present invention relates to a bispecific antibody having a second antigen-binding region comprising a VH region and a VL region of a CD3 antibody according to SEQ ID NOs: 240 and 241 (**huCLB-T3/4**), and a first antigen-binding region comprising a VH region and a VL region of a HER2 antibody of cross-block 1, 2, 3, or 4 as defined herein, such as SEQ ID NOs: 1 and 5 (**169**), 22 and 26 (**025**), 63 and 67 (**153**), or 165 and 169 (**005**).

One embodiment of the present invention relates to a bispecific antibody having a second antigen-binding region comprising a VH region comprising a VH CDR3 sequence of a CD3 antibody according to SEQ ID NO: 234 (**YTH12.5**), and a first antigen-binding region comprising a VH region of a HER2 antibody of cross-block 1, 2, 3, or 4 as defined herein, such as SEQ ID NOs: 4, 25, 66, or 168 (**169**, **025**, **153**, or **005**).

Another embodiment of the present invention relates to a bispecific antibody having a second antigen-binding region comprising a VH region comprising a CDR3 sequence of a CD3 antibody according to SEQ ID NO: 234 (**YTH12.5**) and a VL region comprising a CDR3 sequence of a CD3 antibody according to SEQ ID NO: 235 (**YTH12.5**), and a first antigen-binding region comprising a VH region comprising a CDR3 sequence of a HER2 antibody of cross-block 1, 2, 3, or 4 as defined herein, such as SEQ ID NOs: 4, 25, 66, or 168 (**169**, **025**, **153**, or **005**) and a VL region comprising a CDR3 sequence of a HER2 antibody of cross-block 1, 2, 3, or 4 as defined herein, such as SEQ ID NOs: 7, 28, 69, or 171 (**169**, **025**, **153**, or **005**).

Another embodiment of the present invention relates to a bispecific antibody having a second antigen-binding region comprising a VH region comprising a VH CDR1, CDR2, and CDR3 sequence according to SEQ ID NO: 234 (**YTH12.5**), and a first antigen-binding region of a HER2 antibody of cross-block 1, 2, 3, or 4 as defined herein, such as SEQ ID NOs: 2, 3, and 4 (**169**), SEQ ID NOs: 23, 24, and 25 (**025**), SEQ ID NOs: 64, 65, and 66 (**153**), or SEQ ID NOs: 166, 167, and 168 (**005**).

Another embodiment of the present invention relates to a bispecific antibody having a second antigen-binding region comprising a VH region comprising CDR1, CDR2, and CDR3 sequences of a CD3 antibody according to SEQ ID NO: 234 (**YTH12.5**) and a VL region comprising CDR1, CDR2, and CDR3 sequences of a CD3 antibody according to SEQ ID NO: 235 (**YTH12.5**), and a first antigen-binding region comprising a VH region comprising CDR1, CDR2, and CDR3 sequences of a HER2 antibody of cross-block 1, 2, 3, or 4 as defined herein and a VL region comprising CDR1, CDR2, and CDR3 sequences of a HER2 antibody of cross-block 1, 2, 3, or 4 as defined herein, such as SEQ ID NOs: 2, 3, 4, 6, DAS, and 7 (**169**), SEQ ID NOs: 23, 24, 25, 27, AAS, and 28 (**025**), SEQ ID NOs: 64, 65, 66, 68, DAS, and 69 (**153**), or SEQ ID NOs: 166, 167, 168, 170, GAS, and 171 (**005**).

Another embodiment of the present invention relates to a bispecific antibody having a second antigen-binding region comprising a VH region of a CD3 antibody according to SEQ ID NO: 234 (**YTH12.5**), and a first antigen-binding region of a HER2 antibody of cross-block 1, 2, 3, or 4, such as SEQ ID NOs: 1, 22, 63, or 165 (**169**, **025**, **153**, or **005**).

Another embodiment of the present invention relates to a bispecific antibody having a second antigen-binding region comprising a VH region and a VL region of a CD3 antibody according to SEQ ID NOs: 234 and 235 (**YTH12.5**), and a first antigen-binding region comprising a VH region and a VL region of a HER2 antibody of cross-block 1, 2, 3, or 4 as defined herein, such as SEQ ID NOs: 1 and 5 (**169**), 22 and 26 (**025**), 63 and 67 (**153**), or 165 and 169 (**005**).

One embodiment of the present invention relates to a bispecific antibody having a second antigen-binding region comprising a VH region comprising a VH CDR3 sequence of a CD3 antibody according to SEQ ID NO: 236 (**HUM291**), and a first antigen-binding region comprising a VH region of a HER2 antibody of cross-block 1, 2, 3, or 4 as defined herein, such as SEQ ID NOs: 4, 25, 66, or 168 (**169**, **025**, **153**, or **005**).

Another embodiment of the present invention relates to a bispecific antibody having a second antigen-binding region comprising a VH region comprising a CDR3 sequence of a CD3 antibody according to SEQ ID NO: 236 (**HUM291**) and a VL region comprising a CDR3 sequence of a CD3 antibody according to SEQ ID NO: 237 (**HUM291**), and a first antigen-

binding region comprising a VH region comprising a CDR3 sequence of a HER2 antibody of cross-block 1, 2, 3, or 4 as defined herein, such as SEQ ID NOs: 4, 25, 66, or 168 (**169**, **025**, **153**, or **005**) and a VL region comprising a CDR3 sequence of a HER2 antibody of cross-block 1, 2, 3, or 4 as defined herein, such as SEQ ID NOs: 7, 28, 69, or 171 (**169**, **025**, **153**, or **005**).

Another embodiment of the present invention relates to a bispecific antibody having a second antigen-binding region comprising a VH region comprising a VH CDR1, CDR2, and CDR3 sequence according to SEQ ID NO: 236 (**HUM291**), and a first antigen-binding region of a HER2 antibody of cross-block 1, 2, 3, or 4 as defined herein, such as SEQ ID NOs: 2, 3, and 4 (**169**), SEQ ID NOs: 23, 24, and 25 (**025**), SEQ ID NOs: 64, 65, and 66 (**153**), or SEQ ID NOs: 166, 167, and 168 (**005**).

Another embodiment of the present invention relates to a bispecific antibody having a second antigen-binding region comprising a VH region comprising CDR1, CDR2, and CDR3 sequences of a CD3 antibody according to SEQ ID NO: 236 (**HUM291**) and a VL region comprising CDR1, CDR2, and CDR3 sequences of a CD3 antibody according to SEQ ID NO: 237 (**HUM2915**), and a first antigen-binding region comprising a VH region comprising CDR1, CDR2, and CDR3 sequences of a HER2 antibody of cross-block 1, 2, 3, or 4 as defined herein and a VL region comprising CDR1, CDR2, and CDR3 sequences of a HER2 antibody of cross-block 1, 2, 3, or 4 as defined herein, such as SEQ ID NOs: 2, 3, 4, 6, DAS, and 7 (**169**), SEQ ID NOs: 23, 24, 25, 27, AAS, and 28 (**025**), SEQ ID NOs: 64, 65, 66, 68, DAS, and 69 (**153**), or SEQ ID NOs: 166, 167, 168, 170, GAS, and 171 (**005**).

Another embodiment of the present invention relates to a bispecific antibody having a second antigen-binding region comprising a VH region of a CD3 antibody according to SEQ ID NO: 236 (**HUM291**), and a first antigen-binding region of a HER2 antibody of cross-block 1, 2, 3, or 4, such as SEQ ID NOs: 1, 22, 63, or 165 (**169**, **025**, **153**, or **005**).

Another embodiment of the present invention relates to a bispecific antibody having a second antigen-binding region comprising a VH region and a VL region of a CD3 antibody according to SEQ ID NOs: 236 and 237 (**HUM291**), and a first antigen-binding region comprising a VH region and a VL region of a HER2 antibody of cross-block 1, 2, 3, or 4 as defined herein, such as SEQ ID NOs: 1 and 5 (**169**), 22 and 26 (**025**), 63 and 67 (**153**), or 165 and 169 (**005**).

One embodiment of the present invention relates to a bispecific antibody having a second antigen-binding region comprising a VH region comprising a VH CDR3 sequence of a CD3 antibody according to SEQ ID NO: 238 (**huOKT3-C114S-gLC**), and a first antigen-

binding region comprising a VH region of a HER2 antibody of cross-block 1, 2, 3, or 4 as defined herein, such as SEQ ID NOs: 4, 25, 66, or 168 (**169**, **025**, **153**, or **005**).

Another embodiment of the present invention relates to a bispecific antibody having a second antigen-binding region comprising a VH region comprising a CDR3 sequence of a CD3 antibody according to SEQ ID NO: 238 (**huOKT3-C114S-gLC**) and a VL region comprising a CDR3 sequence of a CD3 antibody according to SEQ ID NO: 239 (**huOKT3-C114S-gLC**), and a first antigen-binding region comprising a VH region comprising a CDR3 sequence of a HER2 antibody of cross-block 1, 2, 3, or 4 as defined herein, such as SEQ ID NOs: 4, 25, 66, or 168 (**169**, **025**, **153**, or **005**) and a VL region comprising a CDR3 sequence of a HER2 antibody of cross-block 1, 2, 3, or 4 as defined herein, such as SEQ ID NOs: 7, 28, 69, or 171 (**169**, **025**, **153**, or **005).**

Another embodiment of the present invention relates to a bispecific antibody having a second antigen-binding region comprising a VH region comprising a VH CDR1, CDR2, and CDR3 sequence according to SEQ ID NO: 238 (**huOKT3-C114S-gLC**), and a first antigen-binding region of a HER2 antibody of cross-block 1, 2, 3, or 4 as defined herein, such as SEQ ID NOs: 2, 3, and 4 (**169**), SEQ ID NOs: 23, 24, and 25 (**025**), SEQ ID NOs: 64, 65, and 66 (**153**), or SEQ ID NOs: 166, 167, and 168 (**005**).

Another embodiment of the present invention relates to a bispecific antibody having a second antigen-binding region comprising a VH region comprising CDR1, CDR2, and CDR3 sequences of a CD3 antibody according to SEQ ID NO: 238 (**huOKT3-C114S-gLC**) and a VL region comprising CDR1, CDR2, and CDR3 sequences of a CD3 antibody according to SEQ ID NO: 239 (**huOKT3-C114S-gLC**), and a first antigen-binding region comprising a VH region comprising CDR1, CDR2, and CDR3 sequences of a HER2 antibody of cross-block 1, 2, 3, or 4 as defined herein and a VL region comprising CDR1, CDR2, and CDR3 sequences of a HER2 antibody of cross-block 1, 2, 3, or 4 as defined herein, such as SEQ ID NOs: 2, 3, 4, 6, DAS, and 7 (**169**), SEQ ID NOs: 23, 24, 25, 27, AAS, and 28 (**025**), SEQ ID NOs: 64, 65, 66, 68, DAS, and 69 (**153**), or SEQ ID NOs: 166, 167, 168, 170, GAS, and 171 (**005**).

Another embodiment of the present invention relates to a bispecific antibody having a second antigen-binding region comprising a VH region of a CD3 antibody according to SEQ ID NO: 238 (**huOKT3-C114S-gLC**), and a first antigen-binding region of a HER2 antibody of cross-block 1, 2, 3, or 4, such as SEQ ID NOs: 1, 22, 63, or 165 (**169**, **025**, **153**, or **005**).

Another embodiment of the present invention relates to a bispecific antibody having a second antigen-binding region comprising a VH region and a VL region of a CD3 antibody according to SEQ ID NOs: 238 and 239 (**huOKT3-C114S-gLC**), and a first antigen-binding region comprising a VH region and a VL region of a HER2 antibody of cross-block 1, 2, 3, or

4 as defined herein, such as SEQ ID NOs: 1 and 5 (**169**), 22 and 26 (**025**), 63 and 67 (**153**), or 165 and 169 (**005**).

In an additional or alternative embodiment the bispecific antibody is a HER2xCD3 bispecific antibody induce T cell mediated cytotoxicity of AU565 as described in Example 21, and binds the same epitopes as at least one of the bispecific antibodies selected from the group consisting of huCLB-T3/4 x HER2-169, huCLB-T3/4 x HER2 153, and huCLB-T3/4xHER2 005 described in Example 21.

In a further embodiment, the first and second antigen-binding regions of the bispecific antibody according to the invention comprise human antibody VH sequences and, optionally, human antibody VL sequences.

In a further embodiment, the first and second antigen-binding regions of the bispecific antibody according to the invention the first and second antigen-binding regions are from heavy-chain antibodies.

In a further embodiment, the first and second antigen-binding regions of the bispecific antibody according to the invention the first and second antigen-binding regions comprise a first and second light chain.

In a further embodiment, the first and second antigen-binding regions of the bispecific antibody according to the invention wherein said first and second light chains are different.

Fc regions

In one aspect of the present invention, the bispecific HER2xCD3 antibody according to the present invention further comprises a first Fc region and a second Fc region which may be comprised in a first and a second Fab-arm which respectively further comprise the first and second antigen-binding regions described above (or vice versa).

In another aspect of the present invention, the bispecific HER2xCD3 antibody comprises a first and a second Fab-arm comprising a first and a second antigen-binding region, respectively. Typically, the first and second antigen-binding region is the HER2 binding domain and the CD3 binding domain, respectively. The bispecific HER2xCD3 antibody further comprises a first and a second Fc region, typically comprising a first and a second heavy chain polypeptide, respectively.

In the one aspect of the present invention, the bispecific HER2xCD3 antibody comprises the first Fab-arm comprising the first antigen-binding region and the first Fc

region, and the second Fab-arm comprising the second antigen-binding region and the second Fc region.

In another aspect of the present invention, the bispecific HER2xCD3 antibody comprises the second Fab-arm comprising the second antigen-binding region and the first Fc region, and the first Fab-arm comprising the first antigen-binding region and the second Fc region.

The first and second Fc-regions may each be of any isotype, including, but not limited to, IgG1, IgG2, IgG3 and IgG4, and may comprise one or more mutations or modifications. In one embodiment, each of the first and second Fc regions is of the IgG4 isotype or derived therefrom, optionally with one or more mutations or modifications. In one embodiment, each of the first and second Fc regions is of the IgG1 isotype or derived therefrom, optionally with one or more mutations or modifications. In another embodiment, one of the Fc regions is of the IgG1 isotype and the other of the IgG4 isotype, or is derived from such respective isotype, optionally with one or more mutations or modifications.

In one embodiment, one or both Fc-regions are effector-function-deficient. For example, the Fc-region(s) may be of an IgG4 isotype, or a non-IgG4 type, *e.g.* IgG1, IgG2 or IgG3, which has been mutated such that the ability to mediate effector functions, such as ADCC, has been reduced or even eliminated. Such mutations have *e.g.* been described in Dall'Acqua WF *et al.*, J Immunol. 177(2):1129-1138 (2006) and Hezareh M, J Virol. ;75(24):12161-12168 (2001). Other exemplary modifications are described in the Examples, *e.g.*, in Example 27.

In one embodiment, one or both Fc-regions comprise an IgG1 wildtype sequence (SEQ ID NO:247, see Example 21).

In one embodiment, one or both of the Fc regions comprise a mutation removing the acceptor site for Asn-linked glycosylation or is otherwise manipulated to change the glycosylation properties. For example, in an IgG1 Fc-region, an N297Q mutation can be used to remove an Asn-linked glycosylation site. Accordingly, in a specific embodiment, one or both Fc-regions comprise an IgG1 wildtype sequence with an N297Q mutation (SEQ ID NO:250, see Example 21).

In a further embodiment, one or both of the Fc regions are glyco-engineered to reduce fucose and thus enhance ADCC, *e.g.* by addition of compounds to the culture media during antibody production as described in US2009317869 or as described in van Berkel *et al*. (2010) Biotechnol. Bioeng. 105:350 or by using FUT8 knockout cells, *e.g.* as described in Yamane-Ohnuki *et al* (2004) Biotechnol. Bioeng 87:614. ADCC may alternatively be optimized using the method described by Umaña *et al*. (1999) Nature Biotech 17:176. In a

further embodiment, one or both of the Fc-regions have been engineered to enhance complement activation, *e.g.* as described in Natsume *et al*. (2009) Cancer Sci. 100:2411.

In one embodiment of the invention, the first or second antigen-binding regions or a part thereof, *e.g.* one or more CDRs, are of a species in the family Camelidae, see WO2010001251, or a species of cartilaginous fish, such as the nurse shark. In one embodiment, the first and second antigen-binding regions or heavy chains are from heavy-chain antibodies.

In one embodiment, the first and/or second Fc-region is conjugated to a drug, a prodrug or a toxin or contains an acceptor group for the same. Such acceptor group may *e.g.* be an unnatural amino acid.

In one aspect, the bispecific antibody of the invention comprises a first Fc-region comprising a first CH3 region, and a second Fc-region comprising a second CH3 region, wherein the sequences of the first and second CH3 regions are different and are such that the heterodimeric interaction between said first and second CH3 regions is stronger than each of the homodimeric interactions of said first and second CH3 regions. More details on these interactions and how they can be achieved are provided in PCT/EP2011/056388, published as WO 2011131746 (Genmab), which is hereby incorporated by reference in its entirety.

As described further herein and in the Examples, a stable bispecific HER2xCD3 molecule can be obtained at high yield using a particular method on the basis of one homodimeric starting HER2 antibody and one homodimeric starting CD3 antibody containing only a few, fairly conservative, asymmetrical mutations in the CH3 regions. Asymmetrical mutations mean that the sequences of said first and second CH3 regions contain amino acid substitutions at non-identical positions.

In one embodiment, the first Fc-region has an amino acid substitution at a position selected from the group consisting of: 366, 368, 370, 399, 405, 407 and 409, and the second Fc-region has an amino acid substitution at a position selected from the group consisting of: 366, 368, 370, 399, 405, 407 and 409, and wherein the first and second Fc-regions are not substituted in the same positions.

In one embodiment, the first Fc-region has an amino acid substitution at position 366, and said second Fc-region has an amino acid substitution at a position selected from the group consisting of: 368, 370, 399, 405, 407 and 409. In one embodiment the amino acid at position 366 is selected from Ala, Asp, Glu, His, Asn, Val, or Gln.

In one embodiment, the first Fc-region has an amino acid substitution at position 368, and said second Fc-region has an amino acid substitution at a position selected from the group consisting of: 366, 370, 399, 405, 407 and 409.

In one embodiment, the first Fc-region has an amino acid substitution at position 370, and said second Fc-region has an amino acid substitution at a position selected from the group consisting of: 366, 368, 399, 405, 407 and 409.

In one embodiment, the first Fc-region has an amino acid substitution at position 399, and said second Fc-region has an amino acid substitution at a position selected from the group consisting of: 366, 368, 370, 405, 407 and 409.

In one embodiment, the first Fc-region has an amino acid substitution at position 405, and said second Fc-region has an amino acid substitution at a position selected from the group consisting of: 366, 368, 370, 399, 407 and 409.

In one embodiment, the first Fc-region has an amino acid substitution at position 407, and said second Fc-region has an amino acid substitution at a position selected from the group consisting of: 366, 368, 370, 399, 405, and 409.

In one embodiment, the first Fc-region has an amino acid substitution at position 409, and said second Fc-region has an amino acid substitution at a position selected from the group consisting of: 366, 368, 370, 399, 405, and 407.

Accordingly, in one embodiment, the sequences of said first and second CH3 regions contain asymmetrical mutations, *i.e.* mutations at different positions in the two CH3 regions, *e.g.* a mutation at position 405 in one of the CH3 regions and a mutation at position 409 in the other CH3 region.

In one embodiment, the first Fc-region has an amino acid other than Lys, Leu or Met, *e.g.* Gly, Ala, Val, Ile, Ser, Thr, Phe, Arg, His, Asp, Asn, Glu, Gln, Pro, Trp, Tyr, or Cys, at position 409 and said second Fc-region has an amino-acid substitution at a position selected from the group consisting of: 366, 368, 370, 399, 405 and 407. In one such embodiment, said first Fc-region has an amino acid other than Lys, Leu or Met, *e.g.* Gly, Ala, Val, Ile, Ser, Thr, Phe, Arg, His, Asp, Asn, Glu, Gln, Pro, Trp, Tyr, or Cys, at position 409 and said second Fc-region has an amino acid other than Phe, *e.g.* Gly, Ala, Val, Ile, Ser, Thr, Lys, Arg, His, Asp, Asn, Glu, Gln, Pro, Trp, Tyr, Cys, Lys, or Leu, at position 405. In a further embodiment hereof, said first Fc-region has an amino acid other than Lys, Leu or Met, *e.g.* Gly, Ala, Val, Ile, Ser, Thr, Phe, Arg, His, Asp, Asn, Glu, Gln, Pro, Trp, Tyr, or Cys, at position 409 and said second Fc-region has an amino acid other than Phe, Arg or Gly, *e.g.* Leu, Ala, Val, Ile, Ser, Thr, Met, Lys, His, Asp, Asn, Glu, Gln, Pro, Trp, Tyr, or Cys, at position 405.

In another embodiment, said first Fc-region comprises a Phe at position 405 and an amino acid other than Lys, Leu or Met, *e.g.* Gly, Ala, Val, Ile, Ser, Thr, Phe, Arg, His, Asp, Asn, Glu, Gln, Pro, Trp, Tyr, or Cys, at position 409 and said second Fc-region comprises an

amino acid other than Phe, *e.g.* Gly, Ala, Val, Ile, Ser, Thr, Lys, Arg, His, Asp, Asn, Glu, Gln, Pro, Trp, Tyr, Leu, Met, or Cys, at position 405 and a Lys at position 409. In a further embodiment hereof, said first Fc-region comprises a Phe at position 405 and an amino acid other than Lys, Leu or Met, *e.g.* Gly, Ala, Val, Ile, Ser, Thr, Phe, Arg, His, Asp, Asn, Glu, Gln, Pro, Trp, Tyr, or Cys, at position 409 and said second Fc-region comprises an amino acid other than Phe, Arg or Gly, *e.g.* Leu, Ala, Val, Ile, Ser, Thr, Met, Lys, His, Asp, Asn, Glu, Gln, Pro, Trp, Tyr, or Cys, at position 405 and a Lys at position 409.

In another embodiment, said first Fc-region comprises a Phe at position 405 and an amino acid other than Lys, Leu or Met, *e.g.* Gly, Ala, Val, Ile, Ser, Thr, Phe, Arg, His, Asp, Asn, Glu, Gln, Pro, Trp, Tyr, or Cys, at position 409 and said second Fc-region comprises a Leu at position 405 and a Lys at position 409. In a further embodiment hereof, said first Fc-region comprises a Phe at position 405 and an Arg at position 409 and said second Fc-region comprises an amino acid other than Phe, Arg or Gly, *e.g.* Leu, Ala, Val, Ile, Ser, Thr, Lys, Met, His, Asp, Asn, Glu, Gln, Pro, Trp, Tyr, or Cys, at position 405 and a Lys at position 409. In another embodiment, said first Fc-region comprises Phe at position 405 and an Arg at position 409 and said second Fc-region comprises a Leu at position 405 and a Lys at position 409.

In a further embodiment, said first Fc-region comprises an amino acid other than Lys, Leu or Met, *e.g.* Gly, Ala, Val, Ile, Ser, Thr, Phe, Arg, His, Asp, Asn, Glu, Gln, Pro, Trp, Tyr, or Cys, at position 409 and said second Fc-region comprises a Lys at position 409, a Thr at position 370 and a Leu at position 405. In a further embodiment, said first Fc-region comprises an Arg at position 409 and said second Fc-region comprises a Lys at position 409, a Thr at position 370 and a Leu at position 405.

In an even further embodiment, said first Fc-region comprises a Lys at position 370, a Phe at position 405 and an Arg at position 409 and said second Fc-region comprises a Lys at position 409, a Thr at position 370 and a Leu at position 405.

In another embodiment, said first Fc-region comprises an amino acid other than Lys, Leu or Met, *e.g.* Gly, Ala, Val, Ile, Ser, Thr, Phe, Arg, His, Asp, Asn, Glu, Gln, Pro, Trp, Tyr, or Cys, at position 409 and said second Fc-region comprises a Lys at position 409 and: a) an Ile at position 350 and a Leu at position 405, or b) a Thr at position 370 and a Leu at position 405.

In another embodiment, said first Fc-region comprises an Arg at position 409 and said second HER2 antibody comprises a Lys at position 409 and: a) an Ile at position 350 and a Leu at position 405, or b) a Thr at position 370 and a Leu at position 405.

In another embodiment, said first Fc-region comprises a Thr at position 350, a Lys at position 370, a Phe at position 405 and an Arg at position 409 and said second HER2

antibody comprises a Lys at position 409 and: a) an Ile at position 350 and a Leu at position 405, or b) a Thr at position 370 and a Leu at position 405.

In another embodiment, said first Fc-region comprises a Thr at position 350, a Lys at position 370, a Phe at position 405 and an Arg at position 409 and said second Fc-region comprises an Ile at position 350, a Thr at position 370, a Leu at position 405 and a Lys at position 409.

In another embodiment, said first Fc-region has an amino acid other than Lys, Leu or Met, *e.g.* Gly, Ala, Val, Ile, Ser, Thr, Phe, Arg, His, Asp, Asn, Glu, Gln, Pro, Trp, Tyr, or Cys, at position 409 and said second Fc-region has an amino acid other than Tyr, Asp, Glu, Phe, Lys, Gln, Arg, Ser or Thr, *e.g.* Leu, Met, Gly, Ala, Val, Ile, His, Asn, Pro, Trp, or Cys, at position 407. In another embodiment, said first Fc-region has an amino acid other than Lys, Leu or Met, *e.g.* Gly, Ala, Val, Ile, Ser, Thr, Phe, Arg, His, Asp, Asn, Glu, Gln, Pro, Trp, Tyr, or Cys, at position 409 and said second Fc-region has an Ala, Gly, His, Ile, Leu, Met, Asn, Val or Trp at position 407.

In another embodiment, said first Fc-region has an amino acid other than Lys, Leu or Met, *e.g.* Gly, Ala, Val, Ile, Ser, Thr, Phe, Arg, His, Asp, Asn, Glu, Gln, Pro, Trp, Tyr, or Cys, at position 409 and said second Fc-region has a Gly, Leu, Met, Asn or Trp at position 407.

In another embodiment, said first Fc-region has a Tyr at position 407 and an amino acid other than Lys, Leu or Met, *e.g.* Gly, Ala, Val, Ile, Ser, Thr, Phe, Arg, His, Asp, Asn, Glu, Gln, Pro, Trp, Tyr, or Cys, at position 409 and said second Fc-region has an amino acid other than Tyr, Asp, Glu, Phe, Lys, Gln, Arg, Ser or Thr, *e.g.* Leu, Met, Gly, Ala, Val, Ile, His, Asn, Pro, Trp, or Cys, at position 407 and a Lys at position 409.

In another embodiment, said first Fc-region has a Tyr at position 407 and an amino acid other than Lys, Leu or Met, *e.g.* Gly, Ala, Val, Ile, Ser, Thr, Phe, Arg, His, Asp, Asn, Glu, Gln, Pro, Trp, Tyr, or Cys, at position 409 and said second Fc-region has an Ala, Gly, His, Ile, Leu, Met, Asn, Val or Trp at position 407 and a Lys at position 409.

In another embodiment, said first Fc-region has a Tyr at position 407 and an amino acid other than Lys, Leu or Met, *e.g.* Gly, Ala, Val, Ile, Ser, Thr, Phe, Arg, His, Asp, Asn, Glu, Gln, Pro, Trp, Tyr, or Cys, at position 409 and said second Fc-region has a Gly, Leu, Met, Asn or Trp at position 407 and a Lys at position 409.

In another embodiment, said first Fc-region has a Tyr at position 407 and an Arg at position 409 and said second Fc-region has an amino acid other than Tyr, Asp, Glu, Phe, Lys, Gln, Arg, Ser or Thr, *e.g.* Leu, Met, Gly, Ala, Val, Ile, His, Asn, Pro, Trp, or Cys, at position 407 and a Lys at position 409.

In another embodiment, said first Fc-region has a Tyr at position 407 and an Arg at position 409 and said second Fc-region has an Ala, Gly, His, Ile, Leu, Met, Asn, Val or Trp at position 407 and a Lys at position 409.

In another embodiment, said first Fc-region has a Tyr at position 407 and an Arg at position 409 and said second Fc-region has a Gly, Leu, Met, Asn or Trp at position 407 and a Lys at position 409.

In one embodiment, the first Fc-region has an amino acid other than Lys, Leu or Met, *e.g.* Gly, Ala, Val, Ile, Ser, Thr, Phe, Arg, His, Asp, Asn, Glu, Gln, Pro, Trp, Tyr, or Cys, at position 409, and the second Fc-region has

(i) an amino acid other than Phe, Leu and Met, *e.g.* Gly, Ala, Val, Ile, Ser, Thr, Lys, Arg, His, Asp, Asn, Glu, Gln, Pro, Trp, Tyr, or Cys, at position 368, or

(ii) a Trp at position 370, or

(iii) an amino acid other than Asp, Cys, Pro, Glu or Gln, *e.g.* Phe, Leu, Met, Gly, Ala, Val, Ile, Ser, Thr, Lys, Arg, His, Asn, Trp, Tyr, or Cys, at position 399 or

(iv) an amino acid other than Lys, Arg, Ser, Thr, or Trp, *e.g.* Phe, Leu, Met, Ala, Val, Gly, Ile, Asn, His, Asp, Glu, Gln, Pro, Tyr, or Cys, at position 366.

In one embodiment, the first Fc-region has an Arg, Ala, His or Gly at position 409, and the second homodimeric protein has

(i) a Lys, Gln, Ala, Asp, Glu, Gly, His, Ile, Asn, Arg, Ser, Thr, Val, or Trp at position 368, or

(ii) a Trp at position 370, or

(iii) an Ala, Gly, Ile, Leu, Met, Asn, Ser, Thr, Trp, Phe, His, Lys, Arg or Tyr at position 399, or

(iv) an Ala, Asp, Glu, His, Asn, Val, Gln, Phe, Gly, Ile, Leu, Met, or Tyr at position 366.

In one embodiment, the first Fc-region has an Arg at position 409, and the second homodimeric protein has

(i) an Asp, Glu, Gly, Asn, Arg, Ser, Thr, Val, or Trp at position 368, or

(ii) a Trp at position 370, or

(iii) a Phe, His, Lys, Arg or Tyr at position 399, or

(iv) an Ala, Asp, Glu, His, Asn, Val, Gln at position 366.

In addition to the above-specified amino-acid substitutions, said first and second homodimeric protein may contain further amino-acid substitutions, deletion or insertions relative to wild-type Fc sequences.

In a further embodiment, said first and second Fab-arms (or heavy-chain constant domains) comprising the first and second Fc regions comprise, except for the specified mutations, a sequence independently selected from the following:
(IgG1m(a)):
GQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKL TVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK (SEQ ID NO:256)
(IgG1m(f)):
GQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSK LTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK (SEQ ID NO:257), and
(IgG1m(ax)):
GQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKL TVDKSRWQQGNVFSCSVMHEGLHNHYTQKSLSLSPGK (SEQ ID NO:258).

In one embodiment, neither said first nor said second Fc-region comprises a Cys-Pro-Ser-Cys sequence in the (core) hinge region.

In a further embodiment, both said first and said second Fc-region comprise a Cys-Pro-Pro-Cys sequence in the (core) hinge region.

In separate and specific embodiments, one or both Fab arms comprise a heavy-chain constant region sequence independently selected from SEQ ID NO: 247, 248, 249, 250, 251, 252, 253, 254, and 255 (see Example 21).

In one particular example, the CD3 antibody is an antibody with a VH region comprising the sequence of SEQ ID NO:234 (VH YTH12.5) and VL region comprising the sequence of SEQ ID NO:235 (VL YTH12.5). Another example is a CD3 antibody with a VH region comprising the sequence of SEQ ID NO:240 (VH huCLB-T3/4) and VL region comprising the sequence of SEQ ID NO:241 (VL huCLB-T3/4).

In one embodiment, the bispecific antibody comprises (i) a first Fab-arm comprising an Fc region and VH and VL sequences, wherein the VH region comprises the amino acid sequence of SEQ ID NO:63, and the VL region comprises the amino acid sequence of SEQ ID NO:67 (**153**), optionally wherein the first Fab-arm comprises an IgG1,κ Fc region having Arg at position 409, or Gln at position 297, or Arg at position 409 and Gln at position 297; and (ii) a second Fab-arm having an Fc region and VH and VL sequences, wherein the VH region comprises the amino acid sequence of SEQ ID NO:171 and the VL region comprises the amino acid sequence of SEQ ID NO:172 (YTH12.5), optionally wherein the second Fab-arm comprises an IgG1,κ Fc region having an Gln at position 297, or Leu at position 405, or Gln at position 297 and Leu at position 405.

In one embodiment, the bispecific antibody comprises (i) a first Fab-arm having an Fc region and VH and VL sequences, wherein the VH region comprises the amino acid sequence of SEQ ID NO:1, and the VL region comprises the amino acid sequence of SEQ ID NO:5 (**169**), optionally wherein the first Fab-arm comprises an IgG1,κ Fc region having Arg at position 409; and (ii) a second Fab-arm having an Fc region and VH and VL sequences, wherein the VH region comprises the amino acid sequence of SEQ ID NO:171 and the VL region comprises the amino acid sequence of SEQ ID NO:172 (YTH12.5), optionally wherein the second Fab-arm comprises an IgG1,κ Fc region having an Gln at position 297, or Leu at position 405, or Gln at position 297 and Leu at position 405.

In one embodiment, the bispecific antibody comprises (i) a first Fab-arm having an Fc region and VH and VL sequences, wherein the VH region comprises the amino acid sequence of SEQ ID NO:63, and the VL region comprises the amino acid sequence of SEQ ID NO:67 (**153**), optionally wherein the first Fab-arm comprises an IgG1,κ Fc region having Arg at position 409, or Gln at position 297, or Arg at position 409 and Gln at position 297; and (ii) a second Fab-arm having an Fc region and VH and VL sequences ,wherein the VH region comprises the amino acid sequence of SEQ ID NO:173 and the VL region comprises the amino acid sequence of SEQ ID NO:174 (huCLB-T3/4), optionally wherein the second Fab-arm comprises an IgG1,κ Fc region having an Gln at position 297, or Leu at position 405, or Gln at position 297 and Leu at position 405.

In one embodiment, the bispecific antibody comprises (i) a first Fab-arm having an Fc region and VH and VL sequences, wherein the VH region comprises the amino acid sequence of SEQ ID NO:1, and the VL region comprises the amino acid sequence of SEQ ID NO:5 (**169**), optionally wherein the first Fab-arm comprises an IgG1,κ Fc region having Arg at position 409; and (ii) a second Fab-arm having an Fc region and VH and VL sequences, wherein the VH region comprises the amino acid sequence of SEQ ID NO:173 and the VL region comprises the amino acid sequence of SEQ ID NO:174 (huCLB-T3/4), optionally wherein the second Fab-arm comprises an IgG1,κ Fc region having an Gln at position 297, or Leu at position 405, or Gln at position 297 and Leu at position 405.

In any of the above embodiments, the first and/or second Fab-arm may further comprise CH1 and/or CL sequences.

In one embodiment the bispecific antibody is selected from the group consisting of: IgG1-HER2-153-K409R x IgG1-YTH12.5-F405L, IgG1-HER2-153-K409R x IgG1-YTH12.5-N297Q-F405L, IgG1-HER2-153-K409R x IgG1-hu-CLB-T3/4-F405L, IgG1-HER2-153-K409R x IgG1-hu-CLB-T3/4-N297Q-F405L, IgG1-HER2-153-N297Q-K409R x IgG1-YTH12.5-F405L, IgG1-HER2-153-N297Q-K409R x IgG1-YTH12.5-N297Q-F405L, IgG1- HER2-153-N297Q-K409R x IgG1-hu-CLB-T3/4-F405L, IgG1- HER2-153-N297Q-K409R x IgG1-hu-CLB-T3/4-

N297Q-F405L, IgG1-HER2-169-K409R x IgG1-hu-CLB-T3/4-F405L, IgG1-HER2-169-K409R x IgG1-hu-CLB-T3/4-N297Q-F405L, IgG1-HER2-169-K409R x IgG1-YTH12.5-F405L and IgG1-HER2-169-K409R x IgG1-YTH12.5-N297Q-F405L, wherein ITL means IgG1,κ having Ile at position 350, Thr at position 370, and Leu at position 405, K409R means IgG1,κ having an Arg at position 409, and F405L means IgG1,κ having a Leu at position 405, N297Q means a Gln at position 297, and wherein the bold numbers refer to antibodies described herein with the VH and VL regions comprising the sequences described in Table 1 and Example 21.

In an additional embodiment, the bispecific antibody induces dose-dependent killing of AU565, NIH-3T3, A431 and A549 cells when determined as described in Example 29, and binds the same epitopes as the bispecific antibody IgG1-HER2-169xIgG1-CLBT3/4.

Bispecific antibody formats

The present invention provides bispecific HER2xCD3 antibodies which efficiently promote T cell-mediated killing of HER2-expressing tumor cells. Depending on the desired functional properties for a particular use, particular antigen-binding regions can be selected from the set of antibodies or antigen-binding regions provided by the present invention or from those antibodies or antigen-binding regions sharing, *e.g.*, an epitope or cross-blocking region with the antibodies or antigen-binding regions provided by the present invention. Many different formats and uses of bispecific antibodies are known in the art, and were recently been reviewed by Chames and Baty (2009) Curr Opin Drug Disc Dev 12: 276.

Exemplary bispecific antibody molecules of the invention comprise (i) a single antibody that has two arms comprising different antigen-binding regions, one with a specificity to a HER2 epitope and one with a specificity to CD3, (ii) a single antibody that has one antigen-binding region or arm specific to a HER2 epitope and a second antigen-binding region or arm specific to a CD3 epitope, (iii) a single chain antibody that has a first specificity to a HER2 epitope and a second specificity to a CD3 epitope, *e.g.*, via two scFvs linked in tandem by an extra peptide linker; (iv) a dual-variable-domain antibody (DVD-Ig), where each light chain and heavy chain contains two variable domains in tandem through a short peptide linkage (Wu *et al.*, Generation and Characterization of a Dual Variable Domain Immunoglobulin (DVD-Ig™) Molecule, In: Antibody Engineering, Springer Berlin Heidelberg (2010)); (v) a chemically-linked bispecific $(Fab')_2$ fragment; (vi) a Tandab, which is a fusion of two single chain diabodies resulting in a tetravalent bispecific antibody that has two binding sites for each of the target antigens; (vii) a flexibody, which is a combination of scFvs with a diabody resulting in a multivalent molecule; (viii) a so called "dock and lock"

molecule, based on the "dimerization and docking domain" in Protein Kinase A, which, when applied to Fabs, can yield a trivalent bispecific binding protein consisting of two identical Fab fragments linked to a different Fab fragment; (ix) a so-called Scorpion molecule, comprising, *e.g.*, two scFvs fused to both termini of a human Fab-arm; and (x) a diabody.

In one embodiment, the bispecific antibody of the present invention is a diabody, a cross-body, or a bispecific antibody obtained via a controlled Fab arm exchange as those described in the present invention.

Examples of different classes of bispecific antibodies include but are not limited to

- IgG-like molecules with complementary CH3 domains to force heterodimerisation
- recombinant IgG-like dual targeting molecules, wherein the two sides of the molecule each contain the Fab fragment or part of the Fab fragment of at least two different antibodies;
- IgG fusion molecules, wherein full length IgG antibodies are fused to extra Fab fragment or parts of Fab fragment;
- Fc fusion molecules, wherein single chain Fv molecules or stabilized diabodies are fused to heavy-chain constant-domains, Fc-regions or parts thereof;
- Fab fusion molecules, wherein different Fab-fragments are fused together;
- ScFv-and diabody-based and heavy chain antibodies (*e.g.*, domain antibodies, nanobodies) wherein different single chain Fv molecules or different diabodies or different heavy-chain antibodies (*e.g.* domain antibodies, nanobodies) are fused to each other or to another protein or carrier molecule.

Examples of IgG-like molecules with complementary CH3 domains molecules include but are not limited to the Triomab/Quadroma (Trion Pharma/Fresenius Biotech), the Knobs-into-Holes (Genentech), CrossMAbs (Roche) and the electrostatically-matched (Amgen), the LUZ-Y (Genentech), the Strand Exchange Engineered Domain body (SEEDbody)(EMD Serono), the Biclonic (Merus) and the DuoBody (Genmab A/S).

Examples of recombinant IgG-like dual targeting molecules include but are not limited to Dual Targeting (DT)-Ig (GSK/Domantis), Two-in-one Antibody (Genentech), Cross-linked Mabs (Karmanos Cancer Center), mAb^2 (F-Star) and CovX-body (CovX/Pfizer).

Examples of IgG fusion molecules include but are not limited to Dual Variable Domain (DVD)-Ig (Abbott), IgG-like Bispecific (ImClone/Eli Lilly), Ts2Ab (MedImmune/AZ) and BsAb (Zymogenetics), HERCULES (Biogen Idec) and TvAb (Roche).

Examples of Fc fusion molecules include but are not limited to ScFv/Fc Fusions (Academic Institution), SCORPION (Emergent BioSolutions/Trubion, Zymogenetics/BMS),

Dual Affinity Retargeting Technology (Fc-DART) (MacroGenics) and Dual(ScFv)$_2$-Fab (National Research Center for Antibody Medicine – China).

Examples of Fab fusion bispecific antibodies include but are not limited to F(ab)$_2$ (Medarex/AMGEN), Dual-Action or Bis-Fab (Genentech), Dock-and-Lock (DNL) (ImmunoMedics), Bivalent Bispecific (Biotecnol) and Fab-Fv (UCB-Celltech).

Examples of ScFv-, diabody-based and domain antibodies include but are not limited to Bispecific T Cell Engager (BiTE) (Micromet, Tandem Diabody (Tandab) (Affimed), Dual Affinity Retargeting Technology (DART) (MacroGenics), Single-chain Diabody (Academic), TCR-like Antibodies (AIT, ReceptorLogics), Human Serum Albumin ScFv Fusion (Merrimack) and COMBODY (Epigen Biotech), dual targeting nanobodies (Ablynx), dual targeting heavy chain only domain antibodies.

Methods of preparing bispecific antibodies

Methods of preparing bispecific antibodies of the present invention include those described in WO 2008119353 (Genmab), WO 2011131746 (Genmab) and reported by van der Neut-Kolfschoten *et al*. (Science. 2007 Sep 14;317(5844):1554-7). Examples of other platforms useful for preparing bispecific antibodies include but are not limited to BiTE (Micromet), DART (MacroGenics), Fcab and Mab^2 (F-star) , Fc-engineered IgG1 (Xencor) or DuoBody (based on Fab arm exchange, Genmab, this application, described below and in, *e.g.*, Example 20).

Traditional methods such as the hybrid hybridoma and chemical conjugation methods (Marvin and Zhu (2005) Acta Pharmacol Sin 26:649) can also be used. Co-expression in a host cell of two antibodies, consisting of different heavy and light chains, leads to a mixture of possible antibody products in addition to the desired bispecific antibody, which can then be isolated by, *e.g.*, affinity chromatography or similar methods.

Strategies favoring the formation of a functional bispecific, product, upon co-expression of different antibody constructs can also be used, *e.g.*, the method described by Lindhofer *et al*. (1995 J Immunol 155:219). Fusion of rat and mouse hydridomas producing different antibodies leads to a limited number of heterodimeric proteins because of preferential species-restricted heavy/light chain pairing. Another strategy to promote formation of heterodimers over homodimers is a "knob-into-hole" strategy in which a protuberance is introduced on a first heavy-chain polypeptide and a corresponding cavity in a second heavy-chain polypeptide, such that the protuberance can be positioned in the cavity at the interface of these two heavy chains so as to promote heterodimer formation and hinder homodimer formation. "Protuberances" are constructed by replacing small amino-acid side-chains from the interface of the first polypeptide with larger side chains.

Compensatory "cavities" of identical or similar size to the protuberances are created in the interface of the second polypeptide by replacing large amino-acid side-chains with smaller ones (US patent 5,731,168). EP1870459 (Chugai) and WO 2009089004 (Amgen) describe other strategies for favoring heterodimer formation upon co-expression of different antibody domains in a host cell. In these methods, one or more residues that make up the CH3-CH3 interface in both CH3 domains are replaced with a charged amino acid such that homodimer formation is electrostatically unfavorable and heterodimerization is electrostatically favorable. WO2007110205 (Merck) describe yet another strategy, wherein differences between IgA and IgG CH3 domains are exploited to promote heterodimerization.

Another *in vitro* method for producing bispecific antibodies has been described in WO 2008119353 (Genmab), wherein a bispecific antibody is formed by "Fab-arm" or "half-molecule" exchange (swapping of a heavy chain and attached light chain) between two monospecific IgG4- or IgG4-like antibodies upon incubation under reducing conditions. The resulting product is a bispecific antibody having two Fab arms which may comprise different sequences.

A preferred method for preparing bispecific HER2xCD3 antibodies of the present invention includes the method described in WO 2011131746 (Genmab) comprising the following steps:

a) providing a first antibody comprising an Fc region , said Fc region comprising a first CH3 region;
b) providing a second antibody comprising a second Fc region , said Fc region comprising a second CH3 region, wherein the first antibody is a HER2 antibody and the second antibody is a CD3 antibody, or vice versa;

wherein the sequences of said first and second CH3 regions are different and are such that the heterodimeric interaction between said first and second CH3 regions is stronger than each of the homodimeric interactions of said first and second CH3 regions;

c) incubating said first antibody together with said second antibody under reducing conditions; and
d) obtaining said bispecific HER2xCD3 antibody.

Without being limited to theory, in step c), the heavy-chain disulfide bonds in the hinge regions of the parent antibodies are reduced and the resulting cysteines are then able to form inter heavy-chain disulfide bond with cysteine residues of another parent antibody molecule (originally with a different specificity). In one embodiment of this method, the reducing conditions in step c) comprise the addition of a reducing agent, *e.g.* a reducing

agent selected from the group consisting of: 2-mercaptoethylamine (2-MEA), dithiothreitol (DTT), dithioerythritol (DTE), glutathione, tris(2-carboxyethyl)phosphine (TCEP), L-cysteine and beta-mercapto-ethanol, preferably a reducing agent selected from the group consisting of: 2-mercaptoethylamine, dithiothreitol and tris(2-carboxyethyl)phosphine. In a further embodiment, step c) comprises restoring the conditions to become non-reducing or less reducing, for example by removal of a reducing agent, *e.g.* by desalting.

Typically, in this method, the first and second antibodes are a HER2 and CD3 antibody binding to epitopes of HER2 and CD3, respectively, and/or comprising different antigen-binding sequences of HER2 and CD3, respectively.

For this method any of the HER2 and CD3 antibodes described above may be used including first and second HER2 and CD3 antibodies, respectively, comprising a first and/or second Fc regions. Examples of such first and second Fc regions, including combination of such first and second Fc regions may include any of thos described above. In a particular embodiment the first and second HER2 and CD3 antibodies, respectively, may be chosen so as to obtain a bispecific antibody as described herein.

In one embodiment of this method, said first and/or second antibodies are full-length antibodies.

The Fc regions of the first and second antibodies may be of any isotype, including, but not limited to, IgG1, IgG2, IgG3 or IgG4. In one embodiment of this method, the Fc regions of both said first and said second antibodies are of the IgG1 isotype. In another embodiment, one of the Fc regions of said antibodies is of the IgG1 isotype and the other of the IgG4 isotype. In the latter embodiment, the resulting bispecific antibody comprises an Fc region of an IgG1 and an Fc region of IgG4 and may thus have interesting intermediate properties with respect to activation of effector functions. A similar product can be obtained if said first and/or said second antibody comprises a mutation removing the acceptor site for Asn-linked glycosylation or is otherwise manipulated to change the glycosylation properties.

In a further embodiment of this method, one or both of the antibodies is glyco-engineered to reduce fucose and thus enhance ADCC, *e.g.* by addition of compounds to the culture media during antibody production as described in US2009317869 or as described in van Berkel *et al*. (2010) Biotechnol. Bioeng. 105:350 or by using FUT8 knockout cells, *e.g.* as described in Yamane-Ohnuki *et al* (2004) Biotechnol. Bioeng 87:614. ADCC may alternatively be optimized using the method described by Umaña *et al*. (1999) Nature Biotech 17:176. In a further embodiment, one or both of the antibodies have been engineered to enhance complement activation, *e.g.* as described in Natsume *et al*. (2009) Cancer Sci. 100:2411.

In a further embodiment of this method, one or both of the antibodies have been engineered to reduce or increase the binding to the neonatal Fc receptor (FcRn) in order to manipulate the serum half-life of the heterodimeric protein. In a further embodiment, one of the antibody starting proteins has been engineered to not bind Protein A, thus allowing to separate the heterodimeric protein from said homodimeric starting protein by passing the product over a protein A column.

In a particular embodiment of this method, the antibody or a part thereof, *e.g.* one or more CDRs, is of a species in the family Camelidae, see WO2010001251, or a species of cartilaginous fish, such as the nurse shark, or is a heavy-chain or domain antibody.

In one embodiment, the first and/or second HER2 antibody is conjugated to a drug, a prodrug or a toxin or contains an acceptor group for the same. Such acceptor group may *e.g.* be an unnatural amino acid.

As described above, the sequences of the first and second CH3 regions of the homodimeric starting antibodies are different and are such that the heterodimeric interaction between said first and second CH3 regions is stronger than each of the homodimeric interactions of said first and second CH3 regions. More details on these interactions and how they can be achieved are provided in PCT/EP2011/056388, published as WO 2011131746 (Genmab), which is hereby incorporated by reference in its entirety.

In particular, a stable bispecific HER2xCD3 molecule can be obtained at high yield using the above method of the invention on the basis of two homodimeric starting antibodies which bind HER2 and CD3, respectively, and contain only a few, fairly conservative, asymmetrical mutations in the CH3 regions. Asymmetrical mutations mean that the sequences of said first and second CH3 regions contain amino acid substitutions at non-identical positions.

In one embodiment of this method, the first antibody has an amino acid substitution at a position selected from the group consisting of: 366, 368, 370, 399, 405, 407 and 409, and the second antibody has an amino acid substitution at a position selected from the group consisting of: 366, 368, 370, 399, 405, 407 and 409, and wherein the first and second antibodies are not substituted in the same positions.

In one embodiment of this method, the first antibody has an amino acid substitution at position 366, and said second antibody has an amino acid substitution at a position selected from the group consisting of: 368, 370, 399, 405, 407 and 409. In one embodiment the amino acid at position 366 is selected from Ala, Asp, Glu, His, Asn, Val, or Gln.

In one embodiment of this method, the first antibody protein has an amino acid substitution at position 368, and said second antibody has an amino acid substitution at a position selected from the group consisting of: 366, 370, 399, 405, 407 and 409.

In one embodiment of this method, the first antibody has an amino acid substitution at position 370, and said second antibody has an amino acid substitution at a position selected from the group consisting of: 366, 368, 399, 405, 407 and 409.

In one embodiment of this method, the first antibody has an amino acid substitution at position 399, and said second antibody has an amino acid substitution at a position selected from the group consisting of: 366, 368, 370, 405, 407 and 409.

In one embodiment of this method, the first antibody has an amino acid substitution at position 405, and said second antibody has an amino acid substitution at a position selected from the group consisting of: 366, 368, 370, 399, 407 and 409.

In one embodiment of this method, the first antibody has an amino acid substitution at position 407, and said second antibody has an amino acid substitution at a position selected from the group consisting of: 366, 368, 370, 399, 405, and 409.

In one embodiment of this method, the first antibody has an amino acid substitution at position 409, and said second antibody has an amino acid substitution at a position selected from the group consisting of: 366, 368, 370, 399, 405, and 407.

Accordingly, in one embodiment of this method, the sequences of said first and second CH3 regions contain asymmetrical mutations, *i.e.* mutations at different positions in the two CH3 regions, *e.g.* a mutation at position 405 in one of the CH3 regions and a mutation at position 409 in the other CH3 region.

In one embodiment of this method, the first antibody has an amino acid other than Lys, Leu or Met at position 409 and said second antibody has an amino-acid substitution at a position selected from the group consisting of: 366, 368, 370, 399, 405 and 407. In one such embodiment, said first antibody has an amino acid other than Lys, Leu or Met at position 409 and said second antibody has an amino acid other than Phe at position 405. In a further embodiment hereof, said first antibody has an amino acid other than Lys, Leu or Met at position 409 and said second antibody has an amino acid other than Phe, Arg or Gly at position 405.

In another embodiment of this method, said first antibody comprises a Phe at position 405 and an amino acid other than Lys, Leu or Met at position 409 and said second antibody comprises an amino acid other than Phe at position 405 and a Lys at position 409. In a further embodiment hereof, said first antibody comprises a Phe at position 405 and an

amino acid other than Lys, Leu or Met at position 409 and said second antibody comprises an amino acid other than Phe, Arg or Gly at position 405 and a Lys at position 409.

In another embodiment of this method, said first antibody comprises a Phe at position 405 and an amino acid other than Lys, Leu or Met at position 409 and said second antibody comprises a Leu at position 405 and a Lys at position 409. In a further embodiment hereof, said first antibody comprises a Phe at position 405 and an Arg at position 409 and said second antibody comprises an amino acid other than Phe, Arg or Gly at position 405 and a Lys at position 409. In another embodiment, said first antibody comprises Phe at position 405 and an Arg at position 409 and said second antibody comprises a Leu at position 405 and a Lys at position 409.

In a further embodiment of this method, said first antibody comprises an amino acid other than Lys, Leu or Met at position 409 and said second homodimeric protein comprises a Lys at position 409, a Thr at position 370 and a Leu at position 405. In a further embodiment, said first homodimeric protein comprises an Arg at position 409 and said second homodimeric protein comprises a Lys at position 409, a Thr at position 370 and a Leu at position 405.

In an even further embodiment of this method, said first antibody comprises a Lys at position 370, a Phe at position 405 and an Arg at position 409 and said second antibody comprises a Lys at position 409, a Thr at position 370 and a Leu at position 405.

In another embodiment of this method, said first antibody comprises an amino acid other than Lys, Leu or Met at position 409 and said second antibody comprises a Lys at position 409 and: a) an Ile at position 350 and a Leu at position 405, or b) a Thr at position 370 and a Leu at position 405.

In another embodiment of this method, said first antibody comprises an Arg at position 409 and said second antibody comprises a Lys at position 409 and: a) an Ile at position 350 and a Leu at position 405, or b) a Thr at position 370 and a Leu at position 405.

In another embodiment of this method, said first antibody comprises a Thr at position 350, a Lys at position 370, a Phe at position 405 and an Arg at position 409 and said second antibody comprises a Lys at position 409 and: a) an Ile at position 350 and a Leu at position 405, or b) a Thr at position 370 and a Leu at position 405.

In another embodiment of this method, said first antibody comprises a Thr at position 350, a Lys at position 370, a Phe at position 405 and an Arg at position 409 and said second comprises an Ile at position 350, a Thr at position 370, a Leu at position 405 and a Lys at position 409.

In another embodiment of this method, said first antibody has an amino acid other than Lys, Leu or Met at position 409 and said second antibody has an amino acid other than Tyr, Asp, Glu, Phe, Lys, Gln, Arg, Ser or Thr at position 407. In another embodiment, said first antibody has an amino acid other than Lys, Leu or Met at position 409 and said second antibody has an Ala, Gly, His, Ile, Leu, Met, Asn, Val or Trp at position 407.

In another embodiment of this method, said first antibody has an amino acid other than Lys, Leu or Met at position 409 and said second antibody has a Gly, Leu, Met, Asn or Trp at position 407.

In another embodiment of this method, said first antibody has a Tyr at position 407 and an amino acid other than Lys, Leu or Met at position 409 and said second antibody has an amino acid other than Tyr, Asp, Glu, Phe, Lys, Gln, Arg, Ser or Thr at position 407 and a Lys at position 409.

In another embodiment of this method, said first antibody has a Tyr at position 407 and an amino acid other than Lys, Leu or Met at position 409 and said second antibody has an Ala, Gly, His, Ile, Leu, Met, Asn, Val or Trp at position 407 and a Lys at position 409.

In another embodiment of this method, said first antibody has a Tyr at position 407 and an amino acid other than Lys, Leu or Met at position 409 and said second antibody has a Gly, Leu, Met, Asn or Trp at position 407 and a Lys at position 409.

In another embodiment of this method, said first antibody has a Tyr at position 407 and an Arg at position 409 and said second antibody has an amino acid other than Tyr, Asp, Glu, Phe, Lys, Gln, Arg, Ser or Thr at position 407 and a Lys at position 409.

In another embodiment of this method, said first antibody has a Tyr at position 407 and an Arg at position 409 and said second antibody has an Ala, Gly, His, Ile, Leu, Met, Asn, Val or Trp at position 407 and a Lys at position 409.

In another embodiment of this method, said first antibody has a Tyr at position 407 and an Arg at position 409 and said second antibody has a Gly, Leu, Met, Asn or Trp at position 407 and a Lys at position 409.

In one embodiment of this method, the first antibody has an amino acid other than Lys, Leu or Met at position 409, and the second antibody has

(i) an amino acid other than Phe, Leu and Met at position 368, or

(ii) a Trp at position 370, or

(iii) an amino acid other than Asp, Cys, Pro, Glu or Gln at position 399, or

(iv) an amino acid other than Lys, Arg, Ser, Thr, or Trp at position 366.

In one embodiment of this method, the first homodimeric protein has an Arg, Ala, His or Gly at position 409, and the second homodimeric protein has

(i) a Lys, Gln, Ala, Asp, Glu, Gly, His, Ile, Asn, Arg, Ser, Thr, Val, or Trp at position 368, or

(ii) a Trp at position 370, or

(iii) an Ala, Gly, Ile, Leu, Met, Asn, Ser, Thr, Trp, Phe, His, Lys, Arg or Tyr at position 399, or

(iv) an Ala, Asp, Glu, His, Asn, Val, Gln, Phe, Gly, Ile, Leu, Met, or Tyr at position 366.

In one embodiment of this method, the first homodimeric protein has an Arg at position 409, and the second homodimeric protein has

(i) an Asp, Glu, Gly, Asn, Arg, Ser, Thr, Val, or Trp at position 368, or

(ii) a Trp at position 370, or

(iii) a Phe, His, Lys, Arg or Tyr at position 399, or

(iv) an Ala, Asp, Glu, His, Asn, Val, Gln at position 366.

In addition to the above-specified amino-acid substitutions, said first and second homodimeric protein may contain further amino-acid substitutions, deletion or insertions relative to wild-type Fc sequences.

In a further embodiment, said first and second CH3 regions, except for the specified mutations, comprise the sequences of IgG1m(a) (SEQ ID NO:256), IgG1m(f) (SEQ ID NO:257), or IgG1m(ax) (SEQ ID NO:258).

Thus, in one embodiment, neither said first nor said second antibody comprises a Cys-Pro-Ser-Cys sequence in the (core) hinge region.

In a further embodiment, both said first and said second antibody comprise a Cys-Pro-Pro-Cys sequence in the (core) hinge region.

The bispecific antibodies of the invention may also be obtained by co-expression of constructs encoding the first and second polypeptides in a single cell. Thus, in a further aspect, the invention relates to a method for producing a bispecific antibody, said method comprising the following steps:

a) providing a first nucleic-acid construct encoding a first polypeptide comprising a first Fc region and a first antigen-binding region of a first antibody heavy chain, said first Fc region comprising a first CH3 region,

b) providing a second nucleic-acid construct encoding a second polypeptide comprising a second Fc region and a second antigen-binding region of a second antibody heavy chain, said second Fc region comprising a first CH3 region,

wherein the sequences of said first and second CH3 regions are different and are such that the heterodimeric interaction between said first and second CH3 regions is

stronger than each of the homodimeric interactions of said first and second CH3 regions, and

wherein said first homodimeric protein has an amino acid other than Lys, Leu or Met at position 409 and said second homodimeric protein has an amino-acid substitution at a position selected from the group consisting of: 366, 368, 370, 399, 405 and 407,

optionally wherein said first and second nucleic acid constructs encode light chain sequences of said first and second antibodies

c) co-expressing said first and second nucleic-acid constructs in a host cell, and

d) obtaining said heterodimeric protein from the cell culture.

Thus, the present invention also relates to a recombinant eukaryotic or prokaryotic host cell which produces a bispecific antibody of the present invention.
In one embodiment of the present invention, the bispecific antibody is obtained by any of the methods according to the present invention.

Suitable expression vectors, including promoters, enhancers, etc., and suitable host cells for the production of antibodies are well-known in the art. Examples of host cells include yeast, bacterial and mammalian cells, such as CHO or HEK cells.

In one embodiment of this method, said first CH3 region has an amino acid other than Lys, Leu or Met at position 409 and said second CH3 region has an amino acid other than Phe at position 405.

In another embodiment of this method, said first CH3 region has an amino acid other than Lys, Leu or Met at position 409 and said second CH3 region has an amino acid other than Phe at position 405, such as other than Phe, Arg or Gly at position 405; or said first CH3 region has an amino acid other than Lys, Leu or Met at position 409 and said second CH3 region has an amino acid other than Tyr, Asp, Glu, Phe, Lys, Gln, Arg, Ser or Thr at position 407.

In some embodiments, said first and second polypeptides are full-length heavy chains of two antibodies that bind different epitopes (*i.e.* said first and second nucleic-acid constructs encode full-length heavy chains of two antibodies that bind different epitopes), and thus the heterodimeric protein is a bispecific antibody. This bispecific antibody can be a heavy-chain antibody, or said host cell may further express one or more nucleic-acid constructs encoding a light-chain. If only one light-chain construct is co-expressed with the heavy chain constructs, then a functional bispecific antibody is only formed if the light chain sequence is such that it can form a functional antigen-binding domain with each of the heavy chains. If two or more different light-chain constructs are co-expressed with the heavy chain, multiple products will be formed.

In further embodiments, the co-expression method according to the invention comprises any of the further features described under the *in vitro* method above.
In a further aspect, the invention relates to an expression vector comprising the first and second nucleic-acid constructs specified herein above. In a further embodiment, the expression vector further comprises a nucleotide sequence encoding the constant region of a light chain, a heavy chain or both light and heavy chains of an antibody, *e.g.* a human antibody.

An expression vector in the context of the present invention may be any suitable vector, including chromosomal, non-chromosomal, and synthetic nucleic acid vectors (a nucleic acid sequence comprising a suitable set of expression control elements). Examples of such vectors include derivatives of SV40, bacterial plasmids, phage DNA, baculovirus, yeast plasmids, vectors derived from combinations of plasmids and phage DNA, and viral nucleic acid (RNA or DNA) vectors. In one embodiment, a HER2 antibody-encoding nucleic acid is comprised in a naked DNA or RNA vector, including, for example, a linear expression element (as described in for instance Sykes and Johnston, Nat Biotech 17, 355-59 (1997)), a compacted nucleic acid vector (as described in for instance US 6,077, 835 and/or WO 00/70087), a plasmid vector such as pBR322, pUC 19/18, or pUC 118/119, a "midge" minimally-sized nucleic acid vector (as described in for instance Schakowski *et al.*, Mol Ther 3, 793-800 (2001)), or as a precipitated nucleic acid vector construct, such as a CaP04-precipitated construct (as described in for instance WO 00/46147, Benvenisty and Reshef, PNAS USA 83, 9551-55 (1986), Wigler *et al.*, Cell 14, 725 (1978), and Coraro and Pearson, Somatic Cell Genetics 7, 603 (1981)). Such nucleic acid vectors and the usage thereof are well known in the art (see for instance US 5,589,466 and US 5,973,972).

Exemplary expression vectors for the antibodies of the invention are also described in Examples 2 and 3.

In one embodiment, the vector is suitable for expression of the HER2 antibody in a bacterial cell. Examples of such vectors include expression vectors such as BlueScript (Stratagene), pIN vectors (Van Heeke & Schuster, J Biol Chem 264, 5503-5509 (1989), pET vectors (Novagen, Madison WI) and the like).

An expression vector may also or alternatively be a vector suitable for expression in a yeast system. Any vector suitable for expression in a yeast system may be employed. Suitable vectors include, for example, vectors comprising constitutive or inducible promoters such as alpha factor, alcohol oxidase and PGH (reviewed in: F. Ausubel *et al.*, ed. Current Protocols in Molecular Biology, Greene Publishing and Wiley InterScience New York (1987), and Grant *et al.*, Methods in Enzymol 153, 516-544 (1987)).

An expression vector may also or alternatively be a vector suitable for expression in mammalian cells, *e.g.* a vector comprising glutamine synthetase as a selectable marker, such as the vectors described in Bebbington (1992) Biotechnology (NY) 10:169-175.

A nucleic acid and/or vector may also comprises a nucleic acid sequence encoding a secretion/localization sequence, which can target a polypeptide, such as a nascent polypeptide chain, to the periplasmic space or into cell culture media. Such sequences are known in the art, and include secretion leader or signal peptides.

The expression vector may comprise or be associated with any suitable promoter, enhancer, and other expression-facilitating elements. Examples of such elements include strong expression promoters (e. g., human CMV IE promoter/enhancer as well as RSV, SV40, SL3-3, MMTV, and HIV LTR promoters), effective poly (A) termination sequences, an origin of replication for plasmid product in E. coli, an antibiotic resistance gene as selectable marker, and/or a convenient cloning site (*e.g.*, a polylinker). Nucleic acids may also comprise an inducible promoter as opposed to a constitutive promoter such as CMV IE.

In one embodiment, the HER2 antibody-encoding expression vector may be positioned in and/or delivered to the host cell or host animal via a viral vector.

In an even further aspect, the invention relates to a host cell comprising the first and second nucleic-acid constructs specified herein above.

Thus the present invention also relates to a recombinant eukaryotic or prokaryotic host cell which produces a bispecific antibody of the present invention, such as a transfectoma.

The first HER2 antibody may be expressedin a recombinant eukaryotic or prokaryotic host cell, such as a transfectoma,

Examples of host cells include yeast, bacterial, and mammalian cells, such as CHO or HEK cells. For example, in one embodiment, the host cell may comprise a first and second nucleic acid construct stably integrated into the cellular genome. In another embodiment, the present invention provides a cell comprising a non-integrated nucleic acid, such as a plasmid, cosmid, phagemid, or linear expression element, which comprises a first and second nucleic acid construct as specified above.

In an even further aspect, the invention relates to a transgenic non-human animal or plant comprising nucleic acids encoding one or two sets of a human heavy chain and a human light chain, wherein the animal or plant produces an bispecific antibody of the invention of the invention.

In a further aspect, the invention relates to a hybridoma which produces an antibody of the invention as defined herein. In an even further aspect, the invention relates to a transgenic non-human animal or plant comprising nucleic acids encoding one or two sets of

a human heavy chain and a human light chain, wherein the animal or plant produces an bispecific antibody of the invention of the invention.

In a further aspect, the invention relates to a method for producing a HER2xCD3 antibody of the invention, said method comprising the steps of

a) culturing a host cell of the invention as described herein above, and

b) purifying the antibody of the invention from the culture media.

Preparation of HER2 and CD3 antibodies

Depending on the method for production of a bispecific antibody according to the present invention, it may be relevant to first produce bivalent, monospecifc antibodies. This may for example be relevant if the bispecific antibody is produced as described above which methods are based on the mixing of two bivalent monospecific antibodies under reducing conditions.

Monoclonal antibodies, such as the HER2 antibody, for use in the present invention, for example to provide an antigen-binding region sharing an epitope or cross-blocking region with an antibody of cross-block groups 1, 2, 3 or 4 may be produced, *e.g.*, by the hybridoma method first described by Kohler *et al.*, Nature 256, 495 (1975), or may be produced by recombinant DNA methods. Monoclonal antibodies may also be isolated from phage antibody libraries using the techniques described in, for example, Clackson *et al.*, Nature 352, 624-628 (1991) and Marks *et al.*, J. Mol. Biol. 222, 581-597 (1991). Monoclonal antibodies may be obtained from any suitable source. Thus, for example, monoclonal antibodies may be obtained from hybridomas prepared from murine splenic B cells obtained from mice immunized with an antigen of interest, for instance in form of cells expressing the antigen on the surface, or a nucleic acid encoding an antigen of interest. Monoclonal antibodies may also be obtained from hybridomas derived from antibody-expressing cells of immunized humans or non-human mammals such as rats, dogs, primates, etc.

In one embodiment, the antibody is a human antibody. Human monoclonal antibodies directed against HER2 or CD3 may be generated using transgenic or transchromosomal mice carrying parts of the human immune system rather than the mouse system. Such transgenic and transchromosomic mice include mice referred to herein as HuMAb® mice and KM mice, respectively, and are collectively referred to herein as "transgenic mice".

The HuMAb® mouse contains a human immunoglobulin gene miniloci that encodes unrearranged human heavy (μ and γ) and κ light chain immunoglobulin sequences, together with targeted mutations that inactivate the endogenous μ and κ chain loci (Lonberg, N. *et*

al., Nature 368, 856-859 (1994)). Accordingly, the mice exhibit reduced expression of mouse IgM or κ and in response to immunization, the introduced human heavy and light chain transgenes, undergo class switching and somatic mutation to generate high affinity human IgG,κ monoclonal antibodies (Lonberg, N. *et al.* (1994), supra; reviewed in Lonberg, N. Handbook of Experimental Pharmacology 113, 49-101 (1994) , Lonberg, N. and Huszar, D., Intern. Rev. Immunol. Vol. 13 65-93 (1995) and Harding, F. and Lonberg, N. Ann. N.Y. Acad. Sci 764 536-546 (1995)). The preparation of HuMAb mice is described in detail in Taylor, L. *et al.*, Nucleic Acids Research 20, 6287-6295 (1992), Chen, J. *et al.*, International Immunology 5, 647-656 (1993), Tuaillon *et al.*, J. Immunol. 152, 2912-2920 (1994), Taylor, L. *et al.*, International Immunology 6, 579-591 (1994), Fishwild, D. *et al.*, Nature Biotechnology 14, 845-851 (1996). See also US 5,545,806, US 5,569,825, US 5,625,126, US 5,633,425, US 5,789,650, US 5,877,397, US 5,661,016, US 5,814,318, US 5,874,299, US 5,770,429, US 5,545,807, WO 98/24884, WO 94/25585, WO 93/1227, WO 92/22645, WO 92/03918 and WO 01/09187.

The HCo7, HCo12, HCo17 and HCo20 mice have a JKD disruption in their endogenous light chain (kappa) genes (as described in Chen *et al.*, EMBO J. 12, 821-830 (1993)), a CMD disruption in their endogenous heavy chain genes (as described in Example 1 of WO 01/14424), and a KCo5 human kappa light chain transgene (as described in Fishwild *et al.*, Nature Biotechnology 14, 845-851 (1996)). Additionally, the Hco7 mice have a HCo7 human heavy chain transgene (as described in US 5,770,429), the HCo12 mice have a HCo12 human heavy chain transgene (as described in Example 2 of WO 01/14424), the HCo17 mice have a HCo17 human heavy chain transgene (as described in Example 2 of WO 01/09187) and the HCo20 mice have a HCo20 human heavy chain transgene. The resulting mice express human immunoglobulin heavy and kappa light chain transgenes in a background homozygous for disruption of the endogenous mouse heavy and kappa light chain loci.

In the KM mouse strain, the endogenous mouse kappa light chain gene has been homozygously disrupted as described in Chen *et al.*, EMBO J. 12, 811-820 (1993) and the endogenous mouse heavy chain gene has been homozygously disrupted as described in Example 1 of WO 01/09187. This mouse strain carries a human kappa light chain transgene, KCo5, as described in Fishwild *et al.*, Nature Biotechnology 14, 845-851 (1996). This mouse strain also carries a human heavy chain transchromosome composed of chromosome 14 fragment hCF (SC20) as described in WO 02/43478. HCo12-Balb/C mice can be generated by crossing HCo12 to KCo5[J/K](Balb) as described in WO/2009/097006.

Splenocytes from these transgenic mice may be used to generate hybridomas that secrete human monoclonal antibodies according to well known techniques.

Further, HER2 antigen-binding regions may be obtained from human antibodies or antibodies from other species identified through display-type technologies, including, without limitation, phage display, retroviral display, ribosomal display, and other techniques, using techniques well known in the art and the resulting molecules may be subjected to additional maturation, such as affinity maturation, as such techniques are well known in the art (see for instance Hoogenboom *et al.*, J. Mol. Biol. 227, 381 (1991) (phage display), Vaughan *et al.*, Nature Biotech 14, 309 (1996) (phage display), Hanes and Plucthau, PNAS USA 94, 4937-4942 (1997) (ribosomal display), Parmley and Smith, Gene 73, 305-318 (1988) (phage display), Scott TIBS 17, 241-245 (1992), Cwirla *et al.*, PNAS USA 87, 6378-6382 (1990), Russel *et al.*, Nucl. Acids Research 21, 1081-1085 (1993), Hogenboom *et al.*, Immunol. Reviews 130, 43-68 (1992), Chiswell and McCafferty TIBTECH 10, 80-84 (1992), and US 5,733,743). If display technologies are utilized to produce antibodies that are not human, such antibodies may be humanized.

The bispecific antibody of the invention can be of any isotype. The choice of isotype typically will be guided by the desired effector functions, such as ADCC induction. Exemplary isotypes are IgG1, IgG2, IgG3, and IgG4. Either of the human light chain constant regions, kappa or lambda, may be used. The effector function of the antibodies of the present invention may be changed by isotype switching to, *e.g.*, an IgG1, IgG2, IgG3, IgG4, IgD, IgA, IgE, or IgM antibody for various therapeutic uses. In one embodiment, both Fc-regions of an antibody of the present invention are of the IgG1 isotype, for instance an IgG1,κ. In one embodiment, the two Fc-regions of a bispecific antibody are of the IgG1 and IgG4 isotypes, respectively. Optionally, the Fc-region may be modified in the hinge and/or CH3 region as described elsewhere herein.

In one embodiment, the bispecific antibody of the invention is a full-length antibody, preferably an IgG1 antibody, in particular an IgG1,κ antibody or a variant thereof. In another embodiment, the bispecific antibody of the invention comprises an antibody fragment or a single-chain antibody. Antibody fragments may *e.g.* be obtained by fragmentation using conventional techniques, and the fragments screened for utility in the same manner as described herein for whole antibodies. For example, $F(ab')_2$ fragments may be generated by treating an antibody with pepsin. The resulting $F(ab')_2$ fragment may be treated to reduce disulfide bridges with a reducing agent, such as dithiothreitol, to produce Fab' fragments. Fab fragments may be obtained by treating an antibody with papain. A $F(ab')_2$ fragment may also be produced by binding Fab' fragments via a thioether bond or a disulfide bond. Antibody fragments may also be generated by expression of nucleic acids encoding such fragments in recombinant cells (see for instance Evans *et al.*, J. Immunol.

Meth. 184, 123-38 (1995)). For example, a chimeric gene encoding a portion of an $F(ab')_2$ fragment could include DNA sequences encoding the C_H1 domain and hinge region of the H chain, followed by a translational stop codon to yield such a truncated antibody fragment molecule.

Bispecific HER2xCD3 antibodies of the invention may also be prepared from single chain antibodies. Single chain antibodies are peptides in which the heavy and light chain Fv regions are connected. In one embodiment, the bispecific antibody of the present invention comprises a single-chain Fv (scFv) wherein the heavy and light chains in the Fv of a HER2 antibody of the present invention are joined with a flexible peptide linker (typically of about 10, 12, 15 or more amino acid residues) in a single peptide chain. Methods of producing such antibodies are described in for instance US 4,946,778, Pluckthun in The Pharmacology of Monoclonal Antibodies, vol. 113, Rosenburg and Moore eds. Springer-Verlag, New York, pp. 269-315 (1994), Bird *et al.*, Science 242, 423-426 (1988), Huston *et al.*, PNAS USA 85, 5879-5883 (1988) and McCafferty *et al.*, Nature 348, 552-554 (1990). A bispecific antibody can then be formed from two V_H and V_L from a single-chain HER2 antibody and a single-chain CD3 antibody, or a polyvalent antibody formed from more than two V_H and V_L chains.

In one embodiment, one or both Fc-regions of the HER2 and CD3 mAbs for producing a bispecific antibody of the invention are effector-function-deficient. In one embodiment, the effector-function-deficient antibody is a human stabilized IgG4 antibody, which has been modified to prevent Fab-arm exchange (van der Neut Kolfschoten *et al.* (2007) Science 317(5844):1554-7). Examples of suitable human stabilized IgG4 antibodies are antibodies, wherein arginine at position 409 in a heavy chain constant region of human IgG4, which is indicated in the EU index described in Kabat *et al.*, is substituted with lysine, threonine, methionine, or leucine, preferably lysine (described in WO2006033386 (Kirin)) and/or wherein the hinge region has been modified to comprise a Cys-Pro-Pro-Cys sequence.

In one embodiment, the stabilized IgG4 antibody is an IgG4 antibody comprising a heavy chain and a light chain, wherein said heavy chain comprises a human IgG4 constant region having a residue selected from the group consisting of: Lys, Ala, Thr, Met and Leu at the position corresponding to 409 and/or a residue selected from the group consisting of: Ala, Val, Gly, Ile and Leu at the position corresponding to 405, and wherein said antibody optionally comprises one or more further substitutions, deletions and/or insertions, but does not comprise a Cys-Pro-Pro-Cys sequence in the hinge region. Preferably, said antibody comprises a Lys or Ala residue at the position corresponding to 409 or the CH3 region of the antibody has been replaced by the CH3 region of human IgG1, of human IgG2 or of human IgG3. See also WO2008145142 (Genmab) and WO 211131746 (Genmab).

In an even further embodiment, the stabilized IgG4 antibody is an IgG4 antibody comprising a heavy chain and a light chain, wherein said heavy chain comprises a human IgG4 constant region having a residue selected from the group consisting of: Lys, Ala, Thr, Met and Leu at the position corresponding to 409 and/or a residue selected from the group consisting of: Ala, Val, Gly, Ile and Leu at the position corresponding to 405, and wherein said antibody optionally comprises one or more further substitutions, deletions and/or insertions and wherein said antibody comprises a Cys-Pro-Pro-Cys sequence in the hinge region. Preferably, said antibody comprises a Lys or Ala residue at the position corresponding to 409 or the CH3 region of the antibody has been replaced by the CH3 region of human IgG1, of human IgG2 or of human IgG3.

In a further embodiment, the effector-function-deficient antibody is an antibody of a non-IgG4 type, *e.g.* IgG1, IgG2 or IgG3 which has been mutated such that the ability to mediate effector functions, such as ADCC, has been reduced or even eliminated. Such mutations have *e.g.* been described in Dall'Acqua WF *et al.*, J Immunol. 177(2):1129-1138 (2006) and Hezareh M, J Virol. ;75(24):12161-12168 (2001).

Conjugates

In a further aspect, the present invention provides a bispecific HER2xCD3 antibody linked or conjugated to one or more therapeutic moieties, such as a cytotoxin, a chemotherapeutic drug, a cytokine, an immunosuppressant, and/or a radioisotope. Such conjugates are referred to herein as "immunoconjugates" or "drug conjugates". Immunoconjugates which include one or more cytotoxins are referred to as "immunotoxins".

Compositions

In a further main aspect, the invention relates to a pharmaceutical composition comprising:

- a bispecific HER2xCD3 antibody as defined herein, and
- a pharmaceutically-acceptable carrier.

The pharmaceutical composition of the present invention may contain one bispecific antibody of the present invention or a combination of different bispecific antibodies of the present invention.

The pharmaceutical compositions may be formulated in accordance with conventional techniques such as those disclosed in Remington: The Science and Practice of Pharmacy, 19th Edition, Gennaro, Ed., Mack Publishing Co., Easton, PA, 1995. A pharmaceutical composition of the present invention may *e.g.* include diluents, fillers, salts, buffers, detergents (e. g., a nonionic detergent, such as Tween-20 or Tween-80), stabilizers (e. g.,

sugars or protein-free amino acids), preservatives, tissue fixatives, solubilizers, and/or other materials suitable for inclusion in a pharmaceutical composition.

Pharmaceutically acceptable carriers include any and all suitable solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonicity agents, antioxidants and absorption delaying agents, and the like that are physiologically compatible with a bispecific antibody of the present invention. Examples of suitable aqueous and nonaqueous carriers which may be employed in the pharmaceutical compositions of the present invention include water, saline, phosphate buffered saline, ethanol, dextrose, polyols (such as glycerol, propylene glycol, polyethylene glycol, and the like), and suitable mixtures thereof, vegetable oils, carboxymethyl cellulose colloidal solutions, tragacanth gum and injectable organic esters, such as ethyl oleate, and/or various buffers. Pharmaceutically acceptable carriers include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. Proper fluidity may be maintained, for example, by the use of coating materials, such as lecithin, by the maintenance of the required particle size in the case of dispersions, and by the use of surfactants.

Pharmaceutical bispecific antibodies of the present invention may also comprise pharmaceutically acceptable antioxidants for instance (1) water soluble antioxidants, such as ascorbic acid, cysteine hydrochloride, sodium bisulfate, sodium metabisulfite, sodium sulfite and the like; (2) oil-soluble antioxidants, such as ascorbyl palmitate, butylated hydroxyanisole, butylated hydroxytoluene, lecithin, propyl gallate, alpha-tocopherol, and the like; and (3) metal chelating agents, such as citric acid, ethylenediamine tetraacetic acid (EDTA), sorbitol, tartaric acid, phosphoric acid, and the like.

Pharmaceutical bispecific antibodies of the present invention may also comprise isotonicity agents, such as sugars, polyalcohols, such as mannitol, sorbitol, glycerol or sodium chloride in the compositions.

The pharmaceutical bispecific antibodies of the present invention may also contain one or more adjuvants appropriate for the chosen route of administration such as preservatives, wetting agents, emulsifying agents, dispersing agents, preservatives or buffers, which may enhance the shelf life or effectiveness of the pharmaceutical composition. The bispecific antibodies of the present invention may be prepared with carriers that will protect the bispecific antibody against rapid release, such as a controlled release formulation, including implants, transdermal patches, and microencapsulated delivery systems. Such carriers may include gelatin, glyceryl monostearate, glyceryl distearate, biodegradable, biocompatible polymers such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid alone or with

a wax, or other materials well known in the art. Methods for the preparation of such formulations are generally known to those skilled in the art.

Sterile injectable solutions may be prepared by incorporating the active compound in the required amount in an appropriate solvent with one or a combination of ingredients *e.g.* as enumerated above, as required, followed by sterilization microfiltration. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle that contains a basic dispersion medium and the required other ingredients *e.g.* from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, examples of methods of preparation are vacuum drying and freeze-drying (lyophilization) that yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

The actual dosage levels of the active ingredients in the pharmaceutical compositions may be varied so as to obtain an amount of the active ingredient which is effective to achieve the desired therapeutic response for a particular patient, composition, and mode of administration, without being toxic to the patient. The selected dosage level will depend upon a variety of pharmacokinetic factors including the activity of the particular compositions of the present invention employed, or the amide thereof, the route of administration, the time of administration, the rate of excretion of the particular compound being employed, the duration of the treatment, other drugs, compounds and/or materials used in combination with the particular compositions employed, the age, sex, weight, condition, general health and prior medical history of the patient being treated, and like factors well known in the medical arts.

The pharmaceutical composition may be administered by any suitable route and mode. In one embodiment, a pharmaceutical composition of the present invention is administered parenterally. "Administered parenterally" as used herein means modes of administration other than enteral and topical administration, usually by injection, and include epidermal, intravenous, intramuscular, intraarterial, intrathecal, intracapsular, intraorbital, intracardiac, intradermal, intraperitoneal, intratendinous, transtracheal, subcutaneous, subcuticular, intraarticular, subcapsular, subarachnoid, intraspinal, intracranial, intrathoracic, epidural and intrasternal injection and infusion.

In one embodiment that pharmaceutical composition is administered by intravenous or subcutaneous injection or infusion.

Uses

In a further main aspect, the invention relates to a bispecific HER2xCD3 antibody of the invention for use as a medicament.

The bispecific antibodies of the invention may be used for a number of purposes. In particular, the antibodies of the invention may be used for the treatment of various forms of cancer, including metastatic cancer and refractory cancer.

In one embodiment, the bispecific antibodies of the invention are used for the treatment of breast cancer, including primary, metastatic, and refractory breast cancer.

In one embodiment, the bispecific antibodies of the invention are used for the treatment of a form of cancer selected from the group consisting of prostate cancer, non-small cell lung cancer, bladder cancer, ovarian cancer, gastric cancer, colorectal cancer, esophageal cancer, squamous cell carcinoma of the head & neck, cervical cancer, pancreatic cancer, testis cancer, malignant melanoma and a soft-tissue cancer (*e.g.* synovial sarcoma).

Similarly, the invention relates to a method for killing a tumor cell expressing HER2, comprising administration, to an individual in need thereof, of an effective amount of an antibody of the invention, such as an antibody drug-conjugate (ADC).

The present invention also relates to a method for inhibiting growth and/or proliferation of one or more tumor cells expressing HER2, comprising administration, to an indicidual in need thereof, of a bispecific antibody of the present invention.

The present invention alto relates to am ethod for treating cancer, comprising

a) selecting a subject suffering from a cancer comprising rumor cells co-expressing HER2, and
b) administering to the subject the bispecific antibody of the present invention or a pharmaceutical composition of the present invention.

In one embodiment, said tumor cell is involved in a form of cancer selected from the group consisting of: breast cancer, prostate cancer, non-small cell lung cancer, bladder cancer, ovarian cancer, gastric cancer, colorectal cancer, esophageal cancer and squamous cell carcinoma of the head & neck, cervical cancer, pancreatic cancer, testis cancer, malignant melanoma, and a soft-tissue cancer (*e.g.*, synovial sarcoma).

In one embodiment, the tumor cell is one that co-expresses HER2, and is a tumor cell involved in breast cancer, colorectal cancer, endometrial/cervical cancer, lung cancer, malignant melanoma, ovarian cancer, pancreatic cancer, prostate cancer, testis cancer, a soft-tissue tumor (*e.g.*, synovial sarcoma), or bladder cancer.

In one aspect, the invention relates to a method for treating cancer in a subject, comprising selecting a subject suffering from a cancer comprising tumor cells expressing HER2, and administering to the subject a bispecific antibody of the invention. In one embodiment, the subject suffers from a cancer selected from the group consisting of breast cancer, colorectal cancer, endometrial/cervical cancer, lung cancer, malignant melanoma,

ovarian cancer, pancreatic cancer, prostate cancer, testis cancer, a soft-tissue tumor (*e.g.*, synovial sarcoma), or bladder cancer.

Also, the invention relates to the use of a bispecific antibody that binds to human HER2 and human CD3 for the preparation of a medicament for the treatment of cancer, such as one of the specific cancer indications mentioned above.

The invention further relates to a bispecific antibody for use in the treatment of cancer, such as one of the cancer indications mentioned above.

In a further embodiment of the methods of treatment of the present invention, the efficacy of the treatment is being monitored during the therapy, *e.g.* at predefined points in time, by determining tumor burden or HER2 expression levels on the relevant tumor cells.

Dosage regimens in the above methods of treatment and uses are adjusted to provide the optimum desired response (*e.g.*, a therapeutic response). For example, a single bolus may be administered, several divided doses may be administered over time or the dose may be proportionally reduced or increased as indicated by the exigencies of the therapeutic situation. Parenteral compositions may be formulated in dosage unit form for ease of administration and uniformity of dosage.

The efficient dosages and the dosage regimens for the bispecific antibodies depend on the disease or condition to be treated and may be determined by the persons skilled in the art. An exemplary, non-limiting range for a therapeutically effective amount of a compound of the present invention is about 0.001-10 mg/kg, such as about 0.001-5 mg/kg, for example about 0.001-2 mg/kg, such as about 0.001-1 mg/kg, for instance about 0.001, about 0.01, about 0.1, about 1 or about 10 mg/kg.

A physician or veterinarian having ordinary skill in the art may readily determine and prescribe the effective amount of the pharmaceutical composition required. For example, the physician or veterinarian could start doses of the bispecific antibody employed in the pharmaceutical composition at levels lower than that required in order to achieve the desired therapeutic effect and gradually increase the dosage until the desired effect is achieved. In general, a suitable daily dose of a bispecific antibody of the present invention will be that amount of the compound which is the lowest dose effective to produce a therapeutic effect. Administration may *e.g.* be parenteral, such as intravenous, intramuscular or subcutaneous. In one embodiment, the bispecific antibodies may be administered by infusion in a weekly dosage of calculated by mg/m^2. Such dosages can, for example, be based on the mg/kg dosages provided above according to the following: dose (mg/kg) x 70: 1.8. Such administration may be repeated, *e.g.*, 1 to 8 times, such as 3 to 5 times. The administration may be performed by continuous infusion over a period of from 2 to 24 hours, such as of from 2 to 12 hours. In one embodiment, the bispecific antibodies

may be administered by slow continuous infusion over a long period, such as more than 24 hours, in order to reduce toxic side effects.

In one embodiment the bispecific antibodies may be administered in a weekly dosage of calculated as a fixed dose for up to 8 times, such as from 4 to 6 times when given once a week. Such regimen may be repeated one or more times as necessary, for example, after 6 months or 12 months. Such fixed dosages can, for example, be based on the mg/kg dosages provided above, with a body weight estimate of 70 kg. The dosage may be determined or adjusted by measuring the amount of bispecific antibody of the present invention in the blood upon administration by for instance taking out a biological sample and using anti-idiotypic antibodies which target the HER2 antigen binding region of the bispecific antibodies of the present invention.

In one embodiment, the bispecific antibodies may be administered by maintenance therapy, such as, *e.g.*, once a week for a period of 6 months or more.

A bispecific antibody may also be administered prophylactically in order to reduce the risk of developing cancer, delay the onset of the occurrence of an event in cancer progression, and/or reduce the risk of recurrence when a cancer is in remission.

The bispecific antibodies of the invention may also be administered in combination therapy, *i.e.*, combined with other therapeutic agents relevant for the disease or condition to be treated. Accordingly, in one embodiment, the antibody-containing medicament is for combination with one or more further therapeutic agent, such as a cytotoxic, chemotherapeutic or anti-angiogenic agent.

Such combined administration may be simultaneous, separate or sequential. For simultaneous administration the agents may be administered as one composition or as separate compositions, as appropriate. The present invention thus also provides methods for treating a disorder involving cells expressing HER2 as described above, which methods comprise administration of a bispecific antibody of the present invention combined with one or more additional therapeutic agents as described below.

In one embodiment, the present invention provides a method for treating a disorder involving cells expressing HER2 in a subject, which method comprises administration of a therapeutically effective amount of a bispecific antibody of the present invention, and optionally at least one additional therapeutic agent, or an antibody binding to a different HER2 epitope than said antibody, to a subject in need thereof.

In one embodiment, the present invention provides a method for treating or preventing cancer, which method comprises administration of a therapeutically effective

amount of a bispecific antibody of the present invention and at least one additional therapeutic agent to a subject in need thereof.

In one embodiment, such an additional therapeutic agent may be selected from an antimetabolite, such as methotrexate, 6-mercaptopurine, 6-thioguanine, cytarabine, fludarabine, 5-fluorouracil, decarbazine, hydroxyurea, asparaginase, gemcitabine or cladribine.

In another embodiment, such an additional therapeutic agent may be selected from an alkylating agent, such as mechlorethamine, thioepa, chlorambucil, melphalan, carmustine (BSNU), lomustine (CCNU), cyclophosphamide, busulfan, dibromomannitol, streptozotocin, dacarbazine (DTIC), procarbazine, mitomycin C, cisplatin and other platinum derivatives, such as carboplatin.

In another embodiment, such an additional therapeutic agent may be selected from an anti-mitotic agent, such as taxanes, for instance docetaxel, and paclitaxel, and vinca alkaloids, for instance vindesine, vincristine, vinblastine, and vinorelbine.

In another embodiment, such an additional therapeutic agent may be selected from a topoisomerase inhibitor, such as topotecan or irinotecan, or a cytostatic drug, such as etoposide and teniposide.

In another embodiment, such an additional therapeutic agent may be selected from a growth factor inhibitor, such as an inhibitor of ErbB1 (EGFR) (such as an EGFR antibody, *e.g.* zalutumumab, cetuximab, panitumumab or nimotuzumab or other EGFR inhibitors, such as gefitinib or erlotinib), another inhibitor of ErbB2 (HER2/neu) (such as a HER2 antibody, *e.g.* trastuzumab, trastuzumab-DM1 or pertuzumab) or an inhibitor of both EGFR and HER2, such as lapatinib).

In another embodiment, such an additional therapeutic agent may be selected from a tyrosine kinase inhibitor, such as imatinib (Glivec, Gleevec STI571) or lapatinib, PTK787/ZK222584.

In another embodiment, the present invention provides a method for treating a disorder involving cells expressing HER2 in a subject, which method comprises administration of a therapeutically effective amount of a bispecific antibody of the present invention and at least one inhibitor of angiogenesis, neovascularization, and/or other vascularization to a subject in need thereof

Examples of such angiogenesis inhibitors are urokinase inhibitors, matrix metalloprotease inhibitors (such as marimastat, neovastat, BAY 12-9566, AG 3340, BMS-275291 and similar agents), inhibitors of endothelial cell migration and proliferation (such as TNP-470, squalamine, 2-methoxyestradiol, combretastatins, endostatin, angiostatin, penicillamine, SCH66336 (Schering-Plough Corp, Madison, NJ), R115777

(Janssen Pharmaceutica, Inc, Titusville, NJ) and similar agents), antagonists of angiogenic growth factors (such as such as ZD6474, SU6668, antibodies against angiogenic agents and/or their receptors (such as VEGF (*e.g.* bevacizumab), bFGF, and angiopoietin-1), thalidomide, thalidomide analogs (such as CC-5013), Sugen 5416, SU5402, antiangiogenic ribozyme (such as angiozyme), interferon α (such as interferon α2a), suramin and similar agents), VEGF-R kinase inhibitors and other anti-angiogenic tyrosine kinase inhibitors (such as SU011248), inhibitors of endothelial-specific integrin/survival signaling (such as vitaxin and similar agents), copper antagonists/chelators (such as tetrathiomolybdate, captopril and similar agents), carboxyamido-triazole (CAI), ABT-627, CM101, interleukin-12 (IL-12), IM862, PNU145156E as well as nucleotide molecules inhibiting angiogenesis (such as antisense-VEGF-cDNA, cDNA coding for angiostatin, cDNA coding for p53 and cDNA coding for deficient VEGF receptor-2).

Other examples of such inhibitors of angiogenesis, neovascularization, and/or other vascularization are anti-angiogenic heparin derivatives (*e.g.*, heperinase III), temozolomide, NK4, macrophage migration inhibitory factor, cyclooxygenase-2 inhibitors, inhibitors of hypoxia-inducible factor 1, anti-angiogenic soy isoflavones, oltipraz, fumagillin and analogs thereof, somatostatin analogues, pentosan polysulfate, tecogalan sodium, dalteparin, tumstatin, thrombospondin, NM-3, combrestatin, canstatin, avastatin, antibodies against other targets, such as anti-alpha-v/beta-3 integrin and anti-kininostatin antibodies.

In one embodiment, a therapeutic agent for use in combination with a bispecific antibody for treating the disorders as described above may be an anti-cancer immunogen, such as a cancer antigen/tumor-associated antigen (*e.g.*, epithelial cell adhesion molecule (EpCAM/TACSTD1), mucin 1 (MUC1), carcinoembryonic antigen (CEA), tumor-associated glycoprotein 72 (TAG-72), gp100, Melan-A, MART-1, KDR, RCAS1, MDA7, cancer-associated viral vaccines (*e.g.*, human papillomavirus vaccines) or tumor-derived heat shock proteins,

In one embodiment, a therapeutic agent for use in combination with a bispecific antibody for treating the disorders as described above may be an anti-cancer cytokine, chemokine, or combination thereof. Examples of suitable cytokines and growth factors include IFNγ, IL-2, IL-4, IL-6, IL-7, IL-10, IL-12, IL-13, IL-15, IL-18, IL-23, IL-24, IL-27, IL-28a, IL-28b, IL-29, KGF, IFNα (*e.g.*, INFα2b), IFNβ, GM-CSF, CD40L, Flt3 ligand, stem cell factor, ancestim, and TNFα. Suitable chemokines may include Glu-Leu-Arg (ELR)-negative chemokines such as IP-10, MCP-3, MIG, and SDF-1α from the human CXC and C-C chemokine families. Suitable cytokines include cytokine derivatives, cytokine variants, cytokine fragments, and cytokine fusion proteins.

In one embodiment, a therapeutic agent for use in combination with a bispecific antibody for treating the disorders as described above may be a cell cycle control/apoptosis

regulator (or "regulating agent"). A cell cycle control/apoptosis regulator may include molecules that target and modulate cell cycle control/apoptosis regulators such as (i) cdc-25 (such as NSC 663284), (ii) cyclin-dependent kinases that overstimulate the cell cycle (such as flavopiridol (L868275, HMR1275), 7-hydroxystaurosporine (UCN-01, KW-2401), and roscovitine (R-roscovitine, CYC202)), and (iii) telomerase modulators (such as BIBR1532, SOT-095, GRN163 and compositions described in for instance US 6,440,735 and US 6,713,055). Non-limiting examples of molecules that interfere with apoptotic pathways include TNF-related apoptosis-inducing ligand (TRAIL)/apoptosis-2 ligand (Apo-2L), antibodies that activate TRAIL receptors, IFNs, and anti-sense Bcl-2.

In one embodiment, a therapeutic agent for use in combination with a bispecific antibody for treating the disorders as described above may be a hormonal regulating agent, such as agents useful for anti-androgen and anti-estrogen therapy. Examples of such hormonal regulating agents are tamoxifen, idoxifene, fulvestrant, droloxifene, toremifene, raloxifene, diethylstilbestrol, ethinyl estradiol/estinyl, an antiandrogene (such as flutaminde/eulexin), a progestin (such as such as hydroxyprogesterone caproate, medroxy-progesterone/provera, megestrol acepate/megace), an adrenocorticosteroid (such as hydrocortisone, prednisone), luteinizing hormone-releasing hormone (and analogs thereof and other LHRH agonists such as buserelin and goserelin), an aromatase inhibitor (such as anastrazole/arimidex, aminoglutethimide/cytraden, exemestane) or a hormone inhibitor (such as octreotide/sandostatin).

In one embodiment, a therapeutic agent for use in combination with a bispecific antibody for treating the disorders as described above may be an anti-anergic agent, such asmolecules that block the activity of CTLA-4, *e.g.* ipilimumab.

In one embodiment, a therapeutic agent for use in combination with a bispecific antibody for treating the disorders as described above may be an anti-cancer nucleic acid or an anti-cancer inhibitory RNA molecule.

Examples of other anti-cancer agents, which may be relevant as therapeutic agents for use in combination with a bispecific antibody according to the invention for treating the disorders as described above are differentiation inducing agents, retinoic acid analogues (such as all trans retinoic acid, 13-cis retinoic acid and similar agents), vitamin D analogues (such as seocalcitol and similar agents), inhibitors of ErbB3, ErbB4, IGF-IR, insulin receptor, PDGFRa, PDGFRbeta, Flk2, Flt4, FGFR1, FGFR2, FGFR3, FGFR4, TRKA, TRKC, RON (such as an anti-RON antibody), Sea, Tie, Tie2, Eph, Ret, Ros, Alk, LTK, PTK7 and similar agents.

Examples of other anti-cancer agents, which may be relevant as therapeutic agents for use in combination with a bispecific antibody according to the invention for treating the disorders as described above are estramustine and epirubicin.

Examples of other anti-cancer agents, which may be relevant as therapeutic agents for use in combination with a bispecific antibody according to the invention for treating the disorders as described above are a HSP90 inhibitor like 17-allyl amino geld-anamycin, antibodies directed against a tumor antigen such as PSA, CA125, KSA, integrins, *e.g.* integrin β1, or inhibitors of VCAM. Examples of other anti-cancer agents, which may be relevant as therapeutic agents for use in combination with a bispecific antibody for treating the disorders as described above are calcineurin-inhibitors (such as valspodar, PSC 833 and other MDR-1 or p-glycoprotein inhibitors), TOR-inhibitors (such as sirolimus, everolimus and rapamcyin), and inhibitors of "lymphocyte homing" mechanisms (such as FTY720), and agents with effects on cell signaling such as adhesion molecule inhibitors (for instance anti-LFA).

In one embodiment, the bispecific antibody of the invention is for use in combination with one or more other therapeutic antibodies, such as ofatumumab, zanolimumab, daratumumab, ranibizumab, nimotuzumab, panitumumab, hu806, daclizumab (Zenapax), basiliximab (Simulect), infliximab (Remicade), adalimumab (Humira), natalizumab (Tysabri), omalizumab (Xolair), efalizumab (Raptiva) and/or rituximab.

In another embodiment, two or more different antibodies of the invention as described herein are used in combination for the treatment of disease. Particularly interesting combinations include two or more non-blocking antibodies. Such combination therapy may lead to binding of an increased number of antibody molecules per cell, which may give increase efficacy, *e.g.* via activation of complement-mediated lysis.

In addition to the above, other embodiments of combination therapies of the invention include the following:

For the treatment of breast cancer, a bispecific antibody or a therapeutic conjugate thereof, in combination with methotrexate, paclitaxel, doxorubicin, carboplatin, cyclophosphamide, daunorubicin, epirubicin, 5-fluorouracil, gemcitabine, ixabepilone, mutamycin, mitoxantrone, vinorelbine, docetaxel, thiotepa, vincristine, capecitabine, an EGFR antibody (*e.g.* zalutumumab, cetuximab, panitumumab or nimotuzumab) or other EGFR inhibitor (such as gefitinib or erlotinib), another HER2 antibody or –conjugate (such as, *e.g.*, trastuzumab, trastuzumab-DM1 or pertuzumab), an inhibitor of both EGFR and HER2 (such as lapatinib), and/or in combination with a HER3 inhibitor.

For the treatment of non-small-cell lung cancer, a bispecific antibody of the invention in combination with EGFR inhibitors, such as an EGFR antibody, *e.g.* zalutumumab, cetuximab, panitumumab or nimotuzumab or other EGFR inhibitors (such as gefitinib or erlotinib), or in combination with an another HER2 agent (such as a HER2 antibody, *e.g.*

trastuzumab, trastuzumab-DM1 or pertuzumab) or in combination with an inhibitor of both EGFR and HER2, such as lapatinib, or in combination with a HER3 inhibitor.

For the treatment of colorectal cancer, a bispecific antibody of the invention in combination with one or more compounds selected from: gemcitabine, bevacizumab, FOLFOX, FOLFIRI, XELOX, IFL, oxaliplatin, irinotecan, 5-FU/LV, Capecitabine, UFT, EGFR targeting agents, such as cetuximab, panitumumab, zalutumumab; VEGF inhibitors, or tyrosine kinase inhibitors such as sunitinib.

For the treatment of prostate cancer, a bispecific antibody in combination with one or more compounds selected from: hormonal/antihormonal therapies; such as antiandrogens, Luteinizing hormone releasing hormone (LHRH) agonists, and chemotherapeutics such as taxanes, mitoxantrone, estramustine, 5FU, vinblastine, and ixabepilone.

Radiotherapy - surgery

In one embodiment, the present invention provides a method for treating a disorder involving cells expressing HER2 in a subject, which method comprises administration of a therapeutically effective amount of a bispecific antibody, such as a HER2xCD3 antibody of the present invention, and radiotherapy to a subject in need thereof.

In one embodiment, the present invention provides a method for treating or preventing cancer, which method comprises administration of a therapeutically effective amount of a bispecific antibody, such as a HER2xCD3 antibody of the present invention, and radiotherapy to a subject in need thereof.

In one embodiment, the present invention provides the use of a bispecific antibody of the present invention, for the preparation of a pharmaceutical composition for treating cancer to be administered in combination with radiotherapy.

Radiotherapy may comprise radiation or associated administration of radiopharmaceuticals to a patient is provided. The source of radiation may be either external or internal to the patient being treated (radiation treatment may, for example, be in the form of external beam radiation therapy (EBRT) or brachytherapy (BT)). Radioactive elements that may be used in practicing such methods include, *e.g.*, radium, cesium-137, iridium-192, americium-241, gold-198, cobalt-57, copper-67, technetium-99, iodide-123, iodide-131, and indium-111.

In a further embodiment, the present invention provides a method for treating or preventing cancer, which method comprises administration to a subject in need thereof of a therapeutically effective amount of a bispecific antibody of the present invention, in combination with surgery.

Diagnostic uses

The bispecific antibodies of the invention may also be used for diagnostic purposes. Thus, in a further aspect, the invention relates to a diagnostic composition comprising a bispecific HER2xCD3 antibody as defined herein, and to its use. In one embodiment, the present invention provides a kit for diagnosis of cancer comprising a container comprising a bispecific HER2xCD3 antibody, and one or more reagents for detecting binding of the antibody to HER2. Reagents may include, for example, fluorescent tags, enzymatic tags, or other detectable tags. The reagents may also include secondary or tertiary antibodies or reagents for enzymatic reactions, wherein the enzymatic reactions produce a product that may be visualized.

The present invention is further illustrated by the following examples, which should not be construed as limiting the scope of the invention.

EXAMPLES

Example 1 - Expression constructs for HER2 and HER2 variants

Fully codon-optimized constructs for expression of full length HER2 (1255 aa, Swissprot P04626), the extracellular domain (ECD) of HER2 (Her2-ECDHis, aa 1-653 with a C-terminal His6 tag), the naturally occurring HER2 splice variant (Her2-delex16, resulting from exon 16 deletion and lacking aa 633-648) and a truncated form of the HER2 receptor (Her2-stumpy, aa 648-1256), were generated. The construct contained suitable restriction sites for cloning and an optimal Kozak sequence (Kozak, M., Gene 1999;234(2):187-208.). The constructs were cloned in the mammalian expression vector pEE13.4 (Lonza Biologics; Bebbington, C.R., *et al.*, Biotechnology (N Y) 1992;10(2):169-75) and fully sequenced to confirm the correctness of the construct.

Example 2 - Expression constructs for Pertuzumab, C1 and F5

Fully codon-optimized constructs for expression of the heavy chain (HC) and the light chain (LC) of the IgG1 antibodies pertuzumab, C1 and F5 in HEK cells, were generated. The variable regions encoded by these constructs are identical to those described in U.S. Patent No. 6,949,245 for pertuzumab heavy chain and light chain and U.S. Patent No. 7,244,826 for C1 and F5 heavy and light chain. For C1 and F5, the mammalian expression vectors p33G1f and p33K or p33L (pcDNA3.3 (Invitrogen)) containing the fully codon optimized constant region for the human IgG1 heavy chain (allotype f), the human kappa light chain or the human lambda light chain, respectively, were used. For pertuzumab, the mammalian expression vectors pG1f (pEE12.4 (Lonza Biologics) and pKappa (pEE6.4 (Lonza Biologics), containing the fully codon-optimized constant region for the human IgG1 heavy chain (allotype f) and the human kappa light chain, respectively, were used.

Trastuzumab (Herceptin®) can be produced in the same manner, using the heavy and light chain sequences described in, *e.g.*, U.S. Patent No. 7,632,924.

The sequence disclosures of U.S. Patent Nos. 6,949,245; 7,244,826 and 7,632,924 are hereby incorporated by reference in their entirities.

Example 3 - Transient expression in HEK-293 or CHO cells

Freestyle™ 293-F (a HEK-293 subclone adapted to suspension growth and chemically defined Freestyle medium, (HEK-293F)) cells were obtained from Invitrogen and transfected with the appropriate plasmid DNA, using 293fectin (Invitrogen) according to the manufacturer's instructions. In the case of antibody expression, the appropriate heavy chain and light chain expression vectors were co-expressed.

pEE13.4Her2, pEE13.4Her2-delex16 and pEE13.4Her2-stumpy were transiently transfected in the Freestyle™ CHO-S (Invitrogen) cell line using Freestyle MAX transfection reagent (Invitrogen). Expression of HER2 and Her2-delex16 was tested by means of FACS analysis as described below.

Example 4 - Stable polyclonal pool expression in NS0

pEE13.4Her2, pEE13.4Her2-delex16 and pEE13.4Her2-stumpy were stably transfected in NS0 cells by nucleofection (Amaxa). A pool of stably transfected cells was established after selection on glutamine dependent growth, based on the integrated glutamine synthetase selection marker (Barnes, L.M., *et al.*, Cytotechnology 2000;32(2):109-123).

Example 5 - Purification of His-tagged HER2

Her2ECDHis was expressed in HEK-293F cells. The His-tag in Her2ECDHis enabled purification with immobilized metal affinity chromatography, since the His-tagged protein binds strongly to the resin beads, while other proteins present in the culture supernatant do not bind strongly.

In this process, a chelator fixed onto the chromatographic resin was charged with Co^{2+} cations. Her2ECDHis containing supernatant was incubated with the resin in batch mode (*i.e.* solution). After incubation, the beads were retrieved from the supernatant and packed into a column. The column was washed in order to remove weakly bound proteins. The strongly bound Her2ECDHis proteins were then eluted with a buffer containing imidazole, which competes with the binding of His to Co^{2+}. The eluent was removed from the protein by buffer exchange on a desalting column.

Example 6 - Immunization procedure of transgenic mice

Antibodies 001, 019, 021, 025, 027, 032, 033, 035, 036, 049, 050, 051, 054, 055, 084, 091, 094, 098, 100, 105, 123 and 124 were derived from the following immunization: three female HCo12 mice, one male and two female HCo12-Balb/C mice, one male HCo17 mouse and one male HCo20 mouse (Medarex, San José, CA, USA) were immunized alternating with $5x10^6$ NS0 cells stably transfected with Her2ECD intraperitoneal (IP) and 20 µg Her2ECDHis protein coupled to the hapten Keyhole Limpet Hemocyanin (KLH) subcutaneous (SC) at the tail base, with an interval of fourteen days. A maximum of eight immunizations was performed per mouse (four IP and four SC immunizations). The first immunization with cells was done in complete Freunds' adjuvant (CFA; Difco Laboratories, Detroit, MI, USA). For all other immunizations, cells were injected IP in PBS and KLH coupled Her2ECDHis was injected SC using incomplete Freunds' adjuvant (IFA; Difco Laboratories, Detroit, MI, USA).

Antibodies 125, 127, 129, 132, 152, 153 and 159 were derived from the following immunization: one male and two female HCo12-Balb/C mice, one female HCo20 mouse, and one female HCo12 mouse (Medarex) were immunized alternating with $5x10^6$ NS0 cells stably transfected with Her2delex16 IP and 20 µg Her2ECDHis protein coupled to the hapten Keyhole Limpet Hemocyanin (KLH) SC at the tail base, with an interval of fourteen days. A maximum of eight immunizations was performed per mouse (four IP and four SC immunizations). The first immunization with cells was done in complete Freunds' adjuvant (CFA; Difco Laboratories, Detroit, MI, USA). For all other immunizations, cells were injected IP in PBS and KLH coupled Her2ECD was injected SC using incomplete Freunds' adjuvant (IFA; Difco Laboratories, Detroit, MI, USA).

Antibody 143, 160, 161, 162, 166 and 169 were derived from the following immunization: one female and one male Hco12 mouse, one female Hco12-Balb/C mouse, one male HCo17 mouse and one male HCo20 mouse (Medarex) were immunized alternating with 20 µg Her2ECDHis protein coupled to the hapten Keyhole Limpet Hemocyanin (KLH), alternating IP and SC at the tail base with an interval of fourteen days. A maximum of eight immunizations was performed per mouse (four IP and four SC immunizations). The first immunization was done IP in complete Freunds' adjuvant (CFA; Difco Laboratories, Detroit, MI, USA). The other immunizations were injected using incomplete Freunds' adjuvant (IFA; Difco Laboratories, Detroit, MI, USA).

Antibodies 005, 006, 041, 044, 059, 060, 067, 072, 093, 106 and 111 were derived from the following immunization procedure: two female HCo12 mice, one female and one male HCo12-Balb/C mouse, one female and one male HCo17 mouse, and two male HCo20 mice (Medarex, San José, CA, USA) were immunized every fortnight, alternating between $5x10^6$ NS0 cells stably transfected with Her2ECDHis intraperitoneal (IP) and 20 µg Her2ECDHis protein coupled to the hapten Keyhole Limpet Hemocyanin (KLH) subcutaneous

(SC) at the tail base. A maximum of eight immunizations was performed per mouse (four IP and four SC immunizations). The first immunization with cells was done in complete Freunds' adjuvant (CFA; Difco Laboratories, Detroit, MI, USA). For all other immunizations, cells were injected IP in PBS and KLH coupled Her2ECD was injected SC using incomplete Freunds' adjuvant (IFA; Difco Laboratories, Detroit, MI, USA).

Antibody 150 was derived from immunization of one female HCo17 mouse (Medarex) alternating with $5x10^6$ NS0 cells stably transfected with Her2delex16 IP and 20 µg Her2ECDHis protein coupled to the hapten Keyhole Limpet Hemocyanin (KLH) SC at the tail base, with an interval of fourteen days. A maximum of eight immunizations was performed (four IP and four SC immunizations). The first immunization with cells was done in complete Freunds' adjuvant (CFA; Difco Laboratories, Detroit, MI, USA). For all other immunizations, cells were injected IP in PBS and KLH coupled Her2ECD was injected SC using incomplete Freunds' adjuvant (IFA; Difco Laboratories, Detroit, MI, USA).

Antibody 163 was derived from immunization of one male HCo20 mouse (Medarex) with 20 µg Her2ECDHis protein coupled to the hapten Keyhole Limpet Hemocyanin (KLH), alternating IP and SC at the tailbase with an interval of fourteen days. A maximum of eight immunizations was performed (four IP and four SC immunizations). The first immunization was done IP in complete Freunds' adjuvant (CFA; Difco Laboratories, Detroit, MI, USA). The other immunizations were injected using incomplete Freunds' adjuvant (IFA; Difco Laboratories, Detroit, MI, USA).

Mice with at least two sequential titers against TC1014-Her2, TC1014-Her2delex16 or TC1014-Her2stumpy in the antigen specific FMAT screening assay (as described in Example 7), were considered positive and fused.

Example 7 - Homogeneous antigen specific screening assay

The presence of HER2 antibodies in sera of immunized mice or HuMab (human monoclonal antibody) hybridoma or transfectoma culture supernatant was determined by homogeneous antigen specific screening assays (four quadrant) using Fluorometric Micro volume Assay Technology (FMAT; Applied Biosystems, Foster City, CA, USA). For this, a combination of 4 cell based assays was used. Binding to TC1014-Her2 (CHO-S cells transiently expressing the HER2 receptor; produced as described above), TC1014-Her2delex16 (CHO-S cells transiently expressing the extracellular domain of Her2-delex (a 16 amino acid deletion mutant of the HER2 receptor; produced as described above) and TC1014-Her2stumpy (CHO-S cells transiently expressing the extracellular stumpy domain of the HER2 receptor; produced as described above) as well as CHO-S wild type cells (negative control cells which do not express HER2) was determined. Samples were added to the cells to allow binding to

HER2. Subsequently, binding of HuMab was detected using a fluorescent conjugate (Goat anti-Human IgG-Cy5; Jackson ImmunoResearch). TH1014-Pertuzumab (produced in HEK-293F cells) was used as a positive control and HuMab®-mouse pooled serum and HuMab-KLH were used as negative controls. The samples were scanned using an Applied Biosystems 8200 Cellular Detection System (8200 CDS) and 'counts x fluorescence' was used as read-out. Samples were stated positive when counts were higher than 50 and counts x fluorescence were at least three times higher than the negative control.

Example 8 - HuMab hybridoma generation

HuMab mice with sufficient antigen-specific titer development (defined as above) were sacrificed and the spleen and lymph nodes flanking the abdominal aorta and vena cava were collected. Fusion of splenocytes and lymph node cells to a mouse myeloma cell line was done by electrofusion using a CEEF 50 Electrofusion System (Cyto Pulse Sciences, Glen Burnie, MD, USA), essentially according to the manufacturer's instructions. Next, the primary wells were sub cloned using the ClonePix system (Genetix, Hampshire, UK). To this end specific primary well hybridoma's were seeded in semisolid medium made from 40% CloneMedia (Genetix, Hampshire, UK) and 60% HyQ 2x complete media (Hyclone, Waltham, USA). The sub clones were retested in the antigen-specific binding assay as described in Example 7 and IgG levels were measured using an Octet (Fortebio, Menlo Park, USA) in order to select the most specific and best producing clone per primary well for further expansion. Further expansion and culturing of the resulting HuMab hybridomas were done based upon standard protocols (*e.g.* as described in Coligan J.E., Bierer, B.E., Margulies, D.H., Shevach, E.M. and Strober, W., eds. Current Protocols in Immunology, John Wiley & Sons, Inc., 2006). Clones derived by this process were designated PC1014.

Example 9 - Mass Spectrometry of purified antibodies

Small aliquots of 0.8 mL antibody containing supernatant from 6-well or Hyperflask stage were purified using PhyTip columns containing Protein G resin (PhyNexus Inc., San Jose, USA) on a Sciclone ALH 3000 workstation (Caliper Lifesciences, Hopkinton, USA). The PhyTip columns were used according to manufacturer's instructions, although buffers were replaced by: Binding Buffer PBS (B.Braun, Medical B.V., Oss, Netherlands) and Elution Buffer 0.1M Glycine-HCl pH 2.7 (Fluka Riedel-de Haën, Buchs, Germany). After purification, samples were neutralized with 2M Tris-HCl, pH 9.0 (Sigma-Aldrich, Zwijndrecht, Netherlands). Alternatively, in some cases larger volumes of culture supernatant were purified using MabSelect SuRe columns (GE Health Care).

After purification, the samples were placed in a 384-well plate (Waters, 100 µl square well plate, part# 186002631). Samples were deglycosylated overnight at 37°C with N-glycosidase F (Roche cat no 11365177001. DTT (15 mg/mL) was added (1 µL/well) and incubated for 1 h at 37°C. Samples (5 or 6 µL) were desalted on an Acquity UPLC™ (Waters, Milford, USA) with a BEH300 C18, 1.7µm, 2.1x 50 mm column at 60°C. MQ water and LC-MS grade acetonitrile (Biosolve, cat no 01204101, Valkenswaard, The Netherlands) with both 0.1% formic acid (Fluka, cat no 56302, Buchs, Germany), were used as Eluens A and B, respectively. Time-of-flight electrospray ionization mass spectra were recorded on-line on a micrOTOF™ mass spectrometer (Bruker, Bremen, Germany) operating in the positive ion mode. Prior to analysis, a 900-3000 m/z scale was calibrated with ES tuning mix (Agilent Technologies, Santa Clara, USA). Mass spectra were deconvoluted with DataAnalysis™ software v. 3.4 (Bruker) using the Maximal Entropy algorithm searching for molecular weights between 5 and 80 kDa.

After deconvolution, the resulting heavy and light chain masses for all samples were compared in order to find duplicate antibodies. This was sometimes due to the presence of an extra light chain, but in the comparison of the heavy chains, the possible presence of C-terminal lysine variants was also taken into account. This resulted in a list of unique antibodies, *i.e.*, a unique combination of specific heavy and light chains. In case duplicate antibodies were found, one unique antibody was selected based on results from other tests.

Example 10 - Sequence analysis of the HER2 antibody variable domains and cloning in expression vectors

Total RNA of the HER2 HuMabs was prepared from $5x10^6$ hybridoma cells and 5'-RACE-Complementary DNA (cDNA) was prepared from 100 ng total RNA, using the SMART RACE cDNA Amplification kit (Clontech), according to the manufacturer's instructions. VH and VL coding regions were amplified by PCR and cloned directly, in frame, in the pG1f and pKappa expression vectors, by ligation independent cloning (Aslanidis, C. and P.J. de Jong, Nucleic Acids Res 1990;18(20): 6069-74). The appropriate heacy chain and light chain vectors were transiently co-expressed in Freestyle™ 293-F cells using 293fectin. Clones derived by this process were designated TH1014 (TH stands for transient HEK cells). For each antibody, 16 VL clones and 8 VH clones were sequenced. Clones with predicted heavy and light chain mass in agreement with the mass of the hybridoma derived material of the same antibody (as determined by mass spectrometry) were selected for further study and expression.

The resulting sequences are shown in Figures 1 and 2 and in the Sequence Listing. Selected sequences are also described in more detail below. CDR sequences were defined according to IMGT (Lefranc MP. *et al.*, Nucleic Acids Research, 27, 209-212, 1999 and

Brochet X. Nucl. Acids Res. 36, W503-508 (2008)). Table 1, Table 2 and Table 3 give an overview of antibody sequence information or germline sequences, and Table 4 shows consensus sequences.

Table 1A and 1B: Heavy chain variable region (VH), light chain variable region (VL) and CDR sequences of HuMabs 169, 050, 084, 025, 091, 129, 127, 159, 098, 153, and 132 (Table 1A) and HuMabs 005, 006, 059, 060, 106, and 111 (Table 1B).

1A:

SEQ ID No:1	VH 169	QVQLVQSGAEVKKPGASVKVSCKASGYTFTNYGISW VRQAPGQGLEWMGWLSAYSGNTIYAQKLQGRVTMT TDTSTTTAYMELRSLRSDDTAVYYCARDRIVVRPDYF DYWGQGTLVTVSS
SEQ ID No:2	VH 169, CDR1	GYTFTNYG
SEQ ID No:3	VH 169, CDR2	LSAYSGNT
SEQ ID No:4	VH 169, CDR3	ARDRIVVRPDYFDY
SEQ ID No:5	VL 169	EIVLTQSPATLSLSPGERATLSCRASQSVSSYLAWYQ QKPGQAPRLLIYDASNRATGIPARFSGSGSGTDFTLTI SSLEPEDFAVYYCQQRSNWPRTFGQGTKVEIK
SEQ ID No:6	VL 169, CDR1	QSVSSY
	VL 169, CDR2	DAS
SEQ ID No:7	VL 169, CDR3	QQRSNWPRT
SEQ ID No:8	VH 050	EVQLLESGGGLVQPGGSLRLSCAASGFTFSSYAMNW VRQAPGKGLEWVSAISGRGGTTYYADSVKGRFTISR DNSKNTLYLQMSSLRAEDTAVYYCAKARANWDYFDY WGQGTLVTVSS
SEQ ID No:9	VH 050, CDR1	GFTFSSYA
SEQ ID No:10	VH 050, CDR2	ISGRGGTT
SEQ ID No:11	VH 050, CDR3	AKARANWDYFDY
SEQ ID No:12	VL 050	DIQMTQSPSSVSASVGDRVTITCRASQGISSWLAWY QHKPGKAPKLLIYAASILQSGVPSRFSGSGSGTDFTL TISSLQPEDFATYYCQQANSFPITFGQGTRLEIK
SEQ ID No:13	VL 050, CDR1	QGISSW
	VL 050, CDR2	AAS
SEQ ID No:14	VL 050, CDR3	QQANSFPIT
SEQ ID No:15	VH 084	QVQLVQSGAEVKKPGSSVKVSCKASGGTFRTYAINW VRQAPGQGLEWMGRINTVLGIVNHAQKFQGRVTITA DKSTNTAYMELNSLRSEDTAVYYCAREKGVDYYYGIE VWGQGTTVTVSS
SEQ ID No:16	VH 084, CDR1	GGTFRTYA
SEQ ID No:17	VH 084, CDR2	INTVLGIV
SEQ ID No:18	VH 084, CDR3	AREKGVDYYYGIEV

SEQ ID No:19	VL 084	DIQMTQSPSSVSASVGDRVTITCRASQGISSWLAWY QHKPGKAPKLLIYVASTLQSGVPSRFSGSGSGTDFTL TISSLQPEDFATYYCQQANSFPLTFGGGTKVEIK
SEQ ID No:20	VL 084, CDR1	QGISSW
	VL 084, CDR2	VAS
SEQ ID No:21	VL 084, CDR3	QQANSFPLT
SEQ ID No:22	VH 025	QVQLQQWGAGLLKPSETLSLTCAVYGGSFSDYYWN WIRQPPGKGLEWIGEIHHSGSTNYNPSLKSRVTISVD TSKNQFSLKLSSVTAADTAVYYCARGYYDSGVYYFDY WAQGTLVTVSS
SEQ ID No:23	VH 025, CDR1	GGSFSDYY
SEQ ID No:24	VH 025, CDR2	IHHSGST
SEQ ID No:25	VH 025, CDR3	ARGYYDSGVYYFDY
SEQ ID No:26	VL 025	DIQMTQSPSSLSASVGDRVTITCRASQGISRWLAWY QQKPEKAPKSLIYAASSLRSGVPSRFSGSGSGTDFTL TISSLQPEDFATYYCQQYNSYPITFGQGTRLEIK
SEQ ID No:27	VL 025, CDR1	QGISRW
	VL 025, CDR2	AAS
SEQ ID No:28	VL 025, CDR3	QQYNSYPIT
SEQ ID No:29	VH 091	QVQLQQWGAGLLKPSETLSLTCAVSGGSFSGYYWT WIRQPPGKGLEWIGEIYHSGDTNYNPSLKSRVTISVD TSKNQFSLKLYSVTAADTAVYYCARLYFGSGIYYLDY WGQGTLVTVSS
SEQ ID No:30	VH 091, CDR1	GGSFSGYY
SEQ ID No:163	VH 091, CDR2	IYHSGDT
SEQ ID No:31	VH 091, CDR3	ARLYFGSGIYYLDY
SEQ ID No:32	VL 091	DIQMTQSPSSLSASVGDRVTITCRASQGISSWLVWY QQKPEKAPKSLIYAASSLQSGVPSRFSGSGSGTDFTL TISSLQPEDFATYYCQQYNSFPPTFGQGTKVEIK
SEQ ID No:33	VL 091, CDR1	QGISSW
	VL 091, CDR2	AAS
SEQ ID No:34	VL 091, CDR3	QQYNSFPPT
SEQ ID No:35	VH 129	QVQLVESGGGVVQPGRSLRLSCAASGFTFSTFAIHW VRQAPGKGLEWVAVISYDGGHKFYADSVKGRFTISR DNSKNTLYLQMNSLRAEDTAMYYCARGLGVWGAFD YWGQGTLVTVSS
SEQ ID No:36	VH 129, CDR1	GFTFSTFA
SEQ ID No:37	VH 129, CDR2	ISYDGGHK
SEQ ID No:38	VH 129, CDR3	ARGLGVWGAFDY
SEQ ID No:39	VL 129	EIVLTQSPATLSLSPGERATLSCRASQSVSSYLAWYQ QKPGQAPRLLIYDASNRATGIPARFSGSGSGTDFTLTI SSLEPEDFAVYYCQQRSNWWTFGQGTKVEIK
SEQ ID No:40	VL 129, CDR1	QSVSSY

	VL 129, CDR2	DAS
SEQ ID No:41	VL 129, CDR3	QQRSNWWT
SEQ ID No:42	VH 127	EVQLVQSGAEVKKPGESLTISCKGSGYSFSIYWIGW VRQMPGKGLEWMGIIFPGDSDIRYSPSFQGQVTISA DKSISTAYLQWSSLKASDTAMYYCARQPGDWSPRH WYFDLWGRGTLVTVSS
SEQ ID No:43	VH 127, CDR1	GYSFSIYW
SEQ ID No:44	VH 127, CDR2	IFPGDSDI
SEQ ID No:45	VH 127, CDR3	ARQPGDWSPRHWYFDL
SEQ ID No:46	VL 127	VIWMTQSPSLLSASTGDRVTISCRMSQGISSYLAWY QQKPGKAPELLIYAASTLQSGVPSRFSGSGSGTDFTL TISYLQSEDFATYYCQQYYSFPLTFGGGTKVEIK
SEQ ID No:47	VL 127, CDR1	QGISSY
	VL 127, CDR2	AAS
SEQ ID No:48	VL 127, CDR3	QQYYSFPLT
SEQ ID No:49	VH 159	EVQLVQSGAEVKKPGESLKISCKGSGYNFTSYWIGW VRQMPGKGLEWMGIIYPGDSDTRYSPSFQGQVTISA DKSISTAYLQWSSLKASDTAMYYCARWGTYYDILTG YFNWFDPWGQGTLVTVSS
SEQ ID No:50	VH 159, CDR1	GYNFTSYW
SEQ ID No:51	VH 159, CDR2	IYPGDSDT
SEQ ID No:52	VH 159, CDR3	ARWGTYYDILTGYFN
SEQ ID No:53	VL 159	DIQMTQSPSSLSASVGDRVTITCRASQGISSWLAWY QQKPEKAPKSLIYAASSLQSGVPSRFSGSGSGTDFTL TISSLQPEDFATYYCQQYYIYPWTFGQGTKVEIK
SEQ ID No:54	VL 159, CDR1	QGISSW
	VL 159, CDR2	AAS
SEQ ID No:55	VL 159, CDR3	QQYYIYPWT
SEQ ID No:56	VH 098	EVQLLESGGGLVQPGGSLRLSCAASGFTFSNYGMSW VRQAPGKGLEWVSAISGSAYSTYYADSVKGRFTISR DNSKNTLWLQMNSLRAEDTAVYYCAKAHYHGSGSYY TLFDYWGQGTLVTVSS
SEQ ID No:57	VH 098, CDR1	GFTFSNYG
SEQ ID No:58	VH 098, CDR2	ISGSAYST
SEQ ID No:59	VH 098, CDR3	AKAHYHGSGSYYTLFDY
SEQ ID No:60	VL 098	DIQMTQSPSSLSASVGDRVTITCRASQGISSWLAWY QQKPEKAPKSLIYAASSLQSGVPSRFSGSGSGTDFTL TISSLQPEDFATYYCQQYNSYPYTFGQGTKLEIK
SEQ ID No:61	VL 098, CDR1	QGISSW
	VL 098, CDR2	AAS
SEQ ID No:62	VL 098, CDR3	QQYNSYPYT

SEQ ID No:63	VH 153	QVQLVESGGGVVQPGRSLRLSCAASGFTFSDYVIHW VRQAPGKGLEWVTVISYDGSNKYYADSVKGRFTISR DNSKNTLYLQMNSLSAEDTAMYYCARGGITGTTGVF DYWGQGTLVTVSS
SEQ ID No:64	VH 153, CDR1	GFTFSDYV
SEQ ID No:65	VH 153, CDR2	ISYDGSNK
SEQ ID No:66	VH 153, CDR3	ARGGITGTTGVFDY
SEQ ID No:67	VL 153	DIQMTQSPSSLSASVGDRVTITCRASQGISSWLAWY QQKPEKAPKSLIYDASSLQSGVPSRFSGSGYGTDFSL TISSLQPEDFAIYYCQQYKSYPITFGQGTRLEIK
SEQ ID No:68	VL 153, CDR1	QGISSW
	VL 153, CDR2	DAS
SEQ ID No:69	VL 153, CDR3	QQYKSYPIT
SEQ ID No:70	VH 132	QVQLVQSGAEVKKPGASVKVSCKASGYTFTSYGISW VRQAPGQGLEWMGWISAYNGNSNYVQKFQGRVTM TTDTTTSTAYMELRSLTSDDTAVYYCAREYSYDSGTY FYYGMDVWGQGTTVTVSS
SEQ ID No:71	VH 132, CDR1	GYTFTSYG
SEQ ID No:72	VH 132, CDR2	ISAYNGNS
SEQ ID No:73	VH 132, CDR3	AREYSYDSGTYFYYGMDV
SEQ ID No:74	VL 132	EIVLTQSPATLSLSPGERATLSCRASQSVSSYLAWYQ QKPGQAPRLLIYDASNRATGIPARFSGSGSGTDFTLTI SSLEPEDFAVYYCQQRSNWPMYTFGQGTKLEIK
SEQ ID No:75	VL 132, CDR1	QSVSSY
	VL 132, CDR2	DAS
SEQ ID No:76	VL 132, CDR3	QQRSNWPMYT

1B)

SEQ ID No: 165	VH 005	EVQLVQSGAEVKKPGESLKISCKASGYSFHFYWIGW VRQMPGKGLEWMGSIYPGDSDTRYRPSFQGQVTISA DKSISTAYLQWTSLKASDTAIYYCARQRGDYYYFYGM DVWGQGTTVTVSS
SEQ ID No: 166	VH 005, CDR1	GYSFHFYW
SEQ ID No: 167	VH 005, CDR2	IYPGDSDT
SEQ ID No: 168	VH 005, CDR3	ARQRGDYYYFYGMDV
SEQ ID No: 169	VL 005	EIVLTQSPGTLSLSPGERATLSCRASQSVSSSYLAWY QQKPGQVPRLLIYGASSRATGIPDRFSGSGSGTDFTL TISRLEPEDFAVYYCQQYGSS-LTFGGGTKVEIK
SEQ ID No: 170	VL 005, CDR1	QSVSSSY
	VL 005, CDR2	GAS
SEQ ID No: 171	VL 005, CDR3	QQYGSSLT

SEQ ID No: 172	VH 006	EVQLLESGGGLVQPGGSLRLSCAASGFTFSNYALIWV RQAPGKGLEWVSIIRGGAGSTYYADSVKGRFTISRD NSKNTLYLQMNSLRAEDTAVYYCAKARIWGPLFDYW GQGTLVTVSS
SEQ ID No: 173	VH 006, CDR1	GFTFSNYA
SEQ ID No: 174	VH 006, CDR2	IRGGAGST
SEQ ID No: 175	VH 006, CDR3	AKARIWGPLFDY
SEQ ID No: 176	VL 006	EIVLTQSPATLSLSPGERATLSCRASQSVSSYLAWYQ QKPGQAPRLLIYDASNRATGIPARFSGSGSGTDFTLTI SSLEPEDFAVYYCQQRSNWPPLTFGGGTKVEIK
SEQ ID No: 177	VL 006, CDR1	QSVSSY
	VL 006, CDR2	DAS
SEQ ID No: 178	VL 006, CDR3	QQRSNWPPLT
SEQ ID No: 179	VH 059	QVQLVQSGAEVKKPGASVRVPCKASGYTFTRYGISW VRQAPGQGLEWMGWISAYNGKTYYAQKLQGRVTMT TDTSTSTAYMELRSLRSDDTAVYYCARSPLLWFEELY FDYWGQGTLVTVSS
SEQ ID No: 180	VH 059, CDR1	GYTFTRYG
SEQ ID No: 181	VH 059, CDR2	ISAYNGKT
SEQ ID No: 182	VH 059, CDR3	ARSPLLWFEELYFDY
SEQ ID No: 183	VL 059	EIVLTQSPGTLSLSPGERATLSCRASQSVSSTYLAWY QQKPGQAPRLLIYGASSRATGIPDRFSGSGSGTDFTL TISRLEPEDFAVYYCQQYGTSLFTFGPGTKVDIK
SEQ ID No: 184	VL 059, CDR1	QSVSSTY
	VL 059, CDR2	GAS
SEQ ID No: 185	VL 059, CDR3	QQYGTSLFT
SEQ ID No: 186	VH 060	EVQLVQSGAEVKKPGESLKISCKGSGYRFTSYWIGW VRQMPGKGLEWMGSIYPGDSYTRNSPSFQGQVTISA DKSIATAYLQWNSLKASDTAMYYCARHAGDFYYFDG LDVWGQGTTVTVSS
SEQ ID No: 187	VH 060, CDR1	GYRFTTSYW
SEQ ID No: 188	VH 060, CDR2	IYPGDSYT
SEQ ID No: 189	VH 060, CDR3	ARHAGDFYYFDGLDV
SEQ ID No: 190	VL 060	EIVLTQSPGTLSLSPGERATLSCRASQSVSSSYLAWY QQKPGQAPRLLIYGASSRATGIPDRFSGSGSGTDFTL TISRLEPEDFAVYYCQQYGSSPPITFGQGTRLEIK
SEQ ID No: 191	VL 060, CDR1	QSVSSSY
	VL 060, CDR2	GAS
SEQ ID No: 192	VL 060, CDR3	QQYGSSPPIT
SEQ ID No: 193	VH 106	EVQLVQSGAEVKKPGESLKISCKGSGYSFTRYWIGW VRQMPGKGLEWMGIIYPGDSDTRYSPSFQGQVTISA DKSISTAYLQWSSLKASDTAMYYCARLTGDRGFDYY SGMDVWGQGTTVTVSS
SEQ ID No: 194	VH 106, CDR1	GYSFTRYW

SEQ ID No: 195	VH 106, CDR2	IYPGDSDT
SEQ ID No: 196	VH 106, CDR3	ARLTGDRGFDYYSGMDV
SEQ ID No: 197	VL 106	EIVLTQSPGTLSLSPGERATLSCRASQSVSSSYLAWY QQKPGQAPRLLIYGASSRATGIPDRFSGSGSGTDFTL TISRLEPEDFAVYYCQQYGSS-FTFGPGTKVDIK
SEQ ID No: 198	VL 106, CDR1	QSVSSSY
	VL 106, CDR2	GAS
SEQ ID No: 199	VL 106, CDR3	QQYGSSFT
SEQ ID No: 200	VH 111	QVQLVQSGAEVKKPGSSVKVSCKASGGTFSSYGISW VRQAPGPGLEWMGRIIPILGIANYAQKFQGRVTITAD KSTNTAYMELSSLRSEDTAVYYCARDQEYSSNWYYW GQGTLVTVSS
SEQ ID No: 201	VH 111, CDR1	GGTFSSYG
SEQ ID No: 202	VH 111, CDR2	IIPILGIA
SEQ ID No: 203	VH 111, CDR3	ARDQEYSSNWYY
SEQ ID No: 204	VL 111	EIVLTQSPGTLSLSPGERATLSCRASQSVRSSYLAWY QQKPGQAPRLLIYGASSRATGIPDRFSGSGSGTDFTL TISRLEPEDFAVYYCQLYGSSPTFGPGTKVDIK
SEQ ID No: 205	VL 111, CDR1	QSVRSSY
	VL 111, CDR2	GAS
SEQ ID No: 206	VL 111, CDR3	QLYGSSPT

Table 2: Mouse origin and heavy and light chain sequence homologies of selected HuMabs.

HuMab:	Mouse:	Strain:	Germline VH:	Germline VL:
169	361494	HCo20	IgHV1-18-01	IgKV3-11-01
050	350633	HCo12	IgHV3-23-01	IgKV1-12-01
084	350615	HCo12-BalbC	IgHV1-69-04	IgKV1-12-01
025	350631	HCo12	IgHV4-34-01	IgKV1D-16-01
091	350630	HCo12	IgHV4-34-01	IgKV1D-16-01
129	359783	HCo12-BalbC	IgHV3-30-3-01	IgKV3-11-01
127	359783	HCo12-BalbC	IgHV5-51-01	IgKV1D-8-01
159	363503	HCo12	IgHV5-51-01	IgKV1D-16-01
098	350659	HCo17	IgHV3-23-01	IgKV1D-16-01
153	359785	HCo12-BalbC	IgHV3-30-3-01	IgKV1D-16-01
132	361487	HCo20	IgHV1-18-01	IgKV3-11-01
005	350611	HCo12-BalbC	IgHV5-51-1	IgKV3-20-01
006	350611	HCo12-BalbC	IgHV3-23-1	IgKV3-11-01
059	350654	HCo17	IgHV1-18-1	IgKV3-20-01

060	350654	HCo17	IgHV5-51-1	IgKV3-20-01
106	350660	HCo17	IgHV5-51-1	IgKV3-20-01
111	350660	HCo17	IgHV1-69-4	IgKV3-20-01

Table 3A and 3B: Heavy chain variable region (VH), light chain variable region (VL) sequences of HuMabs 049, 051, 055, 123, 161, 124, 001, 143, 019, 021, 027, 032, 035, 036, 054, 094 (3A) and HuMabs 041, 150, 067, 072, 163, 093, and 044 (3B). The respective CDRs correspond to those underlined in Figures 1 and 2, for VH and VL sequences, respectively.

3A:

SEQ ID No: 77	VH 049	EVQLLESGGDLVQPGGSLRLSCAASGFTFSSYAMSWVRQAPG KGLEWVSAISGRGGTTYYADSVKGRFTISRDNSKSTLCLQMNS LRAEDTAVYYCAKARANWDYFDYWGQGTLVTVSS
SEQ ID No: 78	VL 049	DIQMTQSPSSVSASVGDRVTITCRASQGISSWLAWYQHKPGK APKLLIYAASILQSGVPSRFSGSGSGTDFTLTISSLRPEDFATYY CQQANSFPITFGQGTRLEIK
SEQ ID No: 79	VH 051	EVQLLESGGDLVQPGGSLRLSCAASGFTFSSYAMSWVRQAPG KGLEWVSAISGRGGTTYYADSVKGRFTISRDNSKSTLCLQMNS LRAEDTAVYYCAKARANWDYFDYWGQGTLVTVSS
SEQ ID No: 80	VL 051	DIQMTQSPSSVSASVGDRVTITCRASQGISSWLAWYQHKPGK APKLLIYAASILQSGVPSRFSGSGSGTDFTLTISSLRPEDFATYY CQQANSFPITFGQGTRLEIK
SEQ ID No: 81	VH 055	EVQLLESGGGLVQPGGSLRLSCAASGFTFSSYAMNWVRQAPG KGLEWVSAISGRGGTTYYADSVKGRFTISRDNSKSTLCLQMNS LRAEDTAVYYCAKARANWDYFDYWGQGTLVTVSS
SEQ ID No: 82	VL 055	DIQMTQSPSSVSASVGDRVTITCRASQGISSWLAWYQHKPGK APKLLIYAASILQSGVPSRFSGSGSGTDFTLTISSLRPEDFATYY CQQANSFPITFGQGTRLEIK
SEQ ID No: 83	VH 123	QVQLVQSGAEVKKPGASVKVSCKAAGYTFTNYGISWVRQAPG QALEWMGWITTYSSNTIYAQKLQGRVTMTTDTSTSTAYMELRS LRSDDTAVYYCARDRVVVRPDYFDYWGQGTLVTVSS
SEQ ID No: 84	VL 123	EIVLTQSPATLSLSPGERATLSCRASQSVSSYLAWYQQKPGQAP RLLIYDTSNRATGIPARFSGSGSGTDFTLTISSLEPEDFAVYYCQ QRSHWPRTFGQGTKVEIK
SEQ ID No: 85	VH 161	QVQLVQSGAEVKKPGASVKVSCKASGYTFTNYGISWVRQAPG QGLEWMGWLSAYSGNTIYAQKLQGRVTMTTDTSTTTAYMELR SLRSDDTAVYYCARDRIVVRPDYFDYWGQGTLVTVSS
SEQ ID No: 86	VL 161	EIVLTQSPATLSLSPGERATLSCRASQSVSSYLAWYQQKPGQAP RLLIYDASNRATGIPARFSGSGSGTDFTLTISSLEPEDFAVYYCQ QRSNWPRTFGQGTKVEIK
SEQ ID No: 87	VH 124	QVQLVQSGAEVKKPGASVKVSCKAAGYTFTNYGISWVRQAPG QGLEWMGWIITYNGNTIYAQRFQDRVTMTTDTSTSTAYMELRS LRSDDTAVYYCARDRIIVRPDYFDYWGQGTLVTVSS
SEQ ID No: 88	VL 124	EIVLTQSPATLSLSPGERATLSCRASQSVSSYLAWYQQKPGQAP RLLIYDASNRATGIPARFSGSGSGTDFTLTISSLEPEDFAVYYCQ QRSNWPRTFGQGTKVEIK

SEQ ID No: 89	VH 001	QVQLQQWGAGLLKPSETLSLTCAVYGGSFSGYYWNWIRQPPG KGLEWIGEINHSGSTNYNPSLKSRVTISVDTSKNQFSLKLSSVT AADTAVYYCARGNYGSGYYYFDLWGRGTQVTVSS
SEQ ID No: 90	VL 001	DIQMTQSPSSLSASVGDRVTITCRASQGISSWLAWYQQKPEK APKSLIFAASSLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYY CQQYISFPITFGQGTRLEIK
SEQ ID No: 91	VH 143	QVQLQQWGAGLLKPSETLSLTCAVYGGSFSGYYWNWIRQPPG KGLEWIGEIHHSGSANYNPSLMSRVTISVDTSKNQFSLQLSSV TAADTAVYYCARGYYGSGYYYFDYWGQGTLVTVSS
SEQ ID No: 92	VL 143	DIQMTQSPSSLSASVGDRVTITCRASQGISSWLAWYQQKPEK APKSLIYAASRLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYY CQQYNSYPITFGQGTRLEIK
SEQ ID No: 93	VH 019	QVQLQQWGAGLLKPSETLSLTCAVYGGSFSDYYWNWIRQPPG KGLEWIGEIHHVGSTNYNPSLKSRVTISVDTSKSQFSLKLSSVT AADTAVYYCARGYYDSGVYYFDYWAQGTLVTVSS
SEQ ID No: 94	VL 019	DIQMTQSPSSLSASVGDRVTITCRASQGISRWLAWYQQKPEK APKSLIYAASSLRSGVPSRFSGSGSGTDFTLTISSLQPEDFATYY CQQYNSYPITFGQGTRLEIK
SEQ ID No: 95	VH 021	QVQLQQWGAGLLKPSETLSLTCAVYGGSFSDYYWNWIRQPPG KGLEWIGEIHHSGSTNYNPSLKSRVTISVDTSKNQFSLKLSSVT AADTAVYYCARGYYASGVYYFDYWGQGTLVTVSS
SEQ ID No: 96	VL 021	DIQMTQSPSSLSASVGDRVTITCRASQGISSWLAWYQQKPEK APKSLIYAASSLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYY CQQYNSYPITFGQGTRLEIK
SEQ ID No: 97	VH 027	QVQLQQWGAGLLKPSETLSLTCAVYGGSFSDYFWNWIRQPPG KGLEWIGEIHHSGSTNYNPSLKSRVTISVDTSKNQFSLNLSSVT AADTAVYYCARGLIGSGYYYFDYWDQGTLVTVSS
SEQ ID No: 98	VL 027	DIQMTQSPSSLSASVGDRVTITCRASQGISSWLAWYQQKPEK APKSLIYAASSLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYY CQQYNSYPITFGQGTRLEIK
SEQ ID No: 99	VH 032	QVQLQQWGAGLLKPSETLSLTCAVYGGSFSGYYWSWIRQPPG KGLEWIGEINHSGDTNYNPSLTSRVTISVDTSKNQFSLKLSSVT AADTAVYYCARLFYGSGIYYFDYWGQGTLVTVSS
SEQ ID No: 100	VL 032	DIQMTQSPSSLSASVGDRVTITCRASQGISSWLAWYQQKPEK APKSLIYATFRLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYY CQQYNSFPPTFGQGTKVEIK
SEQ ID No: 101	VH 035	QVQLQQWGAGLLKPSETLSLTCAIYGGSFSGYYWSWIRQPPG KGLEWIGEINHSGDTNYNPSLTSRVTISVDTSKNQFSLKLSSVT AADTAVYYCARLFYGSGIYYFDYWGQGTLVTVSS
SEQ ID No: 102	VL 035	DIQMTQSPSSLSASVGDRVTITCRASQGISSWLAWYQQKPEK APKSLIYATFRLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYY CQQYNSFPPTFGQGTKVEIK
SEQ ID No: 103	VH 036	QVQLQQWGAGLLKPSETLSLTCAVYGGSFSDYYWSWIRQPPG KGLEWIGEINHSGSTNYNPSLKSRVTISVDTSKNQFSLKLSSVT AADTAVYYCARLYYGSGTYYFDYWGQGTLVTVSS
SEQ ID No: 104	VL 036	DIQMTQSPSSLSASVGDRVTITCRASQGISSWLTWYQQKPEKA PKSLIYAASRLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYC QQYNSFPPTFGQGTKVEIK
SEQ ID No: 105	VH 054	QVQLQQWGAGLLKPSETLSLTCAVYGGSFSGYYWSWIRQPPG KGLEWIGEIHHSGSTNYNPSLKSRVTISVDTSKNQFSLKLSSVT AADTAVYYCARLWYGSGSYYFDYWGQGTLVTVSS
SEQ ID No: 106	VL 054	DIQMTQSPSSLSASVGDRVTITCRASQGISSWLAWYQQKPEK APKSLIYAASSLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYY CQQYNSFPPTFGGGTKVEIK

SEQ ID No: 107	VH 094	QVQLQQWGAGLLKPSETLSLTCAVSGGSFSGYYWTWIRQPPG KGLEWIGEIYHSGDTNYNPSLKSRVTISVDTSKNQFSLKLYSVT AADTAVYYCARLYFGSGIYYLDYWGQGTLVTVSS
SEQ ID No: 108	VL 094	DIQMTQSPSSLSASVGDRVTITCRASQGISSWLVWYQQKPEK APKSLIYAASSLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYY CQQYNSFPPTFGQGTKVEIK
SEQ ID No: 109	VH 105	EVQLLESGGGLVQPGGSLRLSCAASGFTFSNYGMSWVRQAPG KGLEWVSAISGSAYSTYYADSVKGRFTISRDNSKNTLWLQMNS LRAEDTAVYYCAKAHYHGSGSYYTLFDYWGQGTLVTVSS
SEQ ID No: 110	VL 105	DIQMTQSPSSLSASVGDRVTITCRASQGISSWLAWYQQKPEK APKSLIYAASSLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYY CQQYNSYPYTFGQGTKLEIK
SEQ ID No: 111	VH 100	EVQLLESGGGLVQPGGSLRLSCAASGFTFNNYGMNWVRQAPG KGLEWVSAISGTGYSTYYADSVKGRFTISRDNSKNTLYLQMNS LRAEDTAVYYCAKAHYFGSGSYYTLFDYWGQGTLVTVSS
SEQ ID No: 112	VL 100	DIQMTQSPSSLSASVGDRVTITCRASQGISSWLAWYQQKPEK APKSLIYAASSLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYY CQQYNSYPYTFGQGTKLEIK
SEQ ID No: 113	VH 125	EVQLLESGGGLVQPGGSLRLSCAASGFTFTDYAMNWVRQAPG KGLEWVSTISGSGYATYYADSVKGRFTISRDNSKTTLYLQMNS LRAEDTAVYYCAKGHTLGSGSYYTLFDYWGQGTLVTVSS
SEQ ID No: 114	VL 125	DIQMTQSPSSLSASVGDRVTITCRASQGINSWLAWYQQKPEK APKSLIYAASSLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYY CQQYNSYPYTFGQGTKLEIK
SEQ ID No: 115	VH 162	EVQLWESGGGSVQPGGSLRLSCAASGFTFSSYGMSWVRQAP GKGLEWVSGISGSGYSTYYADSVKGRFTISRDNSKNTLYLQMN SLRAEDTAVYYCAKGYYHGSGSYYTSFDYWGQGTLVTVSS
SEQ ID No: 116	VL 162	DIQMTQSPSSLSASVGDRVTITCRASQGISSWLAWYQQKPEK APKSLIYAASSLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYY CQQYNSYPLTFGGGTKVEIK
SEQ ID No: 117	VH 033	QVQLVESGGGVVQTGRSLRLSCAASGFTFSSHAMHWVRQAPG KGLEWVAAISYDGSNKYYADSVKGRFTISRDNSKNTLYLQMNS LRAEDTAVYYCARGDYISSSGVFDYWGQGTLVTVSS
SEQ ID No: 118	VL 033	DIQMTQSPSSLSASVGDRVTITCRASQGISSWLAWYQQKPEK APKSLIYAASSLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYY CQQYNSYPITFGQGTRLEIK
SEQ ID No: 119	VH 160	QVQLVESGGGVVQPGRSLRLSCAASGFTFSSHAMHWVRQAPG KGLEWVAAISYDGSNKYYADSVKGRFTISRDNSKNTMYLQMN SLRAEDTAMCYCARGSITGSTGVFDYWGQGTLVTVSS
SEQ ID No: 120	VL 160	DIQMTQSPSSLSASVGDRVTITCRASQDISSWLAWYQQKPEK APKSLIYAASSLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYY CQQYNSYPITFGQGTRLEIK
SEQ ID No: 121	VH 166	QVQLVESGGGVVQPGRSLRLSCAASGFTFSSYAMHWVRQAPG KGLEWVAVISYDGSNEYYADSVKGRFTISRDNSKNTLYLQMNS LRAEDTAVYYCARGSIIGSTGVFDYWGQGTLVTVSS
SEQ ID No: 122	VL 166	DIQMTQSPSSLSASVGDRVTITCRASQGISNWLAWYQQKPEK APKSLIYDASSLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYY CQQYNSYPITFGQGTRLEIK
SEQ ID No: 123	VH 152	QVQVVESGGGVVQPGRSLRLSCAASGFTFSSYAMHWVRQAPG KGLEWVAVISYDGSYKYYADSVKGRFTISRDNSKNTLYLQMNS LRAEDTAVYYCARGSITGSTGVFDYWGQGTLVTVSS
SEQ ID No: 124	VL 152	DIQMTQSPSSLSASVGDRVTITCRASQGINSWLAWYQQKPEK APKSLIYDASSLQSGVPSRFSGSGSGTDFTLTISSLQPENFATYY CQQYNSYPITFGQGTRLEIK

SEQ ID No: 125	VH 167	QVQLVESGGGVVQPGRSLRLSCAASGFTFSSYAIHWVRQAPG KGLEWVAVISYDGSNKYYADSVKGRFTISRDNSKNTLYLQMNS LRAEDTAVYYCARGSITGSTGVFDYWGQGTLVTVSS
SEQ ID No: 126	VL 167	DIQMTQSPSSLSASVGDRVTITCRASQGISNWLAWYQQKPEK APKSLIYDASSLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYY CQQYNSYPITFGQGTRLEIK

3B:

SEQ ID No: 207	VH 041	EVQLVQSGAEVKKPGESLKISCKGSGYSFTSYWIGWVRQMPG KGLEWMGSIYPGDSHTRYRPSFQGQVTISADKSISTAYLQWSS LKASDTAMYYCARQKGDFYYFFGLDVWGQGTAITVSS
SEQ ID No: 208	VL 041	EIVLTQSPGTLSLSPGERATLSCRASQSVSSSYLAWYQQKPGQ APRLLIYGASSRATGIPDRFSGSGSGTDFTLTISRLEPEDFAVYY CQQYGSSLTFGGGTKVEIK
SEQ ID No: 209	VH 150	EVQLVQSGAEVKKPGESLKISCKGSGYSFTSYWIGWVRQMPG KGLEWMGSIYPGDSHTRYRPSFQGQVTISADKSISTAYLQWSS LKASDTAMYYCARQAGDYYYYNGMDVWGQGTTVTVSS
SEQ ID No: 210	VL 150	EIVLTQSPGTLSLSPGERATLSCRASQSVSSSYLTWYQQKPGQ APRLLIYGASSRATGIPDRFSGSGSGTDFTLTISRLEPEDFAVYY CQQYGSSLTFGGGTKVEIK
SEQ ID No: 211	VH 067	EVQLVQSGAEVKKPGESLKISCKGSGYSFTSYWIGWVRQMPG KGLEWMGIIYPGDSDTRYSPSFQGQVTISVDKSISTAYLQWSS LKASDTAMYYCARQKGDYYYHYGLDVWGQGTTVTVSS
SEQ ID No: 212	VL 067	EIVLTQSPGTLSLSPGERATLSCRASQSVSSSYLAWYQQKPGQ APRLLIYGASSRATGIPDRFSGSGSGTDFTLTISRLEPEDFAVYY CQQYGSSPRLTFGGGTKVEIK
SEQ ID No: 213	VH 072	EVQLVQSGAEVKKPGESLKISCKGSGYSFTSYWIGWVRQMPG KGLEWMGIIYPGDSDTRYSPSFQGQVTISADKSISTAYLQWSS LKASDTAMYYCARQKGDYYYFNGLDVWGQGTTVTVSS
SEQ ID No: 214	VL 072	EIVLTQSPGTLSLSPGERATLSCRASQSVSSSYLAWYQQKPGQ APRLLIYGASSRATGIPDRFSGSGSGTDFTLTISRLEPEDFAVYY CQQYGSSPRLTFGGGTKVEIK
SEQ ID No: 215	VH 163	EVQLVQSGAEVKKPGESLKISCQGSGYRFISYWIGWVRQMPG KGLEWMGRIYPGDSDTRYSPSFQGQVTISVDKSISTAYLQWSS LKASDTAMYYCARQRGDYYYFNGLDVWGQGTTVTVSS
SEQ ID No: 216	VL 163	EIVLTQSPGTLSLSPGERATLSCRASQSVSSSYLAWYQQKPGQ APRLLIYGASSRATGIPDRFSGSGSGTDFTLTISRLEPEDFAVYY CQQYGSSLTFGGGTKVEIK
SEQ ID No: 217	VH 093	EVQLVQSGAEVKKPGESLKISCKGSGYSFTSYWIGWVRQMPG KGLEWMGRIYPGDSDTRYSPSFQGQVTISADKSITTAYLQWSS LRASDTAMYYCARQRGDYYYFFGLDIWGQGTTVTVSL
SEQ ID No: 218	VL 093	EIVLTQSPGTLSLSPGERATLSCRASQSVSSSYLAWYQQKPGQ APRLLIYGASSRATGIPDRFSGSGSGTDFTLTISRLEPEDFAVYY CQQYGSSLTFGGGTKVEIK
SEQ ID No: 219	VH 044	EVQLVQSGAEVKKPGESLKISCKGSGYRFSSYWIGWVRQMPG KGLEWMGSIFPGDSDTRYSPSFQGQVTISADKSITTAYLQWSS LKASDTAMYYCARQAGDYYYYNGMDVWGQGTTVTVSS
SEQ ID No: 220	VL 044	EIVLTQSPGTLSLSPGERATLSCRASQSVSSSYLAWYQQKPGQ APRLLIYGASSRATGIPDRFSGSGSGTDFTLTISRLEPEDFAVYY CQQYGSSLTFGGGTKVEIK

Table 4: Consensus CDRs based on sequence alignments shown in Figures 1 and 2.

SEQ ID No: 9 050-049-051-055	IgHV3-23-1	VH CDR1	GFTFSSYA	
SEQ ID No: 127 050-049-051-055	IgHV3-23-1	VH CDR2	ISGX1GGX2T	Wherein X1=R or S, and X2=T or S; preferably, wherein X1=R and X2=T
SEQ ID No: 11 050-049-051-055	IgHV3-23-1	VH CDR3	AKARANWDYFDY	
SEQ ID No: 128 084	IgHV1-69-04	VH CDR1	GGTFX1X2YA	Wherein X1=R or S, and X2=T or S; preferably, wherein X1=R and X2=T
SEQ ID No: 129 084	IgHV1-69-04	VH CDR2	IX1X2X3LGIX4	Wherein X1=N or I, X2=T or P, X3=V or I, and X4=V or A, preferably, wherein X1=N, X2=T, X3=V, and X4=V
SEQ ID No: 130 084	IgHV1-69-04	VH CDR3	AREKGVDYYYGX1X2	Wherein X1=I or M, X2=E or D; preferably, wherein X1=I, X2=E
SEQ ID No: 131 169-123-161-124	IgHV1-18-1	VH CDR1	GYTFTXYG	Wherein X=N or S, preferably N
SEQ ID No: 132 169-123-161-124	IgHV1-18-1	VH CDR2	IX1X2YX3GNT	Wherein X1=S, T, or I; X2=A or T; X3=S or N; preferably, wherein X1=S, X2=A, and X3=S
SEQ ID No: 133 169-123-161-124	IgHV1-18-1	VH CDR3	ARDRX1X2VRPDYFDY	Wherein X1=I or V, X2=V or I; preferably, wherein X1=I and X2=V
SEQ ID No: 134 025-001-143-019-021-027	IgHV4-34-01	VH CDR1	GGSFSX1YX2	Wherein X1=D or G and X2=Y or F; preferably, wherein X1=D and X2=Y

SEQ ID No: 135 025-001-143-019-021-027	IgHV4-34-01	VH CDR2	IX1HX2GSX3	Wherein X1=H or N, X2=S or V, and X3=T or A; preferably, wherein X1=H, X2=S, and X3=T
SEQ ID No:136 025-001-143-019-021-027	IgHV4-34-01	VH CDR3	ARGX1X2X3SG X4YYFDX5	Wherein X1=Y, N or L; X2=Y or I, X3=D, G or A; X4=V or Y; and X5=Y or L; preferably, wherein X1=Y, X2=Y, X3=D, X4=V, and X5=Y
SEQ ID No:137 091-032-035-036-054-094	IgHV4-34-01	VH CDR1	GGSFSX1YY	Wherein X1=G or D, preferably G
SEQ ID No:138 091-032-035-036-054-094	IgHV4-34-01	VH CDR2	IX1HSGX2T	Wherein X1=Y, N or H; and X2=D or S; preferably, wherein X1=Y and X2=D
SEQ ID No:139 091-032-035-036-054-094	IgHV4-34-01	VH CDR3	ARLX1X2GSGX 3YYX4DY	Wherein X1=Y, F or W; X2=F or Y; X3=I, T or S; and X4=L or F; preferably, wherein X1=Y, X2=F, X3=I, and X4=L
SEQ ID No:140 129	IgHV3-30-01	VH CDR1	GFTFSX1X2A	Wherein X1=T or F, X2=F or Y; preferably, wherein X1=T and X2=F
SEQ ID No:141 129	IgHV3-30-01	VH CDR2	ISYDGX1X2K	Wherein X1=G or S, X2=H or N; preferably, wherein X1=G and X2=H
SEQ ID No:142 129	IgHV3-30-01	VH CDR3	ARGLGVWGX1F DY	Wherein X1=A or Y, preferably A
SEQ ID No:143 098-105-100-125-162	IgHV3-23-01	VH CDR1	GFTFX1X2YX3	Wherein X1=S, N or T; X2=N, D or S; and X3=G or A; preferably, wherein X1=S, X2=N and X3=G
SEQ ID No:144 098-105-100-125-162	IgHV3-23-01	VH CDR2	ISGX1X2X3X4T	Wherein X1=S or T, X2=A or G, X3=Y or G, X4=S or A; preferably, wherein X1=S, X2=A, X3=Y, X4=S

SEQ ID No:145 098-105-100-125-162	IgHV3-23-01	VH CDR3	AKX1X2X3X4G SGSYYTX5FDY	Wherein X1=A or G; X2=H or Y; X3=Y or T; X4=H, F or L; X5=L or S; preferably, wherein X1=A; X2=H; X3=Y; X4=H; X5=L
SEQ ID No:146 127	IgHV5-51-01	VH CDR1	GYSFX1X2YW	Wherein X1=S or T, X2=I or S; preferably, wherein X1=S, X2=I
SEQ ID No:147 127	IgHV5-51-01	VH CDR2	IX1PGDSDX2	Wherein X1=F or Y, X2=I or T; preferably, wherein X1=F, X2=I
SEQ ID No:148 127	IgHV5-51-01	VH CDR3	ARQPGDWSPR HWYFDL	
SEQ ID No:149 159	IgHV5-51-01	VH CDR1	GYXFTSYW	Wherein X=N or S, preferably N
SEQ ID No:51 159	IgHV5-51-01	VH CDR2	IYPGDSDT	
SEQ ID No:52 159	IgHV5-51-01	VH CDR3	ARWGTYYDILT GYFN	
SEQ ID No:71 132	IgHV1-18-01	VH CDR1	GYTFTSYG	
SEQ ID No:150 132	IgHV1-18-01	VH CDR2	ISAYNGNX	Wherein X=S or T, preferably S
SEQ ID No:151 132	IgHV1-18-01	VH CDR3	AREYSYDSGTY FYYGMDV	
SEQ ID No:152 153-033-160-166-152-167	IgHV3-30-03-01	VH CDR1	GFTFSX1X2X3	Wherein X1=D or S, X2=Y or H, X3=V or A; preferably, wherein X1=D, X2=Y, X3=V
SEQ ID No:153 153-033-160-166-152-167	IgHV3-30-03-01	VH CDR2	ISYDGSX1X2	Wherein X1=N or Y, X2=K or E, preferably wherein X1=N and X2=K

SEQ ID No:154 153-033-160-166-152-167	IgHV3-30-03-01	VH CDR3	ARGX1X2X3X4X5X6GX7FDY	Wherein X1=G, D or S; X2=I or Y; X3=T or I; X4=G or S; X5=T or S; X6=T or S; X7=Y or V; preferably, wherein X1=G; X2=I; X3=T; X4=G; X5=T; X6=T; and X7=V
SEQ ID No:13 050-084-049-051-055	IgKV1-12-01	VL CDR1	QGISSW	
050-084-049-051-055	IgKV1-12-01	VL CDR2	XAS	Wherein X=A or V
SEQ ID No:155 050-084-049-051-055	IgKV1-12-01	VL CDR3	QQANSFPXT	Wherein X=I or L
SEQ ID No:6 169-124-161-123	IgKV3-11-01	VL CDR1	QSVSSY	
169-124-161-123	IgKV3-11-01	VL CDR2	DXS	Wherein X=A or T, preferably A
SEQ ID No:156 169-124-161-123	IgKV3-11-01	VL CDR3	QQRSXWPRT	Wherein X=N or H, preferably N
SEQ ID No:157 025-001-019-143-021-027	IgKV1D-16-01	VL CDR1	QGISXW	Wherein X=R or S, preferably R
025-001-019-143-021-027	IgKV1D-16-01	VL CDR2	AAS	
SEQ ID No:164 025-001-019-143-021-027	IgKV1D-16-01	VL CDR3	QQYNSXPIT	Wherein X=Y or F, preferably Y
SEQ ID No:33 091-032-035-036-054-094	IgKV1D-16-01	VL CDR1	QGISSW	
091-032-035-036-054-094	IgKV1D-16-01	VL CDR2	AX1X2	Wherein X1=A or T, and X2=S or F; preferably, wherein X1=A and X2=S

SEQ ID No:158 091-032-035-036-054-094	IgKV1D-16-01	VL CDR3	QQYNSFPPT	
SEQ ID No:159 098-100-105-125-162	IgKV1D-16-01	VL CDR1	QGIXSW	Wherein X=S or N, preferably S
098-100-105-125-162	IgKV1D-16-01	VL CDR2	AAS	
SEQ ID No:160 098-100-105-125-162	IgKV1D-16-01	VL CDR3	QQYNSYPXT	Wherein X=Y or L, preferably Y
SEQ ID No:161 153-152-166-167-160-033	IgKV1D-16-01	VL CDR1	QGIX1X2W	Wherein X1=S or N; X2=S or N; preferably, wherein X1=X2=S
153-152-166-167-160-033	IgKV1D-16-01	VL CDR2	XAS	Wherein X=D or A, preferably D
SEQ ID No:162 153-152-166-167-160-033	IgKV1D-16-01	VL CDR3	QQYXSYPIT	Wherein X=K or N, preferably K
SEQ ID No: 221 005-060-106-041-150-067-072-163-093-044	IgHV5-51-1	VH CDR1	GYX1FX2X3YW	wherein X1=S or R; X2=S, T, H, or I; and X3=S, R, or F; preferably, wherein X2=H or T
SEQ ID No: 222 005-060-106-041-150-067-072-163-093-044	IgHV5-51-1	VH CDR2	IX1PGDSX2T	wherein X1=Y or F; X2=D, Y, or H preferably, wherein X2=D or Y

SEQ ID No: 223 005-060-106-041-150-067-072-163-093-044	IgHV5-51-1	VH CDR3	ARX1X2X3X4X5X6X7X8YX9X10GX11DX12	wherein X1=Q, H, or L; X2= R, A, T, or K; X3=G; X4=D; X5=R or none; X6=G or none; X7=Y or F; X8=Y or D; X9=Y, F, or H; X10=Y, D, S, F, or N; X11=M or L; and X12=V or I; preferably, wherein X1=Q, X2= R or A; X5=X6=none; X7=Y or F; X8=Y; X9=F; X10=Y; and X12=V
SEQ ID No: 224 006	IgHV3-23-1	VH CDR1	GFTFSXYA	wherein X=N or S, preferably N
SEQ ID No: 225 006	IgHV3-23-1	VH CDR2	IX1GX2X3GST	wherein X1=R or S; X2=G or S; and X3=A or G, preferably wherein X1=R; X2=G; and X3=A
SEQ ID No: 226 006	IgHV3-23-1	VH CDR3	AKRIWGPXFDY	wherein X=L or Y, preferably L
SEQ ID No: 227 059	IgHV1-18-1	VH CDR1	GYTFTXYG	wherein X=R or S, preferably R
SEQ ID No: 228 059	IgHV1-18-1	VH CDR2	ISAYNGXT	wherein X=K or N, preferably K
SEQ ID No: 229 059	IgHV1-18-1	VH CDR3	ARSPLLWFEELY FDY	
SEQ ID No:230 111	IgHV1-69-4	VH CDR1	GGTFSSYX	wherein X=G or A, preferably G
SEQ ID No: 202 111	IgHV1-69-4	VH CDR2	IIPILGIA	
SEQ ID No: 231 111	IgHV1-69-4	VH CDR3	ARDQEYSSX1X2X3	wherein X1=N or Y; X2=W or F; and X3=Y or D, preferably wherein X1=N; X2=W; and X3=Y

SEQ ID No: 232 005-059-060-106-111-041-150-067-072-163-093-044	IgKV3-20-01	VL CDR1	QSVX1SX2Y	wherein X1=S or R and X2=S or T
005-059-060-106-111-041-150-067-072-163-093-044	IgKV3-20-01	VL CDR2	GAS	
SEQ ID No: 233 005-059-060-106-111-041-150-067-072-163-093-044	IgKV3-20-01	VL CDR3	QX1YGX2SX3X4X5T	wherein X1=Q or L; X2=S or T; X3=P or none; X4=P, L, R, or none; and X5=L, F, I, or none; preferably, wherein X4=P, L, or none
SEQ ID No: 177 006	IgKV3-11-01	VL CDR1	QSVSSY	
006	IgKV3-11-01	VL CDR2	DAS	
SEQ ID No: 178 006	IgKV3-11-01	VL CDR3	QQRSNWPPLT	

Example 11 - Purification of antibodies

Culture supernatant was filtered over 0.2 µm dead-end filters, loaded on 5 mL MabSelect SuRe columns (GE Health Care) and eluted with 0.1 M sodium citrate-NaOH, pH 3. The eluate was immediately neutralized with 2M Tris-HCl, pH 9 and dialyzed overnight to 12.6 mM NaH2PO4, 140 mM NaCl, pH 7.4 (B.Braun). Alternatively, subsequent to purification, the eluate was loaded on a HiPrep Desalting column and the antibody was exchanged into 12.6 mM NaH2PO4, 140 mM NaCl, pH 7.4 (B.Braun) buffer. After dialysis or exchange of buffer, samples were sterile filtered over 0.2 µm dead-end filters. Purity was determined by SDS-PAGE and concentration was measured by nephelometry and absorbance at 280 nm. Purified antibodies were stored at 4°C. Mass spectrometry was performed to identify the molecular mass of the antibody heavy and light chains expressed by the hybridomas as described in Example 9.

Example 12 – Binding of HER2 clones to tumor cells expressing membrane-bound HER2 measured by means of FACS analysis

The binding of HER2 antibodies to AU565 cells (purchased at ATCC, CRL-2351) and A431 cells (purchased at ATCC, CRL-1555), was tested using flow cytometry (FACS Canto II, BD Biosciences). Qifi analysis (Dako, Glostrup, Denmark) revealed that AU565 cells expressed on average 1,000,000 copies of HER2 protein per cell, whereas A431 cells expressed on average 15,000 copies per cell. Binding of HER2 antibodies was detected using a Phycoerythrin (PE)-conjugated goat-anti-human IgG antibody (Jackson). Trastuzumab (clinical-grade Herceptin®) was used as positive control antibody, and an isotype control antibody was used as negative control antibody. EC_{50} values were determined by means of non-linear regression (sigmoidal dose-response with variable slope) using GraphPad Prism V4.03 software (GraphPad Software, San Diego, CA, USA).

All tested HER2 antibodies bound to HER2 expressed on both AU565 and A431 cells in a dose-dependent manner. For antibodies of cross-block groups 1, 2 and 3, the EC_{50} values for binding varied between 0.336-2.290 µg/mL for AU565 cells and 0.068-1.135 µg/mL for A431 cells (Figure 3A-D). For antibodies of cross-block group 4, the EC_{50} values for binding varied between 0.304-2.678 µg/mL for AU565 cells and 0.106-1.982 µg/mL for A431 cells (Figure 3E and F). Especially on A431 cells, large differences in EC_{50} values were observed between the tested antibodies. However, antibody 098 had the best (*i.e.*, lowest) EC_{50} value on both types of cells. Also some differences in maximum binding levels were observed between different antibodies, on both AU565 and A431 cells. Of the tested cross-block groups 1-3 antibodies, antibody 098 also had the highest maximum binding level on AU565 cells, whereas antibody 025 had the highest maximum binding level on A431 cells. For antibodies of cross-block group 4, antibodies 005 and 006 demonstrated higher maximum binding levels on A431 as compared to other HER2 antibodies.

Example 13 - Binding of HER2 antibodies to membrane-bound HER2 expressed on Rhesus epithelial cells measured by means of FACS analysis

To determine cross-reactivity with Rhesus HER2, the binding of HER2 antibodies to HER2-positive Rhesus epithelial cells (4MBr-5 purchased at ATCC) was tested using flow cytometry (FACS Canto II, BD Biosciences). A Phycoerythrin-conjugated goat-anti-human IgG antibody (Jackson) was used as a secondary conjugate. An isotype control antibody was used as negative control antibody.

All tested HER2 antibodies were cross-reactive with Rhesus monkey HER2 (Figure 4A and B). At both tested concentrations (1 µg/mL and 10 µg/mL), the HER2 antibodies were able to bind specifically to Rhesus monkey HER2. Antibody 127 demonstrated poor binding

at 1 µg/mL concentration, but showed good binding at 10 µg/mL concentration. Antibody 098 had the highest binding level at both antibody concentrations. No binding was observed with the isotype control antibody.

Example 14 - Competition of HER2 antibodies for binding to soluble Her2ECDHis measured in sandwich-ELISA

The optimal coating concentrations of the tested HER2 antibodies and optimal Her2ECDHis concentration were determined in the following manner: ELISA wells were coated overnight at 4°C with HER2 HuMabs serially diluted in PBS (0.125-8 µg/mL in 2-fold dilutions). Next, the ELISA wells were washed with PBST (PBS supplemented with 0.05% Tween-20 [Sigma-Aldrich, Zwijndrecht, The Netherlands]) and blocked for one hour at room temperature (RT) with PBSTC (PBST supplemented 2% [v/v] chicken serum [Gibco, Paisley, Scotland]). The ELISA wells were then washed with PBST and incubated for one hour at RT with Her2ECDHis serially diluted in PBSTC (0.25-2 µg/mL in 2-fold dilutions). Unbound Her2ECDHis was washed away with PBST, and bound Her2ECDHis was incubated for one hour at RT with 0.25 µg/mL biotinylated rabbit-anti-6xhis-biot (Abcam, Cambridge, UK). The plate was thereafter washed with PBST and incubated for one hour with 0.1 µg/mL Streptavidin-poly-HRP (Sanquin, Amsterdam, The Netherlands) diluted in PBST. After washing, the reaction was visualized through a 15 minutes incubation with 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS: one ABTS tablet diluted in 50 mL ABTS buffer (Roche Diagnostics, Almere, The Netherlands)) at RT protected from light. The colorization was stopped by adding an equal volume of oxalic acid (Sigma-Aldrich, Zwijndrecht, The Netherlands). Fluorescence at 405 nm was measured on a microtiter plate reader (Biotek Instruments, Winooski, USA). The antibody concentrations that resulted in sub-optimal binding of each antibody were determined and used for the following cross-block experiments.

Each HER2 antibody was coated to the ELISA wells at the sub-optimal dose that was determined as described above. After blocking of the ELISA wells, the wells were incubated with the predetermined concentration of 1 µg/mL biotinylated Her2ECDHis in the presence or absence of an excess of a second (competitor) HER2 antibody. The ELISA was then performed as described above. Residual binding of Her2ECDHis to the coated antibody was expressed as a percentage relative to the binding observed in the absence of competitor antibody. Percentage competition was then determined as 100 minus the percentage of inhibition. 75% competition was considered as complete cross-block, whereas 25-74% competition was considered as partial cross-block, and 0-24% competition was considered non-blocking.

Cross-block groups 1, 2 and 3:

As shown in Table 5A, all HER2 antibodies of these groups were found to be able to block binding to Her2ECDHis, at least partially, for themselves. After dividing the antibodies into 3 major cross-block groups, all antibodies were tested for competition with at least one representative antibody from each group.

The first group comprised trastuzumab and antibodies 169, 050 and 084, which blocked each other for binding to Her2ECDHis, but did not cross-block antibodies from other groups.

The second group comprised pertuzumab and antibodies 025, 091 and 129, which blocked each other for binding to Her2ECDHis, except for antibodies 129 and 091 which both cross-blocked pertuzumab and 025, but not each other. None of the antibodies of group 2 blocked antibodies from other groups.

A third group comprised antibodies C1, F5, 127, 098, 132, 153 and 159, which did not cross-block any antibody from the other groups. Within this group 3, some variation was observed. Antibody 127 was the only antibody that was able to cross-block all other antibodies in this group for binding to Her2ECDHis; antibody 159 cross-blocked all other antibodies within this group, except 132; clone 098 cross-blocked all antibodies of group 3, except 132 and 153; antibody 153 cross-blocked 127, 132 and 159 for binding to Her2ECDHis, but not 098, C1 or F5; clone 132 cross-blocked 127, 132 and 153. When added as competitor antibodies, F5 and C1 only demonstrated cross-blocking of each other. However, the reverse reaction also revealed competition with antibodies 127, 098 and 159, but not 153 and 132. Possibly, these differences may have resulted from lower affinities of antibodies C1 and F5 for Her2ECDHis.

Values higher than 100% can be explained by avidity effects and the formation of antibody-Her2ECDHis complexes containing two non-competing antibodies.

Cross-block group 4:

As shown in Table 5, all HER2 antibodies of this group competed for binding to Her2ECDHis, at least partially, with themselves. Trastuzumab (clinical grade Herceptin®) and pertuzumab (TH1014-pert, transiently produced in HEK-293 cells) could only compete with themselves, and not with any of the other listed HER2 antibodies of cross-block group 4. C1 and F5 (both transiently produced in HEK-293 cells) competed with each other for binding to Her2ECDHis, but did not compete with other HER2 antibodies of cross-block group 4.

Antibodies 005, 006, 059, 060, 106 and 111 all competed with each other for binding to Her2ECDHis, but did not cross-block with trastuzumab, pertuzumab, C1 or F5. Clones 005, 059, 060 and 106 only blocked 006 when 006 was the competitor antibody. In

the reverse reaction where 006 was immobilized, no blocking was found with 005, 059, 060 or 106. This was possibly a result of the higher apparent affinity of clone 006 compared to 005, 059, 060, 106 and 111, shown in Figures 3A and 3B. Values higher than 100% can be explained by avidity effects and the formation of antibody-Her2ECDHis complexes containing two non-blocking antibodies.

Table 5: Competition and cross-blocking of HER2 antibodies for binding to Her2ECDHis

5A:

Immobilized mAb ↓	Competing mAb →														
	tras	**169**	**050**	**084**	**pert**	**025**	**091**	**129**	**C1**	**F5**	**127**	**159**	**098**	**153**	**132**
Trastuzumab	6	15	6	51	100	107	100	85	103	99	115	90	101	101	101
TH1014-169	19	45	21	73	101	98	105	106	ND	ND	ND	ND	105	102	ND
TH1014-050	13	30	12	74	95	104	98	110	ND	ND	ND	ND	102	104	ND
TH1014-084	74	73	76	20	101	106	104	104	ND	ND	ND	ND	109	98	ND
TH1014-pert	104	100	94	95	9	20	19	39	106	125	116	81	103	100	109
TH1014-025	98	98	100	104	8	18	21	15	ND	ND	ND	ND	102	99	ND
TH1014-091	99	99	95	100	5	13	15	78	ND	ND	ND	ND	98	98	ND
TH1014-129	93	99	97	92	22	55	76	12	ND	ND	ND	ND	106	98	ND
TH1014-C1	89	ND	ND	ND	ND	ND	ND	ND	65	58	73	53	58	77	90
TH1014-F5	197	ND	ND	ND	ND	ND	ND	ND	70	21	62	15	16	80	125
TH1014-127	102	ND	ND	ND	ND	ND	ND	ND	112	88	11	8	58	21	44
TH1014-159	111	ND	ND	ND	112	ND	ND	ND	96	86	15	6	11	40	79
TH1014-098	107	102	100	103	104	108	104	107	125	96	21	9	17	110	142
TH1014-153	134	111	103	107	121	97	102	106	257	96	27	23	115	28	33
TH1014-132	353	ND	ND	ND	288	ND	ND	ND	422	379	30	131	309	41	32
Cross-block group	**1**	**1**	**1**	**1**	**2**	**2**	**2**	**2b**	**3a**	**3a**	**3a**	**3a**	**3a**	**3b**	**3b**

5B:

	Competing mAb: →									
Immobilized mAb ↓	**Tras**	**Pert**	**C1**	**F5**	**106**	**111**	**005**	**006**	**059**	**060**
Trastuzumab	6	100	103	99	114	166	137	110	120	119
TH1014-pert	104	9	106	125	115	145	151	125	132	118
TH1014-C1	89	85	65	58	84	86	98	99	89	93
TH1014-F5	197	178	70	21	129	183	178	192	165	185
PC1014-106	323	275	471	495	26	21	25	25	25	23

PC1014-111	110	102	122	119	75	14	51	10	65	36
PC1014-005	126	115	157	227	54	32	18	15	22	12
PC1014-006	163	136	136	153	127	47	148	20	129	125
PC1014-059	117	107	78	128	23	12	13	11	12	11
PC1014-060	106	99	108	126	37	35	30	6	14	19
Cross-block group	**1**	**2**	**3**	**3**	**4**	**4**	**4**	**4**	**4**	**4**

75 – >100% competition

25 – 74% competition

0 – 24% competition

Depicted values are mean percentages of binding relative to the binding observed in the absence of competitor antibody, of two independent experiments. Competition experiments with HEK produced TH1014-C1 and TH1014-F5 were performed once. Trastuzumab (clinical grade Herceptin®) and HEK-produced pertuzumab (TH1014-pert) were also tested.

Example 15 - Antibody-dependent cell-mediated cytotoxicity (ADCC)

SK-BR-3 cells (purchased at ATCC, HTB-30) were harvested ($5x10^6$ cells), washed (twice in PBS, 1500 rpm, 5 min) and collected in 1 mL RPMI 1640 medium supplemented with 10% cosmic calf serum (CCS) (HyClone, Logan, UT, USA), to which 200 µCi ^{51}Cr (Chromium-51; Amersham Biosciences Europe GmbH, Roosendaal, The Netherlands) was added. The mixture was incubated in a shaking water bath for 1.5 hours at 37°C. After washing of the cells (twice in PBS, 1500 rpm, 5 min), the cells were resuspended in RPMI 1640 medium supplemented with 10% CCS, counted by trypan blue exclusion and diluted to a concentration of $1x10^5$ cells/mL.

Meanwhile, peripheral blood mononuclear cells (PBMCs) were isolated from fresh buffy coats (Sanquin, Amsterdam, The Netherlands) using standard Ficoll density centrifugation according to the manufacturer's instructions (lymphocyte separation medium; Lonza, Verviers, France). After resuspension of cells in RPMI 1640 medium supplemented with 10% CCS, cells were counted by trypan blue exclusion and concentrated to $1x10^7$ cells/mL.

Trastuzumab was produced in CHO cells resulting in an (increased) non-core fucosylation grade of 12.4%, whereas the other HER2 antibodies were produced in HEK cells, resulting on average in 4% non-core fucosylation.

For the ADCC experiment, 50 µL ^{51}Cr-labeled SK-BR-3 cells (5.000 cells) were pre-incubated with 15 µg/mL HER2 antibody (IgG1,κ) in a total volume of 100 µL RPMI medium

supplemented with 10% CCS in a 96-well microtiter plate. After 15 min at RT, 50 µL PBMCs (500,000 cells) were added, resulting in an effector to target ratio of 100:1. The maximum amount of cell lysis was determined by incubating 50 µL ^{51}Cr-labeled SK-BR-3 cells (5,000 cells) with 100 µL 5% Triton-X100. The amount of spontaneous lysis was determined by incubating 5000 ^{51}Cr-labeled SK-BR-3 cells in 150 µL medium, without any antibody or effector cells. The level of antibody-independent cell lysis was determined by incubating 5,000 SK-BR-3 cells with 500,000 PBMCs without antibody. Subsequently, the cells were incubated 4 hr at 37°C, 5% CO_2. To determine the amount of cell lysis, the cells were centrifuged (1,200 rpm, 3 min) and 75 µL of supernatant was transferred to micronic tubes, after which the released ^{51}Cr was counted using a gamma counter. The measured counts per minute (cpm) were used to calculate the percentage of antibody-mediated lysis as follows:
(cpm sample - cpm Ab-independent lysis)/(cpm max. lysis - cpm spontaneous lysis)x 100%

HER2 antibodies from cross-block groups 1 and 2 induced efficient lysis of SK-BR-3 cells through ADCC (Figure 5A). From group 3, antibody 153 was the only antibody that induced efficient ADCC, antibody 132 induced about 10% ADCC, and clones 098, 159 and 127 did not induce ADCC. See Figure 5. All HER2 antibodies from cross-block group 4 induced efficient lysis of SK-BR-3 cells through ADCC (Figure 5B). The average percentage lysis by the different antibodies of cross-block group 4 varied between 15% and 28%, in constrast to trastuzumab (Herceptin®), which showed on average 41% lysis. Without being bound by theory, the higher percentage lysis by trastuzumab possibly resulted from an increased non-core fucosylation grade (12.4%) due to its CHO production, compared to ~4% non-core fucosylation on the other HEK-produced HER2 antibodies, or by recognizing an epitope that induces less internalization of the HER2 receptor-antibody complexes.

Example 16 - Inhibition of ligand-independent proliferation of AU565 cells

HER2 antibodies were tested for their ability to inhibit proliferation of AU565 cells *in vitro*. Due to the high HER2 expression levels on AU565 cells (~1,000,000 copies per cell as described in Example 12), HER2 is constitutively active in these cells and thus not dependent on ligand-induced heterodimerization.

In a 96-well tissue culture plate (Greiner bio-one, Frickenhausen, Germany), 9,000 AU565 cells were seeded per well in the presence of 10 µg/mL HER2 antibody in serum-free cell culture medium. As a control, cells were seeded in serum-free medium without antibody. After 3 days, the amount of viable cells was quantified with Alamarblue (BioSource International, San Francisco, US) according to the manufacturer's instructions.

Fluorescence was monitored using the EnVision 2101 Multilabel reader (PerkinElmer, Turku, Finland) with standard Alamarblue settings. The Alamarblue signal of antibody-treated cells was plotted as a percentage relative to untreated cells. Dunnett`s test was applied for statistical analysis.

The percentage proliferation of AU565 cells after HER2 antibody treatment was compared to untreated cells, which was set to 100%. Of the tested Group 1 antibodies, trastuzumab, 050 and 169 demonstrated significant inhibition of AU565 cell proliferation ($P<0.05$), whereas 084 had no effect. None of the tested antibodies from group 2 (Pertuzumab, 025, 092 and 129) was able to inhibit AU565 cell proliferation. The tested antibodies from group 3 (098 and 153) did not inhibit AU565 proliferation. In contrast, both antibodies induced enhanced proliferation of AU565 cells compared to untreated cells (098 more than 153). See Figure 6. For trastuzumab and pertuzumab, this was in accordance with the results described by Juntilla *et al*. (Cancer Cell 2009;15(5):353-355).

From cross-block group 4, TH1014-F5 significantly enhanced proliferation of AU565 cells indicating that this is an agonistic antibody, whereas none of the other antibodies of cross-block group 4 tested (005, 060 and pertuzumab) had a substantial effect on AU565 proliferation (data not shown). Enhancing proliferation can be an advantage in some therapeutic applications of ADC-conjugates, *e.g.*, where the cytotoxic action of the drug relies on, or is enhanced by, cell proliferation.

Example 17 - Inhibition of ligand-induced proliferation of MCF-7 cells

Since HER2 is an orphan receptor, its signaling is mainly dependent on activation of other ErbB-family members such as EGFR and Her3. Upon ligand binding, these two receptors can bind to and activate the HER2 receptor, resulting in *e.g.* proliferation. Various publications describe that pertuzumab efficiently inhibits Heregulin-β1-induced proliferation (Franklin MC. Cancer Cell 2004 / Landgraf R. BCR 2007). For trastuzumab, it has been described that it has little effect on Heregulin-β1-induced HER2/HER3 heterodimerization and proliferation (Larsen SS., *et al.*, Breast Cancer Res Treat 2000;58:41-56; Agus DB., *et al.*, Cancer Cell 2002;2:127-137; Wehrman *et al.* (2006), *supra*).

To investigate the ability of the present human HER2 antibodies to interfere with Heregulin-β1-induced HER2/HER3 heterodimers, a Heregulin-β1-induced proliferation assay was performed. Therefore, MCF7 cells (purchased at ATCC, HTB-22) expressing ~20.000 HER2 molecules per cell, were seeded in a 96-wells tissue culture plate (Greiner bio-one) (2.500 cells/well) in complete cell culture medium. After 4 hours, the cell culture medium was replaced with starvation medium containing 1% Cosmic Calf Serum (CCS) and 10 µg/mL HER2 antibody. Next, Heregulin-β1 (PeproTech, Princeton Business Park, US) diluted

in 1% CCS containing starvation medium was added to the wells to a final concentration of 1.5 ng/mL. After 4 days incubation, the amount of viable cells was quantified with Alamarblue (BioSource International) according to the manufacturer's instructions. Fluorescence was monitored using the EnVision 2101 Multilabel reader (PerkinElmer) with standard Alamarblue settings. The Alamarblue signal of HER2 antibody-treated ligand-induced cells was plotted as a percentage signal compared to ligand-induced cells incubated without HER2 antibody. Dunnett`s test was applied for statistical analysis.

The percentage of viable MCF7 cells stimulated with Heregulin-β1 and treated with the indicated HER2 antibody, relative to the viable cells after stimulation with Heregulin-β1 in the absence of HER2 antibody, was calculated. There was no MCF-7 proliferation in absence of both Heregulin-β1 and antibody. Antibodies 025, 091, 129, 153 and pertuzumab (TH1014-pert) demonstrated significant inhibition of Heregulin-β1-induced MCF-7 proliferation ($P<0.05$). Also trastuzumab showed some inhibition of Heregulin-β1-induced proliferation of MCF-7 cells, although not as efficient as the other tested HER2 antibodies. It has been reported that domain IV of HER2 is involved in the stabilization of EGFR/HER2 heterodimers, but without details on its contribution to HER2/HER3 heterodimers (Wehrman *et al.*, *supra*). Antibodies 050, 084, 169 and 098 had no statistically significant effect on Heregulin-β1-induced proliferation of MCF-7 cells. See Figure 7. Without being limited to theory, this suggests that these antibodies do not inhibit ligand-induced HER2/HER3 heterodimerization.

Example 18 - Anti-kappa-ETA' assay

To investigate the suitability of HER2 antibodies for an antibody-drug conjugate approach, a generic *in vitro* cell-based killing assay using kappa-directed pseudomonas-exotoxin A (anti-kappa-ETA') was developed. The assay makes use of a high affinity anti-kappa domain antibody conjugated to a truncated form of the pseudomonas-exotoxin A. Upon internalization, the anti-kappa-ETA' domain antibody undergoes proteolysis and disulfide-bond reduction, separating the catalytic from the binding domain. The catalytic domain is transported from the Golgi to the endoplasmic reticulum via the KDEL retention motif, and subsequently translocated to the cytosol where it inhibits protein synthesis and induces apoptosis (ref. Kreitman RJ. BioDrugs 2009;23(1):1-13). In this assay, to identify HER2 antibodies that enable internalization and killing through the toxin, HER2 antibodies are preconjugated with the anti-kappa-ETA' before incubation with HER2-positive cells.

First, the optimal concentration of anti-kappa-ETA' was determined for each cell line, *i.e.* the maximally tolerated dose that does not lead to induction of non-specific cell death. AU565 cells (7,500 cells/well) and A431 cells (2500 cells/well) were seeded in normal cell

culture medium in 96-wells tissue culture plate (Greiner bio-one) and allowed to adhere for at least 4 hours. Next, cells were incubated with 100, 10, 1, 0.1, 0.01, 0.001 and 0 µg/mL anti-kappa-ETA' dilutions in normal cell culture medium. After 3 days, the amount of viable cells was quantified with Alamarblue (BioSource International, San Francisco, US) according to the manufacturer's instruction. Fluorescence was monitored using the EnVision 2101 Multilabel reader (PerkinElmer, Turku, Finland) with standard Alamarblue settings. The highest concentration anti-kappa-ETA' that did not kill the cells by itself was used for following experiments (0.5 µg/mL for AU565 and 1 µg/mL for A431).

Next, antibody-mediated internalization and killing by the toxin was tested for different HER2 antibodies. Cells were seeded as described above. Dilution-series of HER2 antibodies were pre-incubated for 30 minutes with the predetermined concentration anti-kappa-ETA' before adding them to the cells. After 3 days of incubation, the amount of viable cells was quantified as described above. The Alamarblue signal of cells treated with anti-kappa-ETA' conjugated antibodies was plotted compared to cells treated with antibody alone. 23.4 µg/mL Staurosporin was used as positive control for cell killing. An isotype control antibody was used as negative control.

Cross-block groups 1, 2 and 3:

As shown in Figure 8A/B and Table 6A, all anti-kappa-ETA'-conjugated HER2 antibodies were able to kill AU565 cells in a dose-dependent manner. All tested anti-kappa-ETA'-conjugated HER2 antibodies demonstrated better killing of AU565 cells (70.3 – 49.9 %) compared to both anti-kappa-ETA'-conjugated trastuzumab (31.9 %) and anti-kappa-ETA'-conjugated pertuzumab (TH1014-pert) (47.51 %). and the EC_{50} values were increased. 12.12 - 46.49 ng/mL compared to 78.49 ng/mL for anti-kappa-ETA'-conjugated trastuzumab and 117.8 ng/mL for anti-kappa-ETA'-conjugated pertuzumab. Antibody 159 had the highest percentage of cell-kill, and 098 the lowest EC_{50}.

As shown in Figures 8C,D and Table 7A, antibodies 025, 091, 098, 129 and 153 were able to induce effective killing of A431 cells (≥75%). The highest percentage of cell-kill, and lowest EC_{50} was shown by antibody 098. When conjugated to anti-kappa-ETA', trastuzumab and isotype control antibody did not induce killing of A431 cells. Antibodies 169, 084 and pertuzumab induced percentages of cell kill of no more than about 50%. No cell kill was observed with non-conjugated HER2 antibodies.

Cross-block group 4:

As shown in Table 6B, all anti-kappa-ETA'-conjugated HER2 antibodies of cross-block group 4 were able to kill AU565 cells in a dose-dependent manner. (50-72% cell killing).

Aantibodies 005 and 111 demonstrated more than three times improved EC_{50} values (resp. 15.13 and 24.20 ng/mL) compared to trastuzumab (78.49 ng/mL). Non-conjugated HER2 antibodies of cross-block group 4 did not induce killing of AU565 cells at the concentrations tested.

As shown in Table 7B, antibodies 005 and 060 were able to induce effective killing of A431 cells (≥85%) when conjugated to anti-kappa-ETA' Antibodies 005 and 111 demonstrated killing of A431 cells already at low antibody concentrations (10 ng/mL) with EC_{50} values of ~10 ng/mL. No cell kill was observed with non-conjugated HER2 antibodies of cross-block 4.

Table 6: Data shown are EC_{50} values and maximal percentage cell kill of AU565 cells treated with anti-kappa-ETA'-conjugated HER2 antibodies (A, cross-block groups 1, 2, and 3; b, cross-block group 4), measured in one representative experiment. Cell-kill induced by Staurosporin was set as 100% and MFI of untreated cells was set as 0%. Ndet = not detected.

A:

antibody	% cells killed	EC50 ng/mL
PC1014-159	70.3	34.93
PC1014-127	69.0	34.46
PC1014-132	61.6	39.35
PC1014-129	60.8	30.85
PC1014-153	60.3	32.26
PC1014-025	60.0	16.71
PC1014-098	58.7	12.12
PC1014-084	58.1	26.97
PC1014-050	52.4	12.71
PC1014-091	50.6	46.49
PC1014-169	49.9	35.62
TH1014-pert	47.5	117.8
trastuzumab	31.9	78.49
isotype control	Ndet	Ndet

B:

antibody	% cells killed	EC50 ng/mL
PC1014-111	72.0	24.2
PC1014-005	69.7	15.13
PC1014-059	67.0	67.65
PC1014-060	64.3	79.38
PC1014-106	59.1	107.9
PC1014-006	50.4	45.14
Trastuzumab	31.9	78.49
isotype control	Ndet	Ndet

Table 7: Data shown are EC_{50} values and maximal percentage cell kill of A431 cells treated with anti-kappa-ETA'-conjugated HER2 antibodies (A, cross-block groups 1, 2, and 3; b, cross-block group 4), measured in one representative experiment. Cell kill induced by Staurosporin was set as 100% and MFI of untreated cells was set as 0%. "NDet" means not detected.

A:

antibody	% cells killed	EC50 ng/mL
PC1014-025	86.7	~9.77
PC1014-084	50.5	ND
PC1014-091	83.3	~9.86
PC1014-098	87.2	1.65
PC1014-129	75.9	~10.60
PC1014-153	82.4	~10.11
PC1014-169	34.0	ND
TH1014-pert	37.0	61.58
trastuzumab	Ndet	Ndet
isotype control	NDet	NDet

B:

antibody	% cells killed	EC50 ng/mL
PC1014-005	88.5	~ 10.07
PC1014-060	85.0	~ 10.03
Trastuzumab	NDet	NDet
isotype control	NDet	NDet

Example 19 - Internalization of HER2 antibodies measured with an FMAT-based fab-CypHer5E assay

To investigate whether the enhanced killing of AU565 cells by the described HER2 antibodies compared to Trastuzumab (Herceptin®) and pertuzumab in the kappa-toxin-ETA' assay described in the previous Example correlated with enhanced internalization of HER2 antibodies, a fab-CypHer5E-based internalization assay was performed. CypHer5E is a pH-sensitive dye which is non-fluorescent at basic pH (extracellular: culture medium) and fluorescent at acidic pH (intracellular: lysosomes), with an acid dissociation constant (pKa) of 7.3.

AU565 cells were seeded in 384-well tissue culture plates (Greiner bio-one), at a density of 3,000 cells/well in normal cell culture medium supplemented with 240 ng/mL fab-CypHer5E (conjugation of Goat-fab-anti-Human IgG [Jackson] with CypHer5E [GE

Healthcare, Eindhoven, The Netherlands] was made according to manufacturer's instructions). Next, HER2 antibodies were serially diluted in normal cell culture medium, added to the cells and left at room temperature for 9 hours. Mean fluorescent intensities (MFI) of intracellular CypHer5E were measured using the 8200 FMAT (Applied Biosystems, Nieuwerkerk A/D IJssel, The Netherlands) and 'counts x fluorescence' was used as read-out. An isotype control antibody was used as negative control antibody. EC_{50} values and maximal MFI were determined by means of non-linear regression (sigmoidal dose-response with variable slope) using GraphPad Prism V4.03 software (GraphPad Software, San Diego, CA, USA).

Cross-block groups 1, 2 and 3:

The results are shown in Table 8A, depicting the EC_{50} and maximal MFI values for all tested HER2 antibodies of cross-block groups 1, 2, and 3 in the CypHer5E internalization assay with AU565 cells. The maximal MFI values indicate how many HER2 receptors are internalized upon antibody binding. All HER2 antibodies showed higher maximal MFI values (137,904 – 38,801) compared to trastuzumab (35,000) and pertuzumab (TH1014-pert) (32,366), indicating that the tested HER2 antibodies induced enhanced receptor internalization. Notably, antibodies that did not compete for HER2 binding with trastuzumab (Herceptin®) or TH1014-pert induced more receptor internalization compared to antibodies that did compete with trastuzumab and TH1014-pert, with the highest MFI achieved by antibodies 098 and 127. Without being limited to theory, this might be inherent to an inability to inhibit HER2 heterodimerization.

Cross-block group 4:

The results are shown in Table 8B, depicting the EC_{50} values and maximal MFI for all tested HER2 antibodies of cross-block group 4 in the CypHer5E internalization assay with AU565 cells. The maximal MFI values reflect how many HER2 antibodies were internalized upon binding. All tested human HER2 antibodies of cross-block group 4 showed higher maximal MFI values (130.529-57.428) than trastuzumab (Herceptin®) (35.000) and TH1014-pert (35.323), indicating that these antibodies induced enhanced receptor internalization. The enhanced internalization of TH1014-F5 may be a result from its agonistic activity and the induction of HER2-HER2 dimerization (see Example 16).

Table 8: Cypher-5-based internalization assay of HER2 antibodies. Data shown are MFI and EC_{50} values of one representative experiment of two experiments with AU565 cells treated

with fab-CypHer5E-labeled HER2 antibodies. Some EC_{50} values could not be calculated (ND).

A:

Cypher 5			
Antibody	**EC_{50} ng/mL**	**Maximal MFI**	
PC1014-025	30.05	63428	mAbs that compete with Herceptin
PC1014-091	32.99	50711	
PC1014-129	7.15	60302	
TH1014-pert	530	32366	
PC1014-169	ND	38801	mAbs that compete with TH1014-pert
PC1014-084	30.51	71059	
trastuzumab	21.70	35000	
PC1014-098	13.77	134575	mAbs that compete with TH1014-F5
PC1014-127	~9.68	137904	
PC1014-159	ND	92427	
TH1014-F5	22.65	113116	
PC1014-132	11.42	112270	Non-competing mAbs
PC1014-153	~14.91	87531	

B:

Cypher 5		
Antibody	**EC_{50} ng/mL**	**Maximal MFI**
PC1014-006	23.08	130829
PC1014-005	21.37	95117
PC1014-111	35.22	81680
PC1014-059	14.77	77123
PC1014-060	36.16	68184
PC1014-106	68.60	57428
TH1014-F5	22.65	113116
TH1014-pert	~1041	35323
Trastuzumab	21.70	35000

Example 20: Generation of bispecific antibodies by 2-MEA-induced Fab-arm exchange

An *in vitro* method for producing bispecific antibodies is described in WO 2008119353 (Genmab) and reported by van der Neut-Kolfschoten *et al*. (Science. 2007 Sep 14;317(5844):1554-7). Herein, a bispecific antibody was formed by "Fab-arm" or "half-molecule" exchange (swapping of a heavy chain and attached light chain) between two monospecific IgG4- or IgG4-like antibodies upon incubation under mildly reducing conditions. Without being limited to theory, this Fab-arm exchange reaction was the result of a disulfide-bond isomerization reaction wherein the inter heavy-chain disulfide bonds in the hinge regions of monospecific antibodies were reduced and the resulting free cysteines form a new inter heavy-chain disulfide bond with cysteine residues of another antibody molecule with a different specificity. The resulting product was a bispecific antibody having two Fab arms with different sequences.

The knowledge of this natural IgG4 Fab-arm exchange was adapted to generate a method to produce stable IgG1-based bispecific antibodies (WO 2011131746 (Genmab)). The bispecific antibody product generated by this method described below will no longer participate in IgG4 Fab-arm exchange. The basis for this method was the use of complimentary CH3 domains, which promote the formation of heterodimers under specific assay conditions. To enable the production of bispecific antibodies by this method, IgG1 molecules carrying certain mutations in the CH3 domain were generated: in one of the parental IgG1 antibody T350I, K370T and F405L mutations (or minimally F405L) in the other parental IgG1 antibody the K409R mutation.

To generate bispecific antibodies, these two parental antibodies, each antibody at a final concentration of 0.5 mg/mL (equimolar concentration), were incubated with 25 mM 2-mercaptoethylamine-HCl (2-MEA) in a total volume of 100 µL TE at 37°C for 90 min. The reduction reaction is stopped when the reducing agent 2-MEA is removed by using spin columns (Microcon centrifugal filters, 30k, Millipore) according to the manufacturer's protocol.

Example 21 - HER2xCD3 bispecific antibodies tested in an *in vitro* cytotoxicity assay

CD3 is a protein complex that is associated with the T cell receptor α and β chain expressed on mature T cells. Combination of a CD3 specific antibody Fab-arm with a tumor antigen specific antibody Fab-arm in a bispecific antibody would result in the specific targeting of T cells to tumor cells, leading to T cell mediated tumor cell lysis. Likewise, CD3 positive T cells could be targeted to other derailed cells in the body, to infected cells or directly to pathogens.

Various HER2xCD3 bispecific antibodies were generated, combining different HER2 and public domain CD3 antibody sequences. Furthermore b12, a gp120 specific antibody (Barbas, CF. J Mol Biol. 1993 Apr 5;230(3):812-23.) was used as a negative control. Heavy and light chain variable region sequences for the HER2-specific Fab-arm for antibody 153 were SEQ ID NO:63 and 67, respectively, and VH and VL sequences for the HER2-specific Fab-arm of antibody 169 were SEQ ID NO:1 and 5, respectively. The following heavy and light chain variable region sequences for the CD3 specific Fab-arm were used:

YTH12.5 (Routledge *et al*., Eur J Immunol. 1991, 21(11):2717-25, hereby incorporated by reference in its entirety, including sequence disclosures)

SEQ ID NO: 234	VH YTH12.5	EVQLLESGGGLVQPGGSLRLSCAASGFTFS**SFP**MAWVRQAP GKGLEWVSTISTSGGRTYYRDSVKGRFTISRDNSKNTLYLQ MNSLRAEDTAVYYCAK*FRQYSGGFDY*WGQGTLVTVSS
SEQ ID NO: 235	VL YTH12.5	DIQLTQPNSVSTSLGSTVKLSCTLS**SGNIE**NNYVHWYQLYE GRSPTTMIYDDDKRPDGVPDRFSGSIDRSSNSAFLTIHNVAI EDEAIYFC*HSYVSSFNV*FGGGTKLTVL

Sequences highlighted by **bold** represent the CDR1 domains, sequences highlighted by underline represent the CDR2 domains, and sequences highlighted by *italic* represent the CDR3 domains.

HUM291 (humanized antibody visilizumab, sequences retrieved from the NCBI protein database under GenBank accession No.: AAC28464.1, hereby incorporated by reference in its entirety)

SEQ ID NO: 236	VH HUM291	QVQLVQSGAEVKKPGASVKVSCKASGYTFI**SYT**MHWVRQA PGQGLEWMGYINPRSGYTHYNQKLKDKATLTADKSASTAYM ELSSLRSEDTAVYYCAR*SAYYDYDGFAY*WGQGTLVTVSS
SEQ ID NO: 237	VL HUM291	DIQMTQSPSSLSASVGDRVTITCSAS**SSVSY**MNWYQQKPG KAPKRLIYDTSKLASGVPSRFSGSGSGTDFTLTISSLQPEDFA TYYC*QQWSSNPPT*FGGGTKVEIK

Sequences highlighted by **bold** represent the CDR1 domains, sequences highlighted by underline represent the CDR2 domains, and sequences highlighted by *italic* represent the CDR3 domains.

huOKT3-C114S-gLC (used in teplizumab, with an additional C114S mutation in VH; Adair, J. *et al*. 1994. Hum Antibodies Hybridomas 5:41-47, hereby incorporated by reference in its entirety, including sequence disclosures).

SEQ ID NO:238	VH huOKT3-C114S-gLC	QVQLVQSGGGVVQPGRSLRLSCKASGYTFT**RYT**MHWVR QAPGKGLEWIGYINPSRGYTNYNQKVKDRFTISRDNSKNT AFLQMDSLRPEDTGVYFCAR*YYDDHYSLDY*WGQGTPVTV SS

SEQ ID NO:239	VL huOKT3-C114S-gLC	DIQMTQSPSSLSASVGDRVTITCSAS**SSVSY**MNWYQQTP GKAPKRWIYDTSKLASGVPSRFSGSGSGTDYTFTISSLQP EDIATYYC*QQWSSNPFT*FGQGTKLQIT

Sequences highlighted by **bold** represent the CDR1 domains, sequences highlighted by underline represent the CDR2 domains, and sequences highlighted by *italic* represent the CDR3 domains.

huCLB-T3/4 is a humanized version of murine antibody CLB-T3/4 (Parren *et al.*, Res Immunol. 1991, 142(9):749-63, hereby incorporated by reference in its entirety, including sequence disclosures. Briefly, the CLB-T3/4 murine VH and VL sequences as published in Parren *et al*. (1991) were aligned to the human VH and VL repertoires using the IMGT's V-QUEST. The closest human germlines that were found were IGHV3-21*01 for the VH gene and IGKV3-11*01(+IGKJ4*02) for the VL gene. All amino acid residues in the murine VH and VL sequences that differed were replaced by the human equivalent, except for those within the CDR regions of CLB-T3/4. As no related J-region was found for the VH sequence, the common WGQGTLVTVSS sequence was used for the FR4 region of the heavy chain. Both sequences were cloned into the relevant expression vectors and expressed by cotransfection in HEK293F cells.

SEQ ID NO: 240	VH huCLB-T3/4	EVQLVESGGGLVKPGGSLRLSCAASGFTFSSYGMFWVRQ APGKGLEWVATISRYSRYIYYPDSVKGRFTISRDNAKNSLY LQMNSLRAEDTAVYYCARRPLYGSSPDYWGQGTLVTVSS
SEQ ID NO: 241	VL huCLB-T3/4	EIVLTQSPATLSLSPGERATLSCSASSSVTYVHWYQQKPG QAPRLLIYDTSKLASGIPARFSGSGSGTDFTLTISSLEPEDF AVYYCFQGSGYPLTFGSGTKLEMR
SEQ ID NO:242	VH CDR1	GFTFSSYG
SEQ ID NO:243	VH CDR2	ISRYSRYI
SEQ ID NO:244	VH CDR3	ARRPLYGSSPDY
SEQ ID NO:245	VL CDR1	SSVTY
	VL CDR2	DTS
SEQ ID NO:246	VL CDR3	FQGSGYPLT

All antibodies were expressed as IgG1,κ being modified in their Fc regions as follows: IgG1-HER2-153-K409R, IgG1-HER2-169-K409R, IgG1-b12-K409R, IgG1-hu-CLB-T3/4-F405L, IgG1-YTH12.5-F405L, IgG1-HUM291-F405L and IgG1-huOKT3-F405L.

Also, for subsequent experiments, N297Q mutants of the same antibodies were generated to make the Fc-domain of the antibodies inert. An inert Fc-domain prevents the antibody to interact with Fc-receptors present on monocytes, since it removes a

glycosylation site; glycosylation at this site is critical for IgG-Fcgamma receptor interactions (Bolt S *et al.*, *Eur J Immunol* 1993, 23:403-411). Alternatively to the N297Q mutation, residual Fc activity was further removed by combining three sets of mutations from the public domain in one antibody Fc domain. The mutations L234F, L235E, P331S (Oganesyan, Acta Cryst. (2008). D64, 700–704), D265A (Shields JBC (2001) 276(9) 6591–6604) and N297Q were introduced in the K409R and F405L IgG1 backbone. The combinations of these five mutations, designated LFLEDANQPS (SEQ ID NO:251) is used in some of the examples as well. The following Fc sequences for the different Fc-variants were used (mutations are highlighted by underlined letters:

IgG1 heavy chain constant region – WT (SEQ ID NO:247)
ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVV
TVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTP
EVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVS
NKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTT
PPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

IgG1 heavy chain constant region – F405L (SEQ ID NO:248)
>ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSV
VTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISR
TPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKV
SNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKT
TPPVLDSDGSFL<u>L</u>YSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

IgG1 heavy chain constant region – K409R (SEQ ID NO:249)
>ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSV
VTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISR
TPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKV
SNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKT
TPPVLDSDGSFFLYS<u>R</u>LTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

IgG1 heavy chain constant region – N297Q (SEQ ID NO:250)
>ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSV
VTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISR
TPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQY<u>Q</u>STYRVVSVLTVLHQDWLNGKEYKCKV

SNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKT
TPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

IgG1 heavy chain constant region – LFLEDANQPS mut (SEQ ID NO:251)
>ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSV
VTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPEFEGGPSVFLFPPKPKDTLMISR
TPEVTCVVVAVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYQSTYRVVSVLTVLHQDWLNGKEYKCKV
SNKALPASIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKT
TPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

IgG1 heavy chain constant region – F405L N297Q (SEQ ID NO:252)
>ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSV
VTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISR
TPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYQSTYRVVSVLTVLHQDWLNGKEYKCKV
SNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKT
TPPVLDSDGSFLLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

IgG1 heavy chain constant region – K409R N297Q (SEQ ID NO:253)
>ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSV
VTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISR
TPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYQSTYRVVSVLTVLHQDWLNGKEYKCKV
SNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKT
TPPVLDSDGSFFLYSRLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

IgG1 heavy chain constant region – F405L LFLEDANQPS (SEQ ID NO:254)
>ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSV
VTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPEFEGGPSVFLFPPKPKDTLMISR
TPEVTCVVVAVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYQSTYRVVSVLTVLHQDWLNGKEYKCKV
SNKALPASIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKT
TPPVLDSDGSFLLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

IgG1 heavy chain constant region – K409R LFLEDANQPS (SEQ ID NO:255)
>ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSV
VTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPEFEGGPSVFLFPPKPKDTLMISR
TPEVTCVVVAVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYQSTYRVVSVLTVLHQDWLNGKEYKCKV

SNKALPASIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKT
TPPVLDSDGSFFLYSRLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

The following heavy and light chain variable region sequences for the b12, HIV gp120 specific Fab-arm were used (sequence as described by: Barbas, CF. J Mol Biol. 1993 Apr 5;230(3):812-23.)

VH b12 (SEQ ID NO:256)
>QVQLVQSGAEVKKPGASVKVSCQASGYRFSNFVIHWVRQAPGQRFEWMGWINPYNGNKEFSAKFQ
DRVTFTADTSANTAYMELRSLRSADTAVYYCARVGPYSWDDSPQDNYYMDVWGKGTTVIVSS

VL b12 (SEQ ID NO:257)
>EIVLTQSPGTLSLSPGERATFSCRSSHSIRSRRVAWYQHKPGQAPRLVIHGVSNRASGISDRFSGSGS
GTDFTLTITRVEPEDFALYYCQVYGASSYTFGQGTKLERK

Bispecific antibodies from these HER2 and CD3 specific antibodies were generated as described in Example 20.

Specificity for human CD3 was verified by binding of the bispecific HER2xCD3 antibodies to Jurkat (CD3 expressing T cell line) cells using flow cytometry. Bivalent binding of parental IgG1 anti-CD3 antibodies was compared to binding by monospecific parental antibodies. All generated bispecific batches showed good binding to both Jurkat cells albeit with a lower affinity than monospecific bivalent CD3 antibodies (Figure 9A-D).

Simultaneous binding of the bispecific antibody huCLB-T3/4-N297Q-F405L x HER2-169 -N297Q-K409R was shown by co-incubating two cell populations labeled with different fluorescent dyes in the presence of bispecific antibodies or control antibodies. HER2 positive AU565 cells were labeled with CFSE (FITC/FL-1) and CD3 expressing Jurkat cells were labeled with PKH26 (PE/FL-2), according to manufacturer's instructions. Both cell types were then co-incubated for 30 min at 4°C, in the presence of bispecific HER2xCD3 antibodies. Samples were analyzed by flow cytometry on FACS CantoII. A quadrant analysis was performed to detect CSFE/PKH26 double-positive cells.

Only in the presence of bispecific antibody, a population of double-positive cells (doublets) was observed, indicating that these antibodies can bind two cell types simultaneously. Data are summarized in Figure 10A and representative examples of cells treated with bispecific HER2xCD3 (169xCLB-T3/4) and a monospecific control antibody are shown in Figure 10B.

The HER2xCD3 antibodies were then tested in an *in vitro* cytotoxicity assay using AU565 cells with either isolated T cells alone or PBMCs as effector cells. AU565 cells were cultured to near confluency. Cells were washed twice with PBS, and trypsinized for 5 minutes at 37ºC. 12 mL culture medium was added to inactivate trypsin and cells were spun down for 5 min, 800 rpm. Cells were resuspended in 10 mL culture medium and a single cell suspension was made by passing the cells through a cellstrainer. 100 µL of a $5x10^5$ cells/mL suspension was added to each well of a 96-well culture plate, and cells were incubated at least 3 hrs at 37ºC, 5% CO2 to allow adherence to the plate.

Peripheral blood mononuclear cells (PBMC) were isolated from blood from healthy volunteers using Leucosep 30 mL tubes, according to the manufacturer's protocol (Greiner Bio-one). T cells were isolated from PBMC preparations by negative selection using the Untouched Human T-cells Dynabead kit (Dynal). Isolated cells were resuspended in culture medium to a final concentration op $7x10^6$ cells/mL.

Culture medium was removed from the adhered AU565 cells, and replaced with 50 µL/well 2x concentrated antibody-dilution and 50 µL/well $7x10^6$ T cells/mL (ratio effector:target = 7:1). Plates were incubated for 3 days at 37ºC, 5% CO_2. Supernatants were removed and plates were washed twice with PBS. To each well 150 µL culture medium and 15 µL Alamar blue was added. Plates were incubate for 4 hours at 37ºC, 5% CO_2, and absorbance was measured (Envision, Perkin Elmer).

Figure 11 shows that all bispecific HER2xCD3 antibodies induced dose-dependent killing of AU565 cells in an *in vitro* cytoxicity assay with isolated T cells. Killing was critically dependent on the presence of a tumor-targeting Fab-arm (both clone 169 and 153), whereas control antibodies (CD3 monospecific IgG1-YTH12.5, IgG1-huCLB-T3/4, IgG1-Hum291 and IgG1-OKT3 and irrelevant antigen-specific IgG1-b12, and CD3xb12) did not induce T cell cytotoxicity. Bispecific antibodies containing HER2-169 were more potent than those containing HER2-153.

As shown in Figure 17 of Example 27, the N297Q mutation and therefore absence of Fc glycosylation of HER2xCD3 bispecific antibody huCLB-T3/4xHER2-169 did not impact the potential to induce dose dependent cytotoxicity of AU565 cells with PBMC.

Example 22 - HER2 downmodulation

To investigate if enhanced HER2 internalization induced by Group 3 antibodies 098 and 153 and Group 4 antibody 005 also results in enhanced receptor downmodulation, AU565 cells were incubated with HER2 antibodies for 3 days, and analyzed for presence of HER2. AU565 cells were seeded in a 24-wells tissue culture plate (100.000 cells/well) in normal cell culture medium and cultured for 3 days at 37°C in the presence of 10 µg/mL HER2

antibody. After washing with PBS, cells were lysed by incubating 30 min at room temperature with 25 µL Surefire Lysis buffer (Perkin Elmer, Turku, Finland). Total protein levels were quantified using bicinchoninic acid (BCA) protein assay reagent (Pierce) according to the manufacturer's protocol. HER2 protein levels in the lysates were analyzed using a HER2-specific sandwich ELISA. Rabbit-anti-human HER2 intracellular domain antibody (Cell Signaling) was used to capture HER2 and biotinylated goat-anti-human HER2 polyclonal antibody (R&D), followed by streptavidin-poly-HRP, were used to detect bound HER2. The reaction was visualized using 2,2'-azino-bis 3-ethylbenzothiazoline-6-sulfonic acid (ABTS: dilute one ABTS tablet in 50 mL ABTS buffer [Roche Diagnostics, Almere, The Netherlands]) and stopped with oxalic acid (Sigma-Aldrich, Zwijndrecht, The Netherlands). Fluorescence at 405 nm was measured on a microtiter plate reader (Biotek Instruments, Winooski, USA) and the amount of HER2 was expressed as a percentage relative to untreated cells.

The results shown in Figure 12 and Table 10 demonstrate that both tested Group 3 antibodies (098 and 153) induced more than 50% HER2 downmodulation. In contrast, antibodies 025, 169 and Trastuzumab (Herceptin®) barely induced downmodulation (approximately 20% of untreated cells) while antibody 005 induced moderate downmodulation (approximately 30% of untreated cells). This was in line with enhanced internalization observed by antibodies 098, 153, and 005.

Table 10: Antibody induced downmodulation of HER2 depicted as percentage HER2 compared to untreated cells

antibody	% HER2 compared to untreated cells
Herceptin	80
IgG1-1014-169	82
IgG1-1014-025	85
IgG1-1014-098	44
IgG1-1014-153	50
IgG1-1014-005	70
isotype control	108

Example 23 - Colocalization of HER2 antibodies with lysosomal marker LAMP1 analyzed by confocal microscopy.

The HER2 downmodulation assay as described in Example 21 and the CypHer-5E based internalization assay as described in Example 19 indicated that HER2 antibodies from groups 3 and 4 were more efficiently internalized and targeted towards lysosomes compared to antibodies from Groups 1 and 2. To confirm the enhanced lysosomal transport of antibodies from groups 3 and 4, AU565 cells were cultured on glass coverslips and

treated for 18 hours with the indicated antibodies. Cells were fixed, permeabilized and stained with FITC-conjugated goat anti-human IgG1 to visualize antibody and mouse anti-human CD107a (LAMP1) followed by goat anti-mouse IgG-Cy5 to identify lysosomes.

However, in these experiments the confocal imaging was done with settings that allowed discriminating between monospecific and bispecific antibodies but not between different monospecific antibodies, in fact, with these settings monospecific antibodies could hardly be detected. To be able to compare between the different monospecific antibodies, the confocal slides were measured again with increased gain settings, to enhance fluorescence intensity. All other steps of the procedure were the same as described in Example 23.

The results are depicted in Figure 13 and Table 11, and show that the FITC pixel intensity overlapping with Cy5 for various monospecific HER2 antibodies. From each slide three different images were analyzed containing ~ 1, 3 or >5 cells. Significant variation was observed between the different images within each slide. Still, it was evident that antibodies 005, 098 and 153 were more efficiently targeted towards lysosomal compartments, compared to 025, pertuzumab, 169 and Trastuzumab (Herceptin®). This correlated well with the enhanced internalization and receptor degradation induced by these antibodies.

Table 11: Mean FITC pixel intensities overlapping with Cy5 depicted as arbitrary units.

antibody	FITC pixel intensity in lysosomes [arbitrary units]
TH1014-005	0.619
TH1014-098	0.522
TH1014-153	0.409
TH1014-025	0.248
TH1014-pert	0.214
TH1014-169	0.255
Herceptin	0.236

Example 24 - HER2 extracellular domain shuffle human-to-chicken

To further define the HER2 binding regions recognized by antibodies from the four different cross-competition groups described in Example 14, a HER2 extracellular domain shuffle experiment was performed. To this end, a small gene-synthesis library with five constructs was generated, swapping the sequences of domain I, II, III or IV of the extracellular domain of human HER2 to the corresponding sequence of chicken HER2 (Gallus gallus isoform B NCBI: NP_001038126.1): 1) fully human HER2 (Uniprot P04626) hereafter named hu-HER2, 2) hu-HER2 with chicken domain I (replacing amino acids (aa) 1-203 of the human HER2 with the corresponding chicken HER2 region) hereafter named hu-HER2-ch(I), 3) hu-HER2 with chicken domain II (replacing amino acids (aa) 204-330 of the human HER2

with the corresponding chicken HER2 region) hereafter named hu-HER2-ch(II), 4) hu-HER2 with chicken domain III (replacing aa 331-507 of the human HER2 with the corresponding chicken HER2 region) hereafter named hu-HER2-ch(III) and 5) hu-HER2 with chicken domain IV (replacing aa 508-651 of the human HER2 with the corresponding chicken HER2 region) hereafter named hu-HER2-ch(IV). The human and chicken HER2 orthologs show 67% homology in their extracellular domain with 62% homology in domain I, 72% homology in domain II, 63% homology in domain III and 68% homology in domain IV. The constructs were transiently transfected in the Freestyle™ CHO-S (Invitrogen) cell line using Freestyle MAX transfection reagent (Invitrogen) according to the instructions of the manufacturer, and transfected cells were cultured for 20 hours. HER2 antibody binding to the transfected cells was analyzed by means of flow cytometry: The transfected CHO-S cells were harvested, washed with FACS buffer and incubated with 10 µg/mL HER2 antibody (30 minutes on ice). Binding of HER2 antibodies was detected using a Phycoerythrin (PE)-conjugated goat-anti-human IgG antibody (Jackson). To check if expression between different batches was the same, cells were fixed and permeabilized using Cytofix/Cytoperm solution (BD) according manufacturer's instruction and stained with a rabbit-anti-human intracellular HER2 antibody (DAKO) in combination with a secondary PE-conjugated goat-anti-rabbit antibody (Jackson). An isotype control antibody was used as negative control. Fluorescence was measured on a FACSCanto-II (BD) and binding curves were made by means of non-linear regression (sigmoidal dose-response with variable slope) using GraphPad Prism V4.03 software (GraphPad Software, San Diego, CA, USA). Loss of binding was used as read out to identify which HER2 domains were recognized by the different antibodies.

Exemplary binding curves for antibody 153 are shown in Figure 14. All binding results are shown in Table 12. Group 1 HER2 antibodies 050, 084, 169 and Trastuzumab (Herceptin®) showed loss of binding to Hu-HER2-ch(IV), but not to the proteins with one of the remaining domains shuffled, demonstrating that the epitopes of Group 1 mAbs reside in HER2 domain IV. Group 2 antibodies 025, 091, 129 and pertuzumab showed only loss of binding to Hu-HER2-ch(II), indicating that the epitope resides in HER2 domain II. Antibodies 098 and 153 were both defined to Group 3 in cross-competition assays (not shown) but showed some variation in the shuffle experiment. Antibody 098 clearly showed loss of binding to Hu-HER2-ch(I) and a minor decrease in binding to Hu-HER2-ch(II), while 153 showed only loss of binding to Hu-HER2-ch(II). These data suggest that Group 3 mAbs 098 and 153 can also bind, at least partially, to the HER2 domain II, with epitopes that possibly extend into HER2 domain I, as is the case for 098. Antibodies 005, 006, 060 and 111 showed loss of binding upon substitution of HER2 domain III, which demonstrated that the

epitope resides in HER2 domain III. Interestingly, antibodies 059 and 106 demonstrated loss of binding to both hu-HER2-ch(III) and hu-HER2-ch(I), implying that antibodies 059 and 106 recognize a conformational epitope within these two domains.

Table 12: Summary of HER2 antibody binding to different HER2ECD receptor constructs. FL; hu-HER2, I; hu-HER2-ch(I), II; hu-HER2-ch(II), III; hu-HER2-ch(III), IV; hu-HER2-ch(IV). +++ indicates normal binding, ++ indicates reduced EC_{50} but the similar maximal binding compared to binding observed to hu-HER2, + indicates reduced EC_{50} and reduced maximal binding detected compared to binding observed to hu-HER2, - indicates no binding.

		HER2-domain shuffled				
Antibody	Group	FL	I	II	III	IV
Herceptin®	1	+++	+++	+++	+++	-
050	1	+++	+++	+++	+++	-
084	1	+++	+++	+++	+++	-
169	1	+++	+++	+++	+++	+
Pertuzumab	2	+++	+++	+	+++	+++
025	2	+++	+++	-	+++	+++
091	2	+++	+++	-	+++	+++
129	2	+++	+++	-	+++	+++
153	3	+++	+++	-	+++	+++
098	3	+++	-	++	+++	+++
005	4	+++	+++	+++	-	+++
006	4	+++	+++	+++	-	+++
059	4	+++	-	+++	-	+++
060	4	+++	+++	+++	-	+++
106	4	+++	-	+++	-	+++
111	4	+++	+++	+++	-	+++

Example 25 - *In vivo* efficacy of HER2 HuMabs 005, 091, 084 and 169 in NCI-N87 human gastric carcinoma xenografts in SCID mice

The *in vivo* effect of HER2-HuMabs 091 (cross-competition Group 2), 084 and 169 (both cross-competition Group 1), and 005 (cross-block Group 4) on tumor growth and survival in a NCI-N87 human gastric carcinoma xenograft model in female CB.17 severe combined immunodeficiency (SCID) mice was determined. $10x10^6$ NCI-N87 tumor cells in 50%

matrigel were injected s.c. in female SCID mice, 10 mice per group. Eight days after tumor inoculation, intravenous treatment with HER2-HuMabs 005, 091, 084, and 169 or control antibody HuMab-HepC was started. In Figure 15 (A) and (C), this is indicated as day 1, day of treatment initiation. The first dose was at 40 mg/kg, followed by 10 mg/kg on days 4, 8, 11, 15, 18, 22, and 25 after treatment initiation. Tumor volume was determined at least 2 times per week. Volumes (mm^3) were calculated from caliper (PLEXX) measurements as ($width^2$ x length)/2.

The results are depicted in Figure 15A, 15B, 15C and 15D, which show that the mice administered with HuMab 005, 084, 169 and 091 demonstrated slower tumor growth (A) and better survival (B) than the mice that received negative control antibody HuMab-HepC. All treatments were well-tolerated.

Example 26 - Therapeutic treatment of BT-474 breast tumor xenografts in Balb/C nude mice

The effect of therapeutic treatment of five different HER2 HuMabs on human subcutaneous BT-474 breast tumor xenografts in Balb/C nude mice was determined. BT-474 tumor cells were injected 24 to 72 hours after a whole body irradiation with a γ-source (1.8 Gy, Co60, BioMep, France). $2x10^7$ BT-474 cells in 200 µl of RPMI 1640 containing matrigel (50:50, v:v; BD Biosciences) were injected subcutaneously into the right flank of female Balb/C nude mice. Body weight and tumor volume of the mice was recorded twice a week. Tumor volumes (mm^3) were calculated from caliper (PLEXX) measurements as: ($width^2$ x length)/2.

Treatment with HER2 HuMabs was started when the tumors reached a mean volume of 100-200 mm3. Tumor bearing mice were randomized into groups of 8 mice. One group received twice weekly intravenous (i.v.) injections of the control mAb HuMab-HepC. Four other groups received twice weekly i.v. injections of HER2 HuMab 025, 129, 153 and 091, with a first dose of 20 mg/kg and following 9 doses of 5 mg/kg.

The results are depicted in Figure 16A and 16B and show that BT-474 tumor growth was partially inhibited with HuMab 129 and HuMab 153 treatment (about 30 and 50% of inhibition compared to HuMab-HepC control treatment). HuMab-025 and HuMab-091 strongly inhibited the BT-474 tumor growth and the time to reach a tumor volume of 800 mm^3 was significantly delayed by these antibodies. Survival was also improved in the HER2 HuMab receiving mice.

Example 27 - Removing Fc-mediated T cell activation by means of Fc mutations

Monocytes, which are present in PBMCs, express Fc-receptors which can interact with the Fc-domains in the IgG monospecific and bispecific antibodies. In case monospecific CD3 antibodies are used, it is known that such active Fc-domain can cause activation of T-cells. Importantly, if purified T-cells are used as effector cells this Fc-mediated effect is absent due to the absence of monocytes.

In order to remove this activity, a strategy was set up to create antibodies without such Fc-mediated activity. Deglycosylation of antibodies, either post-translational via N-glycanase or genetically via N297Q mutation has been desribed to result in an inert antibody format (Tao M H *et al.*, Immunol 1989, 143; 2595-2601). These Fc modified antibodies were generated to determine the contribution of Fc-mediated activation of T cells. A panel of (bispecific) antibodies with either a N297Q mutation in the Fc-domain or chemically deglycosylated were compared to antibodies with WT Fc regions in a cytotoxity assay with PBMCs (E:T ratio 5:1). The cytotoxicity assay was performed as described in Example 21, however human PBMC were used instead of purified T-cells. Deglycosylation did not compromise the activity of the HER2xCD3 bispecific antibodies in the cytotoxicity assay whereas the Fc-mediated activity of monospecific huCLB-T3/4 was strongly but not completely removed under the tested conditions (Figure 17).

To completely remove the residual Fc activity three sets of mutations from the public domain were combined in one mutant. The mutations L234F, L235E, P331S (Oganesyan Acta Cryst. (2008). D64, 700–704), D265A (Shields JBC (2001) 276(9) 6591–6604) and N297Q were introduced in the K409R and F405L IgG1 backbone. This mutant, designated LFLEDANQPS, did not show any residual Fc-mediated activation of T cells in a cytotoxity assay (same protocol as above) with PBMCs (Figure 17).

Example 28 - Effect of HER2 epitope on HER2xCD3 efficacy

The effect of the binding site on the tumor target (epitope) was determined by generating three bispecific antibodies recognizing different HER2 epitopes combined with a CD3 antibody that was proven to be effective in a bispecific format (Examples 21 and 27). The HER2-clones 005, 153 and 169 are three non-crossblocking antibodies recognizing a spatially segregated part of HER2 as shown in Example 24. These three HER2-clones were combined with CD3 antibody clone huCLB-T3/4, which recognizes human CD3 as a bispecific molecule and tested in a cytotoxicity assay with either human PBMCs or purified human T cells. The assay was performed as described in example 21. An E:T ratio of 1:1 was used for the T-cells assay, a 2:1 ratio was used for the PBMC assay.

As shown in Figure 18, all three bispecific antibodies are able to induce killing of AU565 target cells, albeit with different efficacy. These data show an important effect of the location of the target epitope on the cytotoxic potential of a HER2xCD3 bispecific antibody.

Example 29 - Efficacy of T cell mediated killing depends on HER2 expression levels

Cell lines with different HER2 expression levels were used to study the effect of target density on the efficacy of bispecific HER2xCD3 antibodies. Bispecific antibody 169xhuCLB-T3/4-N297Q was tested in a cytotoxicity assay using A549, A431, 3T3 and AU565 cells. HER2 expression was determined using QIFIKIT® analysis, using the mouse anti-human HER2 (R&D Systems, Cat. MAB1129, Lot IBD0207061) and isotype control antibody (BD, Cat. 555740 Lot 3280) at a concentration of 10 µg/mL. The expression levels are summarized in Table 13.

For the cytotoxity assay the different HER2 expressing cell lines were co-cultured with freshly isolated human T cells with an E:T ratio of 10:1, using the protocol described in Example 21.

The cytotoxic efficacy of 169xCLB-T3/4 was correlated with the HER2 expression of the target cell line (Figure 19). AU565 cells were killed at already very low concentrations of antibody whereas the cells with lowest expression (A549) could hardly be killed in this experimental set up.

Table 13: HER2 expression levels of cell lines used in cytotoxity assay.

Cell line	**HER2 expression (molecules per cell)**
AU565	4x10e5
NIH-3T3	7x10e4
A431	1.4x10e4
A549	1x10e4

Example 30: Characterization of HER2xCD3 bispecific antibody induced T cell activation

As shown in previous examples effective killing of various HER2 expressing tumor cell lines was accomplished by using bispecific HER2xCD3 antibodies. The expression of CD69, a well characterized activation marker of cytotoxic T cells, was monitored in T cells co-cultured with AU565 tumor cells in the presence of bispecific HER2xCD3 antibody for 16h at 37°C. A

dose dependent activation of the T cells as measured by CD69 expression was observed (Figure 20) which correlates with the observed cytotoxicity data shown in Example 21.

In agreement with the cytotoxicity data shown in Example 27, *in vitro* characterization of the T cell response revealed that also in the absence of tumor cells, an Fc-mediated T cell activation could be observed when PBMCs were used as effector cells (Figure 21). This effect was most prominent when a variant with an unmodified Fc of DuoBody HER2 169 x huCLBT3/4 was used and could be reduced by introduction of the N297Q mutation. The Fc mediated activation by monospecific CD3 antibodies could be further reduced by using an LFLEDANQPS Fc-mutant (Figure 21).

An *in vitro* cytotoxicity assay was performed as described in Example 21. Th1/Th2 cytokine detection in the medium of the different wells was performed by collecting supernatant samples of a cytotoxicity assay to measure cytokine release. Undiluted samples were analysed on FACS using the human Th1/Th2 Cytokine detection kit (BD Biosciences, cat# 551809), according to manufacturer's instructions. Cytokine concentration of IL-2, IL-4, IL-6, IL-10, IFN-γ and TNF-α in the samples were calculated based on standard curves of these cytokines. Data was analyzed using Graphpad Prism 5.0 and Excel 2003 software. Three groups of cytokines were analyzed (1) Pro-inflammatory cytokines TNF-α, INF-γ and IL-2 (2) Pro and anti-inflammatory cytokine IL6 and (3) Anti-inflammatory cytokines IL4 and IL10. Cytokine profiles at day 3 generated by T-cells or PBMCs in *in vitro* cytotoxicity assay with DuoBody huCLB-T3/4 x HER2-169 N297Q and all appropriate controls are summarized in Table 14.

Cytokines are upregulated when tumor cells and T-cells were incubated together with DuoBody huCLB-T3/4 x HER2-169 N297Q in contrast to the control antibodies and control treatments (medium and T-cells only).

Incubation of tumor cells and PBMC with DuoBody huCLB-T3/4 x HER2-169 N297Q also resulted in upregulation of the measured cytokines when compared to control antibody IgG1-1014-169 N297Q and IgG1-Herceptin® and control situations (medium and T-cells only). However, incubation of target cells and PBMCs with control antibody DuoBody huCLB-T3/4-N297Qxb12-N297Q and monospecific huCLB-T3/4-N297Q also resulted in upregulation of most cytokines compared to control situations (medium and T-cells only).

Cytokine expression was also followed over time. Cytokine profiles at day 1, 2 and 3 generated by T-cells or PBMCs in *in vitro* cytotoxicity assay with DuoBody™ huCLB-T3/4-N297QxHER2-169-N297Q are depicted in Figure 22. In general, the pro-inflammatory cytokines, TNF-α, INF-γ and IL-2, were pronounced present in experiments were lower antibody concentrations were used (3 ng/mL). In experiments where antibody concentrations were increased with a factor 300, most pro-inflammatory cytokines were

present at lower concentrations. IL-6, the cytokine with pro and anti-inflammatory activity, is hardly secreted by T-cells, but highly secreted by PBMCs upon incubation with tumor cells and DuoBody huCLB-T3/4 x HER2-169 N297Q. Some cytokines are secreted at higher levels at day 1 and/ or day 2 and decrease at day 3, whereas others are secreted at lower levels at day 1 and show increased expression at day 2 and or 3.

Additionally the release of GM-CSF as a measure for T cell activation was measured. This cytokine is not consumed during the activation of T cells and is therefore better suited for the measurement of cytokines levels in long term experiments. To investigate the observed cytokine release in the control samples as described above Fc mutants were compared to WT antibodies. Hereto T cells samples from co-cultures of PBMCs with AU565 tumor cells in the presence of bispecific antibodies were analyzed in a GM-CSF ELISA.

The following protocol was used: An ELISA plate was coated with 100 µL/well 2.0 µg/mL coating antibody (anti-GM-CSF 9.1 Sanquin) in PBS and incubated O/N at room temperature (RT). Plates were then washed 3 times with PBS supplemented with 0.05% Tween (PBST) using an Elisa-washer. Samples were diluted in PBST/0.2%BSA and standard curve samples were prepared (Standard curve: first point 1000 pg/mL, two-fold dilution curve, 10 steps and 2 blanks (Standard GM-CSF = rec GM-CSF Sandoz). 100 µL/well of samples were added to the plate and 10 µL/well of monoclonal biotinylated anti-GM-CSF (monoclonal anti-GM-CSF 16.3 Sanquin) (1 µg/mL) diluted in PBST/0.2%BSA and incubated for 2h at RT on a shaker. After washing 3 times with PBS-T 100 µL/well Strep-poly-HRP diluted in PBST/0.2%BSA (0.1 µg/mL) was added and incubated for 20 minutes at room temperature on a shaker. For detection 1 tablet of ABTS substrate was dissolved in 50 ml ABTS-buffer (Roche) and 100 µL/well of the ABTS solution was added to well and incubated for 15-30 mintues at RT in the dark. The reaction was stopped with 100 µL/well 2% oxalic acid and incubated for 10 min in the dark. Absorbance was read at 405 nm using an EL808-Elisa-reader.

DuoBody HER2 169 x huCLB-T3/4 (both Fc variants, N297Q and LFLEDANQPS) induced a dose-dependent activation of T cells as shown by GM-CSF production (Figure 23). The monospecific control antibody IgG1-HER2-169-N297Q and the irrelevant antibody IgG1-b12 N297Q did not induce T cell activation as expected. As observed in the TH1/TH2 cytokine profile assay monospecific IgG1-huCLB-T3/4 N297Q and DuoBody huCLB-T3/4 x b12 N297Q did induce activation of T cells. The $(Fab')_2$ control and the inactive Fc mutant LFLEDANQPS of IgG1-CLB-T3/4 did not induce T cell activation suggesting that Fc mediated activation of T cells by N297Q mutants is occurring.

Table 14: Cytokine profile measured at day 3 of *in vitro* cytotoxicity assay with A. T-cells or B. PBMCs (incubated with 1000 ng/mL antibody).

A	**Cytokines T-cells (conc. pg/mL)**					
	INFγ	**TNFα**	**IL2**	**IL6**	**IL4**	**IL10**
DuoBody huCLB-T3/4-Q x HER2-169-Q	4268.0	1005.1	2371.3	45.3	32.6	2010.1
DuoBody huCLB-T3/4-Q x B12-Q	0.5	0.3	9.3	0.1	0.1	1.7
IgG1-hCLB-T3/4-Q	1.6	1.7	20.5	0.2	0.3	2.6
IgG1-HER2-169-Q	4.8	0.7	5.1	0.3	0.2	0.5
T-cells only	0.1	0.4	0.2	0.1	0.0	0.3
T-cell medium	3.8	1.0	7.9	0.2	0.1	0.3
B	**Cytokines PBMCs (conc. pg/mL)**					
	INFγ	**TNFα**	**IL2**	**IL6**	**IL4**	**IL10**
DuoBody huCLB-T3/4-Q x HER2-169-Q *	4082.6	1073.3	585.6	4191.9	13.2	2018.2
DuoBody huCLB-T3/4-Q x B12-Q *	1760.2	115.0	10.1	569.4	1.4	592.4
IgG1-hCLB-T3/4-Q	1474.2	92.7	345.2	116.5	1.7	1295.4
IgG1-HER2-169-Q	1.3	1.0	15.5	2.2	0.2	2.8
T-cells only	0.4	0.7	0.3	0.2	0.4	0.4
T-cell medium	1.5	1.0	8.8	0.2	0.3	2.2

* Concentration antibody = 1000 ng/mL

Example 31 - *In vivo* proof of concept

The *in vivo* anti-tumor efficacy of bispecific HER2xCD3 antibody was evaluated, in a subcutaneous NCI-N87 xenograft model, in which human T cells are co-inoculated in the form of unstimulated PBMCs with the tumor cells, analogous to the model described by Brischwein *et al.*, (Mol. Immunol. 43 (2006), 1129–1143). Six to eleven weeks old female NOD-SCID (NOD.CB17-Prkdcscid/NcrCrl) mice were used. PBMC from healthy donors were isolated from a buffy coat as described in Example 21. At day 0, a mixture containing $5x10^6$ cells of both PBMCs (and NCI-N87 cells were inoculated subcutaneously in 200 µL in the

right flank of each mouse (PBMCs from two donors were used in parallel to rule out donor specific artefacts. Within one hour of injection, the mice were sorted into five groups (n=7) and each group was injected intraperitoneally (i.p.) with a single dose of (bispecific) antibody. Treatment groups are shown in Table 15. All antibody samples were supplemented with irrelevant mAb IgG1-b12 to obtain a total antibody concentration of 4 mg/kg per sample.

Tumors were measured twice per week using caliper (PLEXX) until an endpoint tumor volume of 1500 mm^3, tumors showed ulcerations or until the end of the study (day 50). Figure 24A shows that on day 42 tumor outgrowth is inhibited most optimal by bispecific HER2xCD3 antibody effectively at a dose of 0.05 mg/kg.

In Figure 24B, the percentage survival (with tumor sizes smaller then 500 mm3) is shown in a Kaplan-Meier plot. Tumor formation is significantly delayed ($p<0.05$, Log Rank (Mantel-Cox)) in mice treated with HER2 x CD3 antibodies (0.05 mg/kg) compared to control group treated with b12 x CD3 control antibody.

Table 15: Treatment groups and dosing.

Group	**Antibody**	**Dose**
1	DuoBody HER2 169 x CLB-T3/4-N297Q	1 µg (= 0.05 mg/kg)
2	DuoBody b12 x CLB-T3/4-N297Q	80 µg (= 4 mg/kg)
3	Neg control mAb IgG1-b12	80 µg (= 4 mg/kg)

Example 32: Unraveling the requirement of the T350I, K370T and F405L substitutions for Fab-arm exchange engagement of human IgG1

To further identify the determinants in the IgG1 CH3 domain that are required for IgG1 to be engaged in Fab-arm exchange, IgG1 containing the triple mutation T350I-K370T-F405L (ITL) was compared to the double mutants T350I-K370T (IT), T350I-F405L (IL) and K370T-F405L (TL) were studied using antibodies 2F8 and 7D8, respectively described in WO 02/100348 and WO 04/035607. Also the single mutant F405L (L) was tested. 2-MEA was used as a reductant to induce *in vitro* Fab-arm exchange (50 µg of each antibody in 100 µL PBS/25 mM 2-MEA for 90 min at 37°C). For the single mutant F405L antibody, unpurified antibody from supernatant of a transient transfection was used after buffer-exchange to PBS using Amicon Ultra centrifugal devices (30k, Millipore, cat. no. UFC803096). To stop the reduction reaction, the reducing agent 2-MEA was removed by desalting the samples using spin columns. The generation of bispecific antibodies was determined by bispecific binding measured in an ELISA.

The triple (ITL), double mutations (IT, IL and TL) and single mutation (L) were introduced in IgG1-2F8. These mutants were combined with IgG4-7D8, containing a CPSC hinge (wild type) or a stabilized hinge (IgG4-7D8-CPPC), for Fab-arm exchange using 25 mM 2-MEA for 90 min at 37°C. Figures 25A-B show that the IgG1-2F8-IL and -TL mutants showed Fab-arm exchange to the same level as the triple mutant ITL, irrespective of the combined IgG4-7D8 (CPSC or CPPC hinge). In contrast, no bispecific binding was found for the combination with the IgG1-2F8-IT mutant. Figure 25C shows that also the IgG1-2F8-F405L mutant showed Fab-arm exchange, irrespective of the combined IgG4-7D8 (CPSC or CPPC hinge). These data indicate that the F405L mutation is sufficient to engage human IgG1 for Fab-arm exchange under the conditions mentioned above.

Example 33: Determinants at the IgG1 409 position for engagement in 2-MEA-induced Fab-arm exchange in combination with IgG1-ITL

2-MEA can induce Fab-arm exchange between human IgG1-ITL and IgG4-CPPC. The CH3 interface residues of human IgG1 and IgG4 differ at position 409 only: lysine (K) in IgG1 and arginine (R) in IgG4. Therefore, it was tested whether substitution of lysine at position 409 by arginine or any other amino acid (K409X) could enable IgG1 to engage in 2-MEA-induced Fab-arm exchange with IgG1-ITL. Combinations of 10 µg human IgG1-2F8-ITL and 10 µg IgG1-7D8-K409X in 20 µl PBS/25 mM 2-MEA (final concentration of 0.5 mg/mL for each antibody) were incubated for 90 min at 37°C. Unpurified antibodies from supernatants of transient transfections were used after buffer-exchange to PBS using Amicon Ultra centrifugal devices (30k, Millipore, cat. no. UFC803096). After the Fab-arm exchange reaction, 20 µL PBS was added to each sample and the reducing agent was removed by desalting the samples using spin desalting plate. Dilution series of the antibody samples (total antibody concentration 0-20 µg/mL in 3-fold dilutions) were used in an ELISA to measure bispecific binding.

Figure 26A shows the results of bispecific binding upon 2-MEA induced Fab-arm exchange between IgG1-2F8-ITL x IgG1-7D8-K409X. In Figure 26B, the exchange is presented as bispecific binding relative to a purified batch of bispecific antibody derived from a 2-MEA-induced Fab-arm-exchange between IgG1-2F8-ITL and IgG4-7D8-CPPC, which was set to 100%. These data were also scored as (-) no Fab-arm exchange, (+/-) low, (+) intermediate or (++) high Fab-arm exchange, as presented in Table 1. No Fab-arm exchange (-) was found when the 409 position in IgG1-7D8 was K (=wild type IgG1), L or M. Fab-arm exchange was found to be intermediate (+) when the 409 position in IgG1-7D8 was F, I, N or Y and high (++) when the 409 position in IgG1-7D8 was A, D, E, G, H, Q, R, S, T, V or W.

Table 16: 2-MEA-induced Fab-arm exchange between IgG1-2F8-ITL and IgG1-7D8-K409X mutants. The generation of bispecific antibodies after 2-MEA-induced *in vitro* Fab-arm exchange between IgG1-2F8-ITL and IgG1-7D8-K409X mutants was determined by a sandwich ELISA. (-) no, (+/-) low, (+) intermediate, (++) high Fab-arm exchange.

	Fab-arm exchange
IgG1-7D8-K409X	x IgG1-2F8-ITL
A	++
D	++
E	++
F	+
G	++
H	++
I	+
K	-
L	-
M	-
N	+
Q	++
R	++
S	++
T	++
V	++
W	++
Y	+

Example 34: Determinants at the IgG1 405 position for engagement in 2-MEA-induced Fab-arm-exchange in combination with IgG1-K409R

In Example 32 it is described that the F405L mutation is sufficient to enable human IgG1 to engage in Fab-arm-exchange when combined with IgG4-7D8. To further test the determinants at the IgG1 405 position for engagement in 2-MEA-induced Fab-arm-exchange in combination with human IgG1-K409R, all possible IgG1-2F8-F405X mutants (with the exception of C and P) were combined with IgG1-7D8-K409R. The procedure was performed with purified antibodies as described in Example 32.

Figure 27 shows the results of bispecific binding upon 2-MEA-induced Fab-arm-exchange between IgG1-2F8-F405X x IgG1-7D8-K409R. These data were also scored as (-) no Fab-arm exchange, (+/-) low, (+) intermediate or (++) high Fab-arm exchange, as presented in Table 18. No Fab-arm exchange (-) was found when the 405 position in IgG1-2F8 was F (= wild type IgG1). Fab-arm exchange was found to be low (+/-) when the 405 position in IgG1-2F8 was G or R. Fab-arm exchange was found to be high (++) when the 405 position in IgG1-2F8 was A, D, E, H, I, K, L, M, N, Q, S, T, V, W or Y. These data

indicate that particular mutations at the IgG1 405 position allow IgG1 to engage in 2-MEA-induced Fab-arm-exchange when combined with IgG1-K409R.

Table 17: 2-MEA-induced Fab-arm-exchange between IgG1-2F8-F405X mutants and IgG1-7D8-K409R. The generation of bispecific antibodies after 2-MEA-induced *in vitro* Fab-arm-exchange between IgG1-2F8-F405X mutants and IgG1-7D8-K409R was determined by a sandwich ELISA. (-) no, (+/-) low, (+) intermediate, (++) high Fab-arm-exchange.

IgG1-2F8-F405X	Fab-arm-exchange x IgG1-7D8-K409R
A	++
D	++
E	++
F	-
G	+/-
H	++
I	++
K	++
L	++
M	++
N	++
Q	++
R	+/-
S	++
T	++
V	++
W	++
Y	++

Example 35: Determinants at the IgG1 407 position for engagement in 2-MEA-induced Fab-arm-exchange in combination with IgG1-K409R

In the previous Example, it is described that certain single mutations at position F405 are sufficient to enable human IgG1 to engage in Fab-arm-exchange when combined with IgG1-K409R. To test whether other determinants implicated in the Fc:Fc interface positions in the CH3 domain could also mediate the Fab-arm-exchange mechanism, mutagenesis of the IgG1 407 position was performed and the mutants were tested for engagement in 2-MEA-induced Fab-arm-exchange in combination with human IgG1-K409R. All possible IgG1-2F8-Y407X mutants (with the exception of C and P) were combined with IgG1-7D8-K409R. The procedure was performed with purified antibodies.

Figure 28 shows the results of bispecific binding upon 2-MEA-induced Fab-arm-exchange between IgG1-2F8-Y407X x IgG1-7D8-K409R. These data were also scored as (-)

no Fab-arm exchange, (+/-) low, (+) intermediate or (++) high Fab-arm exchange, as presented in Table 19. No Fab-arm exchange (-) was found when the 407 position in IgG1-2F8 was Y (= wild type IgG1), E, K, Q, or R. Fab-arm exchange was found to be low (+/-) when the 407 position in IgG1-2F8 was D, F, I, S or T and intermediate (+) when the 407 position in IgG1-2F8 was A, H, N or V, and high (++) when the 407 position in IgG1-2F8 was G, L, M or W. These data indicate that particular single mutations at the IgG1 407 position allow IgG1 to engage in 2-MEA-induced Fab-arm-exchange when combined with IgG1-K409R.

Table 18: 2-MEA-induced Fab-arm-exchange between IgG1-2F8-Y407X mutants and IgG1-7D8-K409R. The generation of bispecific antibodies after 2-MEA-induced *in vitro* Fab-arm exchange between IgG1-2F8-Y407X mutants and IgG1-7D8-K409R was determined by a sandwich ELISA. (-) no, (+/-) low, (+) intermediate, (++) high Fab-arm-exchange.

IgG1-2F8-Y407X	Fab-arm-exchange x IgG1-7D8-K409R
A	+
D	+/-
E	-
F	+/-
G	++
H	+
I	+/-
K	-
L	++
M	++
N	+
Q	-
R	-
S	+/-
T	+/-
V	+
W	++
Y	-

Example 36: Determinants at the IgG1 368 position for engagement in 2-MEA-induced Fab-arm exchange in combination with IgG1-K409R

Examples 34 and 35 show that certain single mutations at position F405 and Y407 are sufficient to enable human IgG1 to engage in Fab-arm exchange when combined with IgG1-K409R. As illustrated in this example further determinants implicated in the Fc:Fc interface positions in the CH3 domain may also mediate the Fab-arm exchange mechanism. To this

effect mutagenesis of the IgG1 368 position was performed and the mutants were tested for engagement in 2-MEA-induced Fab-arm-exchange in combination with human IgG1-K409R. All possible IgG1-2F8-L368X mutants (with the exception of C and P) were combined with IgG1-7D8-K409R. The procedure was performed with purified antibodies.

Figure 29 shows the results of bispecific binding upon 2-MEA-induced Fab-arm exchange between IgG1-2F8-L368X x IgG1-7D8-K409R. These data were also scored as (-) no Fab-arm exchange, (+/-) low, (+) intermediate or (++) high Fab-arm exchange, as presented in Table 20. No Fab-arm exchange (-) was found when the 368 position in IgG1-2F8 was L (= wild type IgG1), F or M. Fab-arm exchange was found to be low (+/-) when the 368 position in IgG1-2F8 was Y. Fab-arm exchange was found to be intermediate (+) when the 368 position in IgG1-2F8 was K and high (++) when the 368 position in IgG1-2F8 was A, D, E, G, H, I, N, Q, R, S, T, V, or W. These data indicate that particular mutations at the IgG1 368 position allow IgG1 to engage in 2-MEA-induced Fab-arm exchange when combined with IgG1-K409R.

Table 19: 2-MEA-induced Fab-arm exchange between IgG1-2F8-L368X mutants and IgG1-7D8-K409R. The generation of bispecific antibodies after 2-MEA-induced *in vitro* Fab-arm exchange between IgG1-2F8-L368X mutants and IgG1-7D8-K409R was determined by a sandwich ELISA. (-) no, (+/-) low, (+) intermediate or (++) high Fab-arm exchange.

	Fab-arm exchange Fab-arm exchange
IgG1-2F8-L368X	x IgG1-7D8-K409R
A	++
D	++
E	++
F	-
G	++
H	++
I	++
K	+
L	-
M	-
N	++
Q	++
R	++
S	++
T	++
V	++
W	++

Example 37: Determinants at the IgG1 370 position for engagement in 2-MEA-induced Fab-arm exchange in combination with IgG1-K409R

The previous Examples show that certain single mutations at positions F405, Y407 or L368 are sufficient to enable human IgG1 to engage in Fab-arm exchange when combined with IgG1-K409R. As illustrated in this example further determinants implicated in the Fc:Fc interface positions in the CH3 domain may also mediate the Fab-arm exchange mechanism. To this effect mutagenesis of the IgG1 370 position was performed and the mutants were tested for engagement in 2-MEA-induced Fab-arm-exchange in combination with human IgG1-K409R. All possible IgG1-2F8-K370X mutants (with the exception of C and P) were combined with IgG1-7D8-K409R. The procedure was performed with purified antibodies.

Figure 30 shows the results of bispecific binding upon 2-MEA-induced Fab-arm exchange between IgG1-2F8-K370X x IgG1-7D8-K409R. These data were also scored as (-) no Fab-arm exchange, (+/-) low, (+) intermediate or (++) high Fab-arm exchange, as presented in Table 21. No Fab-arm exchange (-) was found when the 370 position in IgG1-2F8 was K (= wild type IgG1), A, D, E, F, G, H, I, L, M, N, Q, R, S, T, V or Y. Only substitution of K370 with W resulted in intermediate Fab-arm exchange (+). These data indicate that only one mutation at the IgG1 370 position (K370W) allows IgG1 to engage in 2-MEA-induced Fab-arm exchange when combined with IgG1-K409R.

Table 20: 2-MEA-induced Fab-arm exchange between IgG1-2F8-K370X mutants and IgG1-7D8-K409R. The generation of bispecific antibodies after 2-MEA-induced *in vitro* Fab-arm exchange between IgG1-2F8-K370X mutants and IgG1-7D8-K409R was determined by a sandwich ELISA. (-) no, (+/-) low, (+) intermediate or (++) high Fab-arm exchange.

	Fab-arm exchange
IgG1-2F8-K370X	x IgG1-7D8-K409R
A	-
D	-
E	-
F	-
G	-
H	-
I	-
K	-
L	-
M	-
N	-
Q	-
R	-
S	-

T	-
V	-
W	+
Y	-

Example 38: Determinants at the IgG1 399 position for engagement in 2-MEA-induced Fab-arm exchange in combination with IgG1-K409R

The preceding Examples show that certain single mutations at positions F405, Y407, L368 or K370 are sufficient to enable human IgG1 to engage in Fab-arm exchange when combined with IgG1-K409R. As illustrated in this example further determinants implicated in the Fc:Fc interface positions in the CH3 domain may also mediate the Fab-arm exchange mechanism. To this effect mutagenesis of the IgG1 399 position was performed and the mutants were tested for engagement in 2-MEA-induced Fab-arm-exchange in combination with human IgG1-K409R. All possible IgG1-2F8-D399X mutants (with the exception of C and P) were combined with IgG1-7D8-K409R. The procedure was performed with purified antibodies as described in Example 33.

Figure 31 shows the results of bispecific binding upon 2-MEA-induced Fab-arm exchange between IgG1-2F8-D399X x IgG1-7D8-K409R. These data were also scored as (-) no, (+/-) low, (+) intermediate or (++) high Fab-arm exchange, as presented in Table 10. No Fab-arm exchange (-) was found when the 399 position in IgG1-2F8 was D (= wild type IgG1), E and Q. Fab-arm exchange was found to be low (+/-) when the 399 position in IgG1-2F8 was V, intermediate (+) when the 399 position in IgG1-2F8 was G, I, L, M, N, S, T or W. Fab-arm exchange was found to be high (++) when the 399 position in IgG1-2F8 was A, F, H, K, R or Y. These data indicate that particular mutations at the IgG1 399 position allow IgG1 to engage in 2-MEA-induced Fab-arm exchange when combined with IgG1-K409R.

Table 21: 2-MEA-induced Fab-arm exchange between IgG1-2F8-D399X mutants and IgG1-7D8-K409R. The generation of bispecific antibodies after 2-MEA-induced *in vitro* Fab-arm exchange between IgG1-2F8-D399X mutants and IgG1-7D8-K409R was determined by a sandwich ELISA. (-) no, (+/-) low, (+) intermediate or (++) high Fab-arm exchange.

IgG1-2F8-D399X	Fab-arm exchange x IgG1-7D8-K409R
A	++
D	-
E	-
F	++

G	+
H	++
I	+
K	++
L	+
M	+
N	+
Q	-
R	++
S	+
T	+
V	+/-
W	+
Y	++

Example 39 - Determinants at the IgG1 366 position for engagement in 2-MEA-induced Fab-arm exchange in combination with IgG1-K409R

Examples 32 to 38 show that certain single mutations at positions F405, Y407, L368, K370 or D399 are sufficient to enable human IgG1 to engage in Fab-arm exchange when combined with IgG1-K409R. As illustrated in this example further determinants implicated in the Fc:Fc interface positions in the CH3 domain may also mediate the Fab-arm exchange mechanism. To this effect mutagenesis of the IgG1 366 position was performed and the mutants were tested for engagement in 2-MEA-induced Fab-arm-exchange in combination with human IgG1-K409R. All possible IgG1-2F8-T366X mutants (with the exception of C and P) were combined with IgG1-7D8-K409R. The procedure was performed with purified antibodies as described in Example 33.

Figure 32 shows the results of bispecific binding upon 2-MEA-induced Fab-arm exchange between IgG1-2F8-T366X x IgG1-7D8-K409R. These data were also scored as (-) no, (+/-) low, (+) intermediate or (++) high Fab-arm exchange, as presented in Table X. No Fab-arm exchange (-) was found when the 366 position in IgG1-2F8 was T (= wild type IgG1), K, R, S or W. Fab-arm exchange was found to be low (+/-) when the 366 position in IgG1-2F8 was F, G, I, L, M or Y, intermediate (+) when the 366 position in IgG1-2F8 was A, D, E, H, N, V or Q. These data indicate that particular mutations at the IgG1 366 position allow IgG1 to engage in 2-MEA-induced Fab-arm exchange when combined with IgG1-K409R.

Table 22: 2-MEA-induced Fab-arm exchange between IgG1-2F8-T366X mutants and IgG1-7D8-K409R

The generation of bispecific antibodies after 2-MEA-induced *in vitro* Fab-arm exchange between IgG1-2F8-T366X mutants and IgG1-7D8-K409R was determined by a sandwich ELISA. (-) no, (+/-) low, (+) intermediate or (++) high Fab-arm exchange.

IgG1-2F8-T366X	Fab-arm exchange x IgG1-7D8-K409R
A	+
D	+
E	+
F	+/-
G	+/-
H	+
I	+/-
K	-
L	+/-
M	+/-
N	+
Q	+
R	-
S	-
T	-
V	+
W	-
Y	+/-

Example 40 – In vivo proof of concept: dose titration

To further test the growth inhibitory effect of the bispecific HER2xCD3 antibody, different antibody doses were tested using the subcutaneous NCI-N87 xenograft model in NOD-SCID mice with subcutaneous (s.c.) co-injection of unstimulated human PBMCs (7 mice per group) as described in Example 31. This time, a single dose of antibody was administered intravenously (i.v.) 1 hour after tumor inoculation. Treatment groups are shown in Table 23.

Control groups showed a donor-specific tumor growth inhibition (alloreaction) in the absence of therapeutic antibody with PBMCs from one of the two donors (data not shown). Therefore, data received with PBMCs from that particular donor were excluded from analysis.

Table 23 Treatment groups and dosing

Group	Antibody	Dose
1	DuoBody HER2 169 x CLB-T3/4-N297Q	0.01 µg (= 0.0005 mg/kg)
2	DuoBody HER2 169 x CLB-T3/4-N297Q	0.1 µg (= 0.005 mg/kg)
3	DuoBody HER2 169 x CLB-T3/4-N297Q	1 µg (= 0.05 mg/kg)
4	DuoBody HER2 169 x CLB-T3/4-N297Q	10 µg (= 0.5 mg/kg)
5	DuoBody b12 x CLB-T3/4-N297Q	10 µg (= 0.5 mg/kg)
6	PBS	

Figure 33A shows that tumor growth was inhibited by 0.05 mg/kg and 0.5 mg/kg HER2 x CD3 N297Q.

Figure 33B shows a Kaplan-Meier plot displaying the percentage of mice with tumors <500 mm^3. Tumor formation is delayed in mice treated with 0.05 mg/kg and 0.5 mg/kg HER2xCD3-N297Q bispecific antibody compared to control mice treated with PBS or b12 x CD3 control antibody.

CLAIMS

1. A bispecific antibody comprising a first antigen-binding region and a second antigen-binding region, which second antigen-binding region binds an epitope on human CD3 and the first antigen-binding region binds an epitope on human epidermal growth factor receptor 2 (HER2) and blocks the binding to HER2, optionally soluble HER2, of a reference antibody selected from the group consisting of:

a) an antibody comprising a VH region comprising the sequence of SEQ ID NO:63 and a VL region comprising the sequence of SEQ ID NO:67 (**153**), and
b) an antibody comprising a variable heavy (VH) region comprising the sequence of SEQ ID NO:1 and a variable light (VL) region comprising the sequence of SEQ ID NO:5 (**169**), and
c) an antibody comprising a VH region comprising the sequence of SEQ ID NO:165 and a VL region comprising the sequence of SEQ ID NO:169 (**005**), and
d) an antibody comprising a VH region comprising the sequence of SEQ ID NO:22 and a VL region comprising the sequence of SEQ ID NO:26 (**025**).

2. The bispecific antibody of claim 1, wherein the first antigen-binding region blocks the binding to soluble HER2 of an antibody of (a).

3. The bispecific antibody of claim 1, wherein the first antigen-binding region blocks the binding to soluble HER2 of an antibody of (b).

4. The bispecific antibody of claim 1, wherein the first antigen-binding regions blocks the binding to soluble HER2 of an antibody of (c).

5. The bispecific antibody of claim 1, wherein the first antigen-binding region blocks the binding to soluble HER2 of an antibody of (d).

6. The bispecific antibody of any one of the preceding claims, wherein the first antigen-binding region comprises VH CDR1, CDR2, and CDR3 sequences selected from the group consisting of

a) SEQ ID NOs: 2, 3 and 4, respectively (**169**);
b) SEQ ID NOS: 9, 10 and 11, respectively (**050**);
c) SEQ ID NOs:16, 17 and 18, respectively (**084**);
d) SEQ ID NOS: 23, 24 and 25, respectively (**025**);

e) SEQ ID NOs:30, 163, and 31, respectively (**091**);
f) SEQ ID NOs: 36, 37 and 38, respectively (**129**):
g) SEQ ID NOS: 43, 44 and 45, respectively (**127**);
h) SEQ ID NOs:50, 51 and 52, respectively (**159**);
i) SEQ ID NOs: 57, 58 and 59, respectively (**098**);
j) SEQ ID NOs:64, 65 and 66, respectively (**153**);
k) SEQ ID NOs: 71, 72 and 73, respectively (**132**);
l) SEQ ID NOs: 166, 167 and 168, respectively (**005**);
m) SEQ OD NOS: 173, 174, and 175, respectively (**006**);
n) SEQ ID NOS: 180, 181, and 182, respectively (**059**);
o) SEQ ID NOS:187, 188, and 189, respectively (**060**);
p) SEQ ID NOS:194, 195, and 196, respectively (**106**); and
q) SEQ ID NOS:201, 202, and 203, respectively (**111**).

7. A bispecific antibody comprising a first antigen-binding region and a second antigen-binding region, which second antigen-binding region binds an epitope on human CD3 and the first antigen-binding region binds an epitope on HER2, wherein the first antigen-binding region comprises a VH region and a VL region selected from the group consisting of

a) a VH region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:2, 3 and 4, respectively; and a VL region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:6, DAS, and SEQ ID NO:7, respectively (**169**);
b) a VH region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:9, 10 and 11, respectively; and a VL region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:13, AAS, and SEQ ID NO:14, respectively (**050**);
c) a VH region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:16, 17 and 18, respectively; and a VL region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:20, VAS, and SEQ ID NO:21, respectively (**084**);
d) a VH region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:23, 24 and 25, respectively; and a VL region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:27, AAS, and SEQ ID NO:28, respectively (**025**);
e) a VH region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:30, 163 and 31, respectively; and a VL region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:33, AAS, and SEQ ID NO:34, respectively (**091**);
f) a VH region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:36, 37 and 38, respectively; and a VL region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:40, DAS, and SEQ ID NO:41, respectively (**129**);

g) a VH region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:43, 44 and 45, respectively; and a VL region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:47, AAS, and SEQ ID NO:48, respectively (**127**);
h) a VH region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:50, 51 and 52, respectively; and a VL region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:54, AAS, and SEQ ID NO:55, respectively (**159**);
i) a VH region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:57, 58 and 59, respectively; and a VL region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:60, AAS, and SEQ ID NO:61, respectively (**098**);
j) a VH region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:64, 65 and 66, respectively; and a VL region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:68, DAS, and SEQ ID NO:69, respectively (**153**);
k) a VH region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:71, 72 and 73, respectively; and a VL region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:75, DAS, and SEQ ID NO:76, respectively (**132**);
l) a VH region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:166, 167 and 168, respectively; and a VL region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NO: 170, GAS and SEQ ID NO:171, respectively (**005**);
m) a VH region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs: 173, 174 and 175, respectively; and a VL region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:177, DAS, and SEQ ID NO:178, respectively (**006**);
n) a VH region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:180, 181 and 182, respectively; and a VL region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:184, GAS, and SEQ ID NO:185, respectively (**059**);
o) a VH region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:187, 188 and 189, respectively; and a VL region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs: 191, GAS, and SEQ ID NO:192, respectively (**060**);
p) a VH region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:194, 195 and 196, respectively; and a VL region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:198, GAS, and SEQ ID NO:199, respectively (**106**);
q) a VH region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:201, 202 and 203, respectively; and a VL region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:205, GAS, and SEQ ID NO:206, respectively (**111**).

8. The bispecific antibody of claim 7, wherein the first antigen-binding region comprises a VH region and a VL region selected from the group consisting of

a) a VH region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:2, 3 and 4, respectively; and a VL region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:6, DAS, and SEQ ID NO:7, respectively (**169**);
b) a VH region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:23, 24 and 25, respectively; and a VL region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:27, AAS, and SEQ ID NO:28, respectively (**025**);
c) a VH region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:64, 65 and 66, respectively; and a VL region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:68, DAS, and SEQ ID NO:69, respectively (**153**), and
d) a VH region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:166, 167 and 168, respectively; and a VL region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs: 170, GAS, and SEQ ID NO:171, respectively (**005**).

9. A bispecific antibody comprising a first antigen-binding region and a second antigen-binding region, which second antigen-binding region binds an epitope on human CD3 and the first antigen-binding region binds HER2 and comprises a VH region comprising the CDR1, CDR2, and CDR3 sequences of SEQ ID NOs: 2, 3 and 4, respectively (**169**), and, optionally a VL region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:6, DAS, and SEQ ID NO:7, respectively (**169**).

10. A bispecific antibody comprising a first antigen-binding region and a second antigen-binding region, which second antigen-binding region binds an epitope on human CD3 and the first antigen-binding region binds HER2 and comprises a VH region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:64, 65 and 66, respectively; and, optionally, a VL region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:68, DAS, and SEQ ID NO:69, respectively (**153**).

11. A bispecific antibody comprising a first antigen-binding region and a second antigen-binding region, which second antigen-binding region binds an epitope on human CD3 and the first antigen-binding region binds HER2 and comprises a VH region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:166, 167 and 168, respectively; and, optionally, a VL region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:170, GAS, and SEQ ID NO:171, respectively (**005**).

12. A bispecific antibody comprising a first antigen-binding region and a second antigen-binding region, which second antigen-binding region binds an epitope on human CD3 and

the first antigen-binding region binds HER2 and comprises a VH region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:23, 24 and 25, respectively; and, optionally, a VL region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOs:27, AAS, and SEQ ID NO:28, respectively (**025**).

13. A bispecific antibody comprising a first antigen-binding region and a second antigen-binding region, which second antigen-binding region binds an epitope on human CD3 and the first antigen-binding region binds HER2 and comprises a VH region comprising the sequence of SEQ ID NO:1 and, optionally a VL region comprising SEQ ID NOs:5 (**169**).

14. A bispecific antibody comprising a first antigen-binding region and a second antigen-binding region, which second antigen-binding region binds an epitope on human CD3 and the first antigen-binding region binds HER2 and comprises a VH region comprising the sequence of SEQ ID NO:63 and, optionally a VL region comprising SEQ ID NOs:67 (**153**).

15. A bispecific antibody comprising a first antigen-binding region and a second antigen-binding region, which second antigen-binding region binds an epitope on human CD3 and the first antigen-binding region binds HER2 and comprises a VH region comprising the sequence of SEQ ID NO:165, and, optionally, a VL region comprising the sequence of SEQ ID NO:169 (**005**).

16. A bispecific antibody comprising a first antigen-binding region and a second antigen-binding region, which second antigen-binding region binds CD3 and the first antigen-binding region binds HER2 and comprises a VH region comprising SEQ ID NO:22, and, optionally, a VL region comprising the sequence of SEQ ID NO:26 (**025**).

17. A bispecific antibody of any one of the preceding claims, wherein the second antigen-binding region blocks the binding to CD3 of a reference antibody selected from the group consisting of

a) an antibody comprising a VH region comprising the sequence of SEQ ID NO:240 and a VL region comprising the sequence of SEQ ID NO:241 (**huCLB-T3/4**);
b) an antibody comprising a VH region comprising the sequence of SEQ ID NO:234 and a VL region comprising the sequence of SEQ ID NO:235 (**YTH12.5**);
c) an antibody comprising a VH region comprising the sequence of SEQ ID NO:238 and VL region comprising the sequence of SEQ ID NO:239 (**huOKT3-C114S-gLC**); and

d) an antibody comprising a VH region comprising the sequence of SEQ ID NO:236 and a VL region comprising the sequence of SEQ ID NO:237 (**HUM291**).

18. A bispecific antibody of any one of the preceding claims, wherein the second antigen-binding region blocks the binding to CD3 of a reference antibody comprising a VH region comprising the sequence of SEQ ID NO:240 and a VL region comprising the sequence of SEQ ID NO:241 (**huCLB-T3/4**).

19. A bispecific antibody of any one of the preceding claims, wherein the second antigen-binding region comprises a VH region selected from the group consisting of:

a) a VH region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NOS:242, 243 and 244, respectively (**huCLB-T3/4**);
b) a VH region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NO:234 (**YTH12.5**);
c) a VH region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NO:238 (**huOKT3-C114S-gLC**); and
d) a VH region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NO:236 (**HUM291**).

20. A bispecific antibody of any one of the preceding claims, wherein the second antigen-binding region comprises a VH region comprising the CDR1, CDR2 and CDR3 sequences of SEQ ID NO:242, 243 and 244, respectively (**huCLB-T3/4**);

21. A bispecific antibody comprising a second antigen-binding region comprising the VH CDR3 sequence of SEQ ID NO:244 (**huCLB-T3/4**) and a second antigen-binding region comprising rhe VH CDR3 sequence of SEQ ID NO:4 (**169**).

22. The bispecific antibody of claim 21, wherein the second antigen-binding region comprises the VL CDR3 sequence of SEQ ID NO:246 (**huCLB-T3/4**) and the second antigen-binding region comprises the VL CDR3 sequence of SEQ ID NO:7 (**169**).

23. The bispecific antibody of any preceding claim, wherein the second antigen-binding region comprises the VH CDR1, CDR2, and CDR3 sequences of SEQ ID NOs:242, 243 and 244, respectively; and, optionally, the VL CDR1, CDR2 and CDR3 sequences of 245, DTS and 246, respectively (**huCLB-T3/4).**

24. The bispecific antibody of any preceding claim, wherein the second antigen-binding region comprises a VH region comprising the sequence of SEQ ID NO: 240 and, optionally, a VL region comprising the sequence of SEQ ID NO:241 (**huCLB-T3/4**).

25. The bispecific antibody of any of the preceding claims, wherein said bispecific antibody further comprises a first Fc region and a second Fc region.

26. The bispecific antibody of any one of the preceding claims, comprising a first Fab-arm comprising the first antigen-binding region and a first Fc-region, and a second Fab-arm comprising the second antigen-binding region and a second Fc-region.

27. The bispecific antibody of any one of claims 1-24, comprising a first Fab-arm comprising the second antigen-binding region and a first Fc-region, and a second Fab-arm comprising the first antigen-binding region and a second Fc-region.

28. The bispecific antibody of any one of claims 23 and 24, wherein the isotypes of the first and second Fc-regions are independently selected from IgG1, IgG2, IgG3, and IgG4.

29. The bispecific antibody of claim 28, wherein the isotypes of the first and second Fc-regions are independently selected from IgG1 and IgG4.

30. The bispecific antibody of claim 29, wherein one of the first and second Fc-regions is of an IgG1 isotype and one is of an IgG4 isotype.

31. The bispecific antibody of claim 29, wherein the isotypes of the first and second Fc-region are IgG1.

32. The bispecific antibody of any one of the preceding claims, which is effector-function deficient.

33. The bispecific antibody of any one of claims 26 to 32, wherein the first Fc-region has an amino acid substitution at a position selected from the group consisting of 409, 366, 368, 370, 399, 405 and 407, and said second Fc-region has an amino acid substitution at a position selected from the group consisting of 405, 366, 368, 370, 399, 407, and 409, and wherein said first Fc-region and said second Fc-region are not substituted in the same positions.

34. The bispecific antibody of any one of claims 26 to 33, wherein the first Fc-region has an amino acid other than Lys, Leu or Met at position 409 and the second Fc-region has an amino acid substitution at a position selected from the group consisting of 405, 366, 368, 370, 399 and 407.

35. The bispecific antibody of any one of claims 26 to 33, wherein the first Fc-region has an amino acid other than Lys, Leu or Met at position 409 and the second Fc-region has an amino acid other than Phe at position 405.

36. The bispecific antibody of any one of claims 26 to 33, wherein the first Fc-region has an amino acid other than Lys, Leu or Met at position 409 and the second Fc-region has an amino acid other than Phe, Arg or Gly at position 405.

37. The bispecific antibody of any one of claims 26 to 33, wherein the first Fc-region comprises a Phe at position 405 and an amino acid other than Lys, Leu or Met at position 409 and said second Fc-region comprises an amino acid other than Phe at position 405 and a Lys at position 409.

38. The bispecific antibody of any one of claims 26 to 33, wherein the first Fc-region comprises a Phe at position 405 and an amino acid other than Lys, Leu or Met at position 409 and the second Fc-region comprises an amino acid other than Phe, Arg or Gly at position 405 and a Lys at position 409.

39. The bispecific antibody of any one of claims 26 to 33, wherein the first Fc-region comprises a Phe at position 405 and an amino acid other than Lys, Leu or Met at position 409 and the second Fc-region comprises a Leu at position 405 and a Lys at position 409.

40. The bispecific antibody of any one of claims 26 to 33, wherein the first Fc-region comprises a Phe at position 405 and an Arg at position 409 and said second Fc-region comprises an amino acid other than Phe, Arg or Gly at position 405 and a Lys at position 409.

41. The bispecific antibody of any one of claims 26 to 33, wherein the first Fc-region comprises Phe at position 405 and an Arg at position 409 and the second Fc-region comprises a Leu at position 405 and a Lys at position 409.

42. The bispecific antibody of any one of claims 26 to 33, wherein the first Fc-region comprises an amino acid other than Lys, Leu or Met at position 409 and the second Fc-region comprises a Lys at position 409, a Thr at position 370 and a Leu at position 405.

43. The bispecific antibody of any one of claims 26 to 33, wherein the first Fc-region comprises an Arg at position 409 and the second Fc-region comprises a Lys at position 409, a Thr at position 370 and a Leu at position 405.

44. The bispecific antibody of any one of claims 26 to 33, wherein the first Fc-region comprises a Lys at position 370, a Phe at position 405 and an Arg at position 409 and the second Fc-region comprises a Lys at position 409, a Thr at position 370 and a Leu at position 405.

45. The bispecific antibody of any one of claims 26 to 33, wherein the first Fc-region has an amino acid other than Lys, Leu or Met at position 409 and the second Fc-region has an amino acid other than Tyr, Asp, Glu, Phe, Lys, Gln, Arg, Ser or Thr at position 407.

46. The bispecific antibody of any one of claims 26 to 33, wherein the first Fc-region has an amino acid other than Lys, Leu or Met at position 409 and the second Fc-region has an Ala, Gly, His, Ile, Leu, Met, Asn, Val or Trp at position 407.

47. The bispecific antibody of any one of claims 26 to 33, wherein the first Fc-region has an amino acid other than Lys, Leu or Met at position 409 and the second Fc-region has a Gly, Leu, Met, Asn or Trp at position 407.

48. The bispecific antibody of any one of claims 26 to 33, wherein the first Fc-region has a Tyr at position 407 and an amino acid other than Lys, Leu or Met at position 409 and the second Fc-region has an amino acid other than Tyr, Asp, Glu, Phe, Lys, Gln, Arg, Ser or Thr at position 407 and a Lys at position 409.

49. The bispecific antibody of any one of claims 26 to 332, wherein the first Fc-region has a Tyr at position 407 and an amino acid other than Lys, Leu or Met at position 409 and the second Fc-region has an Ala, Gly, His, Ile, Leu, Met, Asn, Val or Trp at position 407 and a Lys at position 409.

50. The bispecific antibody of any one of claims 26 to 33, wherein the first Fc-region has a Tyr at position 407 and an amino acid other than Lys, Leu or Met at position 409 and the second Fc-region has a Gly, Leu, Met, Asn or Trp at position 407 and a Lys at position 409.

51. The bispecific antibody of any one of claims 26 to 33, wherein the first Fc-region has a Tyr at position 407 and an Arg at position 409 and the second Fc-region has an amino acid other than Tyr, Asp, Glu, Phe, Lys, Gln, Arg, Ser or Thr at position 407 and a Lys at position 409.

52. The bispecific antibody of any one of claims 26 to 33, wherein the first Fc-region has a Tyr at position 407 and an Arg at position 409 and the second Fc-region has an Ala, Gly, His, Ile, Leu, Met, Asn, Val or Trp at position 407 and a Lys at position 409.

53. The bispecific antibody of any one of claims 26 to 33, wherein the first Fc-region has a Tyr at position 407 and an Arg at position 409 and the second Fc-region has a Gly, Leu, Met, Asn or Trp at position 407 and a Lys at position 409.

54. The bispecific antibody of any one of claims 26 to 33, wherein the first Fc-region has an amino acid other than Lys, Leu or Met at position 409, and the second Fc-region has

(i) an amino acid other than Phe, Leu and Met at position 368,

(ii) a Trp at position 370, or

(iii) an amino acid other than Asp, Cys, Pro, Glu or Gln at position 399, or

(iv) an amino acid other than Lys, Arg, Ser, Thr, or Trp at position 366.

55. The bispecific antibody of any one of claims 26 to 33, wherein the first Fc-region has an Arg, Ala, His or Gly at position 409, and the second homodimeric protein has

(i) a Lys, Gln, Ala, Asp, Glu, Gly, His, Ile, Asn, Arg, Ser, Thr, Val, or Trp at position 368, or

(ii) a Trp at position 370,

(iii) an Ala, Gly, Ile, Leu, Met, Asn, Ser, Thr, Trp, Phe, His, Lys, Arg or Tyr at position 399, or

(iv) an Ala, Asp, Glu, His, Asn, Val, Gln, Phe, Gly, Ile, Leu, Met, or Tyr at position 366.

56. The bispecific antibody of any one of claims 26 to 33, wherein the first Fc-region has an Arg at position 409, and the second Fc-region has

(i) an Asp, Glu, Gly, Asn, Arg, Ser, Thr, Val, or Trp at position 368, or

(ii) a Trp at position 370, or

(iii) a Phe, His, Lys, Arg or Tyr at position 399, or

(iv) an Ala, Asp, Glu, His, Asn, Val, Gln at position 366.

57. The bispecific antibody of any one of claims 26 to 56, wherein said first and second CH3 regions, except for the specified mutations, comprise the sequence of SEQ ID NO:256 (IgG1m(a)).

58. The bispecific antibody of any one of claims 26 to 57, wherein neither said first nor said second Fc-region comprises a Cys-Pro-Ser-Cys sequence in the hinge region.

59. The bispecific antibody of any one of claims 26 to 57, wherein both of said first and said second Fc-region comprise a Cys-Pro-Pro-Cys sequence in the hinge region.

60. The bispecific antibody of any one of claims 26 to 58, wherein the first and second Fc-regions are human antibody Fc-regions.

61. The bispecific antibody of any one of claims 26 to 60, wherein said first and second Fc region, except for the specified mutations, comprise a sequence independently selected from the group consisting of SEQ ID NOS: 247, 248, 249, 250, 251, 252, 253, 254, and 255..

62. The bispecific antibody of any one of the preceding claims, wherein the first and second antigen-binding regions comprise human antibody VH sequences and, optionally, human antibody VL sequences.

63. The bispecific antibody of any one of the preceding claims, wherein the first and second antigen-binding regions are from heavy-chain antibodies.

64. The bispecific antibody of any one of claims 1 to 63, wherein the first and second antigen-binding regions comprise a first and second light chain.

65. The bispecific antibody of claim 64, wherein said first and second light chains are different.

66. The bispecific antibody of any one of claims 26 to 65, wherein the first and/or the second Fc-region comprises a mutation removing the acceptor site for Asn-linked glycosylation.

67. The bispecific antibody of any one of the preceding claims, which is conjugated to one or more other moieties, such as a drug, radioisotope, cytokine or cytotoxic moiety, or contains one or more acceptor group for the same.

68. The bispecific antibody of claim 67, which is conjugated to a cytokine selected from the group consisting of IL-2, IL-4, IL-6, IL-7, IL-10, IL-12, IL-13, IL-15, IL-18, IL-23, IL-24, IL-27, IL-28a, IL-28b, IL-29, KGF, IFNα, IFNβ, IFNγ, GM-CSF, CD40L, Flt3 ligand, stem cell factor, ancestim, and TNFα.

69. An *in vitro* method for generating a bispecific antibody, said method comprising the steps of:

a) providing a first antibody binding to an epitope on HER2 and comprising a first Fc region, said Fc region comprising a first CH3 region,
b) providing a second antibody binding to an epitope on human CD3 and comprising a second Fc region, said Fc region comprising a second CH3 region,
c) incubating said first antibody together with said second antibody under reducing conditions, and
d) obtaining said bispecific antibody,

wherein the sequences of said first and second CH3 regions are different and are such that the heterodimeric interaction between said first and second CH3 regions is stronger than each of the homodimeric interactions of said first and second CH3 regions.

70. The method of claim 69, wherein the first antibody blocks the binding to soluble human epidermal growth factor receptor 2 (HER2) of an antibody comprising:

a) a VH region comprising the sequence of SEQ ID NO:1 and a VL region comprising the sequence of SEQ ID NO:5 (**169**),
b) a VH region comprising the sequence of SEQ ID NO:63 and a VL region comprising the sequence of SEQ ID NO:67 (**153**),
c) a VH region comprising the sequence of SEQ ID NO:165 and a VL region comprising the sequence of SEQ ID NO:169 (**005**), and
d) a VH region comprising the sequence of SEQ ID NO:22 and a VL region comprising the sequence of SEQ ID NO:26 (**025**).

71. The method of any one of claims 69 and 70, wherein the first and second Fc-regions comprise the amino acid substitutions according to any one of claims 33 to 56.

72. A bispecific antibody obtainable by the method of any one of claims 69 to 71.

73. A recombinant eukaryotic or prokaryotic host cell which produces a bispecific antibody as defined in any one of claims 1 to 68.

74. A pharmaceutical composition comprising a bispecific antibody as defined in any one of claims 1 to 68 and a pharmaceutically acceptable carrier.

75. The bispecific antibody of any one of claims 1 to 68 for use as a medicament.

76. The bispecific antibody of any one of claims 1 to 68 for use in the treatment of cancer.

77. The bispecific antibody for the use of claim 76, wherein the cancer is selected from the group consisting of breast cancer, prostate cancer, non-small cell lung cancer, bladder cancer, ovarian cancer, gastric cancer, colorectal cancer, esophageal cancer, squamous cell carcinoma of the head and neck, cervical cancer, pancreatic cancer, testis cancer, malignant melanoma and soft-tissue cancer.

78. The bispecific antibody for the use of any one of claims 76 to 77, wherein the bispecific antibody is for the treatment of cancer in combination with one or more further therapeutic agent, such as a chemotherapeutic agent.

79. Use of the bispecific antibody of any one of the claims 1 to 68 for the manufacture of a medicament for treatment of cancer, optionally comprising the further features of claim 77 and/or 78.

80. A method for inhibiting growth and/or proliferation of one or more tumor cells expressing HER2, comprising administration, to an individual in need thereof, of a bispecific antibody according to any one of claims 1 to 68.

81. A method for treating cancer, comprising

a) selecting a subject suffering from a cancer comprising tumor cells co-expressing HER2, and
b) administering to the subject the bispecific antibody according to any one of claims 1-72.

82. The method of claim 81, wherein the cancer is selected from the group consisting of breast cancer, colorectal cancer, endometrial/cervical cancer, lung cancer, malignant melanoma, ovarian cancer, pancreatic cancer, prostate cancer, testis cancer, a soft-tissue tumor such as synovial sarcoma, and bladder cancer.

83. A method for producing a bispecific antibody of any one of claims 1 to 68, said method comprising the steps of
a) culturing a host cell of claim 73, and
b) purifying the bispecific antibody from the culture media.

Figure 1A

IgHV3-23-01 / IGHJ4-02 – VH alignment (Group 1)

```
IgHV1-23-01 EVQLLESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQAPGKGLEWVSAISGSGGSTYYADSVKG
TH1014-050  EVQLLESGGGLVQPGGSLRLSCAASGFTFSSYAMNWVRQAPGKGLEWVSAISGRGGTTYYADSVKG
VH1014-049  EVQLLESGGDLVQPGGSLRLSCAASGFTFSSYAMSWVRQAPGKGLEWVSAISGRGGTTYYADSVKG
VH1014-051  EVQLLESGGDLVQPGGSLRLSCAASGFTFSSYAMSWVRQAPGKGLEWVSAISGRGGTTYYADSVKG
VH1014-055  EVQLLESGGGLVQPGGSLRLSCAASGFTFSSYAMNWVRQAPGKGLEWVSAISGRGGTTYYADSVKG
Consensus   EVQLLESGGXLVQPGGSLRLSCAASGFTFSSYAMXWVRQAPGKGLEWVSAISGXGGXTYYADSVKG

IgHV1-23-01 RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAK------YFDYWGQGTLVTVSS
TH1014-050  RFTISRDNSKNTLYLQMSSLRAEDTAVYYCAKARANWDYFDYWGQGTLVTVSS
VH1014-049  RFTISRDNSKSTLCLQMNSLRAEDTAVYYCAKARANWDYFDYWGQGTLVTVSS
VH1014-051  RFTISRDNSKSTLCLQMNSLRAEDTAVYYCAKARANWDYFDYWGQGTLVTVSS
VH1014-055  RFTISRDNSKSTLCLQMNSLRAEDTAVYYCAKARANWDYFDYWGQGTLVTVSS
Consensus   RFTISRDNSKXTLXLQMXSLRAEDTAVYYCAKARANWDYFDYWGQGTLVTVSS
```

Figure 1B

IgHV1-69-04 / IGHJ6-02 – VH alignment (Group 1)

```
IgHV1-69-04 QVQLVQSGAEVKKPGSSVKVSCKASGGTFSSYAISWVRQAPGQGLEWMGRIIPILGIANYAQKFQG
TH1014-084  QVQLVQSGAEVKKPGSSVKVSCKASGGTFRTYAINWVRQAPGQGLEWMGRINTVLGIVNHAQKFQG
Consensus   QVQLVQSGAEVKKPGSSVKVSCKASGGTFXXYAISWVRQAPGQGLEWMGRIXXXLGIXNXAQKFQG

IgHV1-69-04 RVTITADKSTSTAYMELSSLRSEDTAVYYCAR--------GMDVWGQGTTVTVSS
TH1014-084  RVTITADKSTNTAYMELNSLRSEDTAVYYCAREKGVDYYYGIEVWGQGTTVTVSS
Consensus   RVTITADKSTXTAYMELXSLRSEDTAVYYCAREKGVDYYYGXXVWGQGTTVTVSS
```

Figure 1C

IgHV1-18-01 / IGHJ4-02 – VH alignment (Group 1)

```
IgHV1-18-01 QVQLVQSGAEVKKPGASVKVSCKASGYTFTSYGISWVRQAPGQGLEWMGWISAYNGNTNYAQKLQG
TH1014-169  QVQLVQSGAEVKKPGASVKVSCKASGYTFTNYGISWVRQAPGQGLEWMGWLSAYSGNTIYAQKLQG
VH1014-123  QVQLVQSGAEVKKPGASVKVSCKAAGYTFTNYGISWVRQAPGQALEWMGWITTYSSNTIYAQKLQG
VH1014-161  QVQLVQSGAEVKKPGASVKVSCKASGYTFTNYGISWVRQAPGQGLEWMGWLSAYSGNTIYAQKLQG
VH1014-124  QVQLVQSGAEVKKPGASVKVSCKAAGYTFTNYGISWVRQAPGQGLEWMGWIITYNGNTIYAQRFQD
Consensus   QVQLVQSGAEVKKPGASVKVSCKASGYTFTXYGISWVRQAPGQXLEWMGWIXXYXGNTXYAQXXQG

IgHV1-18-01 RVTMTTDTSTSTAYMELRSLRSDDTAVYYCAR--------YFDYWGQGTLVTVSS
TH1014-169  RVTMTTDTSTTTAYMELRSLRSDDTAVYYCARDRIVVRPDYFDYWGQGTLVTVSS
VH1014-123  RVTMTTDTSTSTAYMELRSLRSDDTAVYYCARDRVVVRPDYFDYWGQGTLVTVSS
VH1014-161  RVTMTTDTSTTTAYMELRSLRSDDTAVYYCARDRIVVRPDYFDYWGQGTLVTVSS
VH1014-124  RVTMTTDTSTSTAYMELRSLRSDDTAVYYCARDRIIVRPDYFDYWGQGTLVTVSS
Consensus   RVTMTTDTSTXTAYMELRSLRSDDTAVYYCARDRXXVRPDYFDYWGQGTLVTVSS
```

Figure 1D

IgHV4-34-01 / IGHJ4-02 – VH alignment (Group 2, No. 1)

```
IgHV4-34-01 QVQLQQWGAGLLKPSETLSLTCAVYGGSFSGYYWSWIRQPPGKGLEWIGEINHSGSTNYNPSLKSR
TH1014-025  QVQLQQWGAGLLKPSETLSLTCAVYGGSFSDYYWNWIRQPPGKGLEWIGEIHHSGSTNYNPSLKSR
VH1014-001  QVQLQQWGAGLLKPSETLSLTCAVYGGSFSGYYWNWIRQPPGKGLEWIGEINHSGSTNYNPSLKSR
VH1014-143  QVQLQQWGAGLLKPSETLSLTCAVYGGSFSGYYWNWIRQPPGKGLEWIGEIHHSGSANYNPSLMSR
VH1014-019  QVQLQQWGAGLLKPSETLSLTCAVYGGSFSDYYWNWIRQPPGKGLEWIGEIHHVGSTNYNPSLKSR
VH1014-021  QVQLQQWGAGLLKPSETLSLTCAVYGGSFSDYYWNWIRQPPGKGLEWIGEIHHSGSTNYNPSLKSR
VH1014-027  QVQLQQWGAGLLKPSETLSLTCAVYGGSFSDYFWNWIRQPPGKGLEWIGEIHHSGSTNYNPSLKSR
Consensus   QVQLQQWGAGLLKPSETLSLTCAVYGGSFSXYXWXWIRQPPGKGLEWIGEIXHXGSXNYNPSLXSR

IgHV4-34-01 VTISVDTSKNQFSLKLSSVTAADTAVYYCAR--------YFDYWGQGTLVTVSS
TH1014-025  VTISVDTSKNQFSLKLSSVTAADTAVYYCARGYYDSGVYYFDYWAQGTLVTVSS
VH1014-001  VTISVDTSKNQFSLKLSSVTAADTAVYYCARGNYGSGYYYFDLWGRGTQVTVSS
VH1014-143  VTISVDTSKNQFSLQLSSVTAADTAVYYCARGYYGSGYYYFDYWGQGTLVTVSS
VH1014-019  VTISVDTSKSQFSLKLSSVTAADTAVYYCARGYYDSGVYYFDYWAQGTLVTVSS
VH1014-021  VTISVDTSKNQFSLKLSSVTAADTAVYYCARGYYASGVYYFDYWGQGTLVTVSS
VH1014-027  VTISVDTSKNQFSLNLSSVTAADTAVYYCARGLIGSGYYYFDYWDQGTLVTVSS
Consensus   VTISVDTSKXQFSLXLSSVTAADTAVYYCARGXXXSGXYYFDXWXXGTXVTVSS
```

Figure 1E

IgHV4-34-01 / IGHJ4-02 – VH alignment (Group 2, No. 2)

```
IgHV4-34-01 QVQLQQWGAGLLKPSETLSLTCAVYGGSFSGYYWSWIRQPPGKGLEWIGEINHSGSTNYNPSLKSR
TH1014-091  QVQLQQWGAGLLKPSETLSLTCAVSGGSFSGYYWTWIRQPPGKGLEWIGEIYHSGDTNYNPSLKSR
VH1014-032  QVQLQQWGAGLLKPSETLSLTCAVYGGSFSGYYWSWIRQPPGKGLEWIGEINHSGDTNYNPSLTSR
VH1014-035  QVQLQQWGAGLLKPSETLSLTCAIYGGSFSGYYWSWIRQPPGKGLEWIGEINHSGDTNYNPSLTSR
VH1014-036  QVQLQQWGAGLLKPSETLSLTCAVYGGSFSDYYWSWIRQPPGKGLEWIGEINHSGSTNYNPSLKSR
VH1014-054  QVQLQQWGAGLLKPSETLSLTCAVYGGSFSGYYWSWIRQPPGKGLEWIGEIHHSGSTNYNPSLKSR
VH1014-094  QVQLQQWGAGLLKPSETLSLTCAVSGGSFSGYYWTWIRQPPGKGLEWIGEIYHSGDTNYNPSLKSR
Consensus   QVQLQQWGAGLLKPSETLSLTCAXXGGSFSXYYWXWIRQPPGKGLEWIGEIXHSGXTNYNPSLXSR

IgHV4-34-01 VTISVDTSKNQFSLKLSSVTAADTAVYYCAR--------YFDYWGQGTLVTVSS
TH1014-091  VTISVDTSKNQFSLKLYSVTAADTAVYYCARLYFGSGIYYLDYWGQGTLVTVSS
VH1014-032  VTISVDTSKNQFSLKLSSVTAADTAVYYCARLFYGSGIYYFDYWGQGTLVTVSS
VH1014-035  VTISVDTSKNQFSLKLSSVTAADTAVYYCARLFYGSGIYYFDYWGQGTLVTVSS
VH1014-036  VTISVDTSKNQFSLKLSSVTAADTAVYYCARLYYGSGTYYFDYWGQGTLVTVSS
VH1014-054  VTISVDTSKNQFSLKLSSVTAADTAVYYCARLWYGSGSYYFDYWGQGTLVTVSS
VH1014-094  VTISVDTSKNQFSLKLYSVTAADTAVYYCARLYFGSGIYYLDYWGQGTLVTVSS
Consensus   VTISVDTSKNQFSLKLXSVTAADTAVYYCARLXXGSGXYYXDYWGQGTLVTVSS
```

Figure 1F

IgHV3-30-3-01 / IGHJ4-02 – VH alignment (Group 2)

```
IgHV1-30-…  QVQLVESGGGVVQPGRSLRLSCAASGFTFSSYAMHWVRQAPGKGLEWVAVISYDGSNKYYADSVKGR
TH1014-129  QVQLVESGGGVVQPGRSLRLSCAASGFTFSTFAIHWVRQAPGKGLEWVAVISYDGGHKFYADSVKGR
Consensus   QVQLVESGGGVVQPGRSLRLSCAASGFTFSXXAXHWVRQAPGKGLEWVAVISYDGXXKXYADSVKGR

IgHV3-30-…  FTISRDNSKNTLYLQMNSLRAEDTAVYYCAR------YFDYWGQGTLVTVSS
TH1014-129  FTISRDNSKNTLYLQMNSLRAEDTAMYYCARGLGVWGAFDYWGQGTLVTVSS
Consensus   FTISRDNSKNTLYLQMNSLRAEDTAXYYCARGLGVWGXFDYWGQGTLVTVSS
```

Figure 1G

IgHV3-23-1 / IGHJ4-02 – VH alignment (Group 3a)

```
IgHV3-23-1   EVQLLESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQAPGKGLEWVSAISGSGGSTYYADSVKG
TH1014-098   EVQLLESGGGLVQPGGSLRLSCAASGFTFSNYGMSWVRQAPGKGLEWVSAISGSAYSTYYADSVKG
VH1014-105   EVQLLESGGGLVQPGGSLRLSCAASGFTFSNYGMSWVRQAPGKGLEWVSAISGSAYSTYYADSVKG
VH1014-100   EVQLLESGGGLVQPGGSLRLSCAASGFTFNNYGMNWVRQAPGKGLEWVSAISGTGYSTYYADSVKG
VH1014-125   EVQLLESGGGLVQPGGSLRLSCAASGFTFTDYAMNWVRQAPGKGLEWVSTISGSGYATYYADSVKG
VH1014-162   EVQLWESGGGSVQPGGSLRLSCAASGFTFSSYGMSWVRQAPGKGLEWVSGISGSGYSTYYADSVKG
Consensus    EVQLXESGGGXVQPGGSLRLSCAASGFTFXXYXMXWVRQAPGKGLEWVSXISGXXXXTYYADSVKG

IgHV3-23-1   RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAK----------YFDYWGQGTLVTVSS
TH1014-098   RFTISRDNSKNTLWLQMNSLRAEDTAVYYCAKAHYHGSGSYYTLFDYWGQGTLVTVSS
VH1014-105   RFTISRDNSKNTLWLQMNSLRAEDTAVYYCAKAHYHGSGSYYTLFDYWGQGTLVTVSS
VH1014-100   RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKAHYFGSGSYYTLFDYWGQGTLVTVSS
VH1014-125   RFTISRDNSKTTLYLQMNSLRAEDTAVYYCAKGHTLGSGSYYTLFDYWGQGTLVTVSS
VH1014-162   RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKGYYHGSGSYYTSFDYWGQGTLVTVSS
Consensus    RFTISRDNSKXTLXLQMNSLRAEDTAVYYCAKXXXXGSGSYYTXFDYWGQGTLVTVSS
```

Figure 1H

IgHV5-51-01 / IGHJ2-01 – VH alignment (Group 3a, No. 1)

```
IgHV5-51-01  EVQLVQSGAEVKKPGESLKISCKGSGYSFTSYWIGWVRQMPGKGLEWMGIIYPGDSDTRYSPSFQG
TH1014-127   EVQLVQSGAEVKKPGESLTISCKGSGYSFSIYWIGWVRQMPGKGLEWMGIIFPGDSDIRYSPSFQG
Consensus    EVQLVQSGAEVKKPGESLXISCKGSGYSFXXYWIGWVRQMPGKGLEWMGIIXPGDSDXRYSPSFQG

IgHV5-51-01  QVTISADKSISTAYLQWSSLKASDTAMYYCAR----------YFDLWGRGTLVTVSS
TH1014-127   QVTISADKSISTAYLQWSSLKASDTAMYYCARQPGDWSPRHWYFDLWGRGTLVTVSS
Consensus    QVTISADKSISTAYLQWSSLKASDTAMYYCARQPGDWSPRHWYFDLWGRGTLVTVSS
```

Figure 1I

IgHV5-51-01-01 / IGHJ5-02 – VH alignment (Group 3a, No. 2)

```
IgHV5-51-01  EVQLVQSGAEVKKPGESLKISCKGSGYSFTSYWIGWVRQMPGKGLEWMGIIYPGDSDTRYSPSFQG
TH1014-159   EVQLVQSGAEVKKPGESLKISCKGSGYNFTSYWIGWVRQMPGKGLEWMGIIYPGDSDTRYSPSFQG
Consensus    EVQLVQSGAEVKKPGESLKISCKGSGYXFTSYWIGWVRQMPGKGLEWMGIIYPGDSDTRYSPSFQG

IgHV5-51-01  QVTISADKSISTAYLQWSSLKASDTAMYYCAR----------NWFDPWGQGTLVTVSS
TH1014-159   QVTISADKSISTAYLQWSSLKASDTAMYYCARWGTYYDILTGYFNWFDPWGQGTLVTVSS
Consensus    QVTISADKSISTAYLQWSSLKASDTAMYYCARWGTYYDILTGYFNWFDPWGQGTLVTVSS
```

Figure 1J

IgHV1-18-01 / IGHJ6-02 – VH alignment (Group 3b)

```
IgHV1-18-01 QVQLVQSGAEVKKPGASVKVSCKASGYTFTSYGISWVRQAPGQGLEWMGWISAYNGNTNYAQKLQG
TH1014-132  QVQLVQSGAEVKKPGASVKVSCKASGYTFTSYGISWVRQAPGQGLEWMGWISAYNGNSNYVQKFQG
Consensus   QVQLVQSGAEVKKPGASVKVSCKASGYTFTSYGISWVRQAPGQGLEWMGWISAYNGNXNYXQKXQG

IgHV1-18-01 RVTMTTDTSTSTAYMELRSLRSDDTAVYYCAR----------GMDVWGQGTTVTVSS
TH1014-132  RVTMTTDTTTSTAYMELRSLTSDDTAVYYCAREYSYDSGTYFYYGMDVWGQGTTVTVSS
Consensus   RVTMTTDTSTSTAYMELRSLRSDDTAVYYCAREYSYDSGTYFYYGMDVWGQGTTVTVSS
```

Figure 1K

IgHV3-30-3-01 / IGHJ4-02 – VH alignment (Group 3b)

```
IgHV3-30…   QVQLVESGGGVVQPGRSLRLSCAASGFTFSSYAMHWVRQAPGKGLEWVAVISYDGSNKYYADSVKG
TH1014-153  QVQLVESGGGVVQPGRSLRLSCAASGFTFSDYVIHWVRQAPGKGLEWVTVISYDGSNKYYADSVKG
VH1014-033  QVQLVESGGGVVQTGRSLRLSCAASGFTFSSHAMHWVRQAPGKGLEWVAAISYDGSNKYYADSVKG
VH1014-160  QVQLVESGGGVVQPGRSLRLSCAASGFTFSSHAMHWVRQAPGKGLEWVAAISYDGSNKYYADSVKG
VH1014-166  QVQLVESGGGVVQPGRSLRLSCAASGFTFSSYAMHWVRQAPGKGLEWVAVISYDGSNEYYADSVKG
VH1014-152  QVQVVESGGGVVQPGRSLRLSCAASGFTFSSYAMHWVRQAPGKGLEWVAVISYDGSYKYYADSVKG
VH1014-167  QVQLVESGGGVVQPGRSLRLSCAASGFTFSSYAIHWVRQAPGKGLEWVAVISYDGSNKYYADSVKG
Consensus   QVQLVESGGGVVQXGRSLRLSCAASGFTFSXXXXHWVRQAPGKGLEWVXXISYDGSXXYYADSVKG

IgHV3-30…   RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAR--------YFDYWGQGTLVTVSS
TH1014-153  RFTISRDNSKNTLYLQMNSLSAEDTAMYYCARGGIGTTGVFDYWGQGTLVTVSS
VH1014-033  RFTISRDNSKNTLYLQMNSLRAEDTAVYYCARGDYISSSGVFDYWGQGTLVTVSS
VH1014-160  RFTISRDNSKNTMYLQMNSLRAEDTAMCYCARGSITGSTGVFDYWGQGTLVTVSS
VH1014-166  RFTISRDNSKNTLYLQMNSLRAEDTAVYYCARGSIIGSTGVFDYWGQGTLVTVSS
VH1014-152  RFTISRDNSKNTLYLQMNSLRAEDTAVYYCARGSITGSTGVFDYWGQGTLVTVSS
VH1014-167  RFTISRDNSKNTLYLQMNSLRAEDTAVYYCARGSITGSTGVFDYWGQGTLVTVSS
Consensus   RFTISRDNSKNTXYLQMNSLXAEDTAXXYCARGXXXXXGXFDYWGQGTLVTVSS
```

Figure 1L

IgHV5-51-1 / IGHJ6-02 – VH alignment

```
IgHV5-51-1   EVQLVQSGAEVKKPGESLKISCKGSGYSFTSYWIGWVRQMPGKGLEWMGIIYPGDSDTRYSPSFQ
TH1014-005   EVQLVQSGAEVKKPGESLKISCKASGYSFHFYWIGWVRQMPGKGLEWMGSIYPGDSDTRYRPSFQ
TH1014-060   EVQLVQSGAEVKKPGESLKISCKGSGYRFTSYWIGWVRQMPGKGLEWMGSIYPGDSYTRNSPSFQ
TH1014-106   EVQLVQSGAEVKKPGESLKISCKGSGYSFTRYWIGWVRQMPGKGLEWMGIIYPGDSDTRYSPSFQ
VH1014-041   EVQLVQSGAEVKKPGESLKISCKGSGYSFTSYWIGWVRQMPGKGLEWMGSIYPGDSHTRYRPSFQ
VH1014-150   EVQLVQSGAEVKKPGESLKISCKGSGYSFTSYWIGWVRQMPGKGLEWMGSIYPGDSHTRYRPSFQ
VH1014-067   EVQLVQSGAEVKKPGESLKISCKGSGYSFTSYWIGWVRQMPGKGLEWMGIIYPGDSDTRYSPSFQ
VH1014-072   EVQLVQSGAEVKKPGESLKISCKGSGYSFTSYWIGWVRQMPGKGLEWMGIIYPGDSDTRYSPSFQ
VH1014-163   EVQLVQSGAEVKKPGESLKISCQGSGYRFISYWIGWVRQMPGKGLEWMGRIYPGDSDTRYSPSFQ
VH1014-093   EVQLVQSGAEVKKPGESLKISCKGSGYSFTSYWIGWVRQMPGKGLEWMGRIYPGDSDTRYSPSFQ
VH1014-044   EVQLVQSGAEVKKPGESLKISCKGSGYRFSSYWIGWVRQMPGKGLEWMGSIFPGDSDTRYSPSFQ
Consensus    EVQLVQSGAEVKKPGESLKISCXXSGYXFXXYWIGWVRQMPGKGLEWMGXIXPGDSXTRXXPSFQ

IgHV5-51-1   GQVTISADKSISTAYLQWSSLKASDTAMYYCAR----------GMDVWGQGTTVTVSS
TH1014-005   GQVTISADKSISTAYLQWTSLKASDTAIYYCARQRGD--YYYFYGMDVWGQGTTVTVSS
TH1014-060   GQVTISADKSIATAYLQWNSLKASDTAMYYCARHAGD--FYYFDGLDVWGQGTTVTVSS
TH1014-106   GQVTISADKSISTAYLQWSSLKASDTAMYYCARLTGDRGFDYYSGMDVWGQGTTVTVSS
VH1014-041   GQVTISADKSISTAYLQWSSLKASDTAMYYCARQKGD--FYYFFGLDVWGQGTAITVSS
VH1014-150   GQVTISADKSISTAYLQWSSLKASDTAMYYCARQAGD--YYYYNGMDVWGQGTTVTVSS
VH1014-067   GQVTISVDKSISTAYLQWSSLKASDTAMYYCARQKGD--YYYHYGLDVWGQGTTVTVSS
VH1014-072   GQVTISADKSISTAYLQWSSLKASDTAMYYCARQKGD--YYYFNGLDVWGQGTTVTVSS
VH1014-163   GQVTISVDKSISTAYLQWSSLKASDTAMYYCARQRGD--YYYFNGLDVWGQGTTVTVSS
VH1014-093   GQVTISADKSITTAYLQWSSLRASDTAMYYCARQRGD--YYYFFGLDIWGQGTTVTVSL
VH1014-044   GQVTISADKSITTAYLQWSSLKASDTAMYYCARQAGD--YYYYNGMDVWGQGTTVTVSS
Consensus    GQVTISXDKSIXTAYLQWXSLXASDTAXYYCARXXXXXXXXXYXXGXDXWGQGTXXTVSX
```

Figure 1M

IgHV3-23-1 / IGHJ4-02 – VH alignment

```
IgHV3-23-1   EVQLLESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQAPGKGLEWVSAISGSGGSTYYADSVKG
TH1014-006   EVQLLESGGGLVQPGGSLRLSCAASGFTFSNYALIWVRQAPGKGLEWVSIIRGGAGSTYYADSVKG
Consensus    EVQLLESGGGLVQPGGSLRLSCAASGFTFSXYAXXWVRQAPGKGLEWVSXIXGXXGSTYYADSVKG

IgHV3-23-1   RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAK------YFDYWGQGTLVTVSS
TH1014-006   RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKARIWGPLFDYWGQGTLVTVSS
Consensus    RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKARIWGPXFDYWGQGTLVTVSS
```

Figure 1N

IgHV1-18-1 / IGHJ4-02 – VH alignment

```
IgHV1-18-1   QVQLVQSGAEVKKPGASVKVSCKASGYTFTSYGISWVRQAPGQGLEWMGWISAYNGNTNYAQKLQG
TH1014-059   QVQLVQSGAEVKKPGASVRVPCKASGYTFTRYGISWVRQAPGQGLEWMGWISAYNGKTYYAQKLQG
Consensus    QVQLVQSGAEVKKPGASVXVXCKASGYTFTXYGISWVRQAPGQGLEWMGWISAYNGXTXYAQKLQG

IgHV1-18-1   RVTMTTDTSTSTAYMELRSLRSDDTAVYYCAR---------YFDYWGQGTLVTVSS
TH1014-059   RVTMTTDTSTSTAYMELRSLRSDDTAVYYCARSPLLWFEELYFDYWGQGTLVTVSS
Consensus    RVTMTTDTSTSTAYMELRSLRSDDTAVYYCARSPLLWFEELYFDYWGQGTLVTVSS
```

Figure 1O

IgHV1-69-4 / IGHJ4-02 – VH alignment

```
IgHV1-69-4   QVQLVQSGAEVKKPGSSVKVSCKASGGTFSSYAISWVRQAPGQGLEWMGRIIPILGIANYAQKFQG
TH1014-111   QVQLVQSGAEVKKPGSSVKVSCKASGGTFSSYGISWVRQAPGPGLEWMGRIIPILGIANYAQKFQG
Consensus    QVQLVQSGAEVKKPGSSVKVSCKASGGTFSSYXISWVRQAPGXGLEWMGRIIPILGIANYAQKFQG

IgHV1-69-4   RVTITADKSTSTAYMELSSLRSEDTAVYYCAR------YFDYWGQGTLVTVSS
TH1014-111   RVTITADKSTNTAYMELSSLRSEDTAVYYCARDQEYSSNWYYWGQGTLVTVSS
Consensus    RVTITADKSTXTAYMELSSLRSEDTAVYYCARDQEYSSXXXYWGQGTLVTVSS
```

Figure 2A

IgKV1-12-01 / IGKJ5-01 – VL alignment (Group 1)

```
IgKV1-12-01 DIQMTQSPSSVSASVGDRVTITCRASQGISSWLAWYQQKPGKAPKLLIYAASSLQSGVPSRFSGSG
VL1014-050  DIQMTQSPSSVSASVGDRVTITCRASQGISSWLAWYQHKPGKAPKLLIYAASILQSGVPSRFSGSG
VL1014-084  DIQMTQSPSSVSASVGDRVTITCRASQGISSWLAWYQHKPGKAPKLLIYVASTLQSGVPSRFSGSG
VL1014-049  DIQMTQSPSSVSASVGDRVTITCRASQGISSWLAWYQHKPGKAPKLLIYAASILQSGVPSRFSGSG
VL1014-051  DIQMTQSPSSVSASVGDRVTITCRASQGISSWLAWYQHKPGKAPKLLIYAASILQSGVPSRFSGSG
VL1014-055  DIQMTQSPSSVSASVGDRVTITCRASQGISSWLAWYQHKPGKAPKLLIYAASILQSGVPSRFSGSG
Consensus   DIQMTQSPSSVSASVGDRVTITCRASQGISSWLAWYQXKPGKAPKLLIYXASXLQSGVPSRFSGSG

IgKV1-12-01 SGTDFTLTISSLQPEDFATYYCQQANSFPITFGQGTRLEIK
VL1014-050  SGTDFTLTISSLQPEDFATYYCQQANSFPITFGQGTRLEIK
VL1014-084  SGTDFTLTISSLQPEDFATYYCQQANSFPLTFGGGTKVEIK
VL1014-049  SGTDFTLTISSLRPEDFATYYCQQANSFPITFGQGTRLEIK
VL1014-051  SGTDFTLTISSLRPEDFATYYCQQANSFPITFGQGTRLEIK
VL1014-055  SGTDFTLTISSLRPEDFATYYCQQANSFPITFGQGTRLEIK
Consensus   SGTDFTLTISSLXPEDFATYYCQQANSFPXTFGXGTRXEIK
```

Figure 2B

IgKV3-11-01 / IGKJ1-01 – VL alignment (Group 1)

```
IgKV3-11-01 EIVLTQSPATLSLSPGERATLSCRASQSVSSYLAWYQQKPGQAPRLLIYDASNRATGIPARFSGSG
VL1014-169  EIVLTQSPATLSLSPGERATLSCRASQSVSSYLAWYQQKPGQAPRLLIYDASNRATGIPARFSGSG
VL1014-124  EIVLTQSPATLSLSPGERATLSCRASQSVSSYLAWYQQKPGQAPRLLIYDASNRATGIPARFSGSG
VL1014-161  EIVLTQSPATLSLSPGERATLSCRASQSVSSYLAWYQQKPGQAPRLLIYDASNRATGIPARFSGSG
VL1014-123  EIVLTQSPATLSLSPGERATLSCRASQSVSSYLAWYQQKPGQAPRLLIYDTSNRATGIPARFSGSG
Consensus   EIVLTQSPATLSLSPGERATLSCRASQSVSSYLAWYQQKPGQAPRLLIYDXSNRATGIPARFSGSG

IgKV3-11-01 SGTDFTLTISSLEPEDFAVYYCQQRSNWPWTFGQGTKVEIK
VL1014-169  SGTDFTLTISSLEPEDFAVYYCQQRSNWPRTFGQGTKVEIK
VL1014-124  SGTDFTLTISSLEPEDFAVYYCQQRSNWPRTFGQGTKVEIK
VL1014-161  SGTDFTLTISSLEPEDFAVYYCQQRSNWPRTFGQGTKVEIK
VL1014-123  SGTDFTLTISSLEPEDFAVYYCQQRSHWPRTFGQGTKVEIK
Consensus   SGTDFTLTISSLEPEDFAVYYCQQRSXWPRTFGQGTKVEIK
```

Figure 2C

IgKV1D-16-01 / IGKJ5-01– VL alignment (Group 2, No. 1)

```
IgKV1D-16 …  DIQMTQSPSSLSASVGDRVTITCRASQGISSWLAWYQQKPEKAPKSLIYAASSLQSGVPSRFSGSG
VL1014-025   DIQMTQSPSSLSASVGDRVTITCRASQGISRWLAWYQQKPEKAPKSLIYAASSLRSGVPSRFSGSG
VL1014-001   DIQMTQSPSSLSASVGDRVTITCRASQGISSWLAWYQQKPEKAPKSLIFAASSLQSGVPSRFSGSG
VL1014-019   DIQMTQSPSSLSASVGDRVTITCRASQGISRWLAWYQQKPEKAPKSLIYAASSLRSGVPSRFSGSG
VL1014-143   DIQMTQSPSSLSASVGDRVTITCRASQGISSWLAWYQQKPEKAPKSLIYAASRLQSGVPSRFSGSG
VL1014-021   DIQMTQSPSSLSASVGDRVTITCRASQGISSWLAWYQQKPEKAPKSLIYAASSLQSGVPSRFSGSG
VL1014-027   DIQMTQSPSSLSASVGDRVTITCRASQGISSWLAWYQQKPEKAPKSLIYAASSLQSGVPSRFSGSG
Consensus    DIQMTQSPSSLSASVGDRVTITCRASQGISXWLAWYQQKPEKAPKSLIXAASXLXSGVPSRFSGSG

IgKV1D-16 …  SGTDFTLTISSLQPEDFATYYCQQYNSYPITFGQGTRLEIK
VL1014-025   SGTDFTLTISSLQPEDFATYYCQQYNSYPITFGQGTRLEIK
VL1014-001   SGTDFTLTISSLQPEDFATYYCQQYISFPITFGQGTRLEIK
VL1014-019   SGTDFTLTISSLQPEDFATYYCQQYNSYPITFGQGTRLEIK
VL1014-143   SGTDFTLTISSLQPEDFATYYCQQYNSYPITFGQGTRLEIK
VL1014-021   SGTDFTLTISSLQPEDFATYYCQQYNSYPITFGQGTRLEIK
VL1014-027   SGTDFTLTISSLQPEDFATYYCQQYNSYPITFGQGTRLEIK
Consensus    SGTDFTLTISSLQPEDFATYYCQQYNSXPITFGQGTRLEIK
```

Figure 2D

IgKV1D-16-01 / IGKJ1-01– VL alignment (Group 2, No. 2)

```
IgKV1D-16 …  DIQMTQSPSSLSASVGDRVTITCRASQGISSWLAWYQQKPEKAPKSLIYAASSLQSGVPSRFSGSG
VL1014-091   DIQMTQSPSSLSASVGDRVTITCRASQGISSWLVWYQQKPEKAPKSLIYAASSLQSGVPSRFSGSG
VL1014-032   DIQMTQSPSSLSASVGDRVTITCRASQGISSWLAWYQQKPEKAPKSLIYATFRLQSGVPSRFSGSG
VL1014-035   DIQMTQSPSSLSASVGDRVTITCRASQGISSWLAWYQQKPEKAPKSLIYATFRLQSGVPSRFSGSG
VL1014-036   DIQMTQSPSSLSASVGDRVTITCRASQGISSWLTWYQQKPEKAPKSLIYAASRLQSGVPSRFSGSG
VL1014-054   DIQMTQSPSSLSASVGDRVTITCRASQGISSWLAWYQQKPEKAPKSLIYAASSLQSGVPSRFSGSG
VL1014-094   DIQMTQSPSSLSASVGDRVTITCRASQGISSWLVWYQQKPEKAPKSLIYAASSLQSGVPSRFSGSG
Consensus    DIQMTQSPSSLSASVGDRVTITCRASQGISSWLXWYQQKPEKAPKSLIYAXXXLQSGVPSRFSGSG

IgKV1D-16 …  SGTDFTLTISSLQPEDFATYYCQQYNSYPWTFGQGTKVEIK
VL1014-091   SGTDFTLTISSLQPEDFATYYCQQYNSFPPTFGQGTKVEIK
VL1014-032   SGTDFTLTISSLQPEDFATYYCQQYNSFPPTFGQGTKVEIK
VL1014-035   SGTDFTLTISSLQPEDFATYYCQQYNSFPPTFGQGTKVEIK
VL1014-036   SGTDFTLTISSLQPEDFATYYCQQYNSFPPTFGQGTKVEIK
VL1014-054   SGTDFTLTISSLQPEDFATYYCQQYNSFPPTFGGGTKVEIK
VL1014-094   SGTDFTLTISSLQPEDFATYYCQQYNSFPPTFGQGTKVEIK
Consensus    SGTDFTLTISSLQPEDFATYYCQQYNSFPPTFGXGTKVEIK
```

Figure 2E

IgKV1D-16-01 / IGKJ2-01 – VL alignment (Group 3a)

```
IgKV1D-16 … DIQMTQSPSSLSASVGDRVTITCRASQGISSWLAWYQQKPEKAPKSLIYAASSLQSGVPSRFSGSG
VL1014-098  DIQMTQSPSSLSASVGDRVTITCRASQGISSWLAWYQQKPEKAPKSLIYAASSLQSGVPSRFSGSG
VL1014-100  DIQMTQSPSSLSASVGDRVTITCRASQGISSWLAWYQQKPEKAPKSLIYAASSLQSGVPSRFSGSG
VL1014-105  DIQMTQSPSSLSASVGDRVTITCRASQGISSWLAWYQQKPEKAPKSLIYAASSLQSGVPSRFSGSG
VL1014-125  DIQMTQSPSSLSASVGDRVTITCRASQGINSWLAWYQQKPEKAPKSLIYAASSLQSGVPSRFSGSG
VL1014-162  DIQMTQSPSSLSASVGDRVTITCRASQGISSWLAWYQQKPEKAPKSLIYAASSLQSGVPSRFSGSG
Consensus   DIQMTQSPSSLSASVGDRVTITCRASQGIXSWLAWYQQKPEKAPKSLIYAASSLQSGVPSRFSGSG

IgKV1D-16 … SGTDFTLTISSLQPEDFATYYCQQYNSYPYTFGQGTKLEIK
VL1014-098  SGTDFTLTISSLQPEDFATYYCQQYNSYPYTFGQGTKLEIK
VL1014-100  SGTDFTLTISSLQPEDFATYYCQQYNSYPYTFGQGTKLEIK
VL1014-105  SGTDFTLTISSLQPEDFATYYCQQYNSYPYTFGQGTKLEIK
VL1014-125  SGTDFTLTISSLQPEDFATYYCQQYNSYPYTFGQGTKLEIK
VL1014-162  SGTDFTLTISSLQPEDFATYYCQQYNSYPLTFGGGTKVEIK
Consensus   SGTDFTLTISSLQPEDFATYYCQQYNSYPXTFGXGTKXEIK
```

Figure 2F

IgKV1D-16-01 / IGKJ5-01 – VL alignment (Group 3b)

```
IgKV1D-16 … DIQMTQSPSSLSASVGDRVTITCRASQGISSWLAWYQQKPEKAPKSLIYAASSLQSGVPSRFSGSG
VL1014-153  DIQMTQSPSSLSASVGDRVTITCRASQGISSWLAWYQQKPEKAPKSLIYDASSLQSGVPSRFSGSG
VL1014-152  DIQMTQSPSSLSASVGDRVTITCRASQGINSWLAWYQQKPEKAPKSLIYDASSLQSGVPSRFSGSG
VL1014-166  DIQMTQSPSSLSASVGDRVTITCRASQGISNWLAWYQQKPEKAPKSLIYDASSLQSGVPSRFSGSG
VL1014-167  DIQMTQSPSSLSASVGDRVTITCRASQGISNWLAWYQQKPEKAPKSLIYDASSLQSGVPSRFSGSG
VL1014-160  DIQMTQSPSSLSASVGDRVTITCRASQDISSWLAWYQQKPEKAPKSLIYAASSLQSGVPSRFSGSG
VL1014-033  DIQMTQSPSSLSASVGDRVTITCRASQGISSWLAWYQQKPEKAPKSLIYAASSLQSGVPSRFSGSG
Consensus   DIQMTQSPSSLSASVGDRVTITCRASQGIXXWLAWYQQKPEKAPKSLIYXASSLQSGVPSRFSGSG

IgKV1D-16 … SGTDFTLTISSLQPEDFATYYCQQYNSYPITFGQGTRLEIK
VL1014-153  YGTDFSLTISSLQPEDFAIYYCQQYKSYPITFGQGTRLEIK
VL1014-152  SGTDFTLTISSLQPENFATYYCQQYNSYPITFGQGTRLEIK
VL1014-166  SGTDFTLTISSLQPEDFATYYCQQYNSYPITFGQGTRLEIK
VL1014-167  SGTDFTLTISSLQPEDFATYYCQQYNSYPITFGQGTRLEIK
VL1014-160  SGTDFTLTISSLQPEDFATYYCQQYNSYPITFGQGTRLEIK
VL1014-033  SGTDFTLTISSLQPEDFATYYCQQYNSYPITFGQGTRLEIK
Consensus   XGTDFXLTISSLQPEXFAXYYCQQYXSYPITFGQGTRLEIK
```

Figure 2G

IgKV3-20-01 / IGKJ4-01 – VL alignment

```
IgKV3-20-01 EIVLTQSPGTLSLSPGERATLSCRASQSVSSSYLAWYQQKPGQAPRLLIYGASSRATGIPDRFSGS
VL1014-005  EIVLTQSPGTLSLSPGERATLSCRASQSVSSSYLAWYQQKPGQVPRLLIYGASSRATGIPDRFSGS
VL1014-059  EIVLTQSPGTLSLSPGERATLSCRASQSVSSTYLAWYQQKPGQAPRLLIYGASSRATGIPDRFSGS
VL1014-060  EIVLTQSPGTLSLSPGERATLSCRASQSVSSSYLAWYQQKPGQAPRLLIYGASSRATGIPDRFSGS
VL1014-106  EIVLTQSPGTLSLSPGERATLSCRASQSVSSSYLAWYQQKPGQAPRLLIYGASSRATGIPDRFSGS
VL1014-111  EIVLTQSPGTLSLSPGERATLSCRASQSVRSSYLAWYQQKPGQAPRLLIYGASSRATGIPDRFSGS
VL1014-041  EIVLTQSPGTLSLSPGERATLSCRASQSVSSSYLAWYQQKPGQAPRLLIYGASSRATGIPDRFSGS
VL1014-150  EIVLTQSPGTLSLSPGERATLSCRASQSVSSSYLIWYQQKPGQAPRLLIYGASSRATGIPDRFSGS
VL1014-067  EIVLTQSPGTLSLSPGERATLSCRASQSVSSSYLAWYQQKPGQAPRLLIYGASSRATGIPDRFSGS
VL1014-072  EIVLTQSPGTLSLSPGERATLSCRASQSVSSSYLAWYQQKPGQAPRLLIYGASSRATGIPDRFSGS
VL1014-163  EIVLTQSPGTLSLSPGERATLSCRASQSVSSSYLAWYQQKPGQAPRLLIYGASSRATGIPDRFSGS
VL1014-093  EIVLTQSPGTLSLSPGERATLSCRASQSVSSSYLAWYQQKPGQAPRLLIYGASSRATGIPDRFSGS
VL1014-044  EIVLTQSPGTLSLSPGERATLSCRASQSVSSSYLAWYQQKPGQAPRLLIYGASSRATGIPDRFSGS
Consensus   EIVLTQSPGTLSLSPGERATLSCRASQSVXSXYLXWYQQKPGQXPRLLIYGASSRATGIPDRFSGS

IgKV3-20-01 GSGTDFTLTISRLEPEDFAVYYCQQYGSSP-LTFGGGTKVEIK
VL1014-005  GSGTDFTLTISRLEPEDFAVYYCQQYGSS--LTFGGGTKVEIK
VL1014-059  GSGTDFTLTISRLEPEDFAVYYCQQYGTS-LFTFGPGTKVDIK
VL1014-060  GSGTDFTLTISRLEPEDFAVYYCQQYGSSPPITFGQGTRLEIK
VL1014-106  GSGTDFTLTISRLEPEDFAVYYCQQYGSS--FTFGPGTKVDIK
VL1014-111  GSGTDFTLTISRLEPEDFAVYYCQLYGSSP--TFGPGTKVDIK
VL1014-041  GSGTDFTLTISRLEPEDFAVYYCQQYGSS--LTFGGGTKVEIK
VL1014-150  GSGTDFTLTISRLEPEDFAVYYCQQYGSS--LTFGGGTKVEIK
VL1014-067  GSGTDFTLTISRLEPEDFAVYYCQQYGSSPRLTFGGGTKVEIK
VL1014-072  GSGTDFTLTISRLEPEDFAVYYCQQYGSSPRLTFGGGTKVEIK
VL1014-163  GSGTDFTLTISRLEPEDFAVYYCQQYGSS--LTFGGGTKVEIK
VL1014-093  GSGTDFTLTISRLEPEDFAVYYCQQYGSS--LTFGGGTKVEIK
VL1014-044  GSGTDFTLTISRLEPEDFAVYYCQQYGSS--LTFGGGTKVEIK
Consensus   GSGTDFTLTISRLEPEDFAVYYCQXYGXSXXXTFGXGTXXXIK
```

Figure 2H

IgKV3-11-01 / IGKJ4-01 – VL alignment

IgKV3-11-01	EIVLTQSPATLSLSPGERATLSCRASQSVSSYLAWYQQKPGQAPRLLIYDASNRATGIPARFSGS
VL1014-006	EIVLTQSPATLSLSPGERATLSCRASQSVSSYLAWYQQKPGQAPRLLIYDASNRATGIPARFSGS
Consensus	EIVLTQSPATLSLSPGERATLSCRASQSVSSYLAWYQQKPGQAPRLLIYDASNRATGIPARFSGS
IgKV3-11-01	GSGTDFTLTISSLEPEDFAVYYCQQRSNWPPLTFGGGTKVEIK
VL1014-006	GSGTDFTLTISSLEPEDFAVYYCQQRSNWPPLTFGGGTKVEIK
Consensus	GSGTDFTLTISSLEPEDFAVYYCQQRSNWPPLTFGGGTKVEIK

Figure 3A

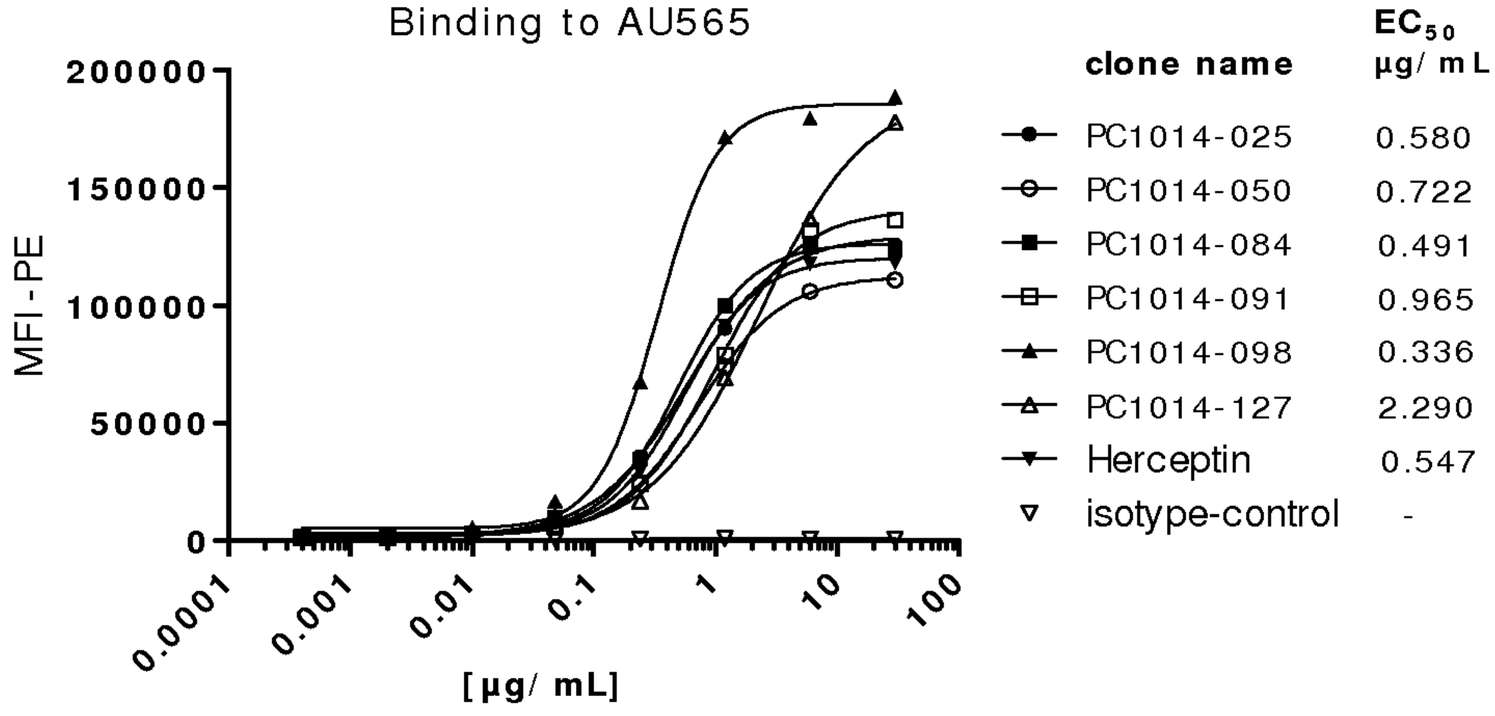


Figure 3B

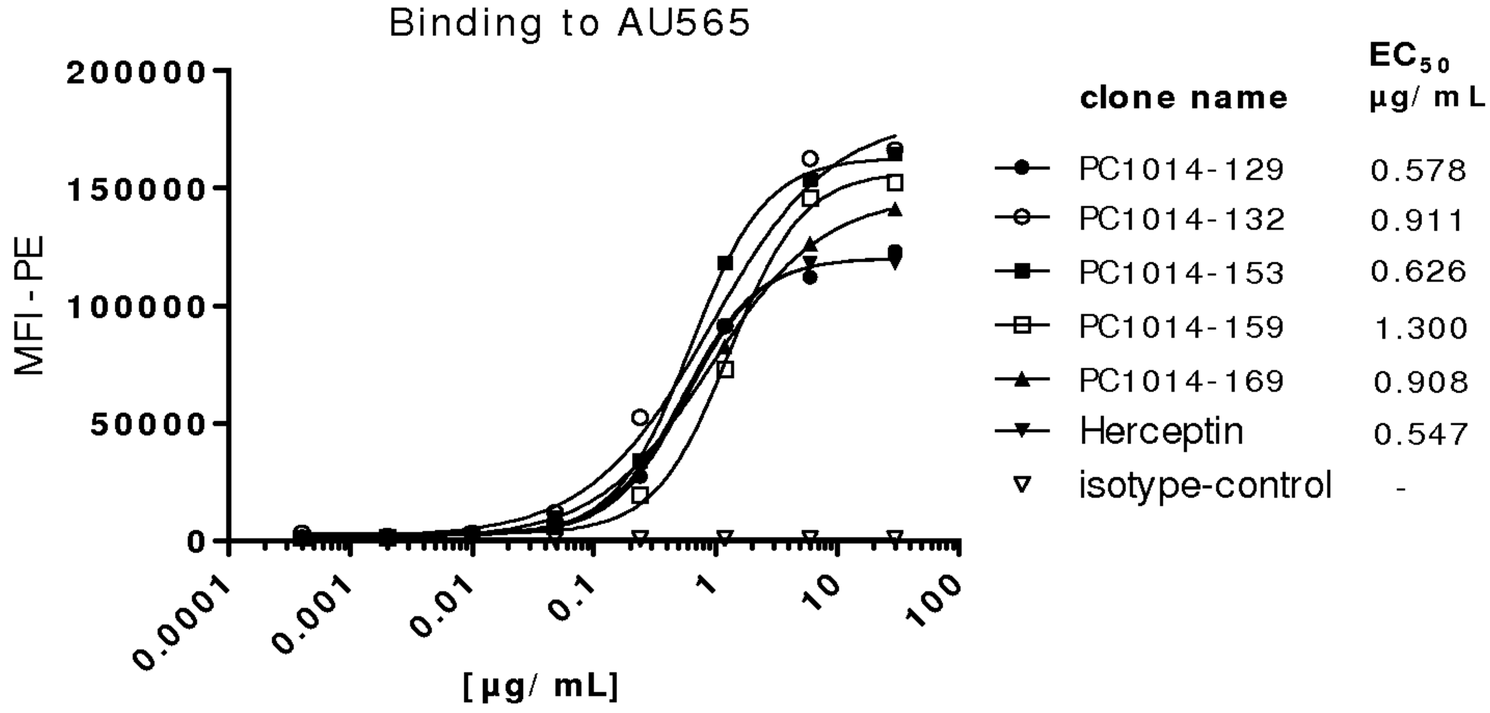


Figure 3C

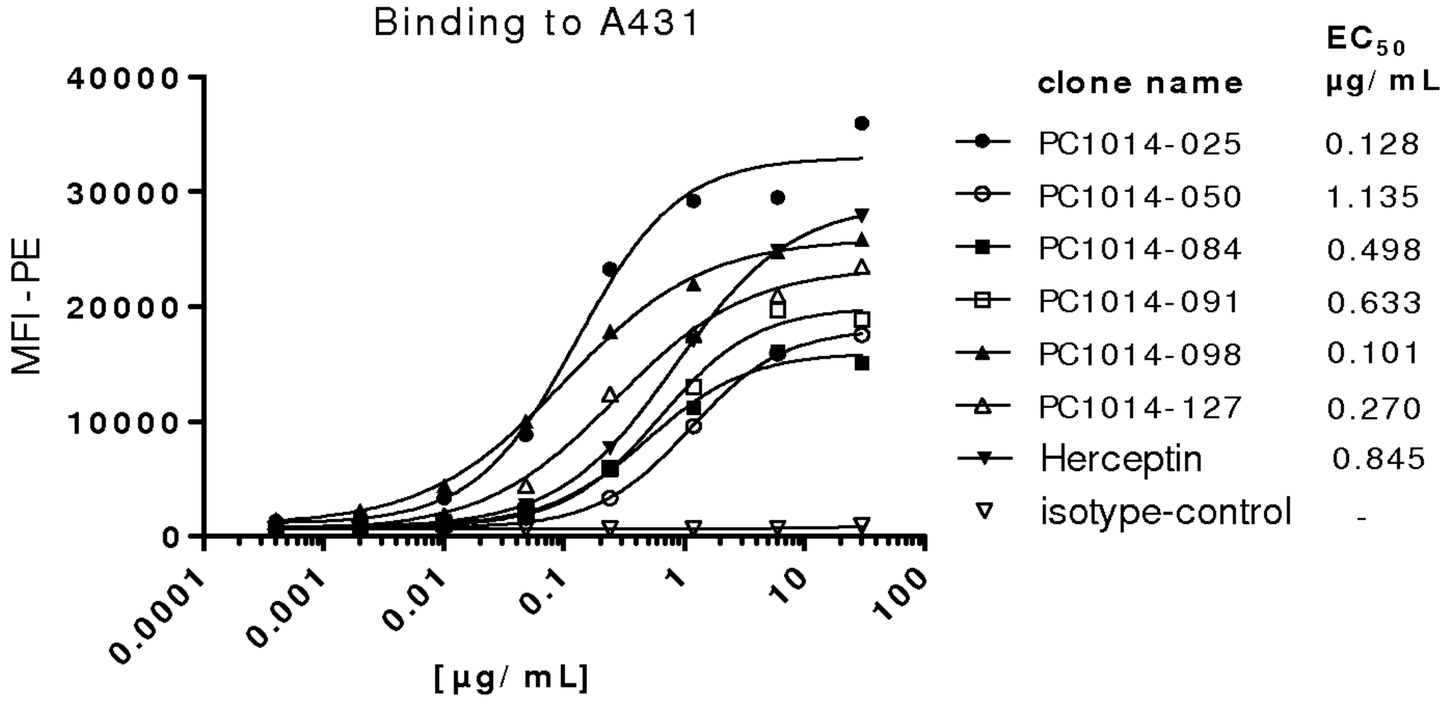


Figure 3D

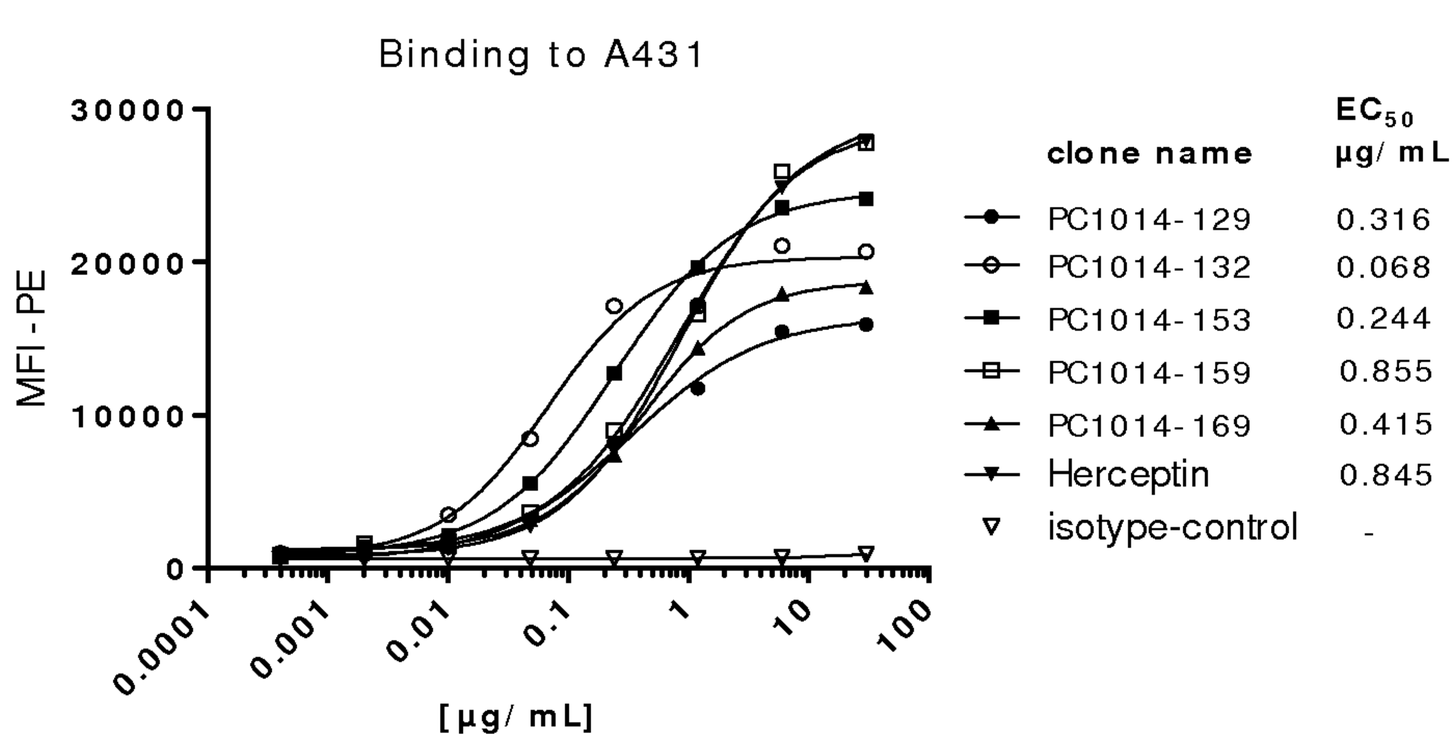


Figure 3E

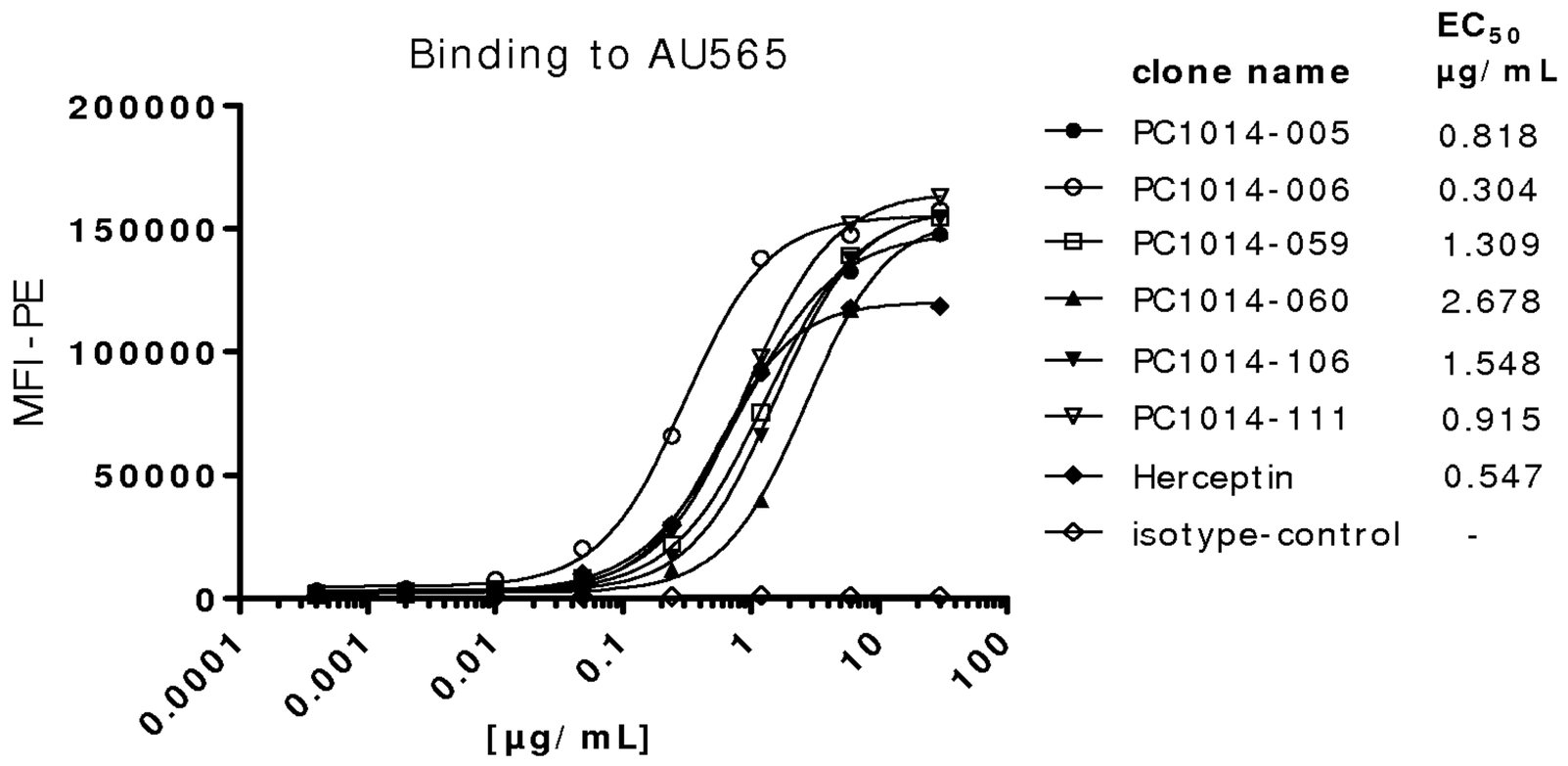


Figure 3F

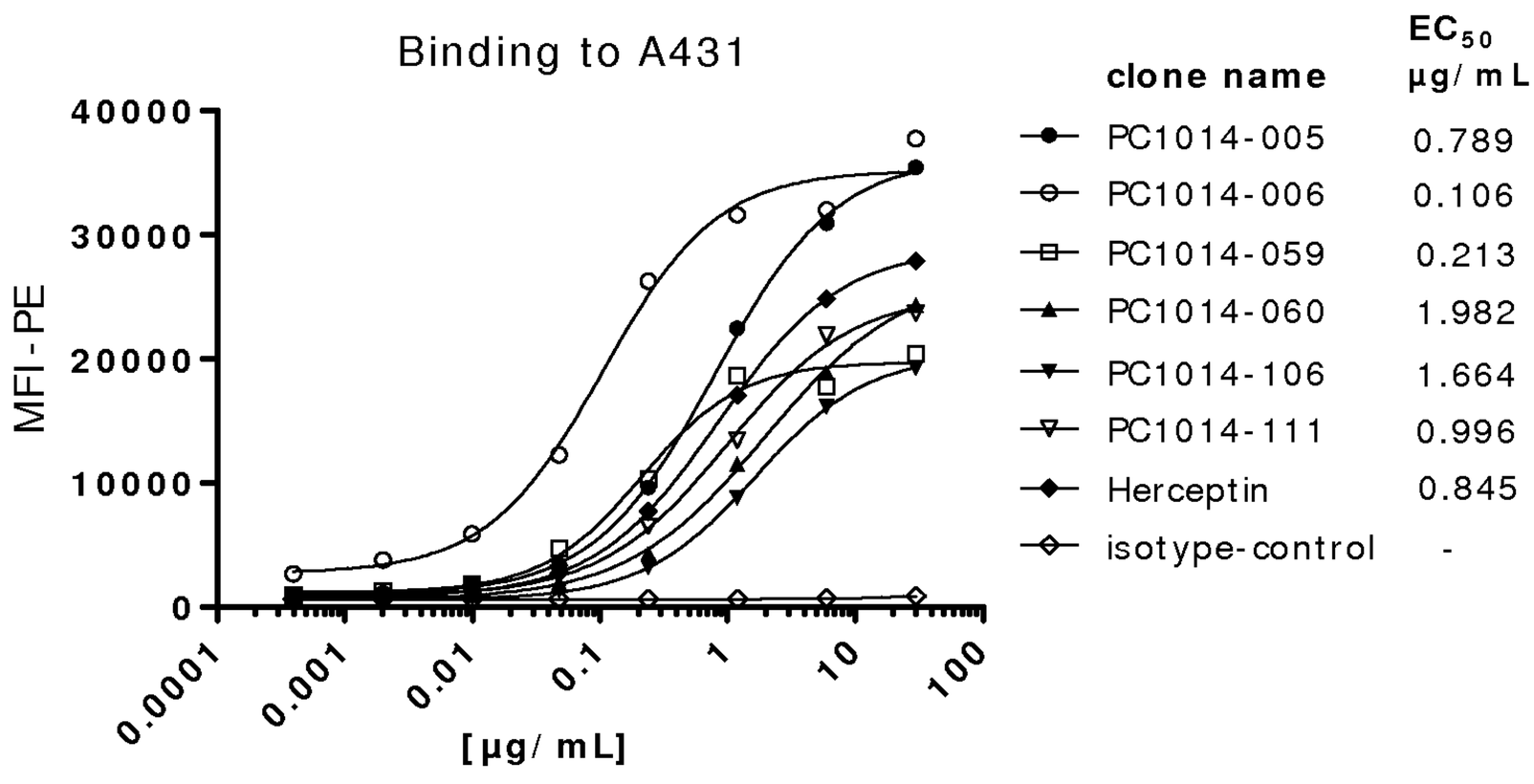


Figure 4A

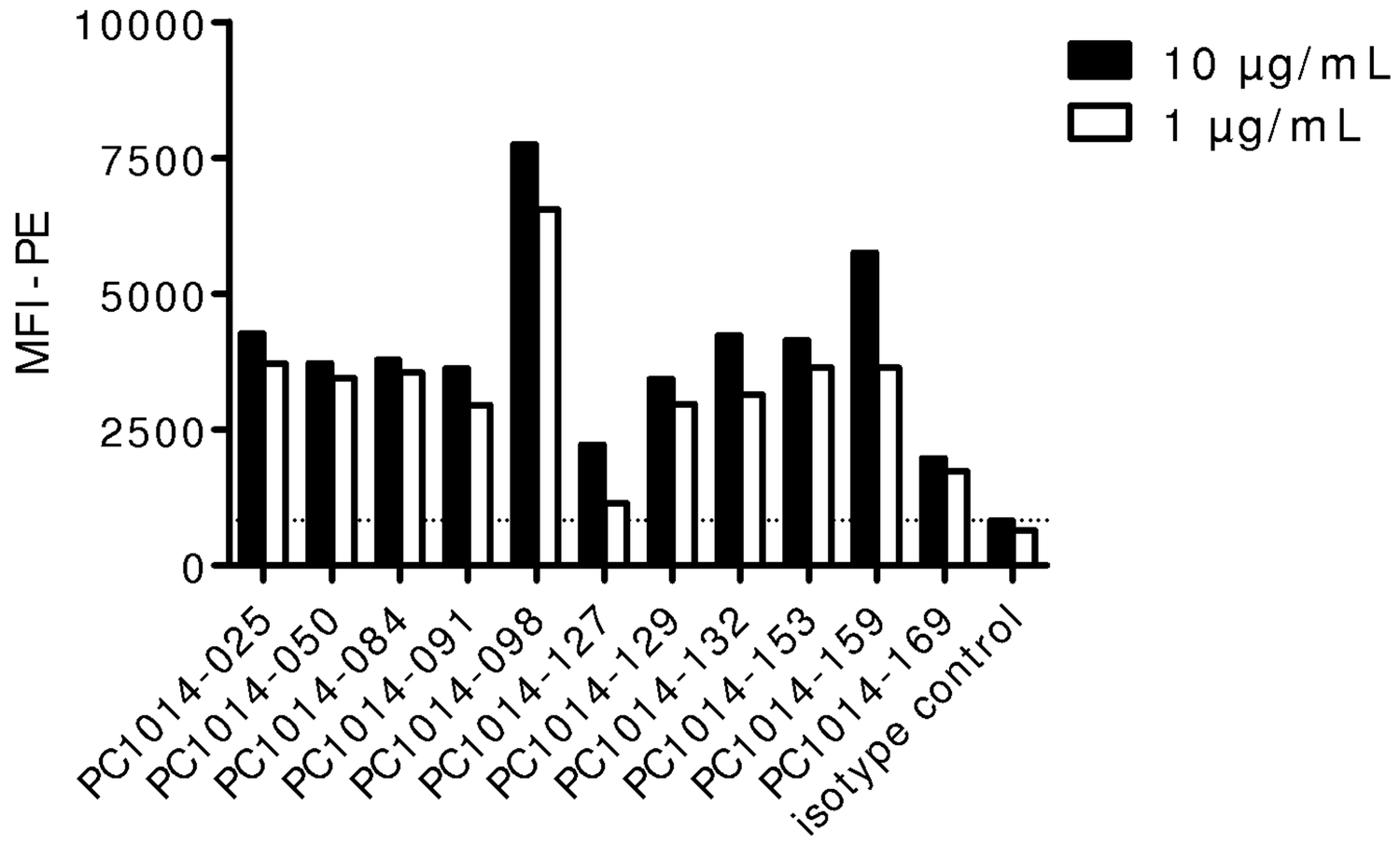


Figure 4B

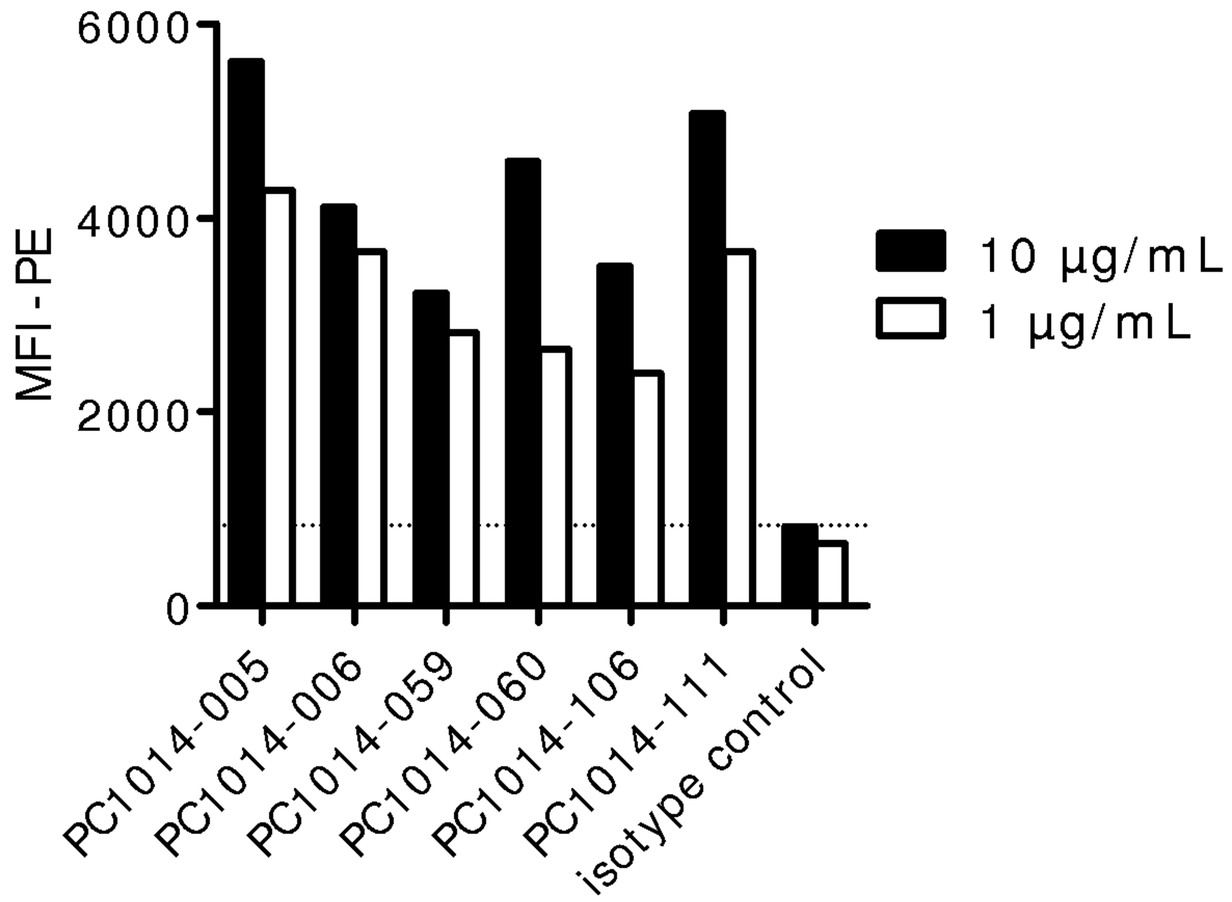


Figure 5A

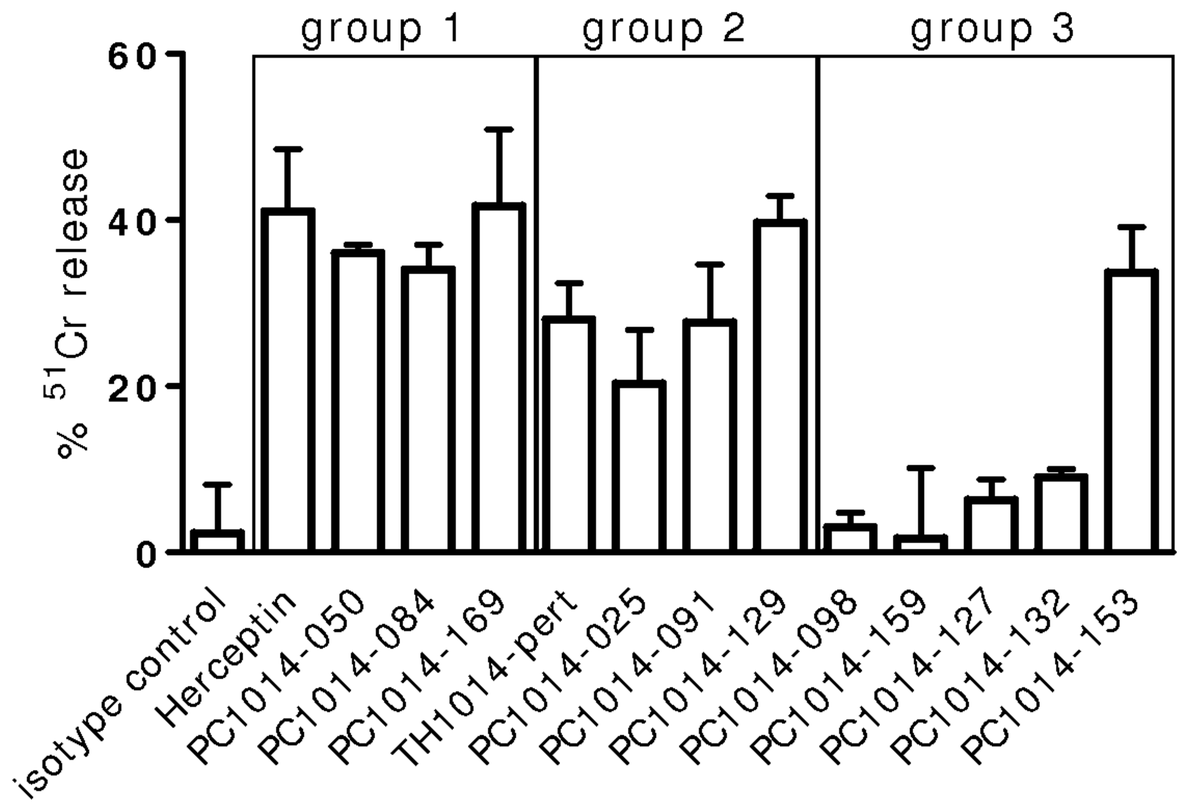


Figure 5B

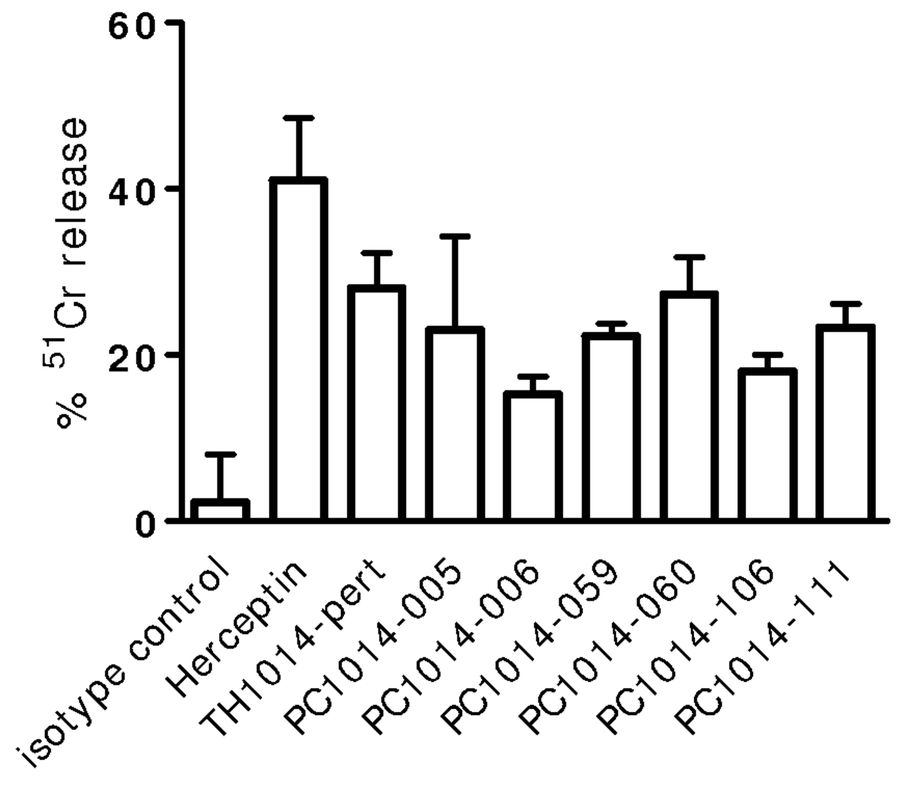


Figure 6

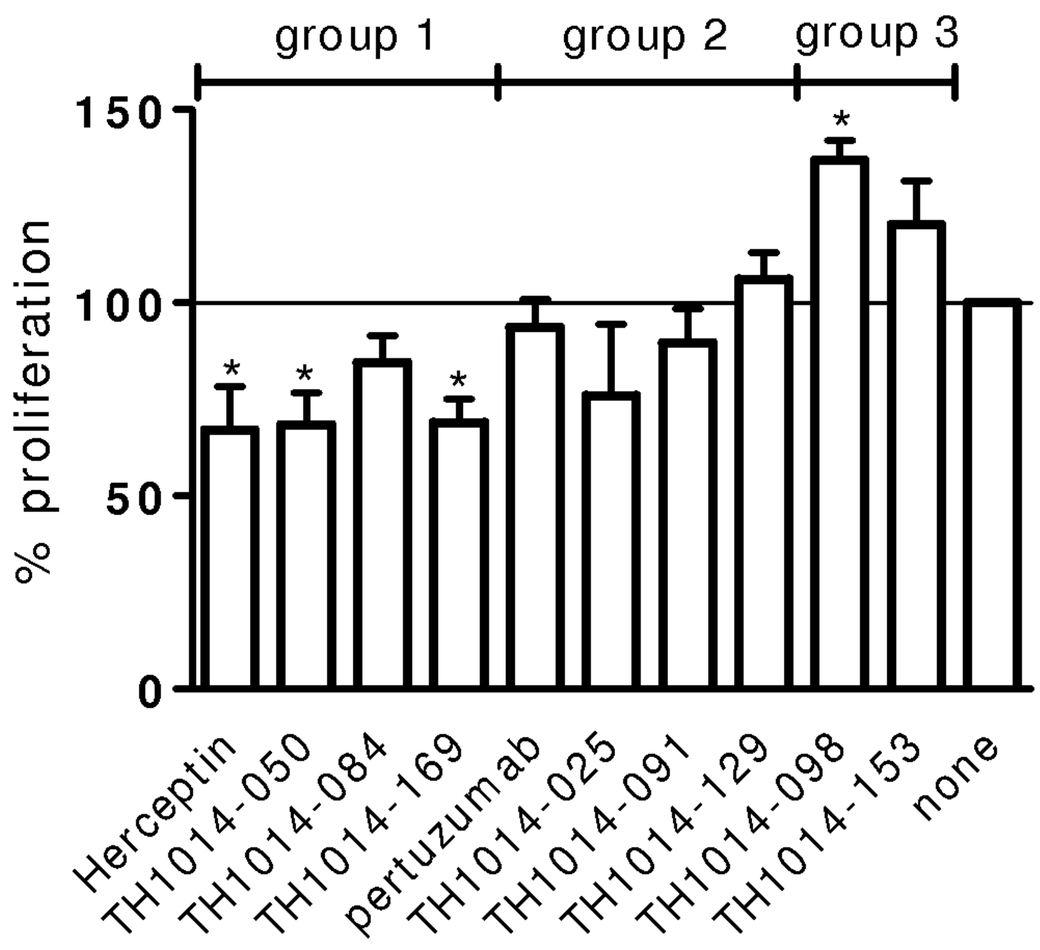


Figure 7

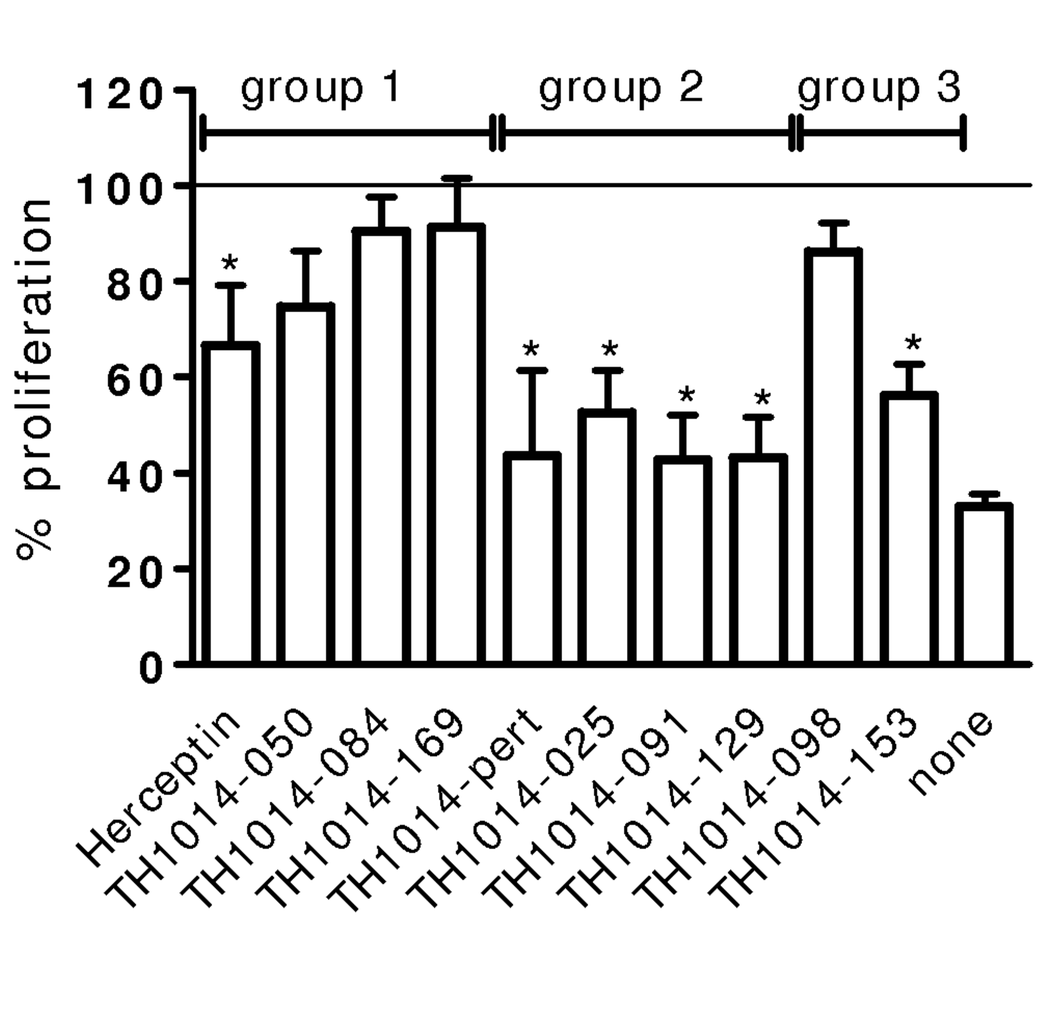


Figure 8A

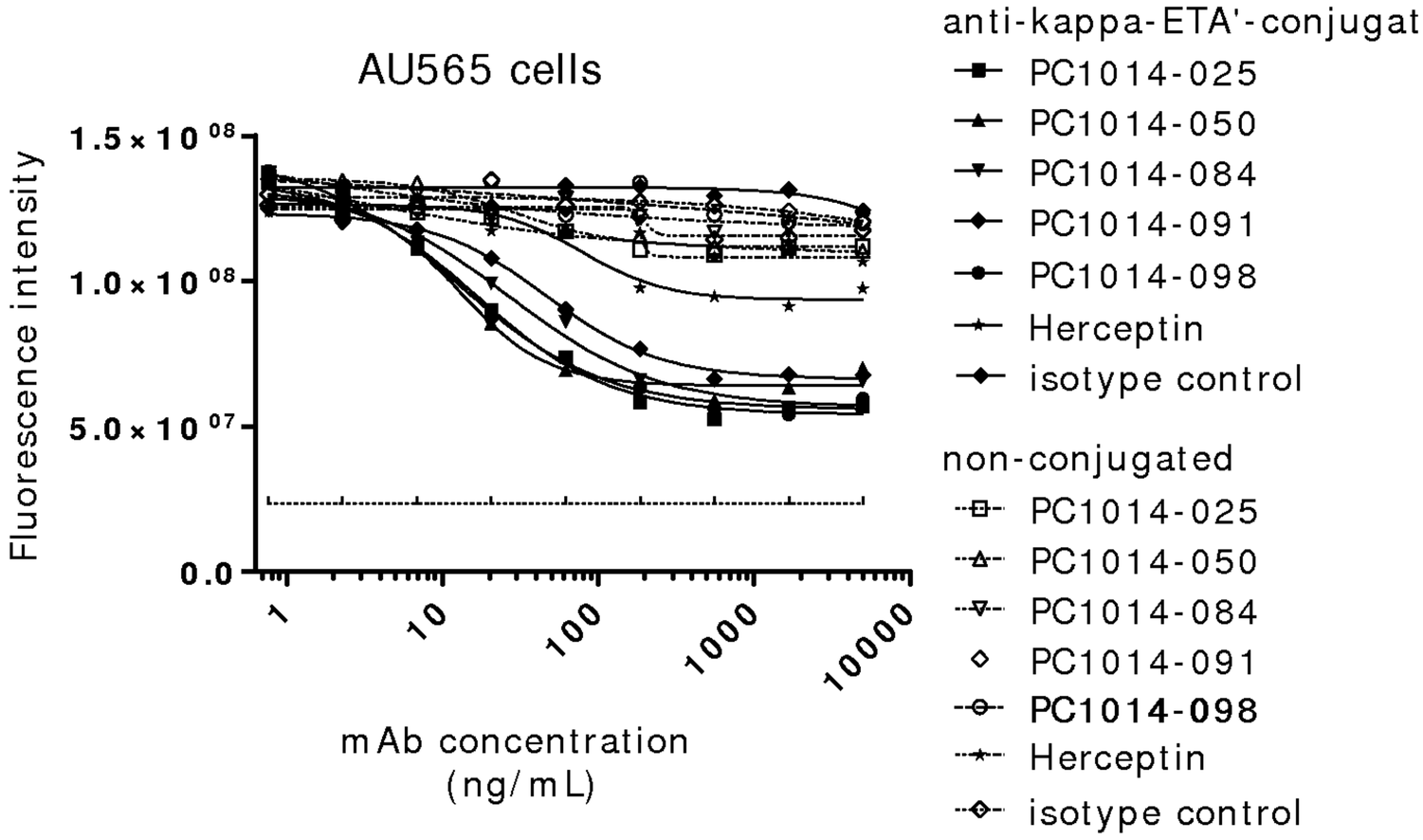


Figure 8B

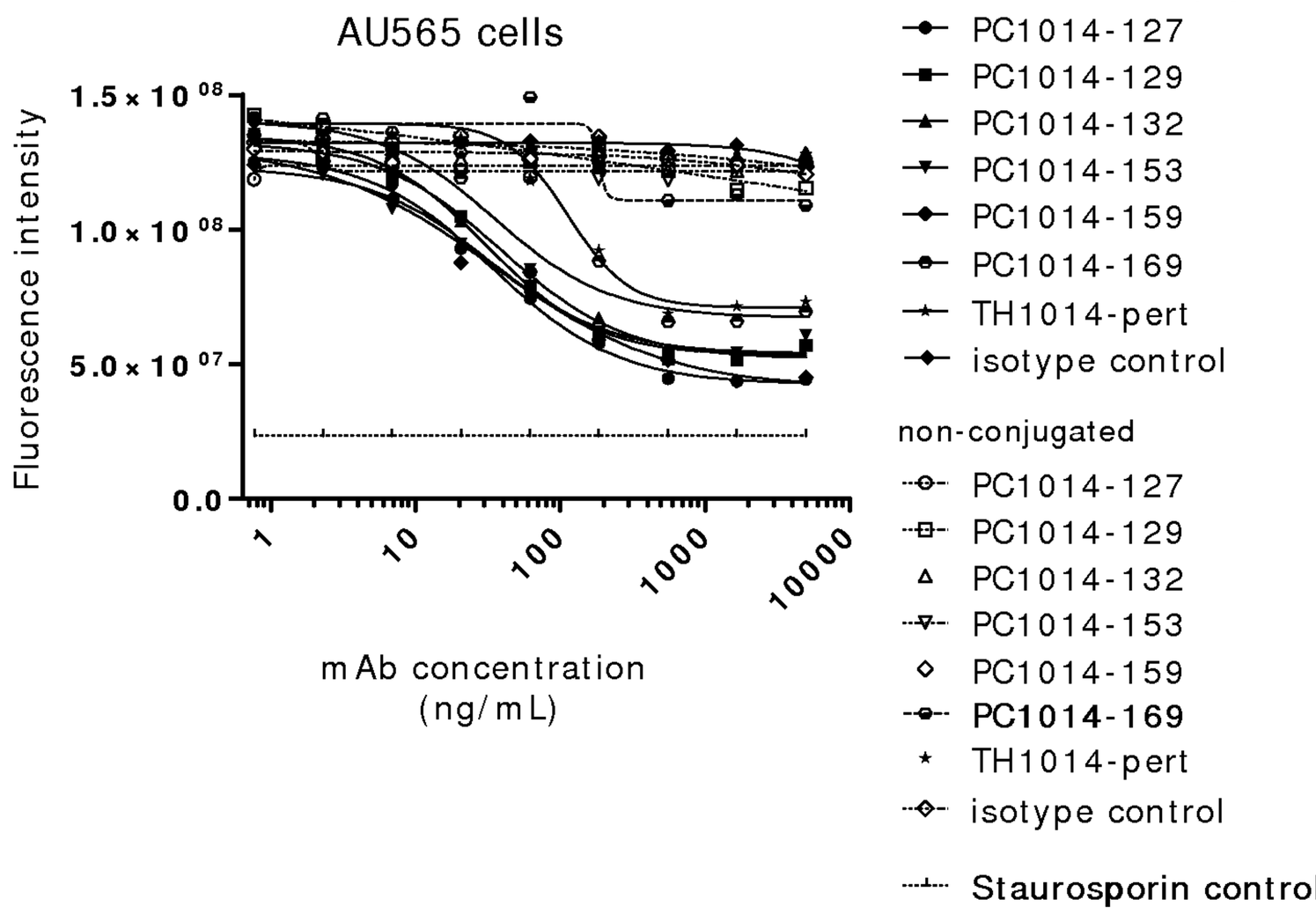


Figure 8C

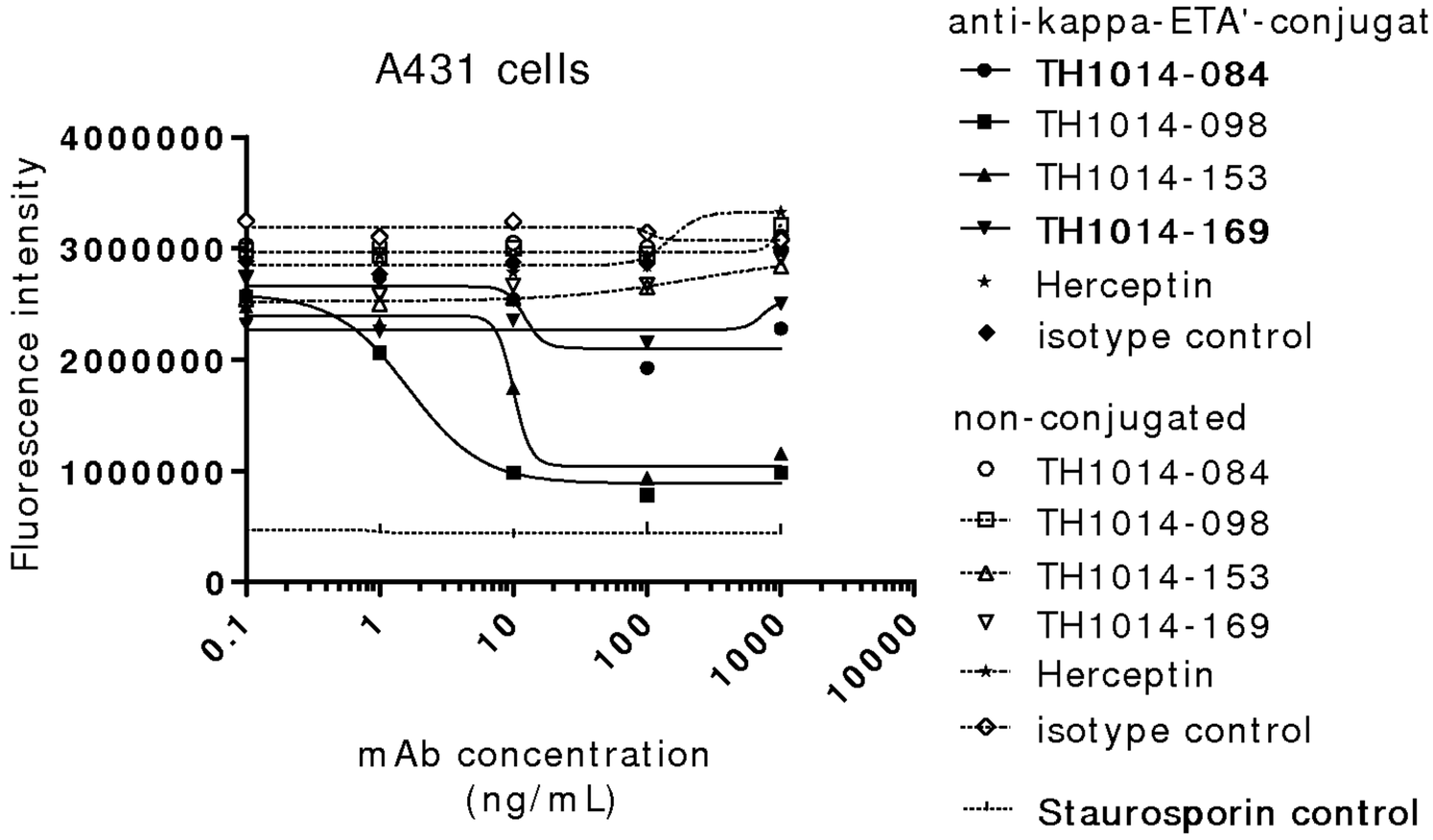


Figure 8D

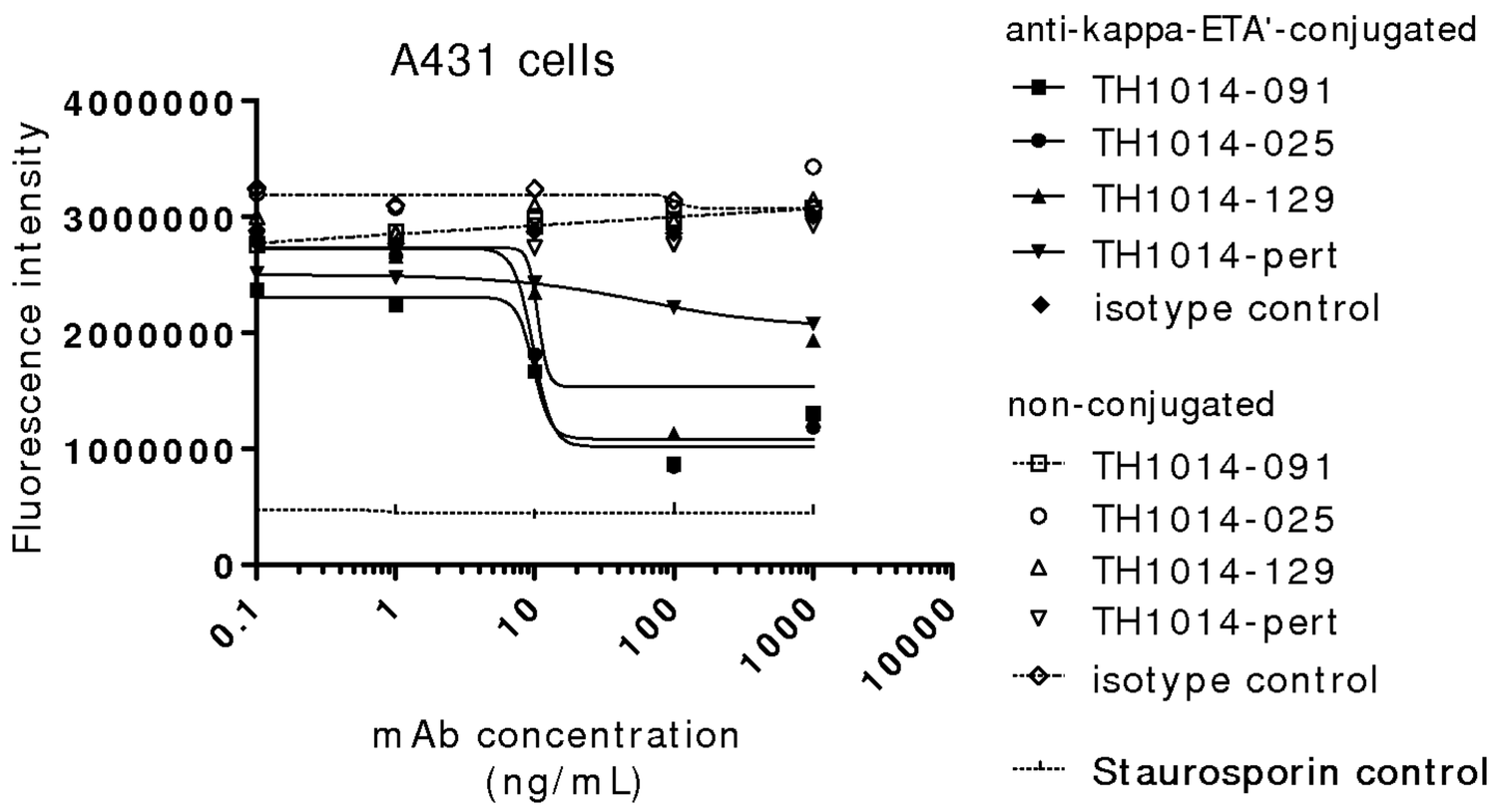


Figure 9A

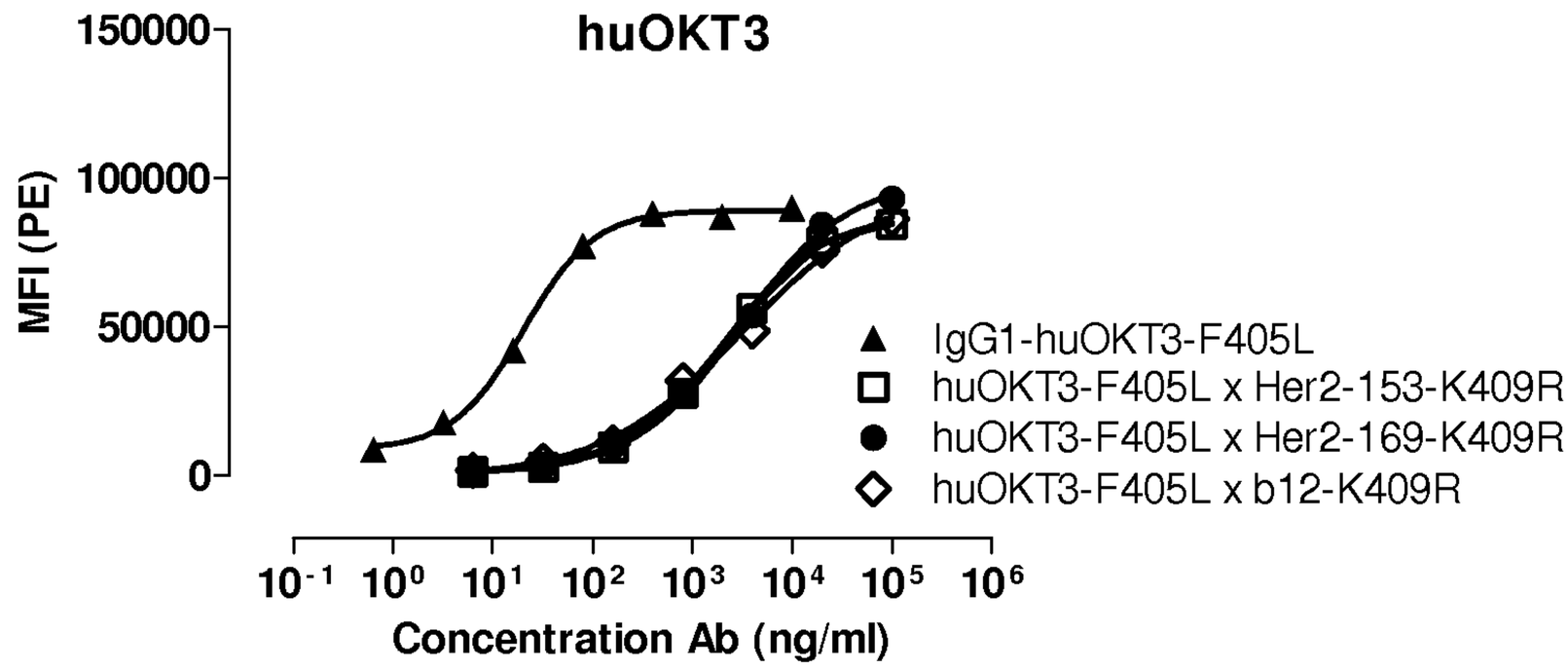


Figure 9B

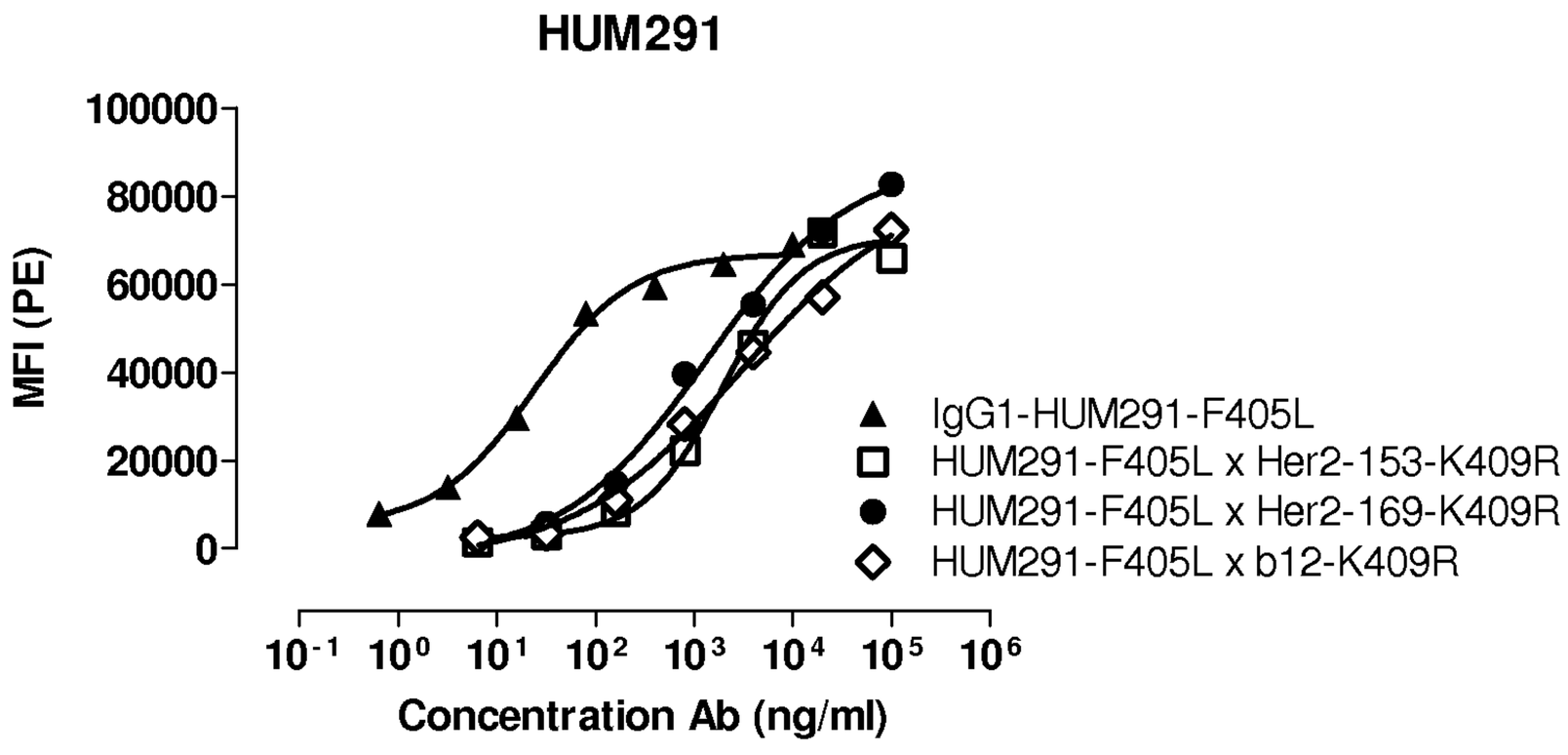


Figure 9C

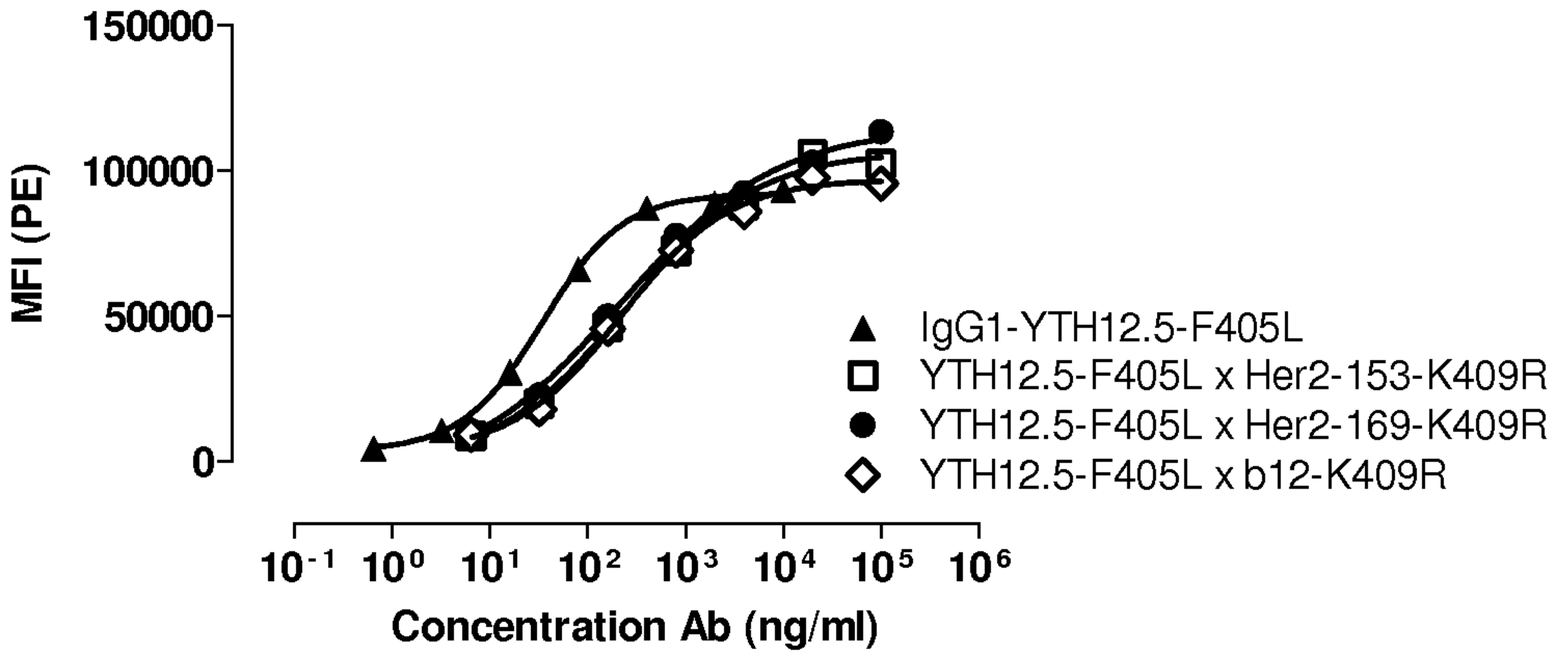


Figure 9D

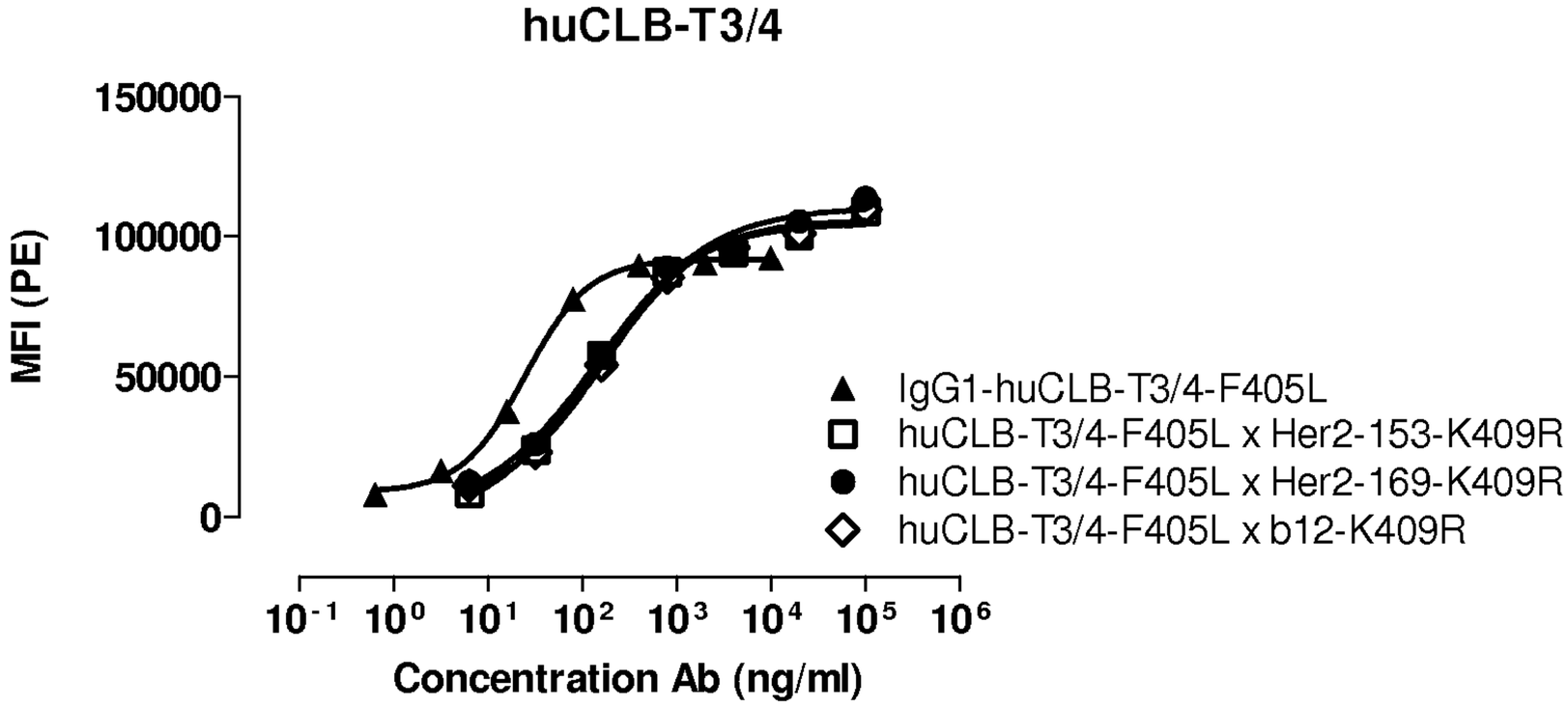


Figure 10A

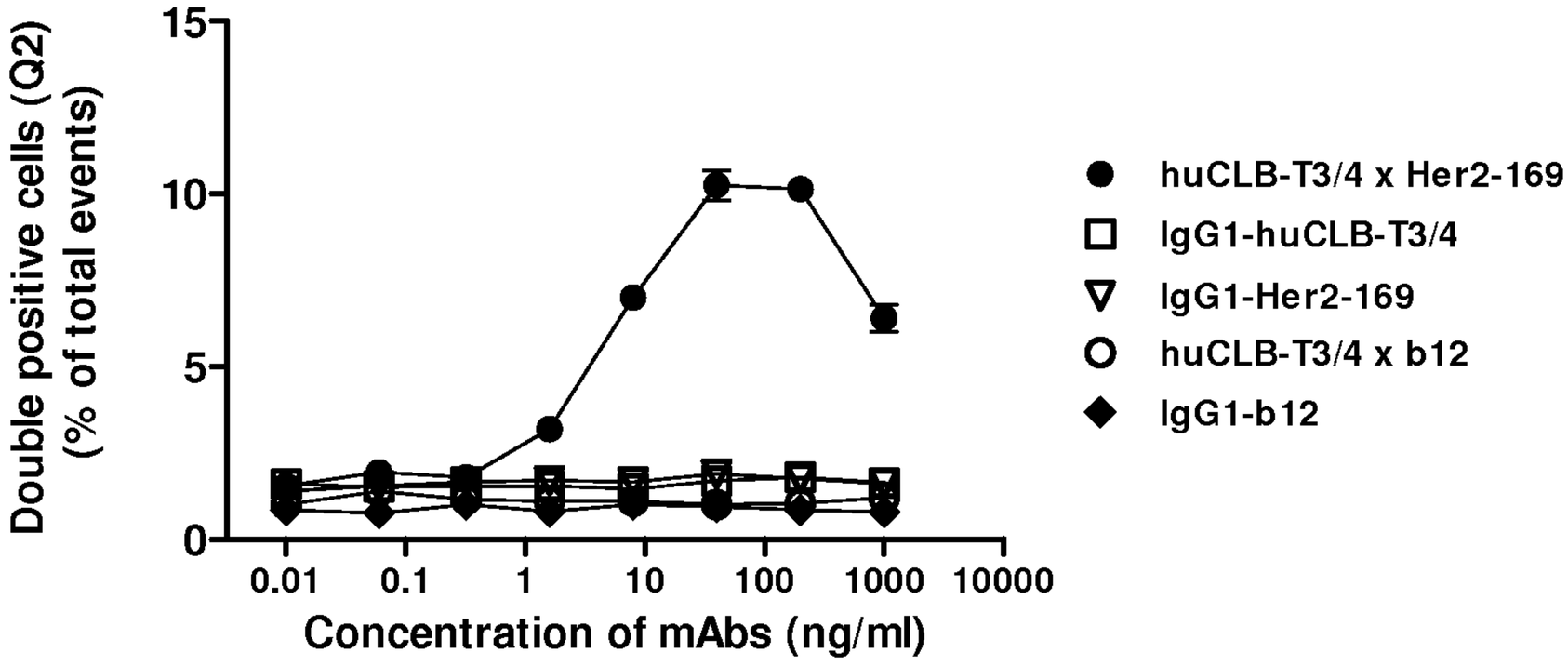


Figure 10B

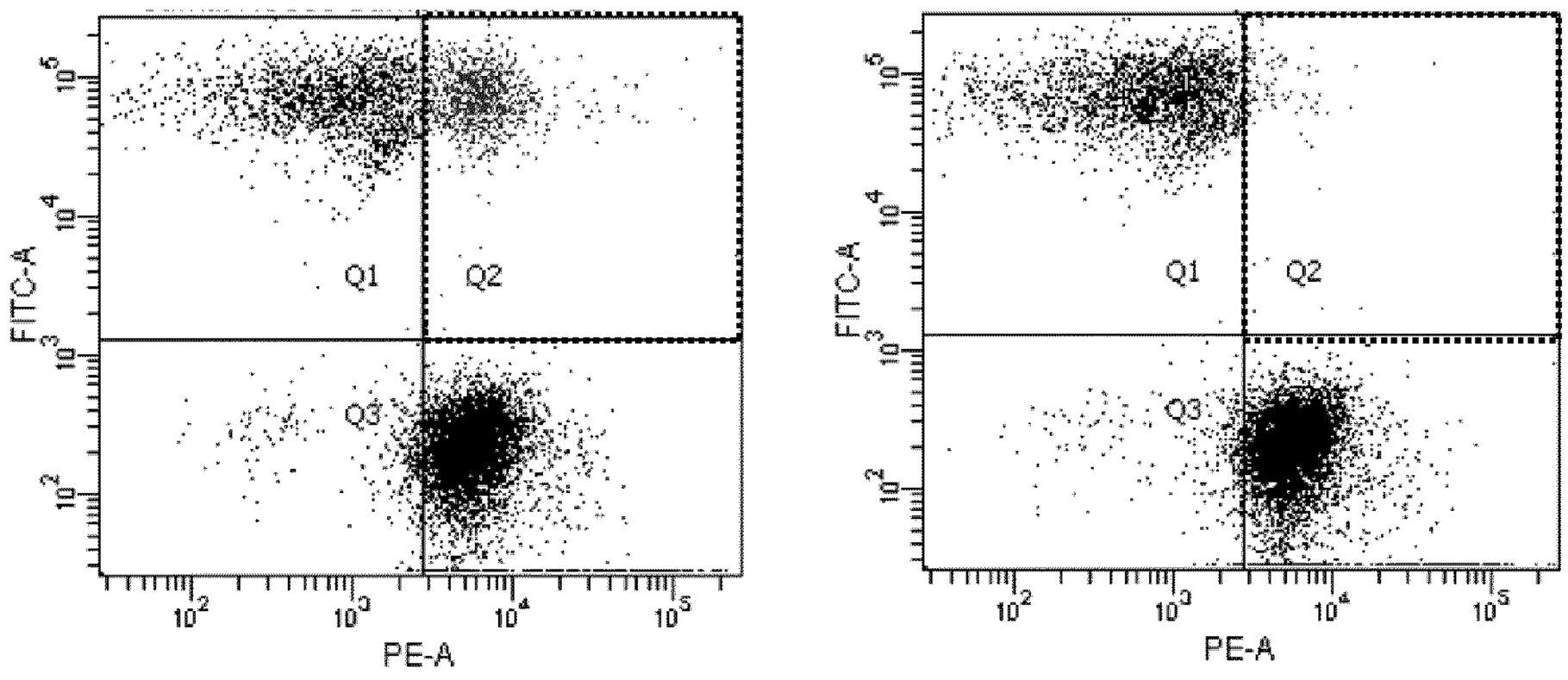


Figure 11A

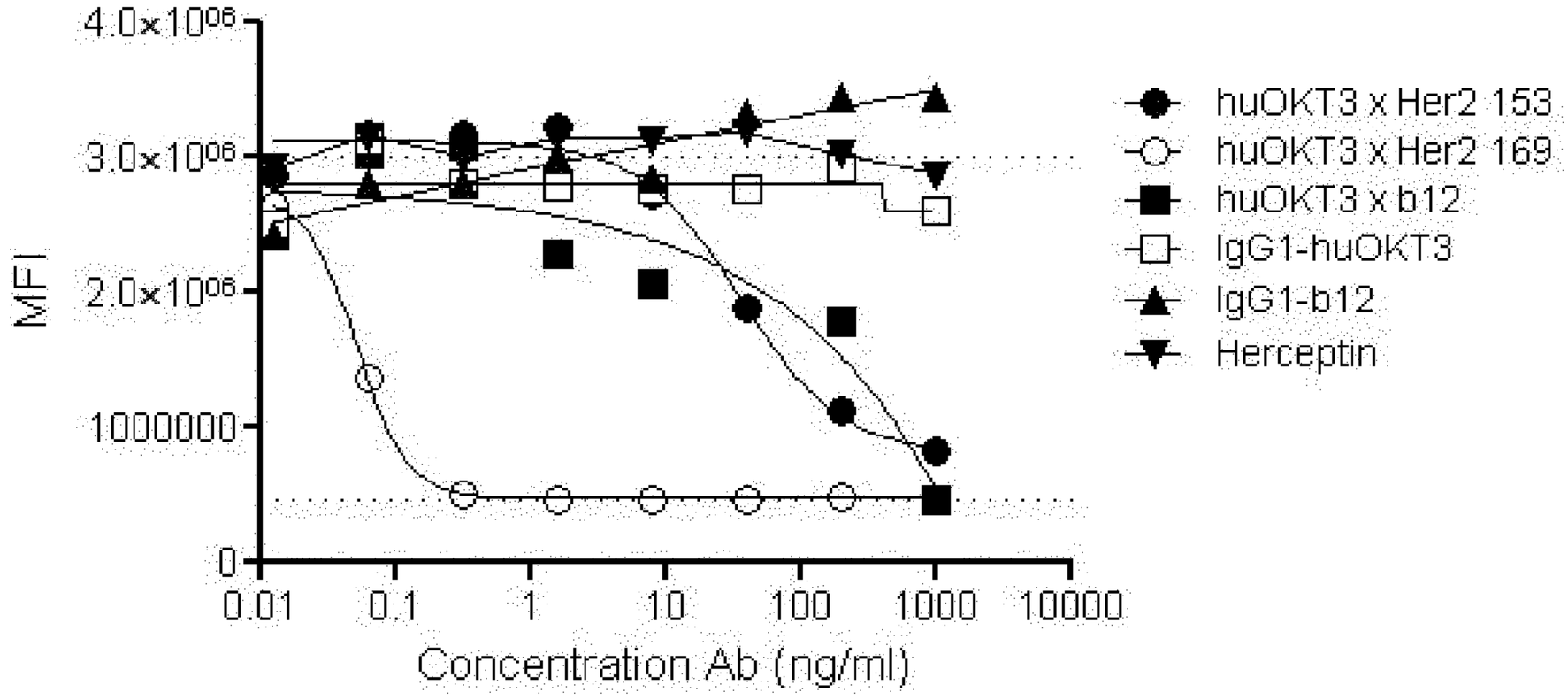


Figure 11B

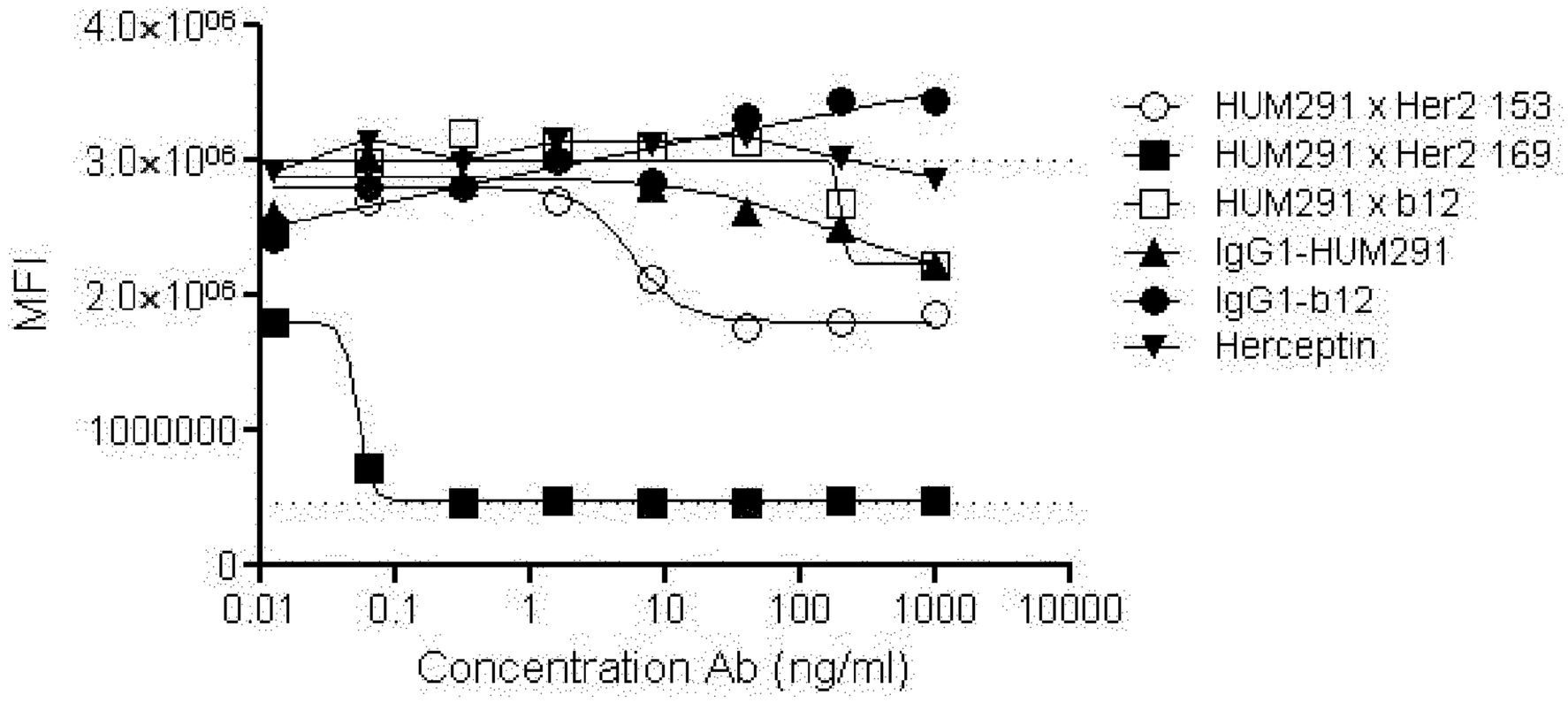


Figure 11C

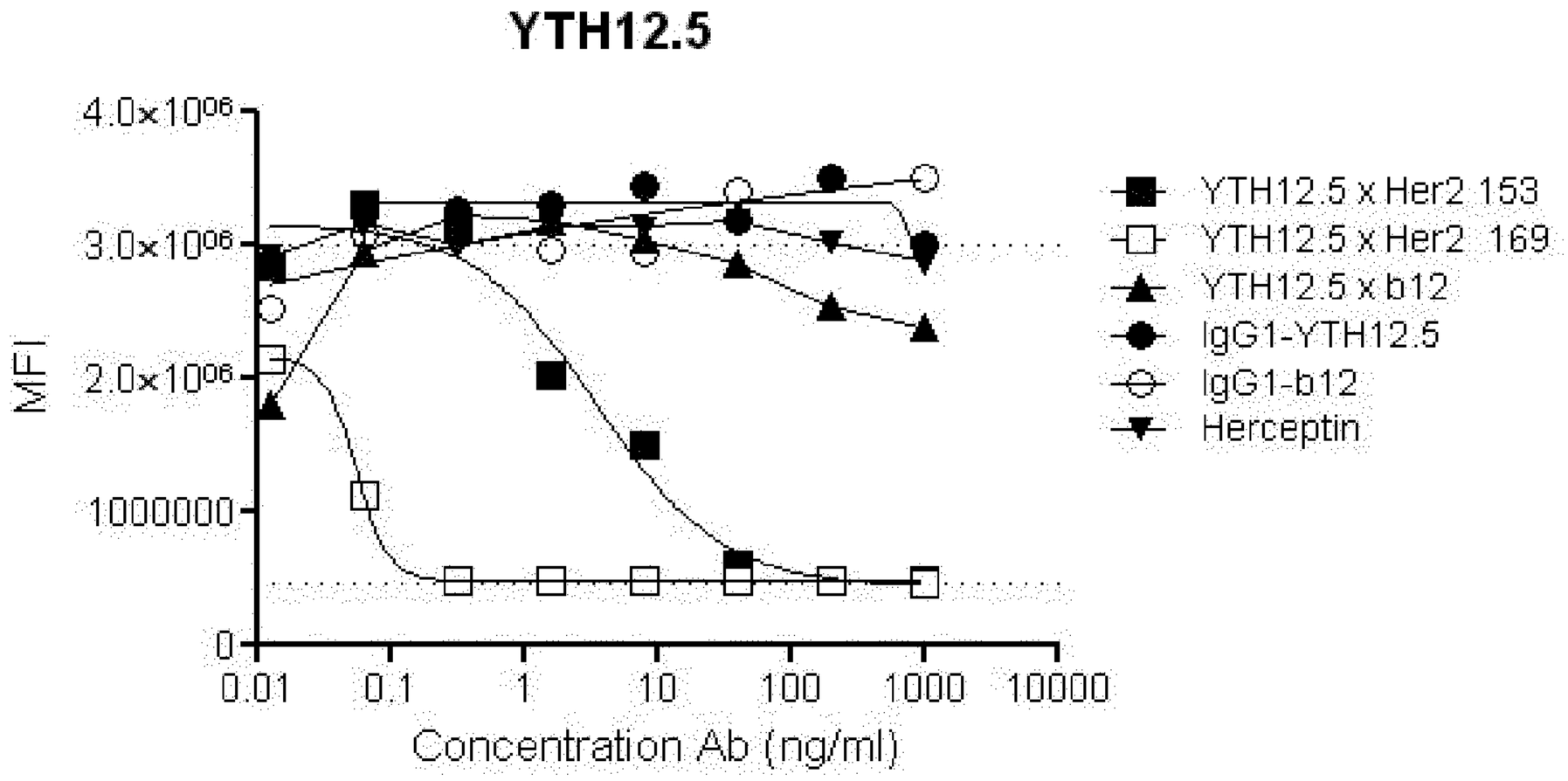


Figure 11D

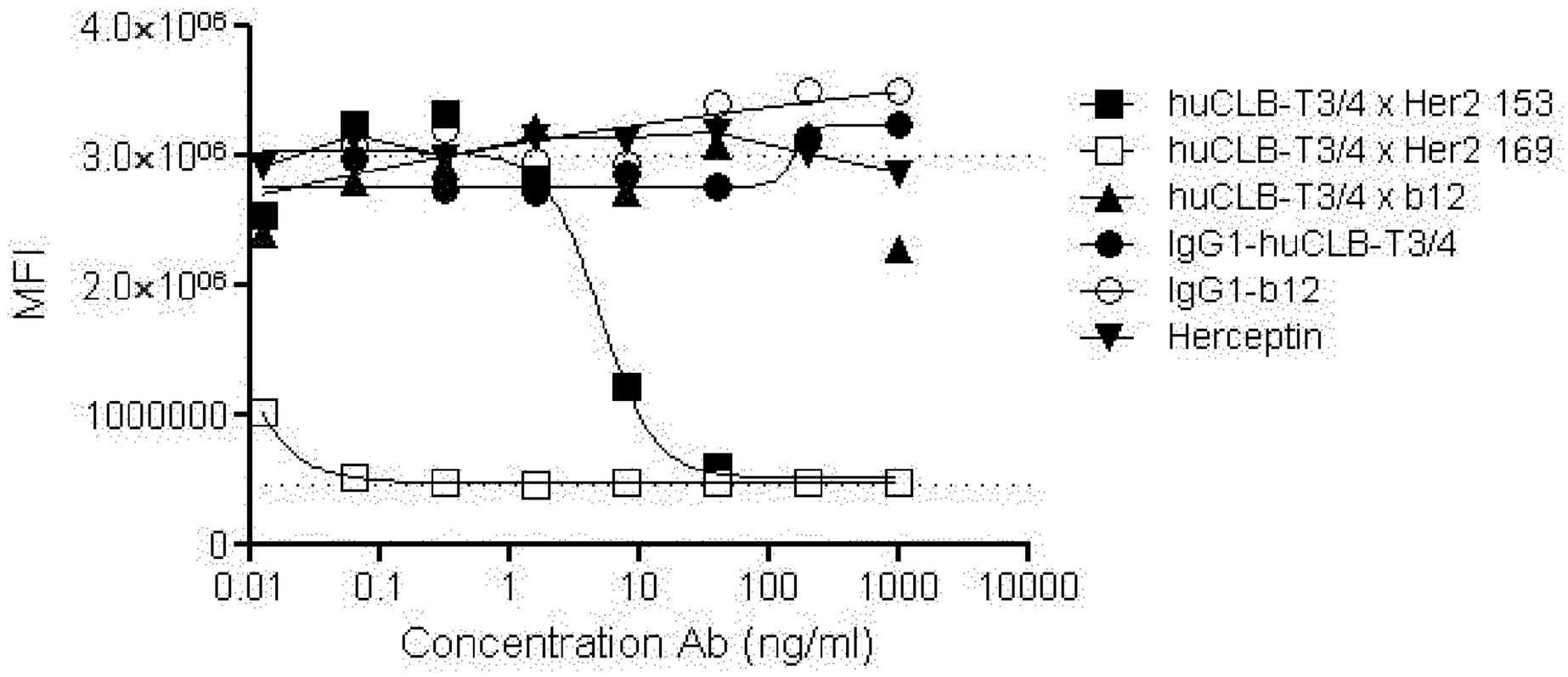


Figure 12

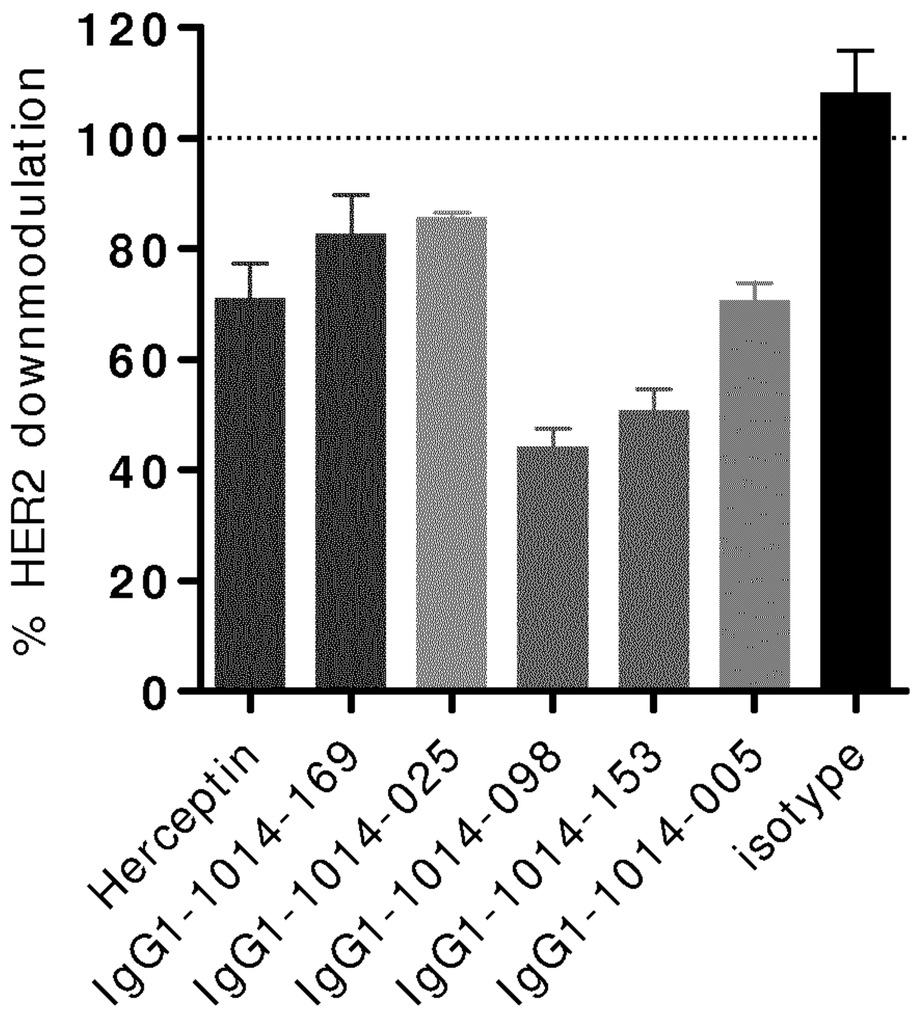


Figure 13

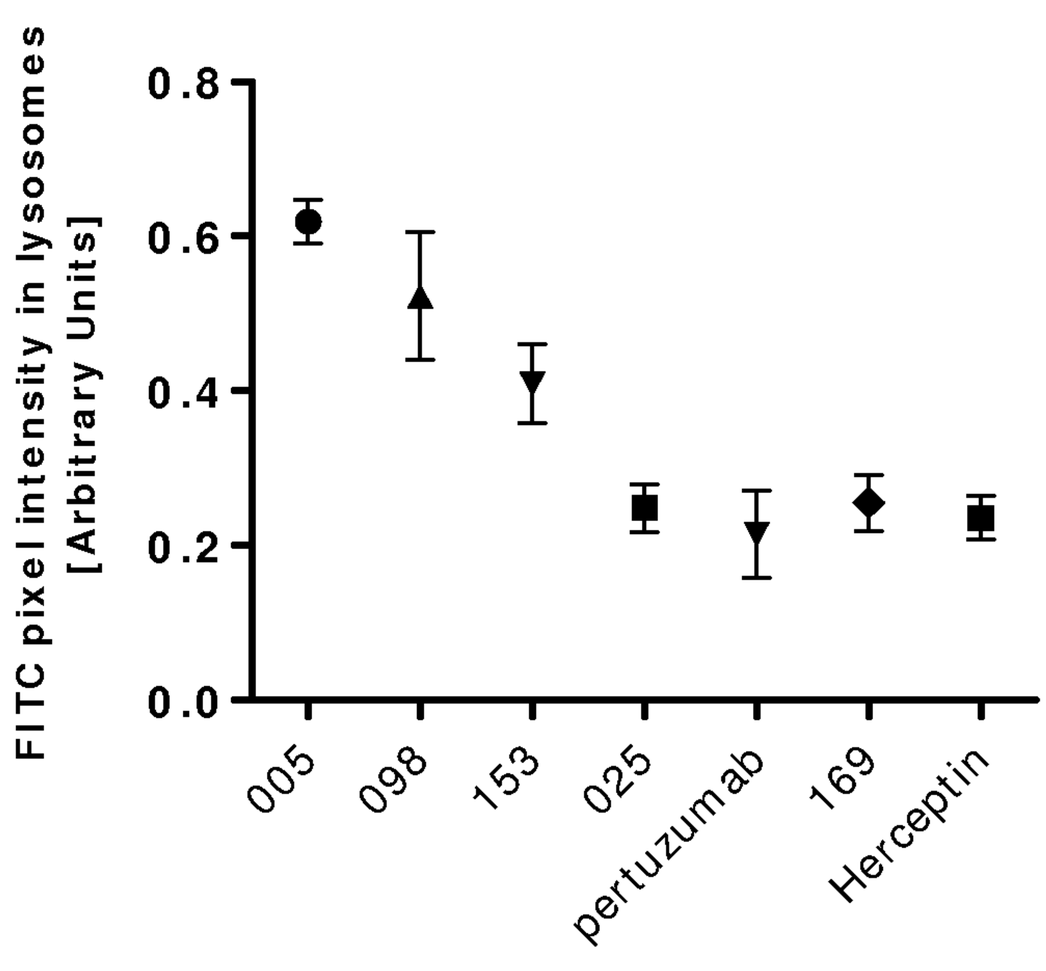


Figure 14

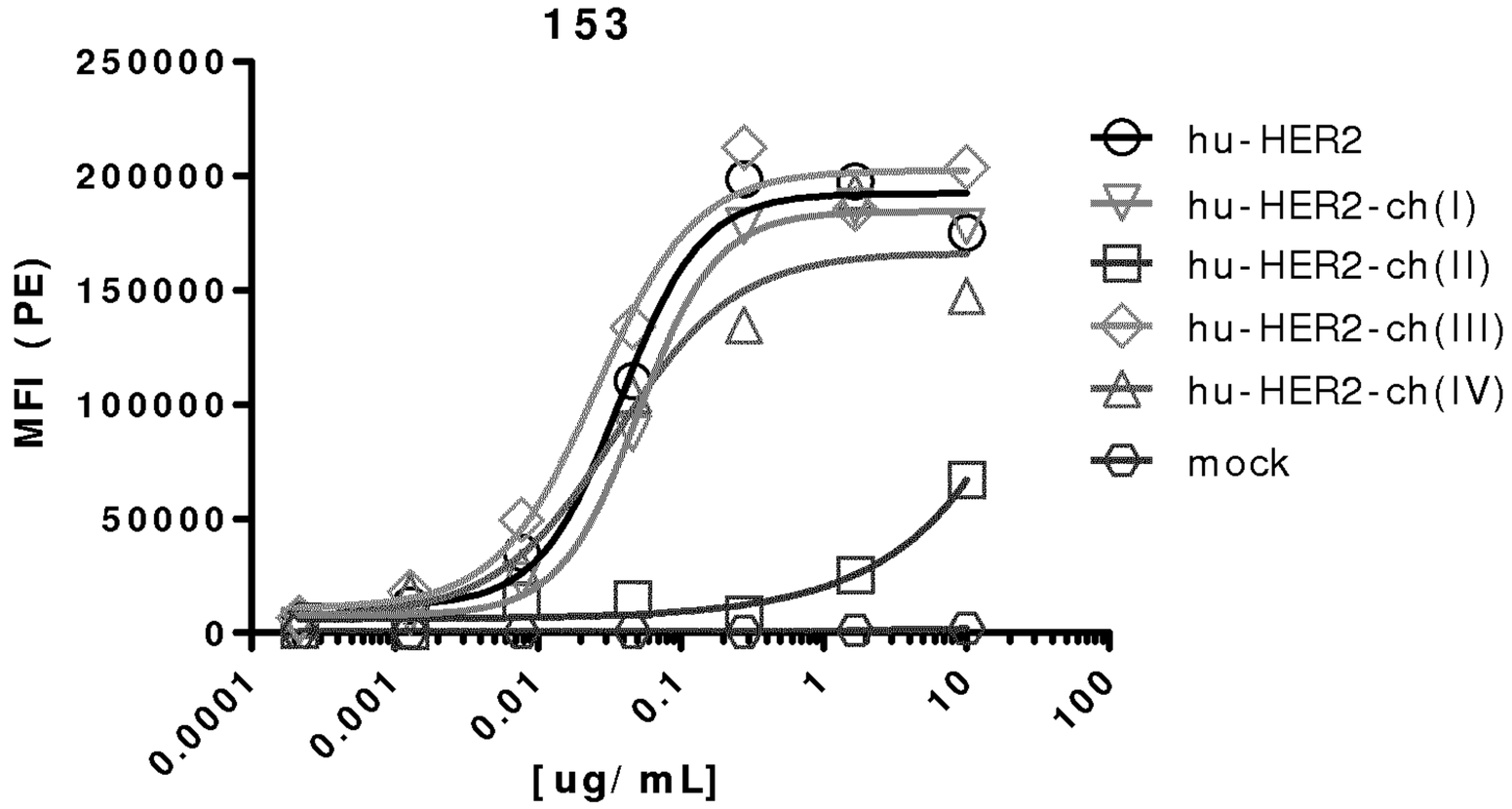

153
250000
200000
150000
100000
50000
0
MFI (PE)
0.0001
0.001
0.01
0.1
1
10
100
[ug/ mL]
hu-HER2
hu-HER2-ch(I)
hu-HER2-ch(II)
hu-HER2-ch(III)
hu-HER2-ch(IV)
mock

Figure 15A

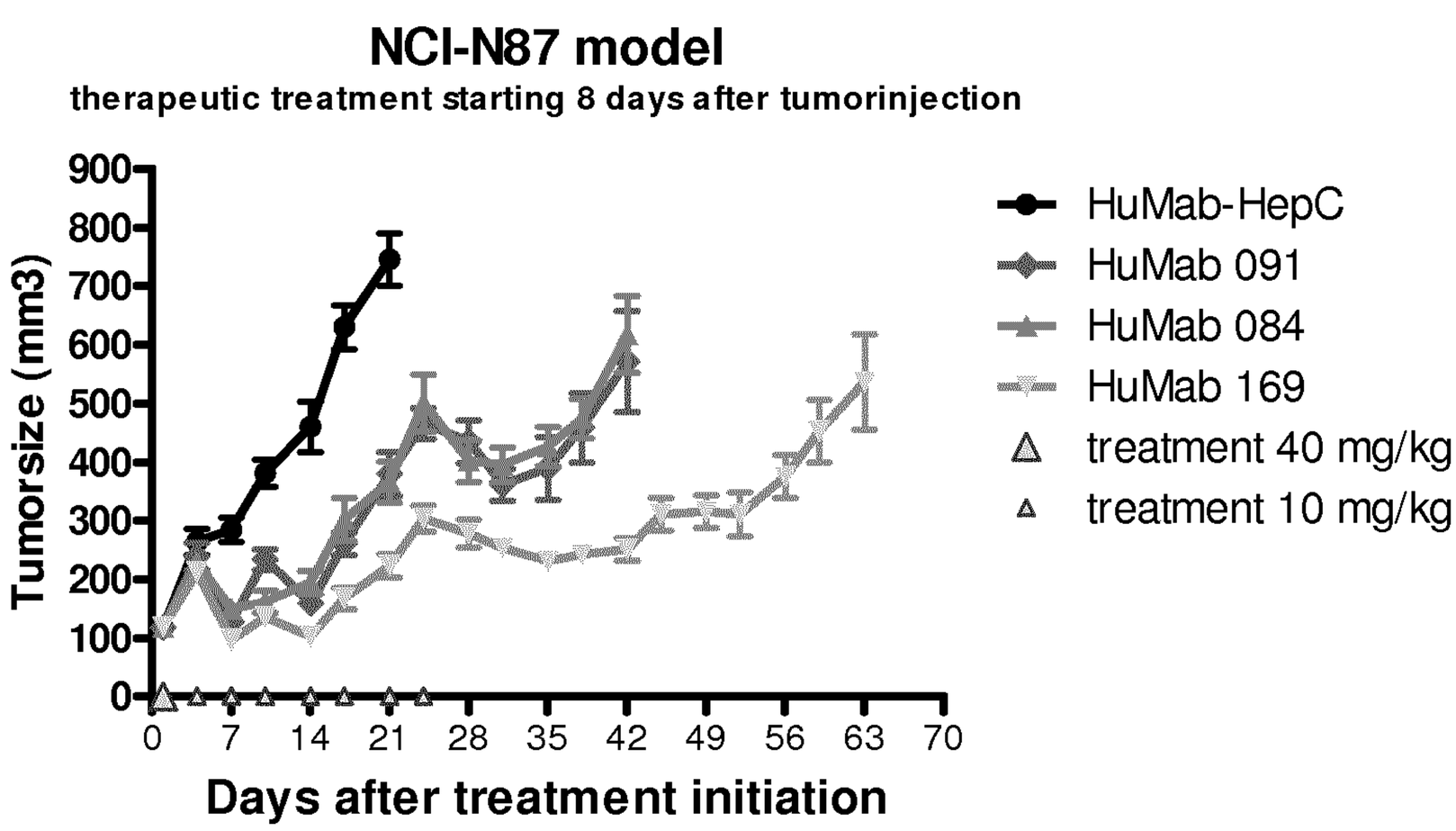


Figure 15B

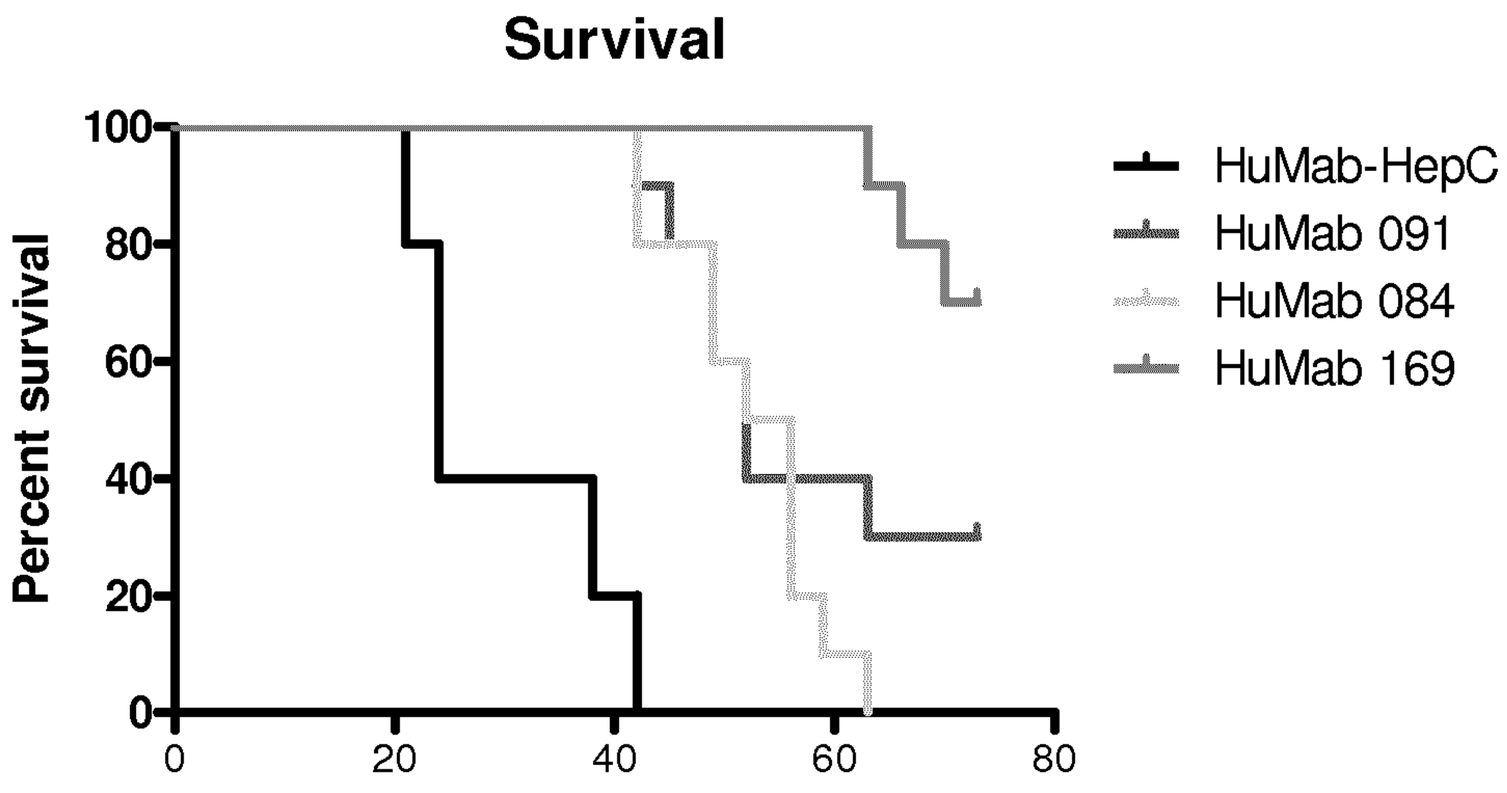


Figure 15C

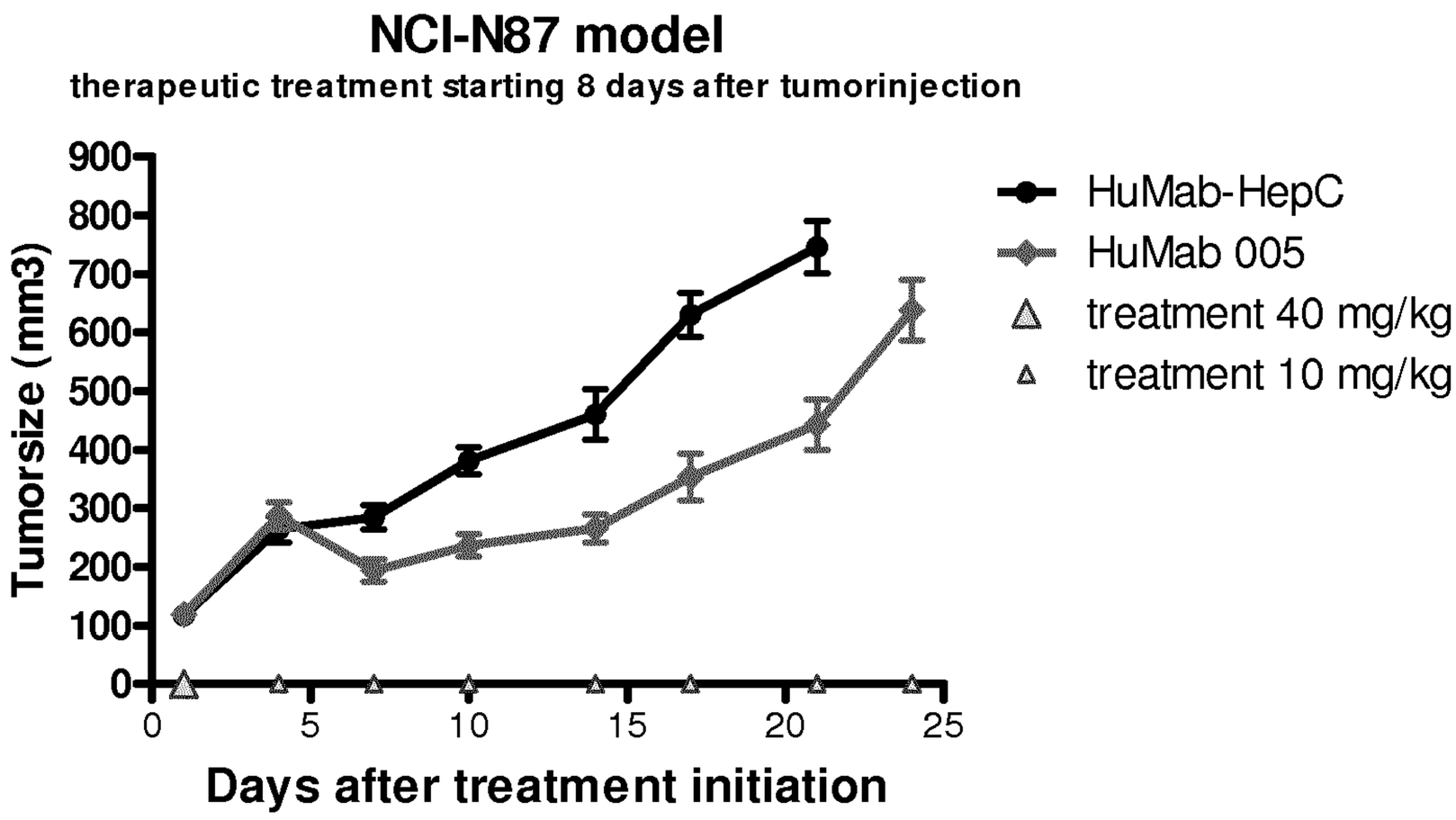


Figure 15D

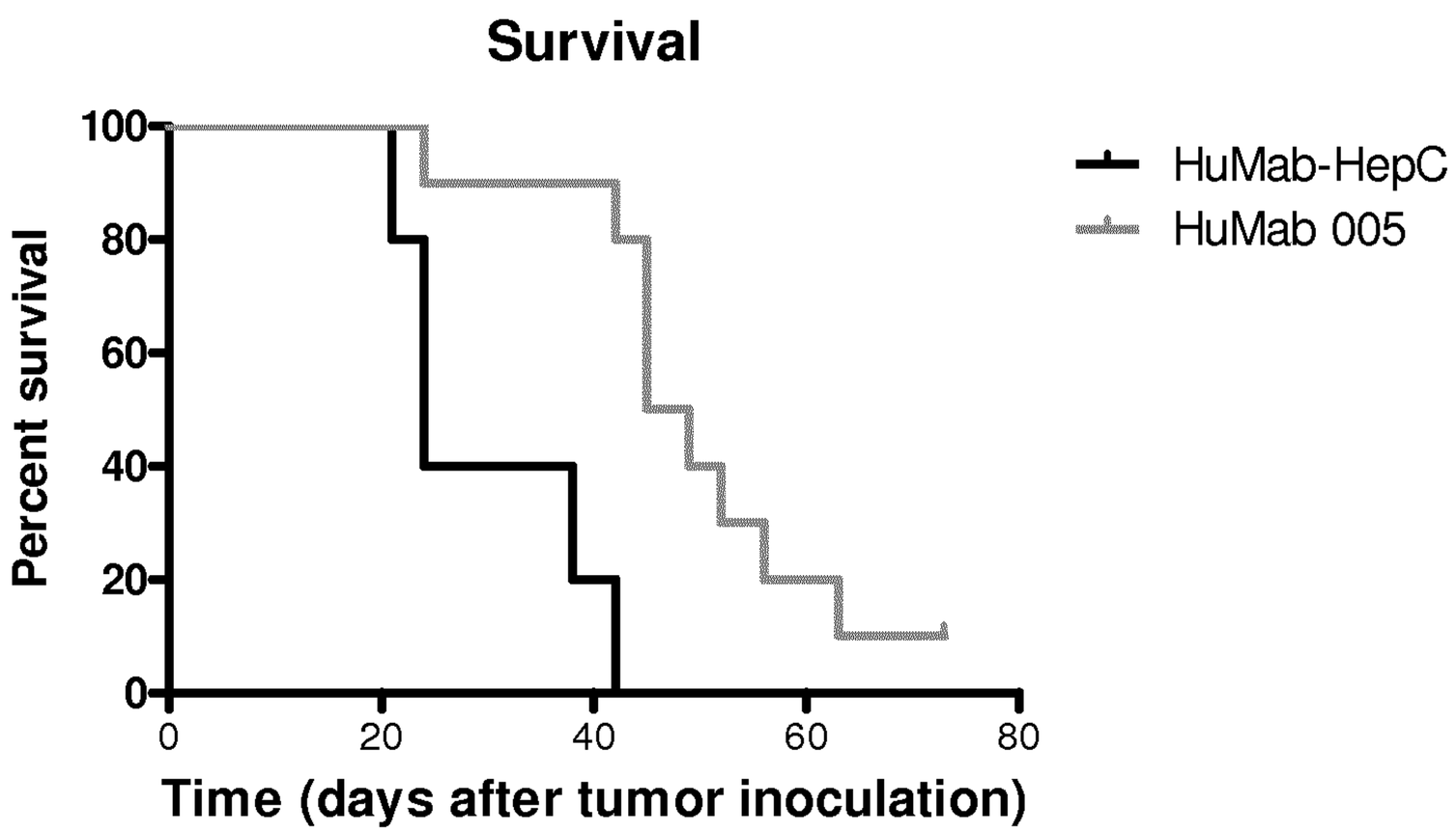


Figure 16A

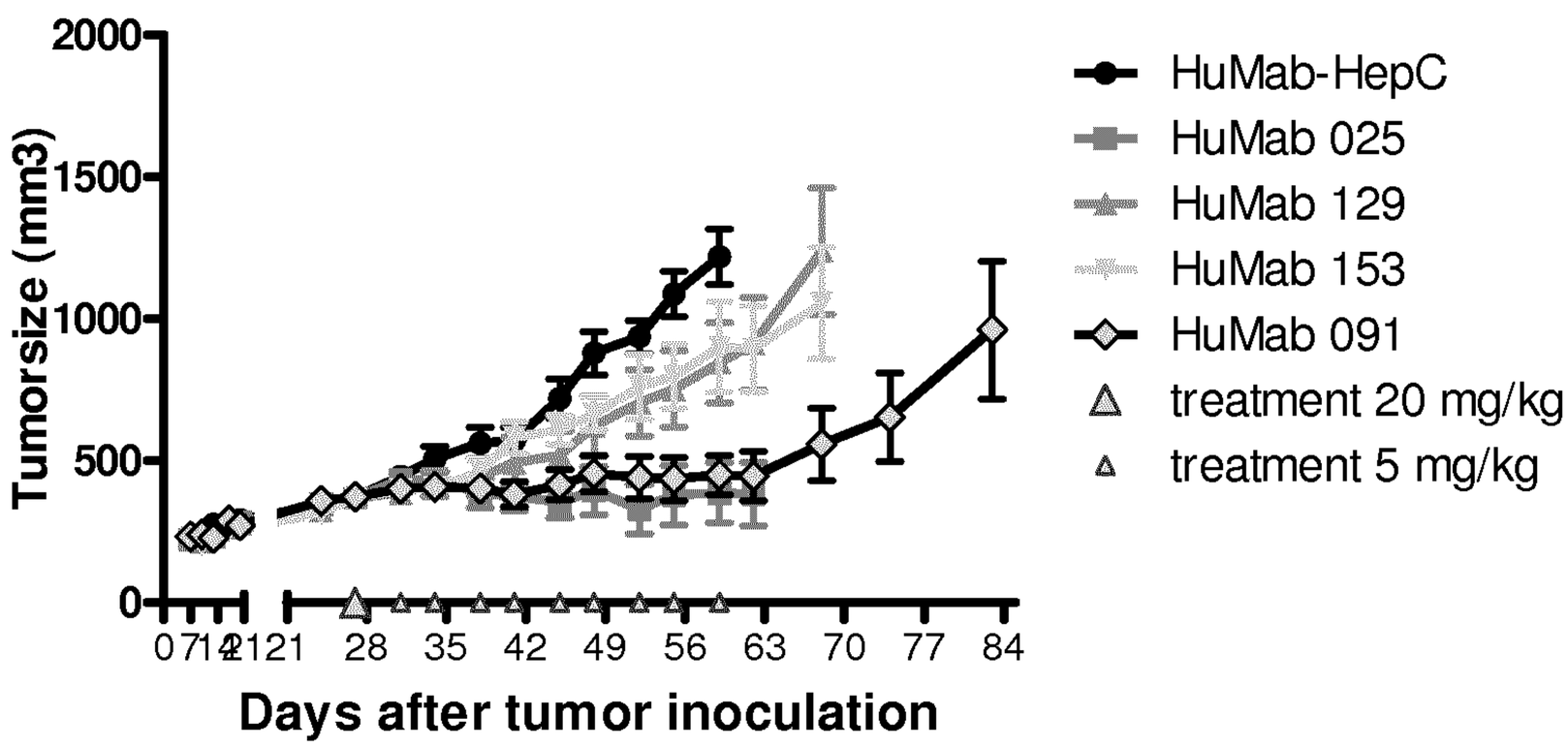


Figure 16B

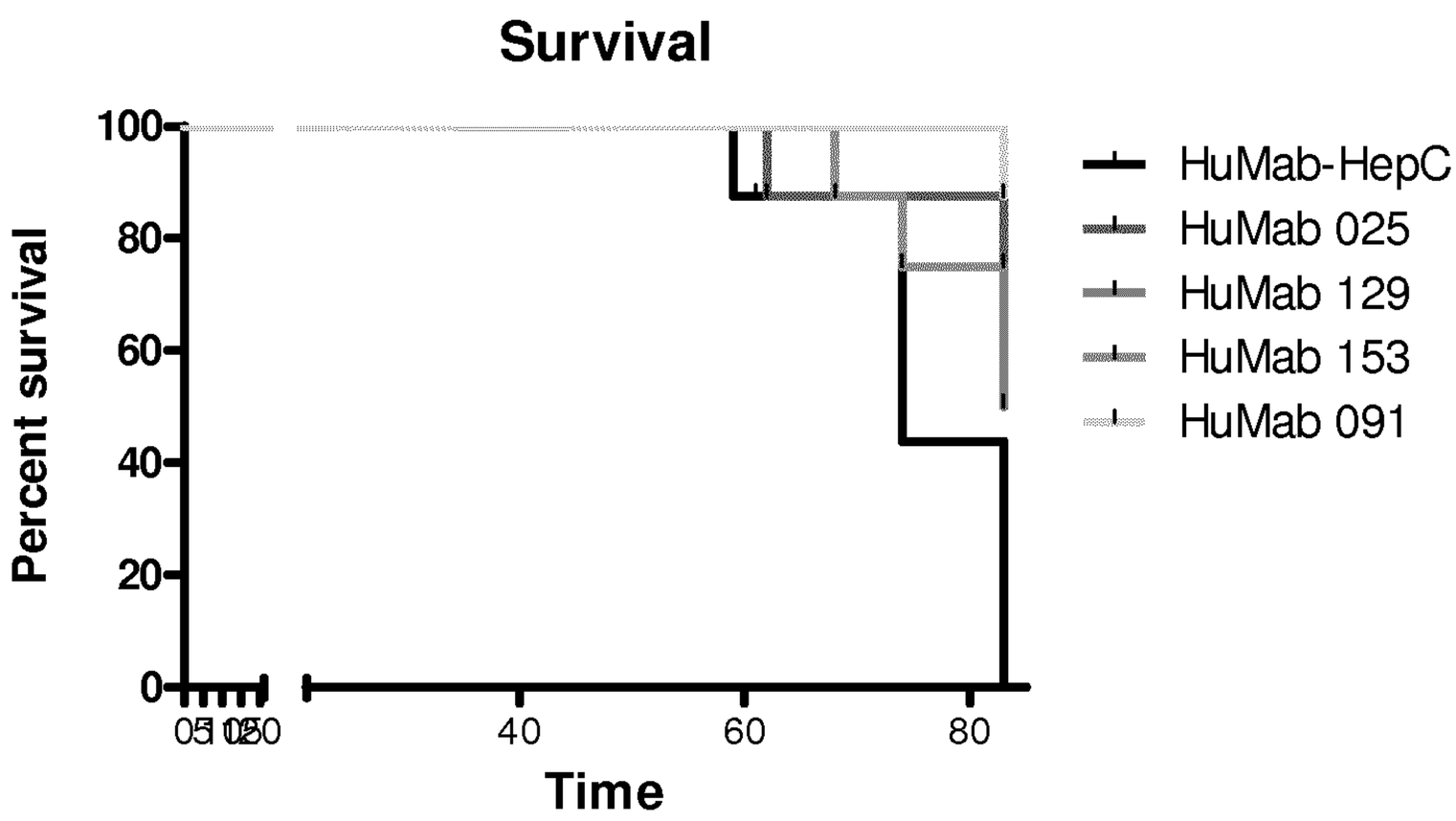


Figure 17

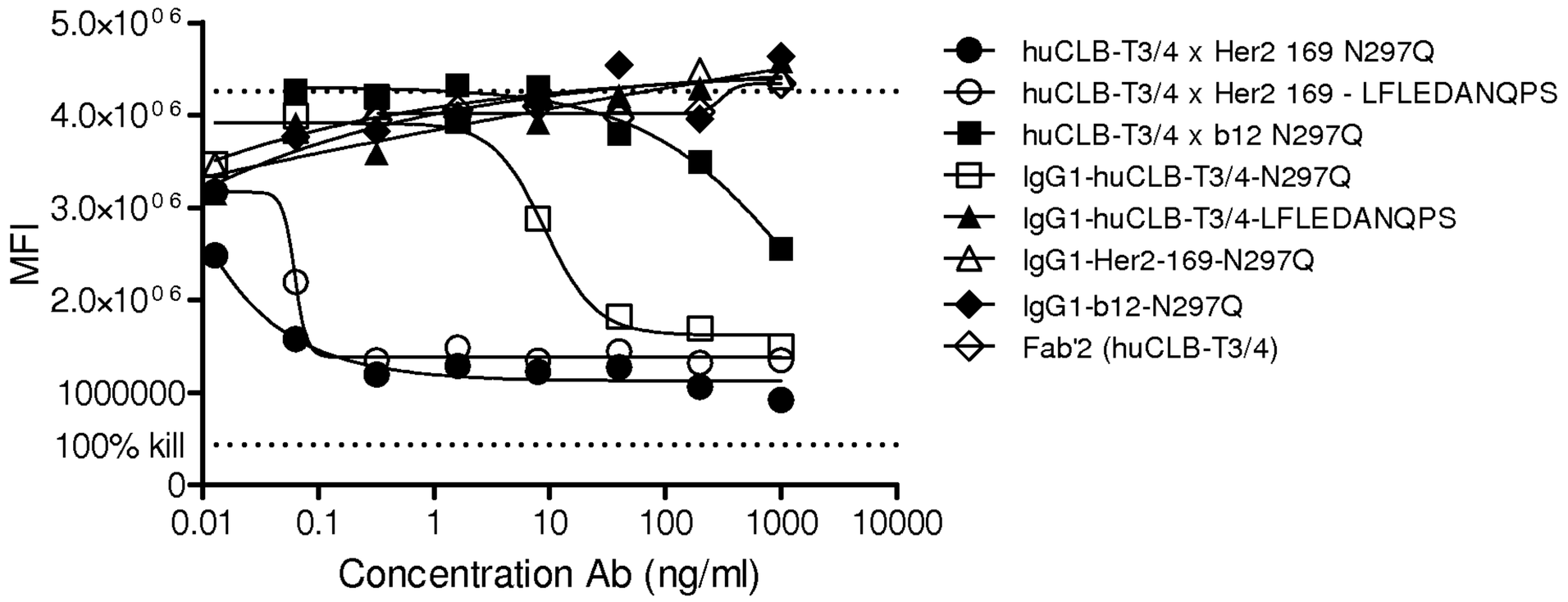


Figure 18A

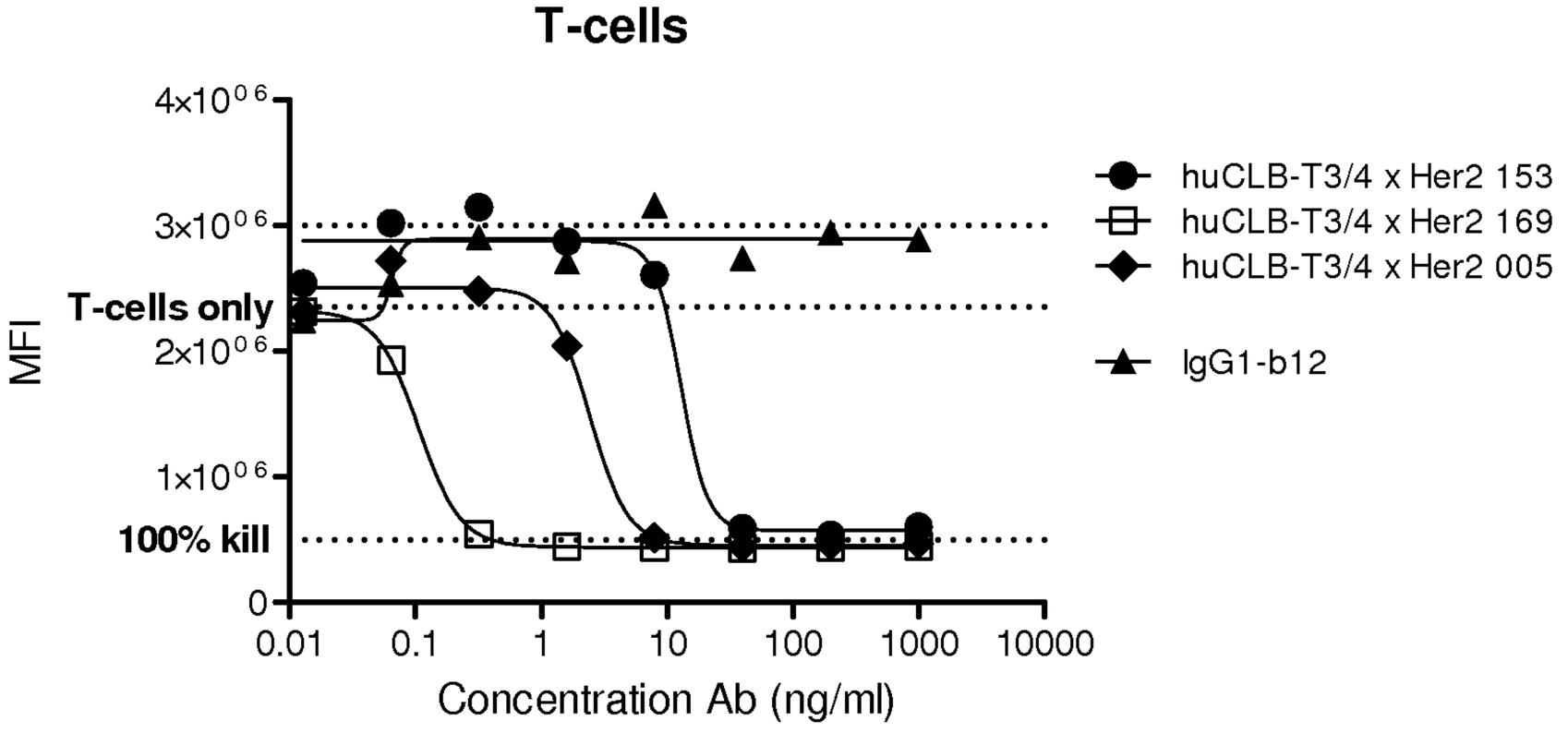


Figure 18B

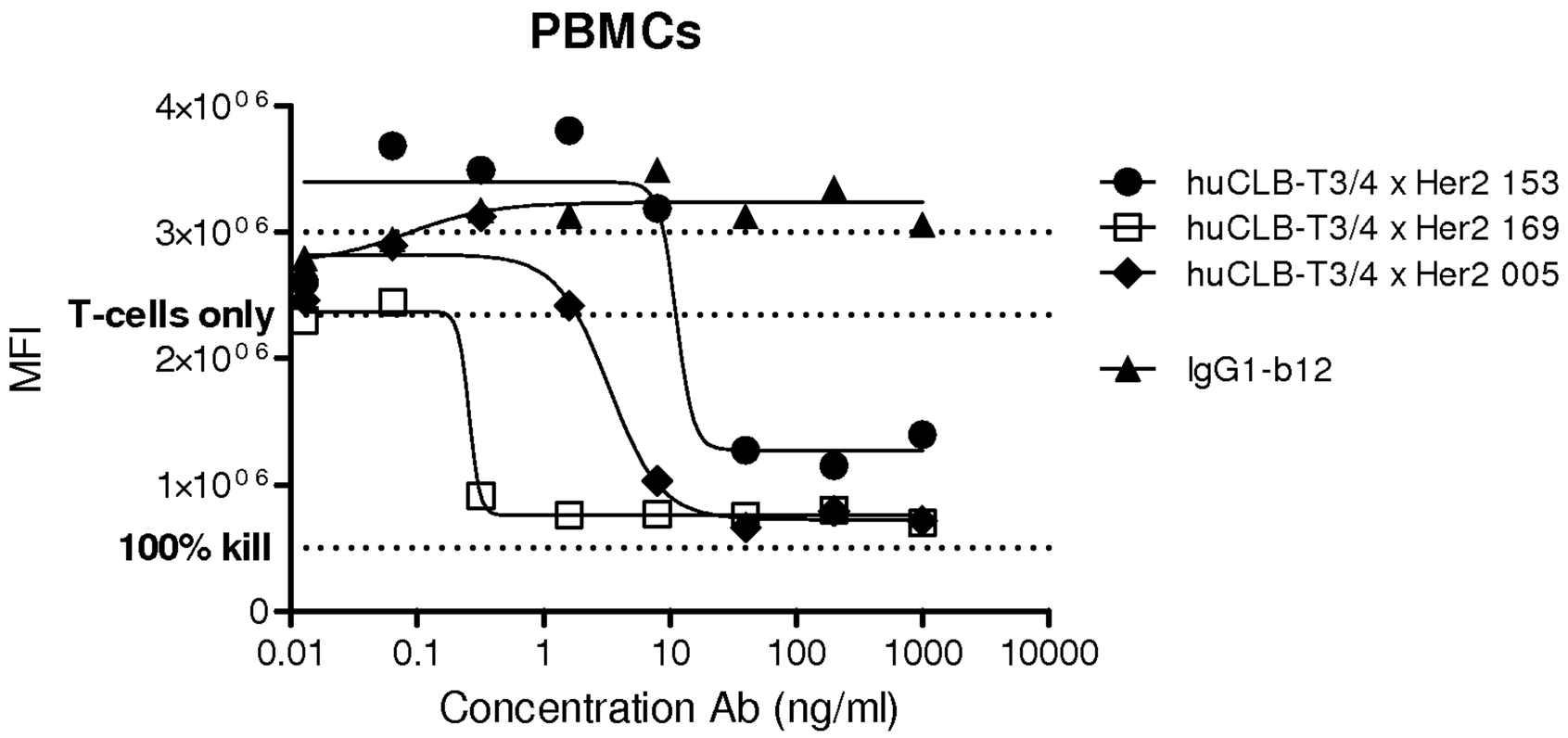


Figure 19

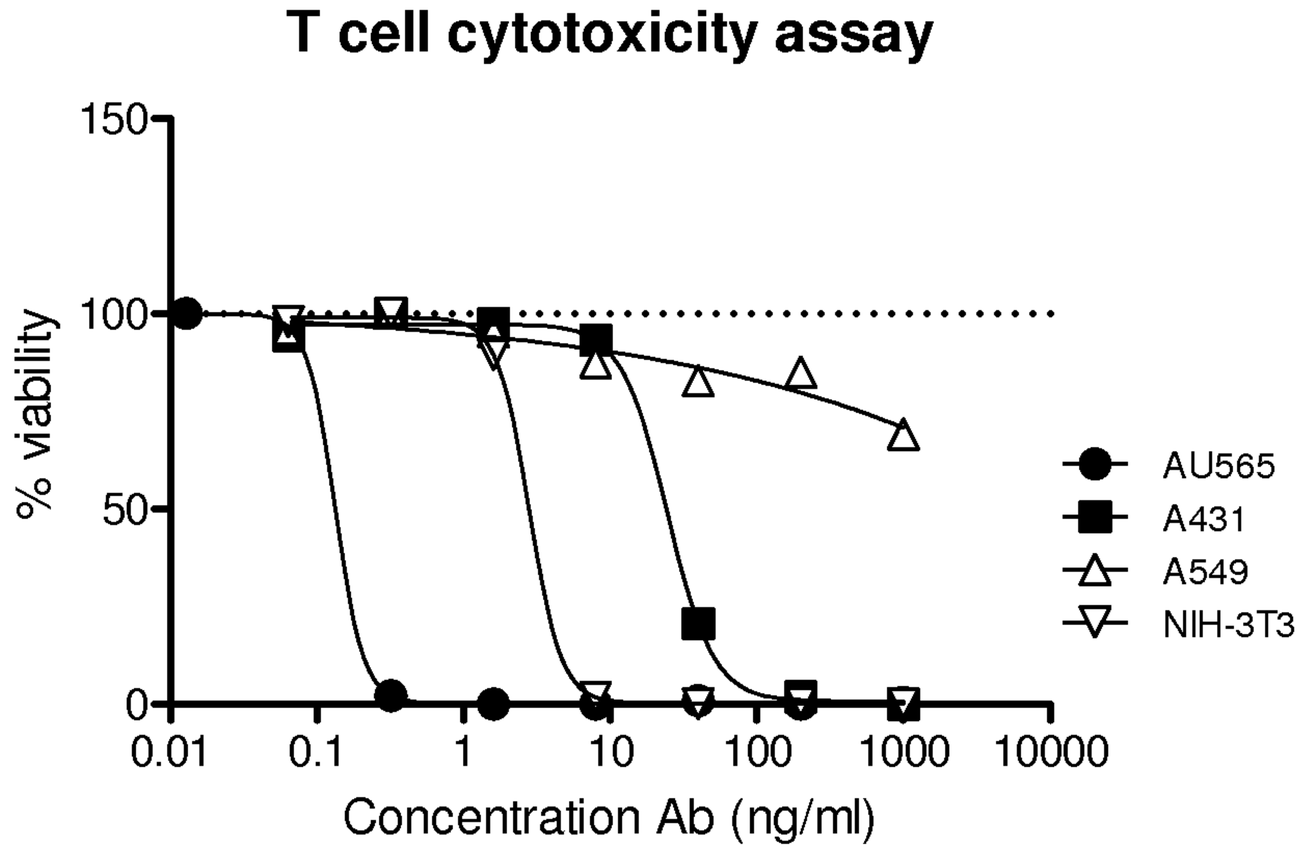


Figure 20

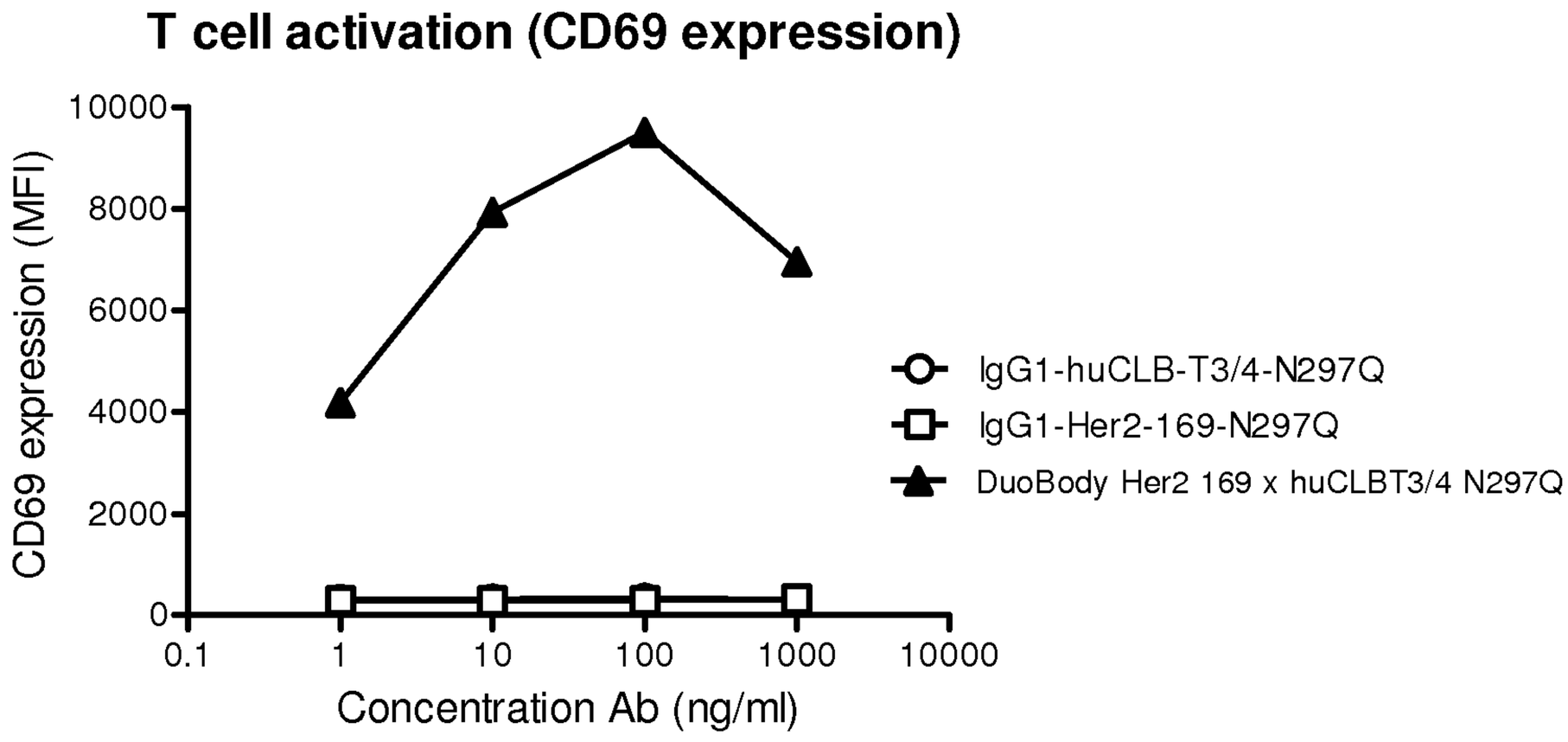


Figure 21

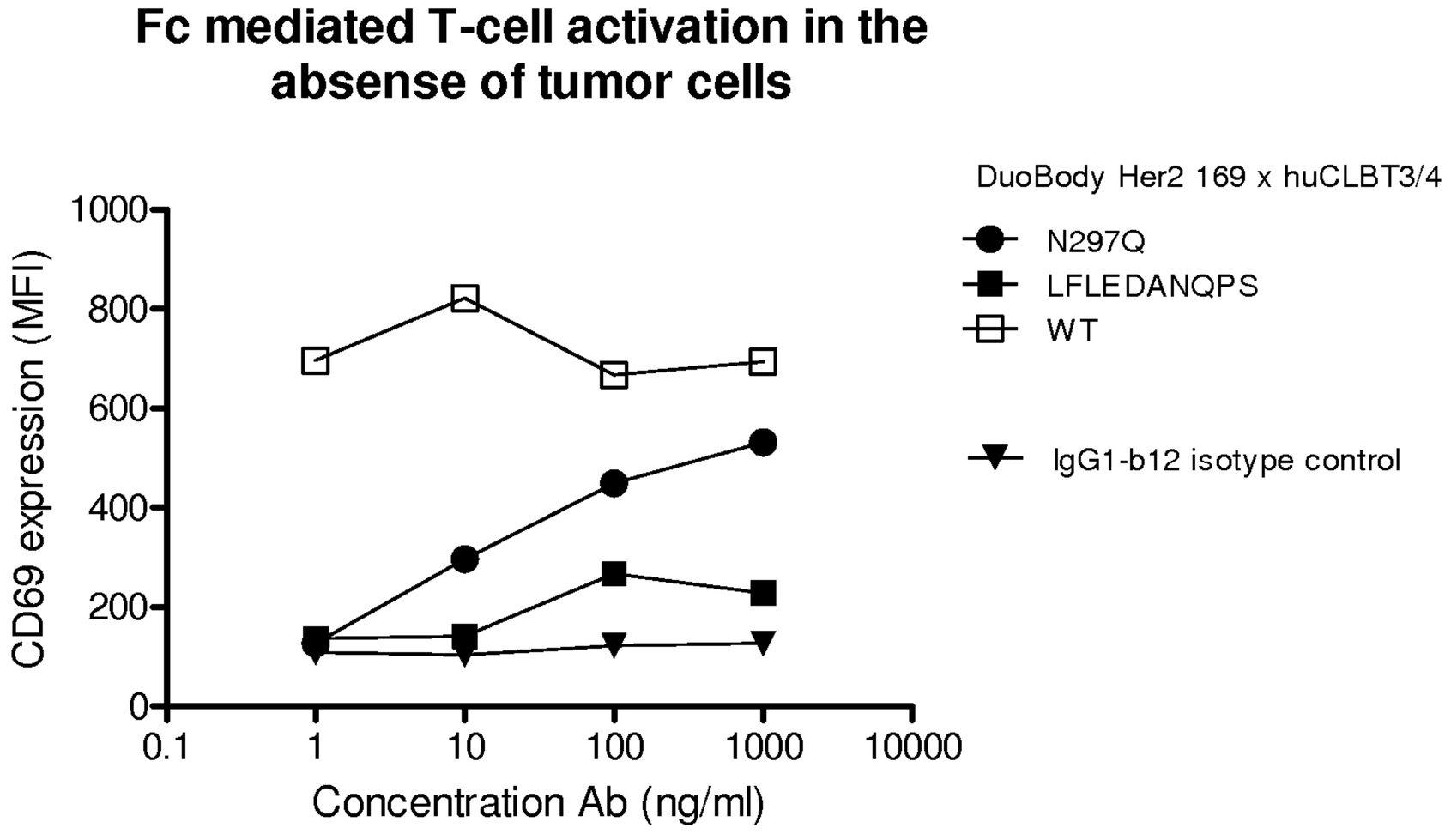


Figure 22

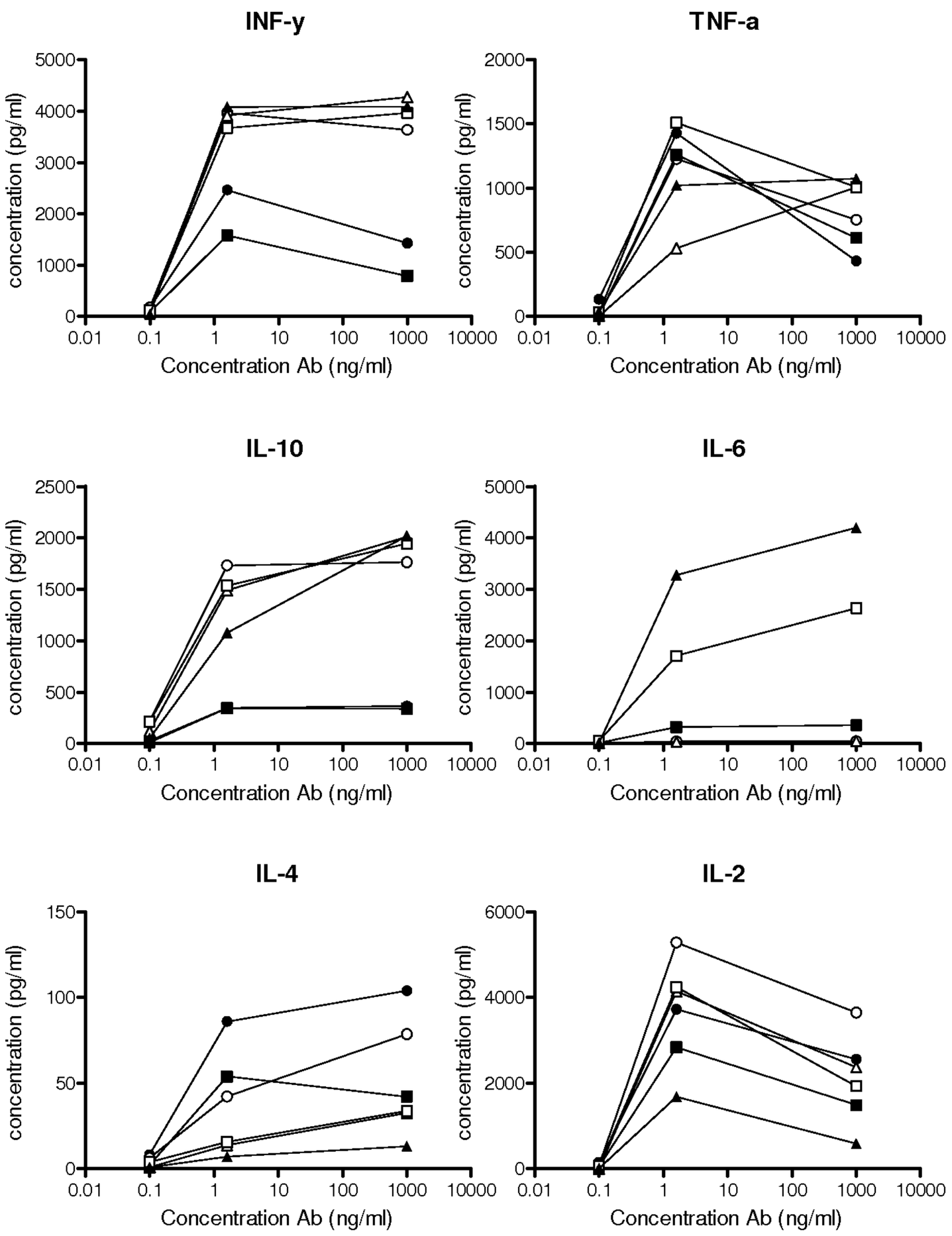

INF-y
TNF-a
IL-10
IL-6
IL-4
IL-2
concentration (pg/ml)
Concentration Ab (ng/ml)
0.01
0.1
1
10
100
1000
10000
DuoBody Her2 169 x huCLBT3/4 N297Q
T=1 day
T=2 days
T=3 days
T-cells
T=1 day
T=2 days
T=3 days
PBMCs

Figure 23

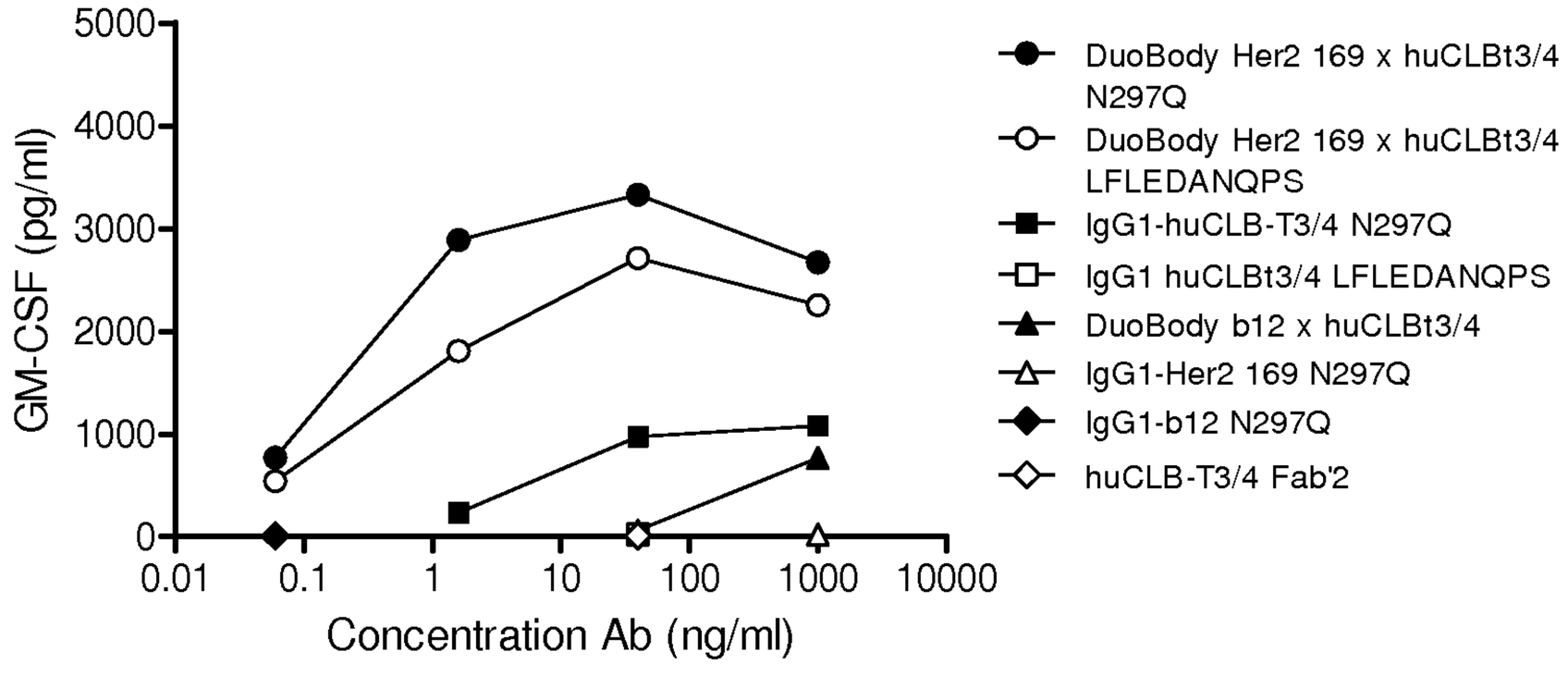


Figure 24A

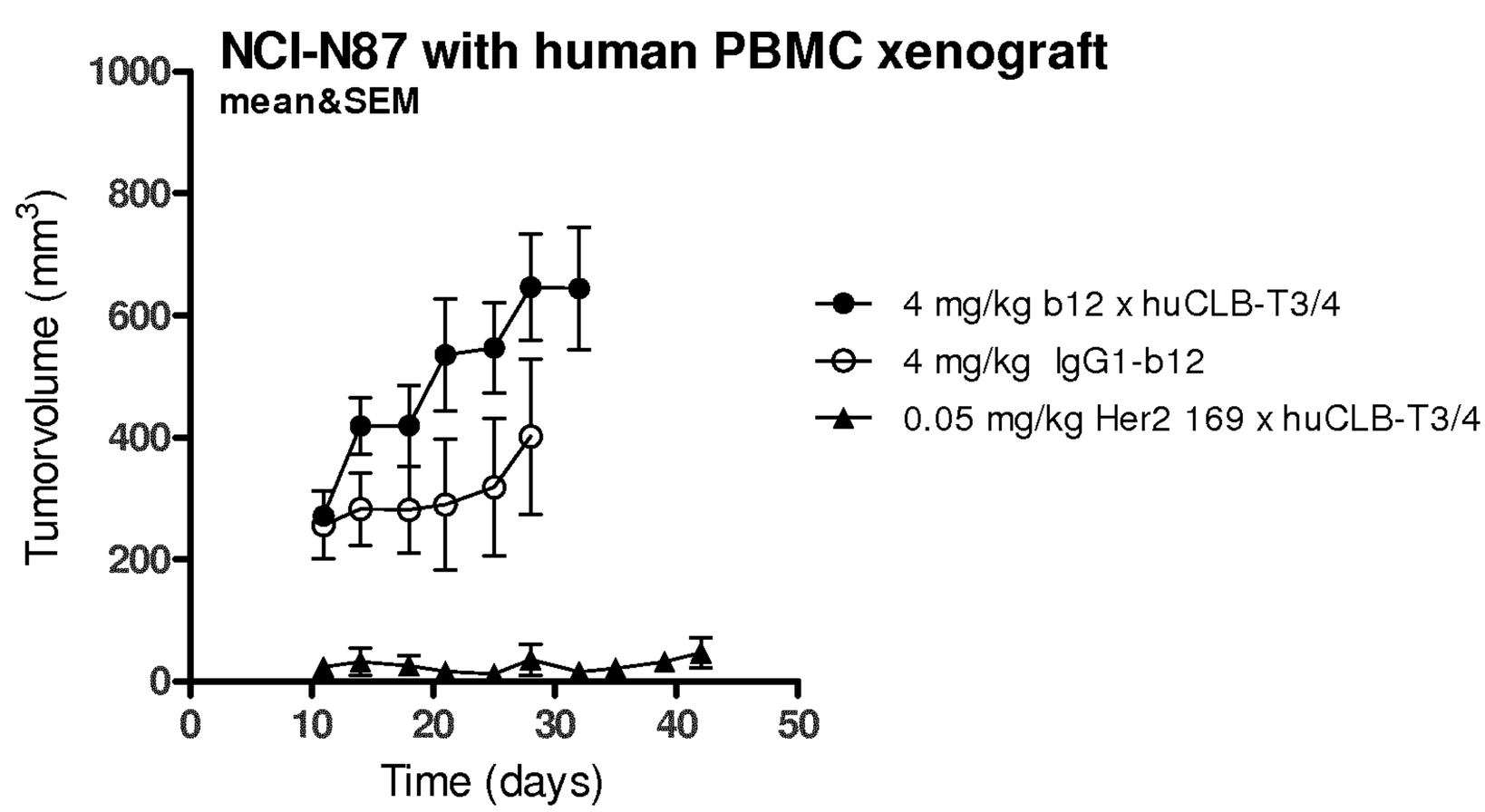


Figure 24B

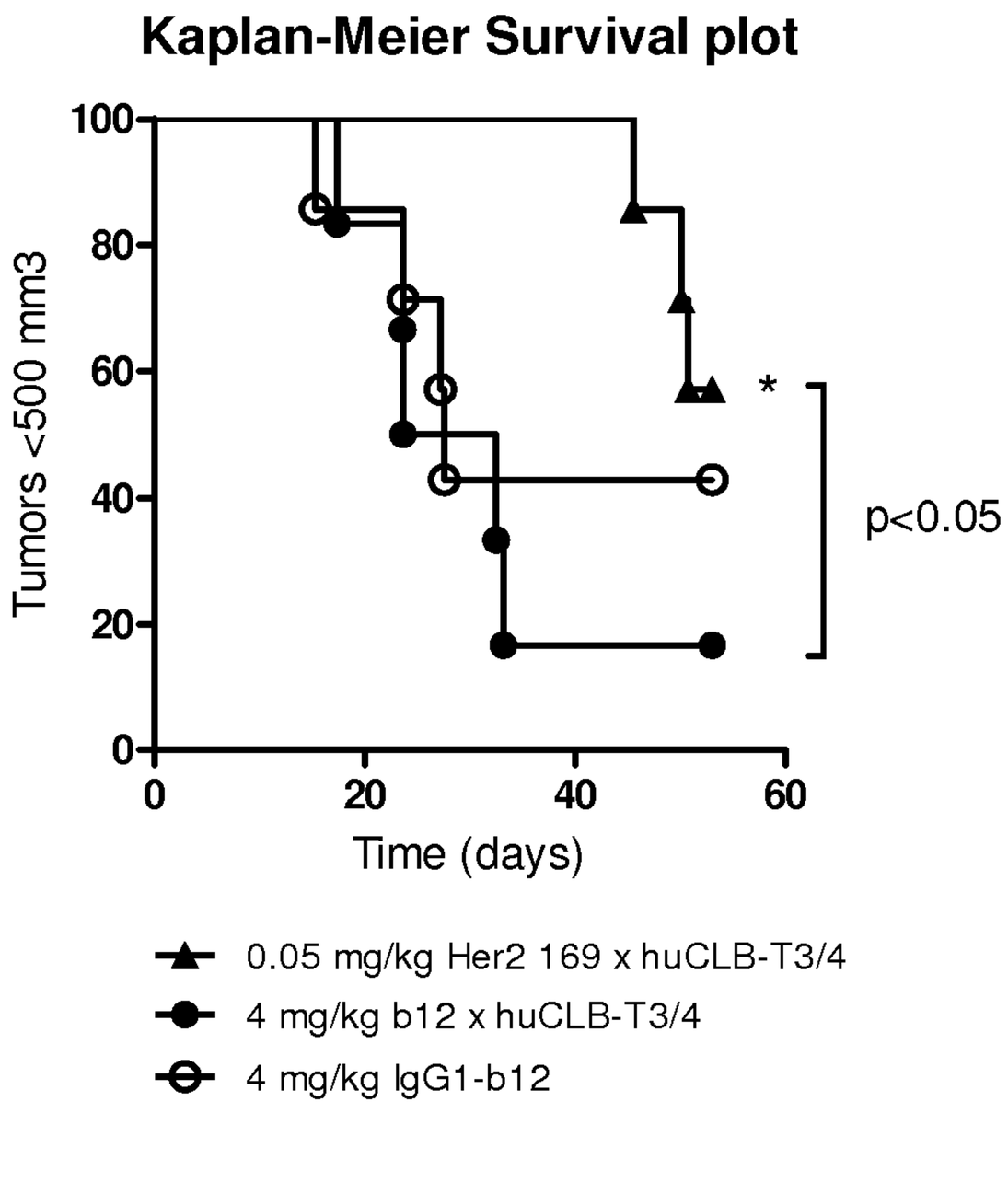


Figure 25A

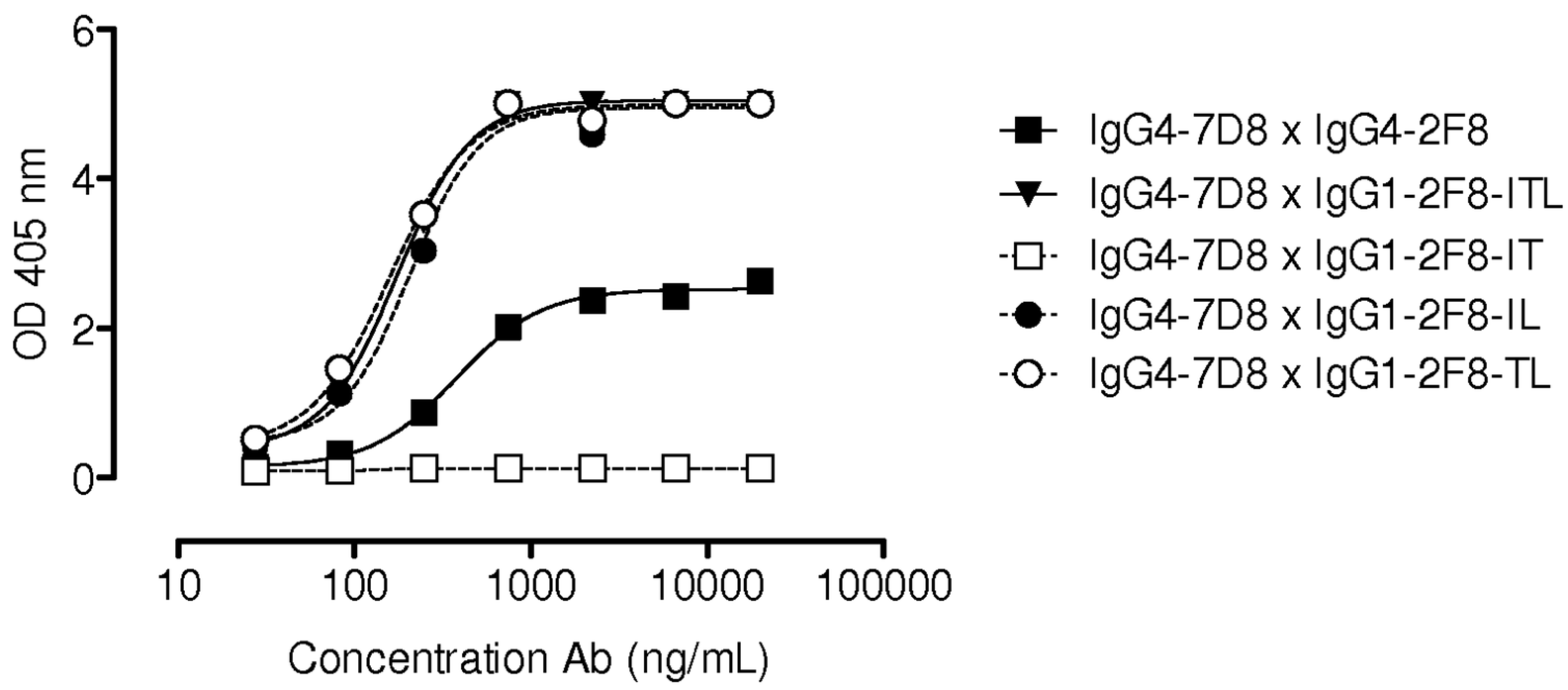


Figure 25B

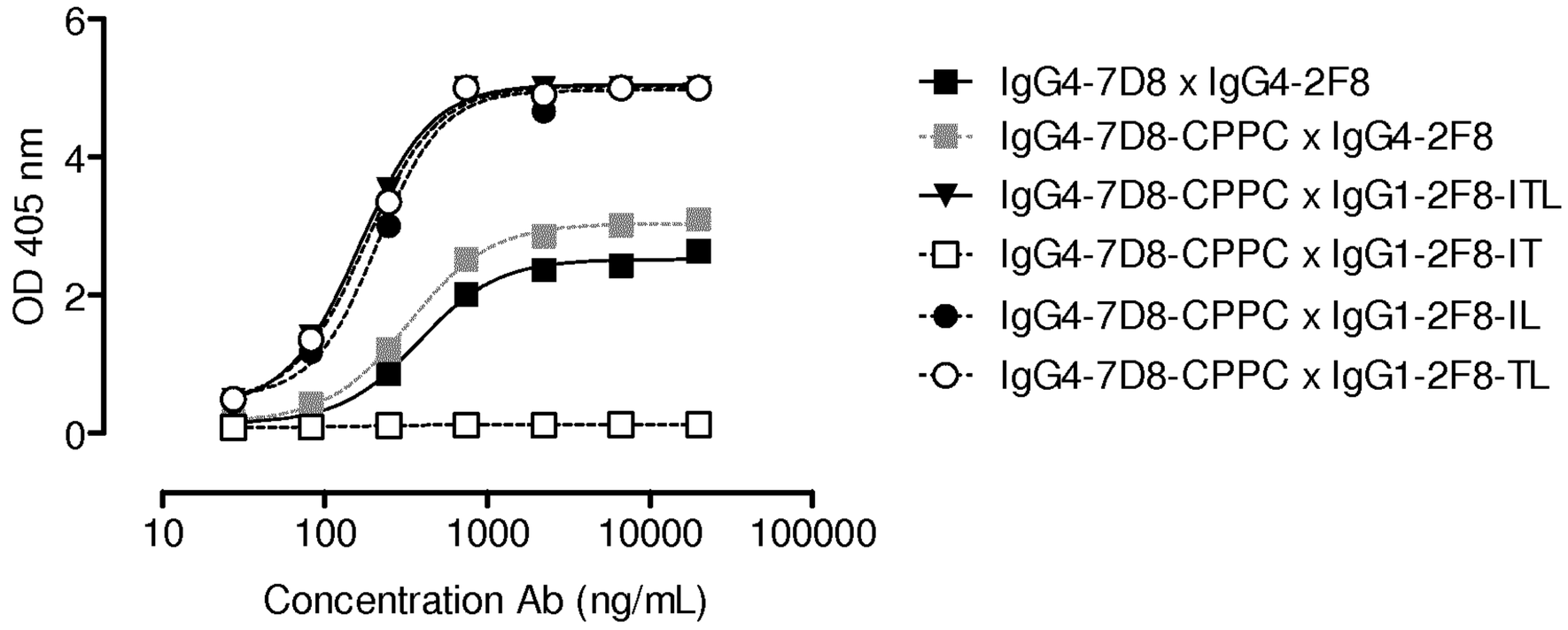


Figure 25C

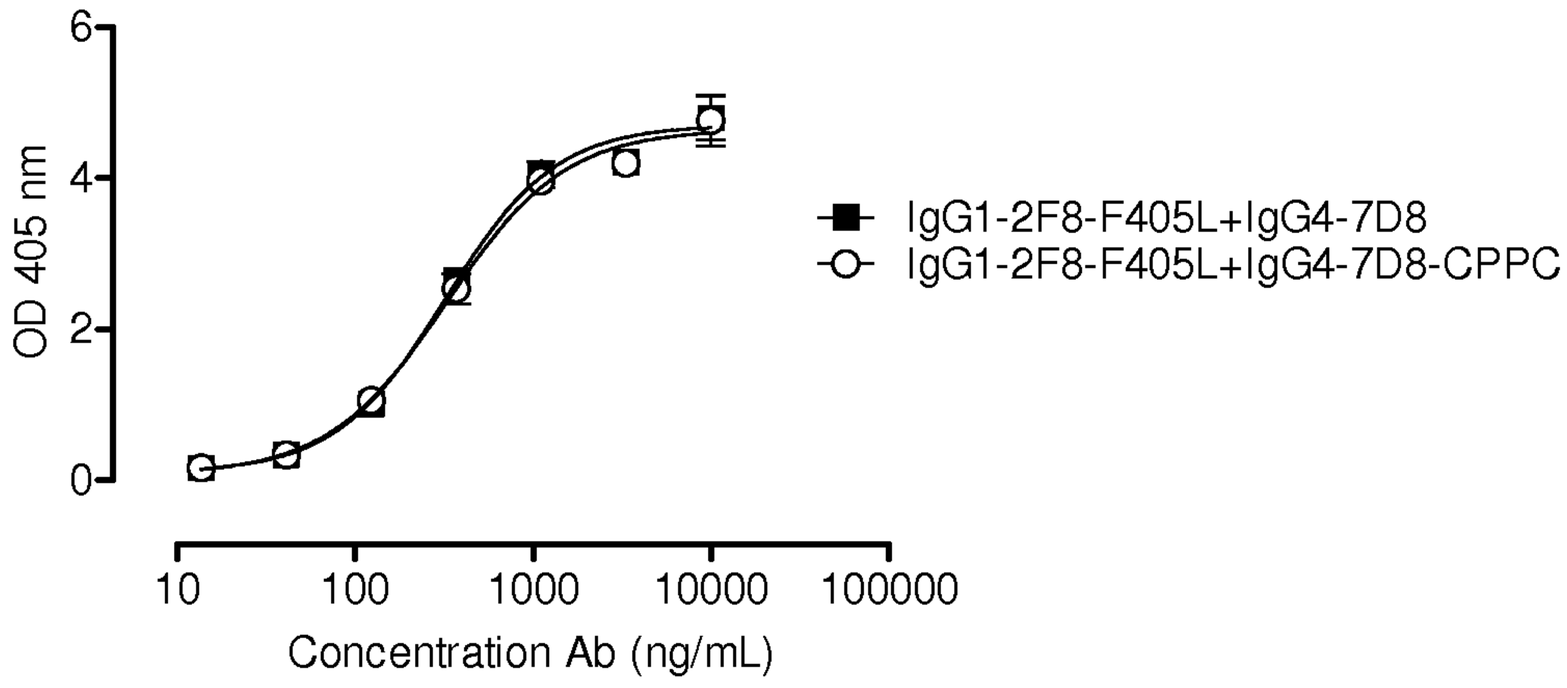


Figure 26A

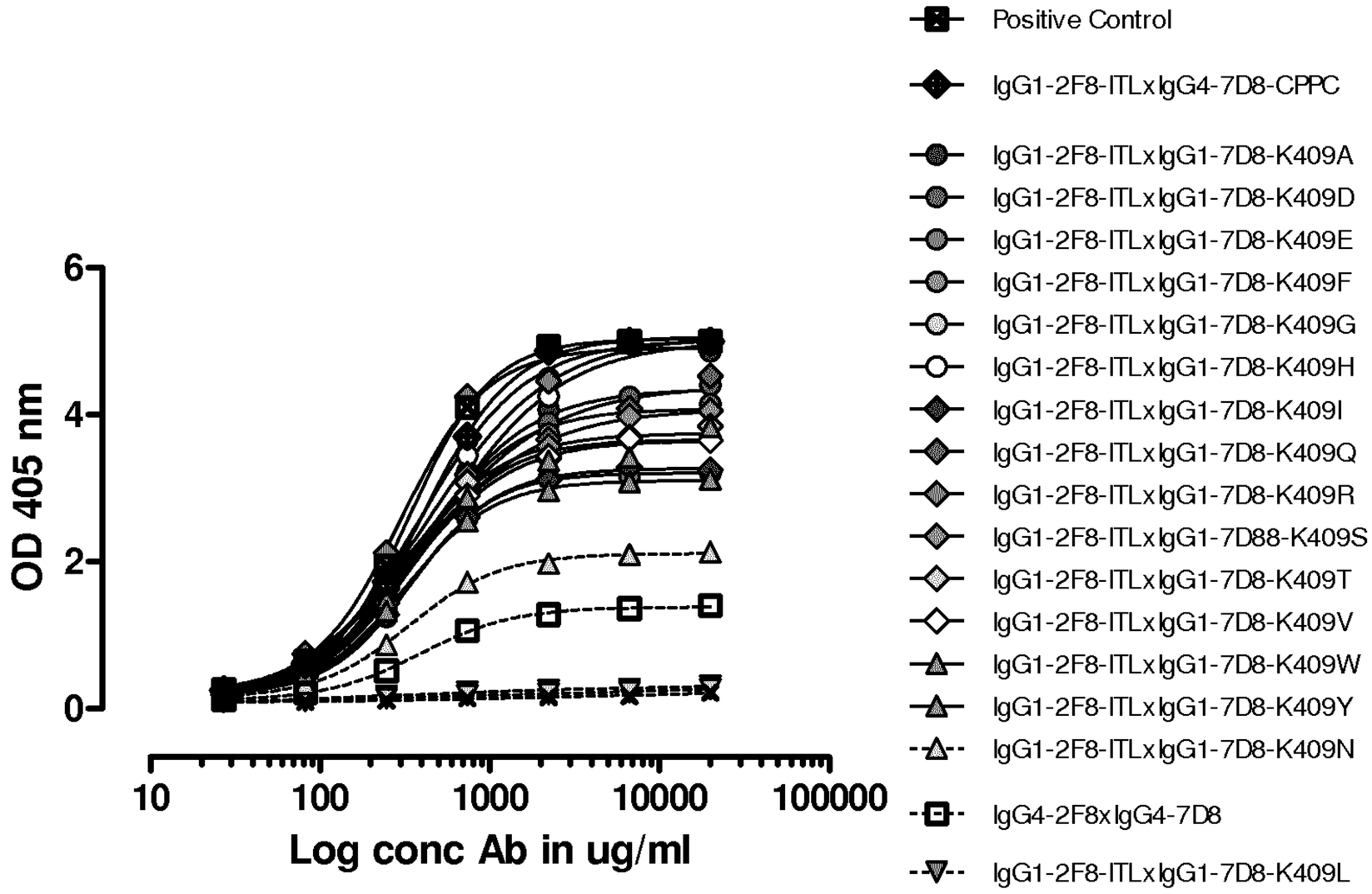


Figure 26B

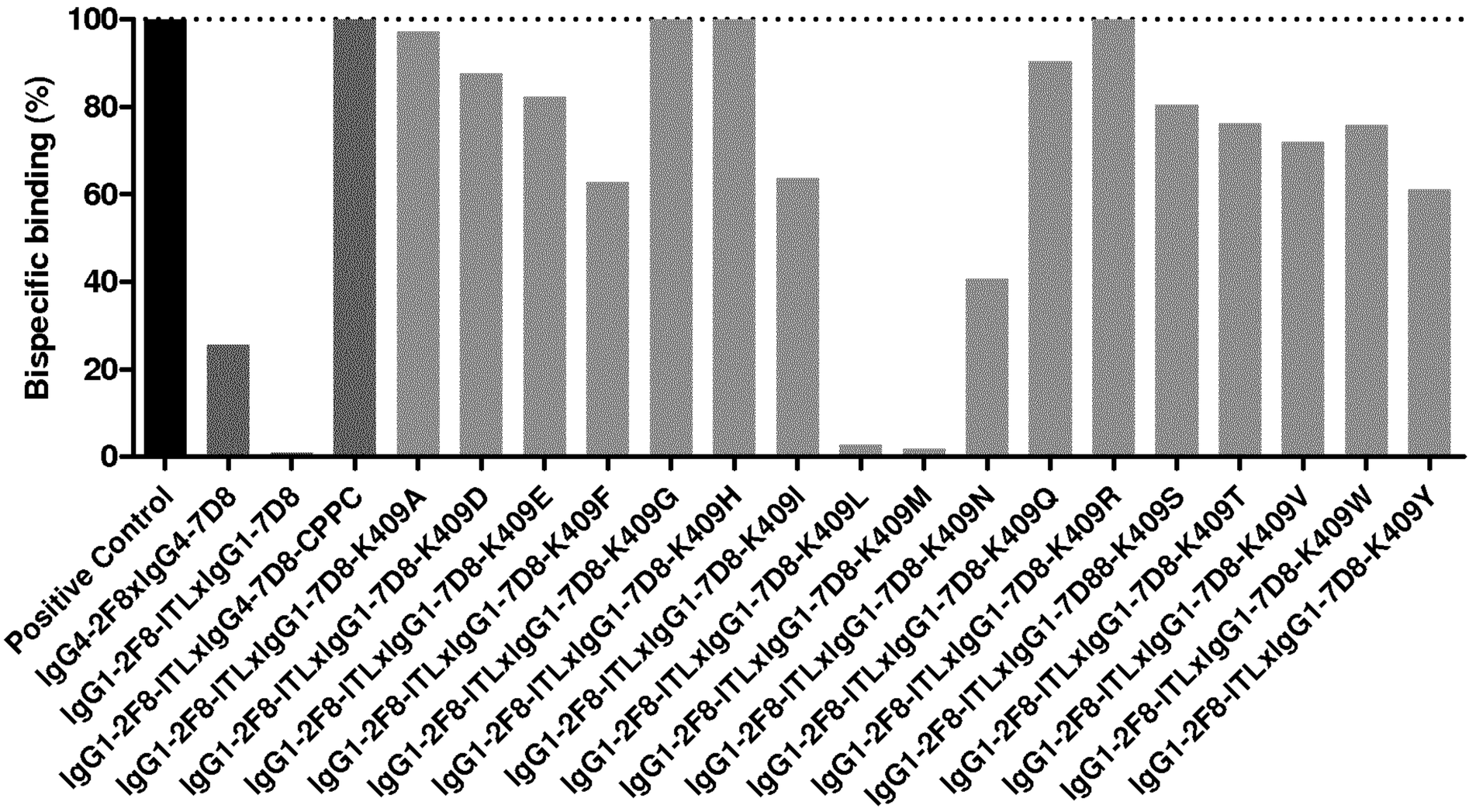


Figure 27A

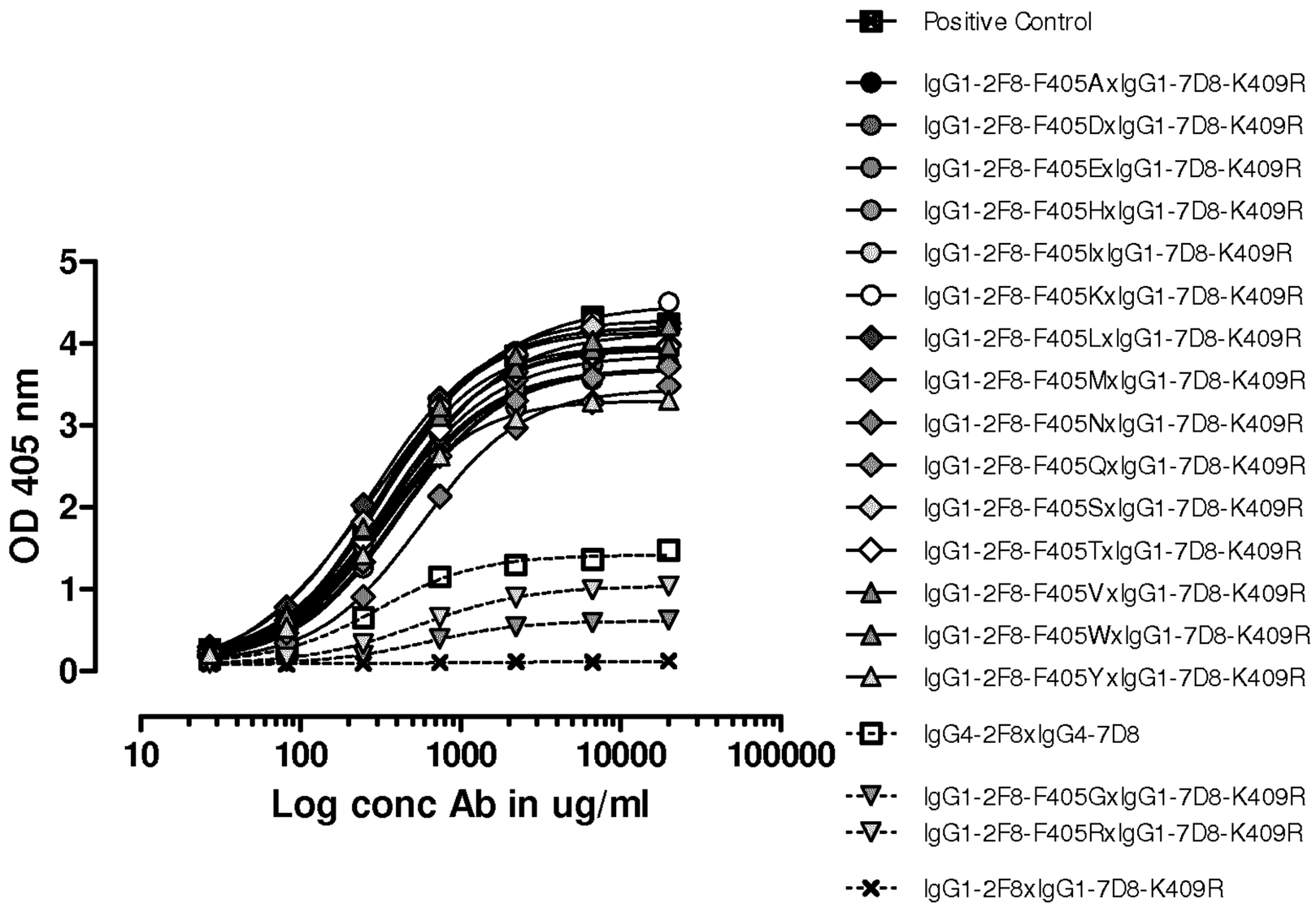


Figure 27B

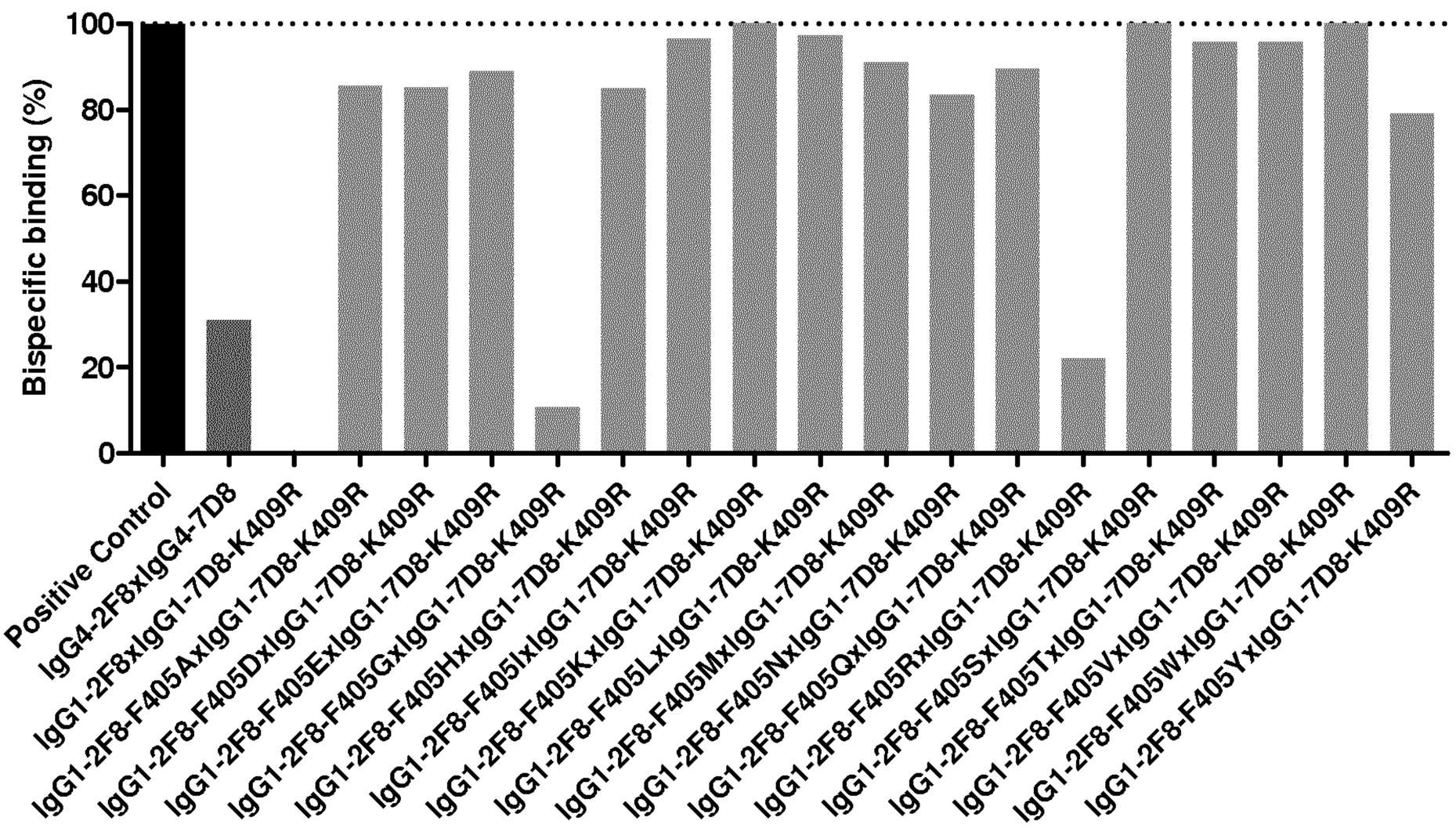


Figure 28A

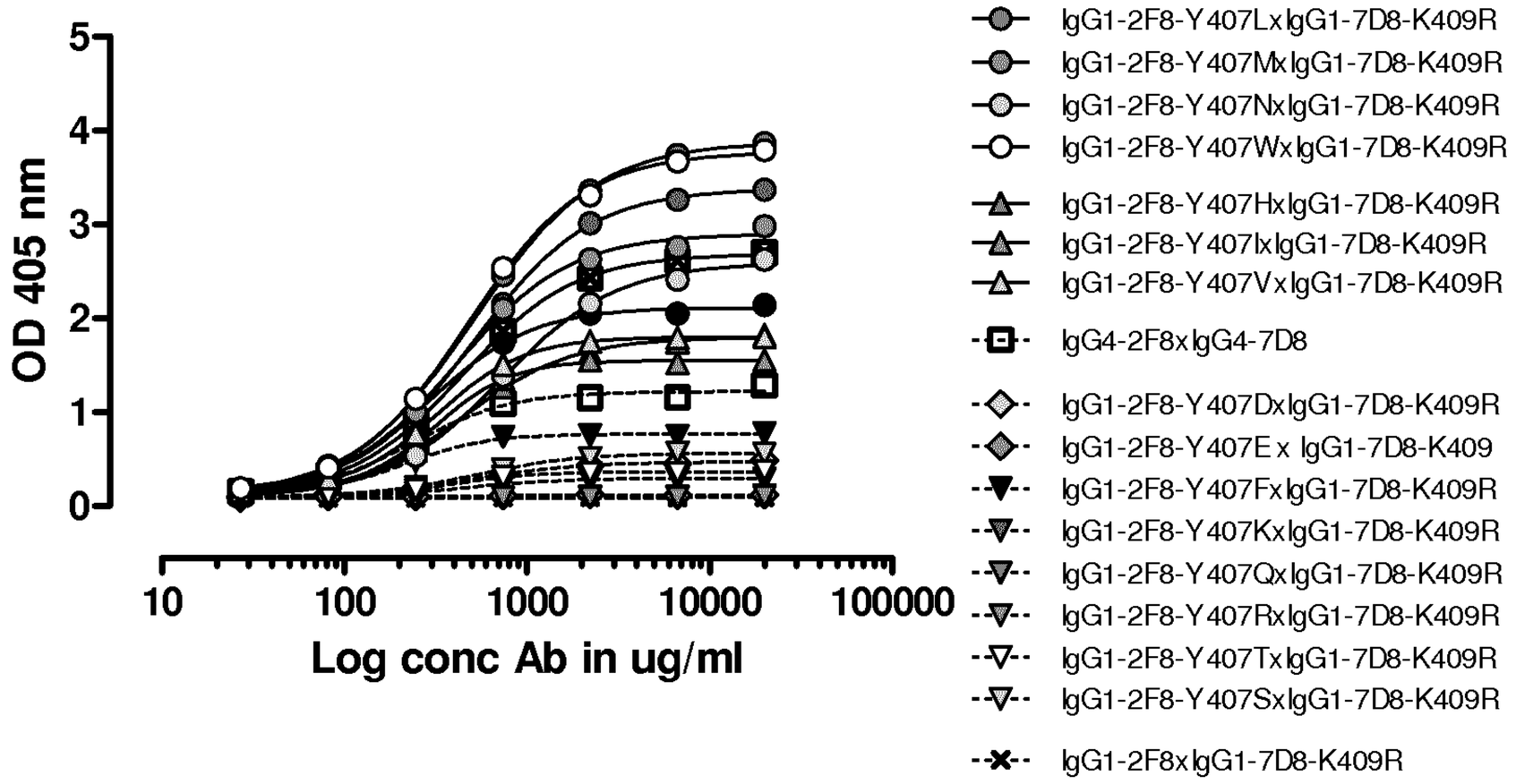


Figure 28B

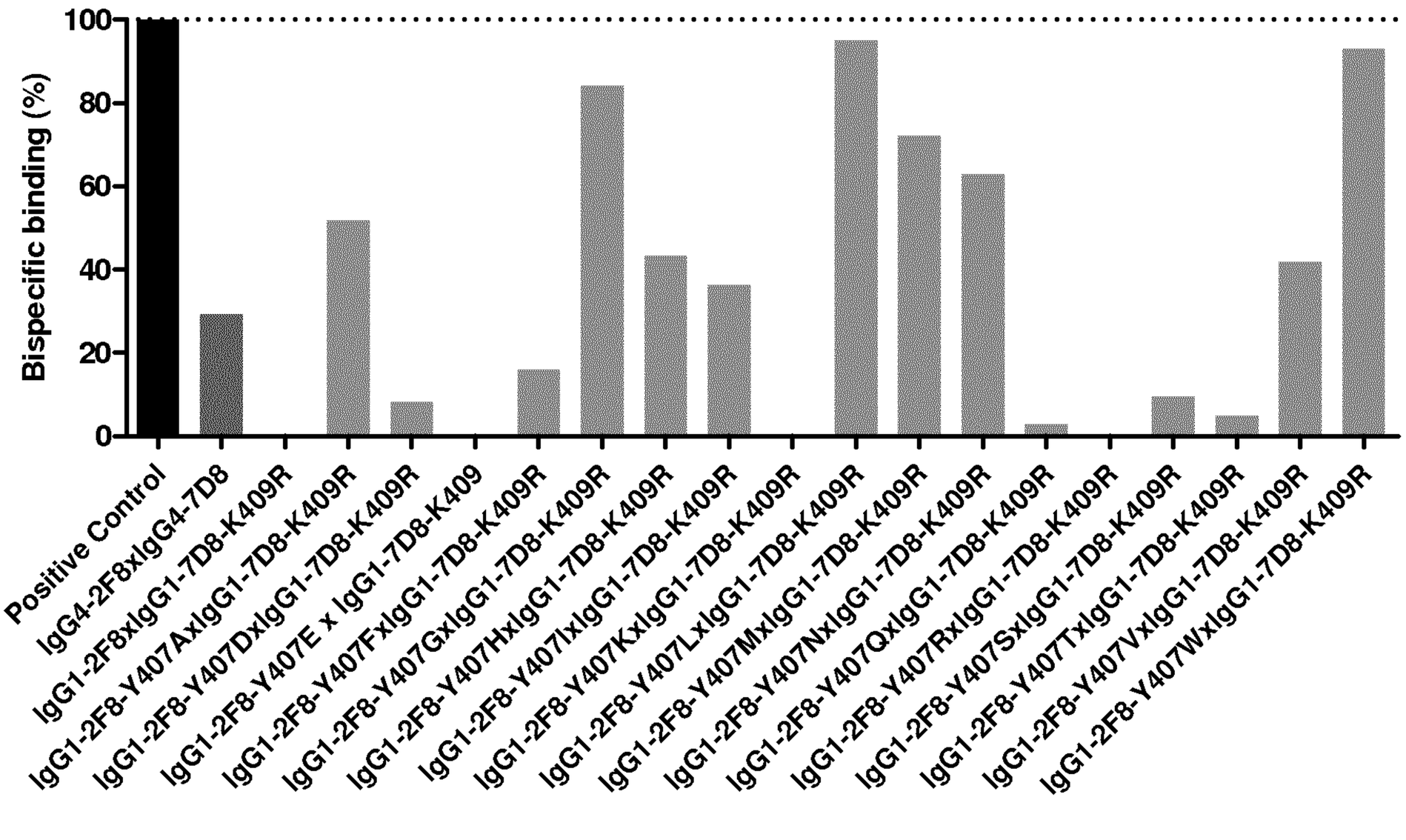


Figure 29A

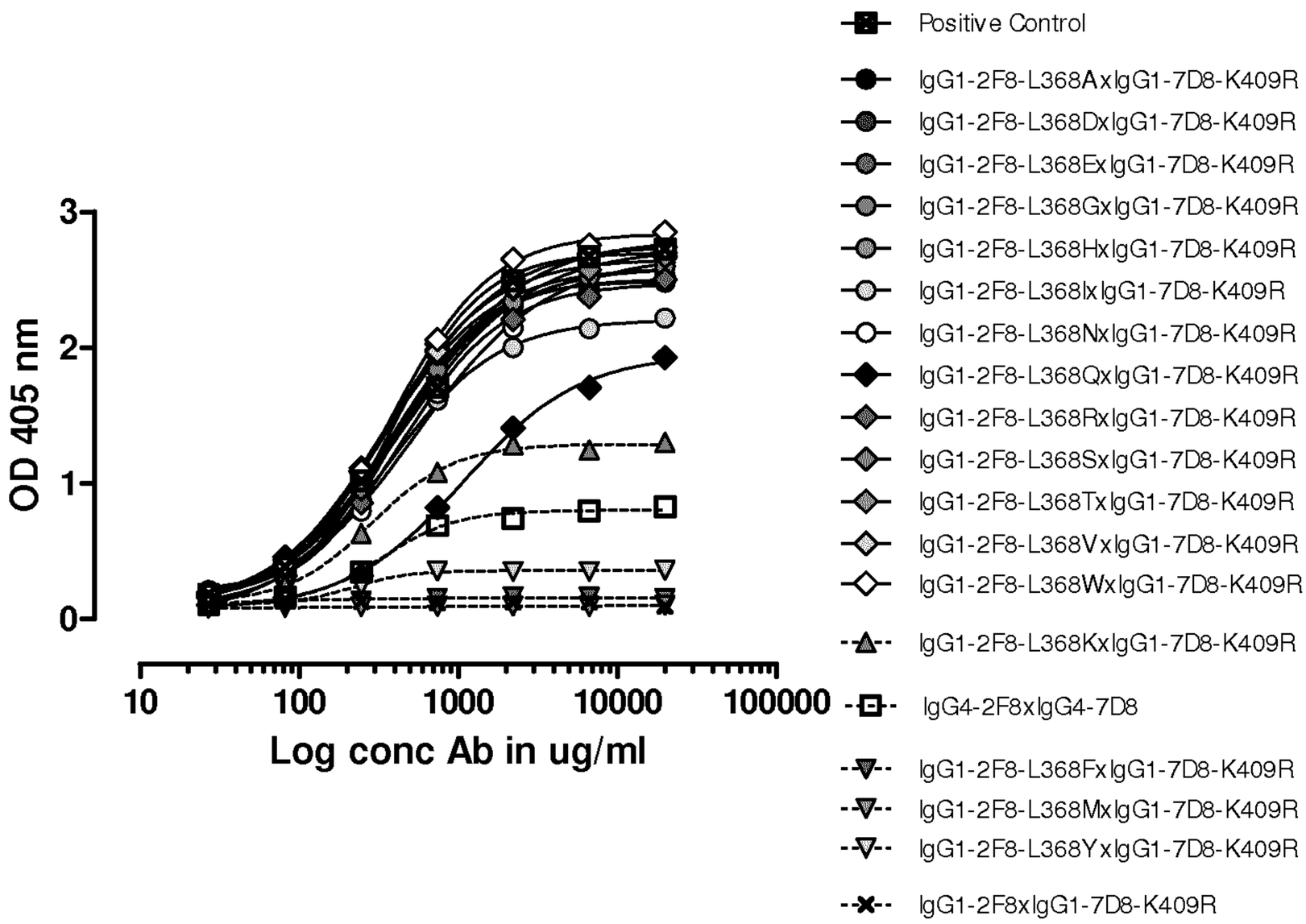


Figure 29B

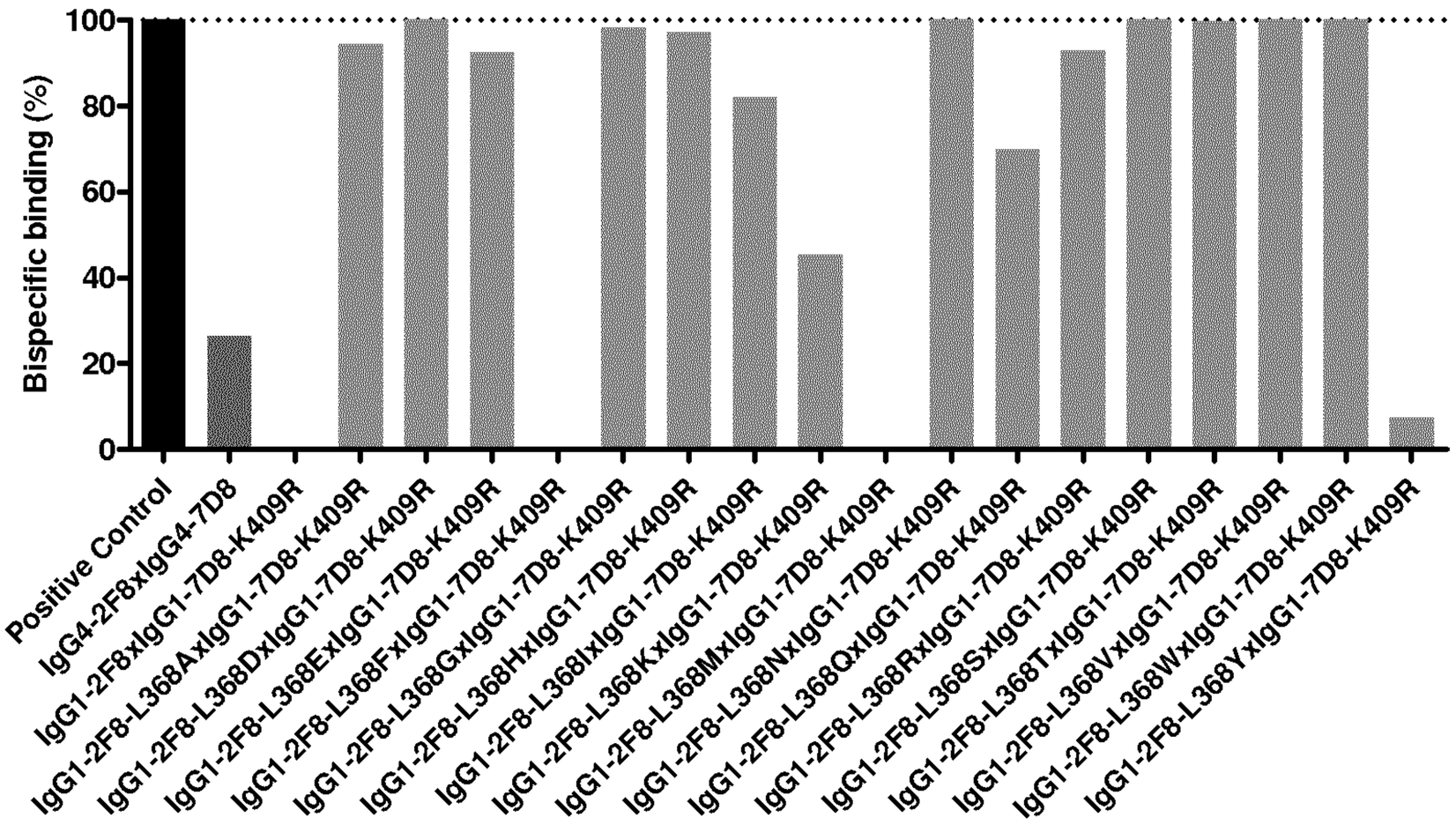
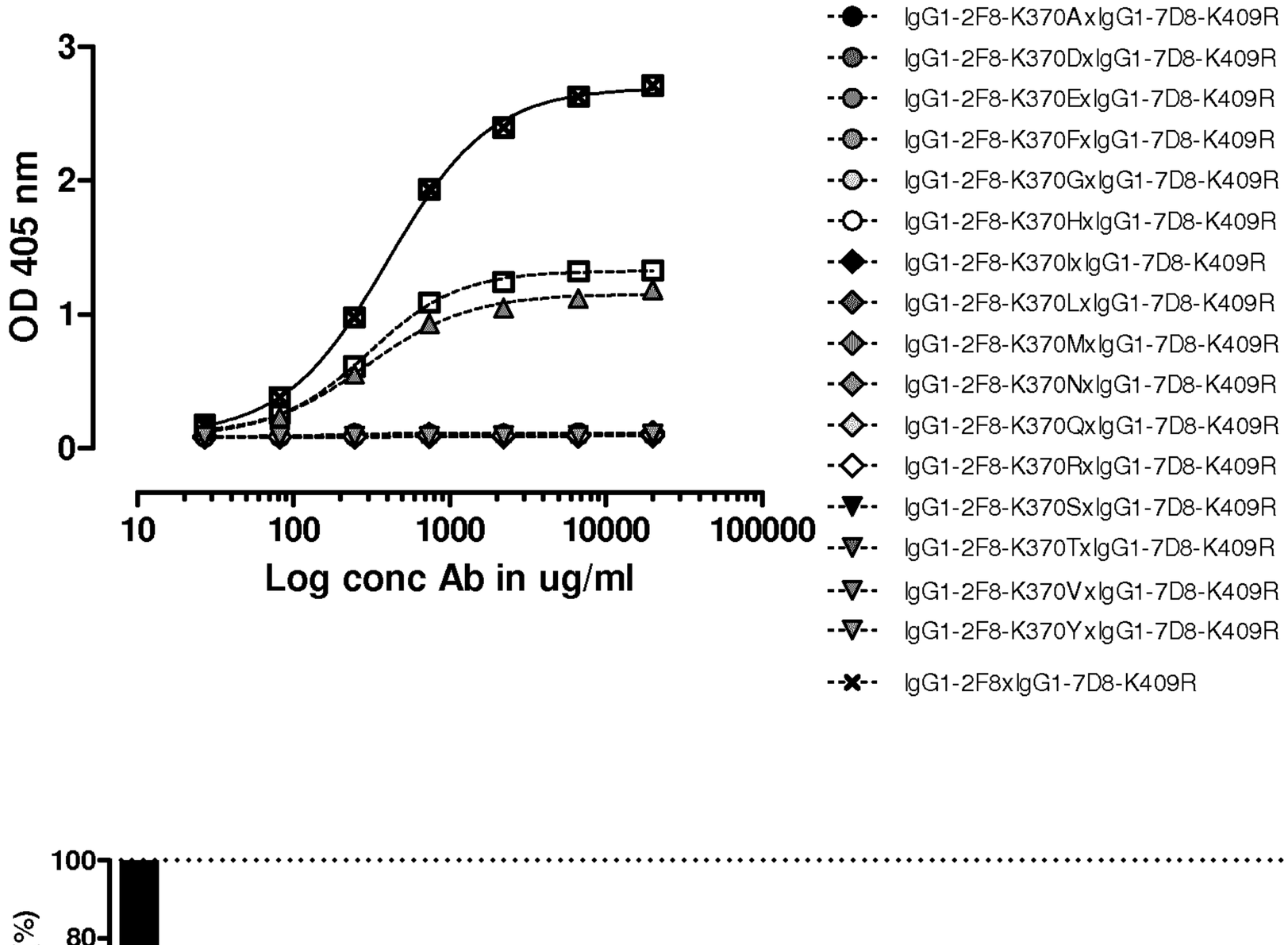


Figure 30

A


OD 405 nm
0
1
2
3
10
100
1000
10000
100000
Log conc Ab in ug/ml
Positive Control
IgG4-2F8xIgG4-7D8
IgG1-2F8-K370WxIgG1-7D8-K409R
IgG1-2F8-K370AxIgG1-7D8-K409R
IgG1-2F8-K370DxIgG1-7D8-K409R
IgG1-2F8-K370ExIgG1-7D8-K409R
IgG1-2F8-K370FxIgG1-7D8-K409R
IgG1-2F8-K370GxIgG1-7D8-K409R
IgG1-2F8-K370HxIgG1-7D8-K409R
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IgG1-2F8-K370LxIgG1-7D8-K409R
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IgG1-2F8-K370NxIgG1-7D8-K409R
IgG1-2F8-K370QxIgG1-7D8-K409R
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IgG1-2F8-K370TxIgG1-7D8-K409R
IgG1-2F8-K370VxIgG1-7D8-K409R
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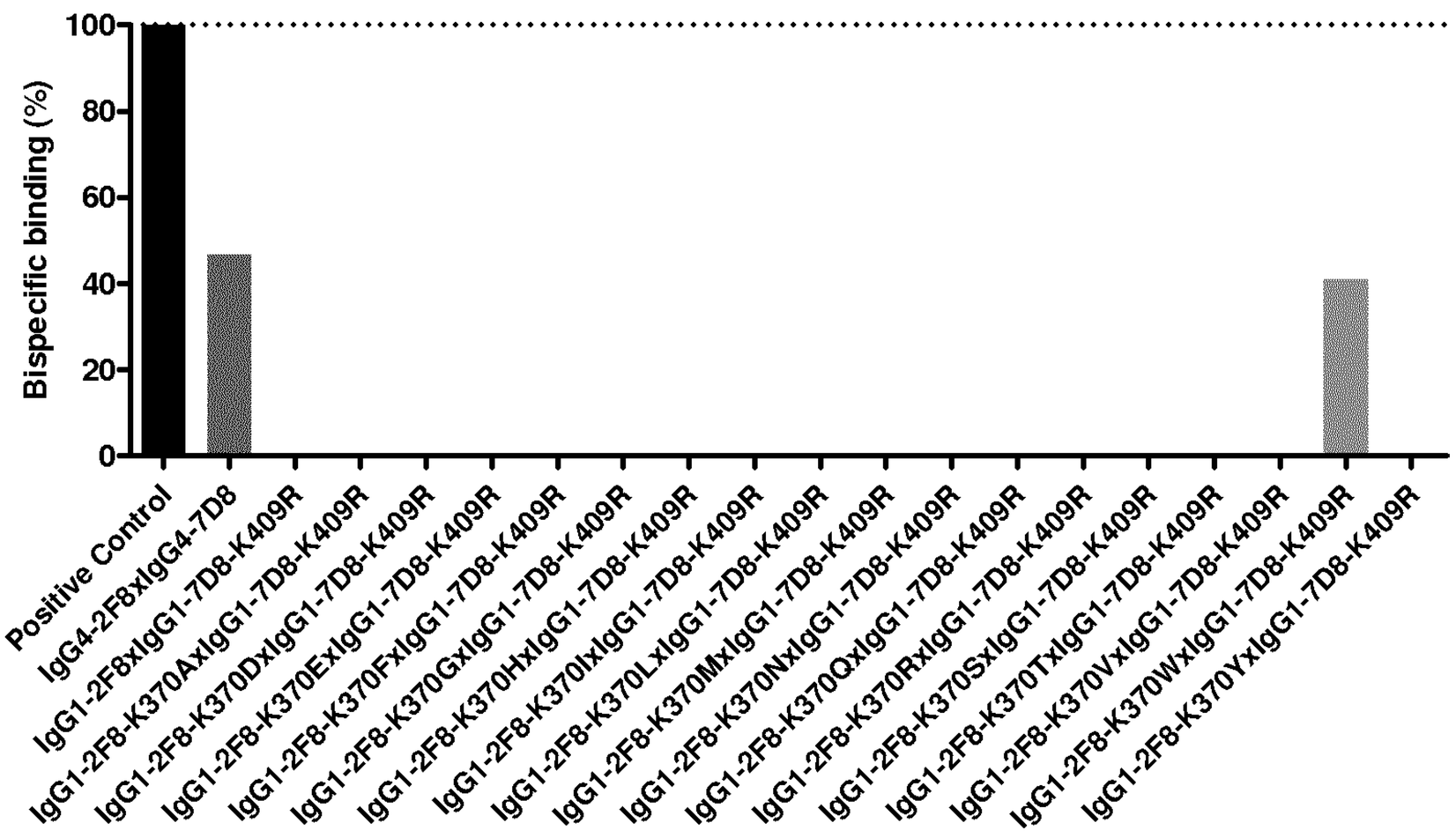
IgG1-2F8xIgG1-7D8-K409R

B


Bispecific binding (%)
0
20
40
60
80
100
Positive Control
IgG4-2F8xIgG4-7D8
IgG1-2F8xIgG1-7D8-K409R
IgG1-2F8-K370AxIgG1-7D8-K409R
IgG1-2F8-K370DxIgG1-7D8-K409R
IgG1-2F8-K370ExIgG1-7D8-K409R
IgG1-2F8-K370FxIgG1-7D8-K409R
IgG1-2F8-K370GxIgG1-7D8-K409R
IgG1-2F8-K370HxIgG1-7D8-K409R
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IgG1-2F8-K370VxIgG1-7D8-K409R
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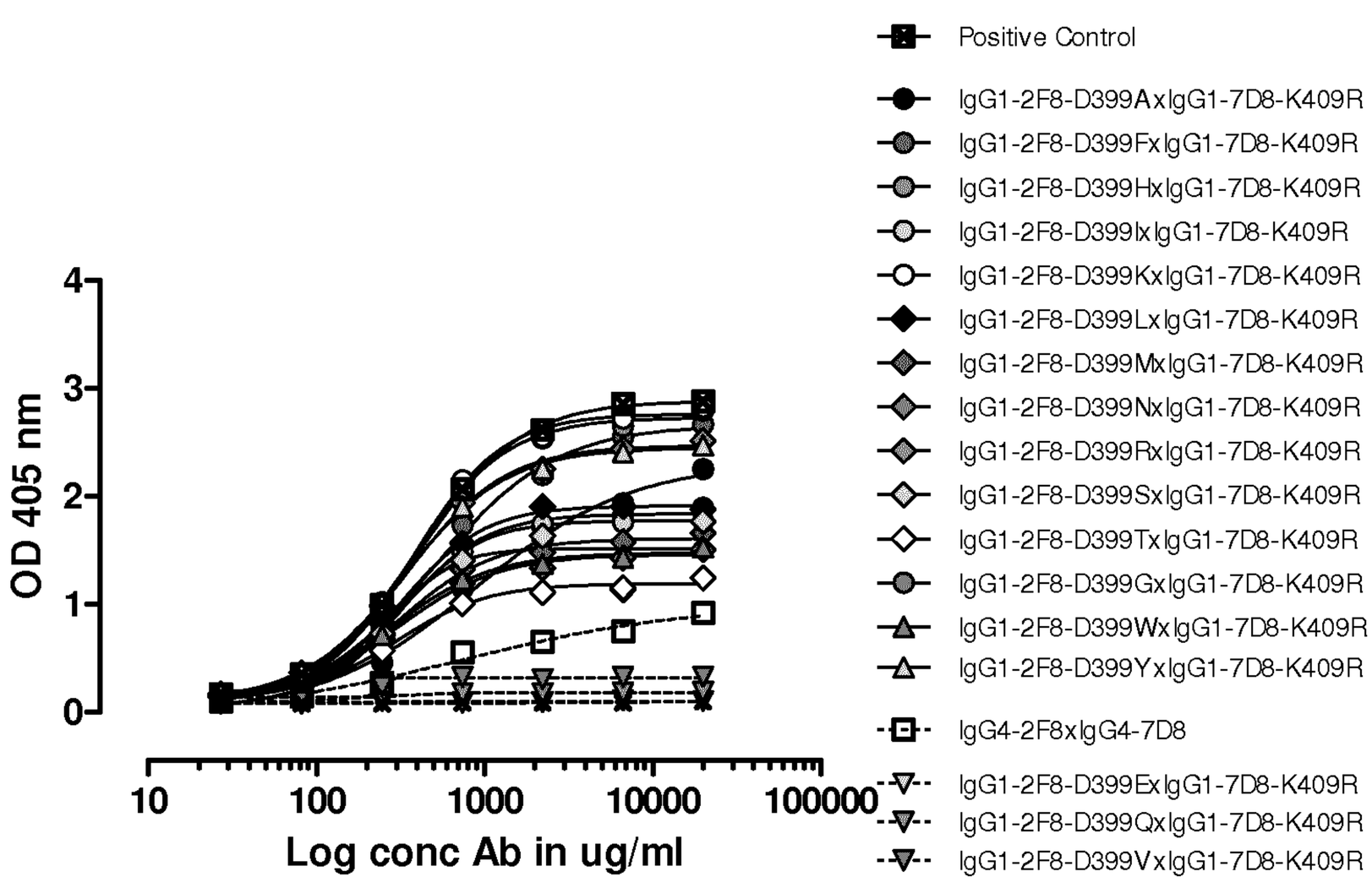
IgG1-2F8-K370YxIgG1-7D8-K409R

Figure 31

A


OD 405 nm
0
1
2
3
4
10
100
1000
10000
100000
Log conc Ab in ug/ml
Positive Control
IgG1-2F8-D399AxIgG1-7D8-K409R
IgG1-2F8-D399FxIgG1-7D8-K409R
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IgG1-2F8-D399KxIgG1-7D8-K409R
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IgG1-2F8-D399NxIgG1-7D8-K409R
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IgG1-2F8-D399YxIgG1-7D8-K409R
IgG4-2F8xIgG4-7D8
IgG1-2F8-D399ExIgG1-7D8-K409R
IgG1-2F8-D399QxIgG1-7D8-K409R
IgG1-2F8-D399VxIgG1-7D8-K409R
IgG1-2F8xIgG1-7D8-K409R

B

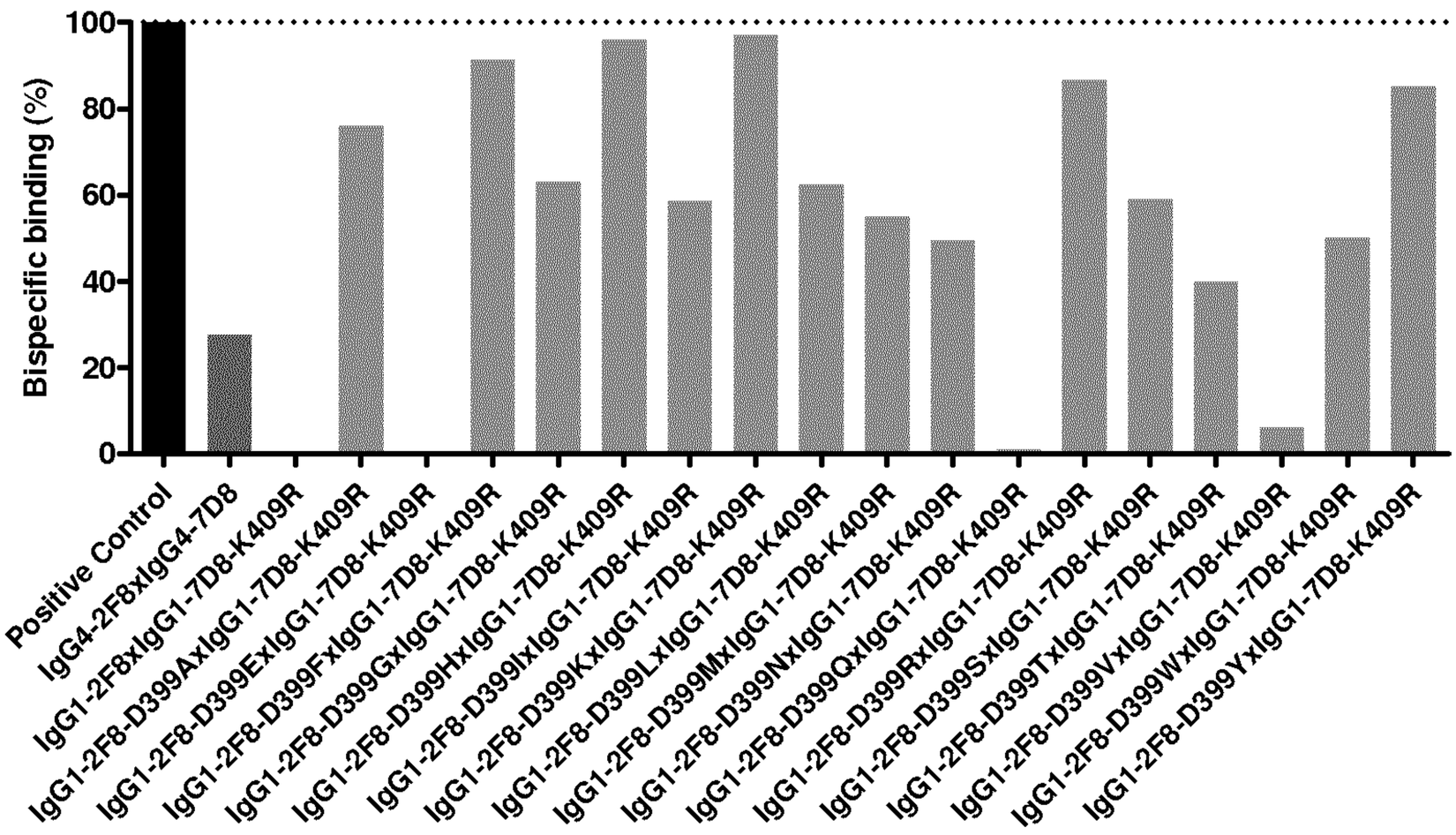

Bispecific binding (%)
0
20
40
60
80
100
Positive Control
IgG4-2F8xIgG4-7D8
IgG1-2F8xIgG1-7D8-K409R
IgG1-2F8-D399AxIgG1-7D8-K409R
IgG1-2F8-D399ExIgG1-7D8-K409R
IgG1-2F8-D399FxIgG1-7D8-K409R
IgG1-2F8-D399GxIgG1-7D8-K409R
IgG1-2F8-D399HxIgG1-7D8-K409R
IgG1-2F8-D399IxIgG1-7D8-K409R
IgG1-2F8-D399KxIgG1-7D8-K409R
IgG1-2F8-D399LxIgG1-7D8-K409R
IgG1-2F8-D399MxIgG1-7D8-K409R
IgG1-2F8-D399NxIgG1-7D8-K409R
IgG1-2F8-D399QxIgG1-7D8-K409R
IgG1-2F8-D399RxIgG1-7D8-K409R
IgG1-2F8-D399SxIgG1-7D8-K409R
IgG1-2F8-D399TxIgG1-7D8-K409R
IgG1-2F8-D399VxIgG1-7D8-K409R
IgG1-2F8-D399WxIgG1-7D8-K409R
IgG1-2F8-D399YxIgG1-7D8-K409R

Figure 32A

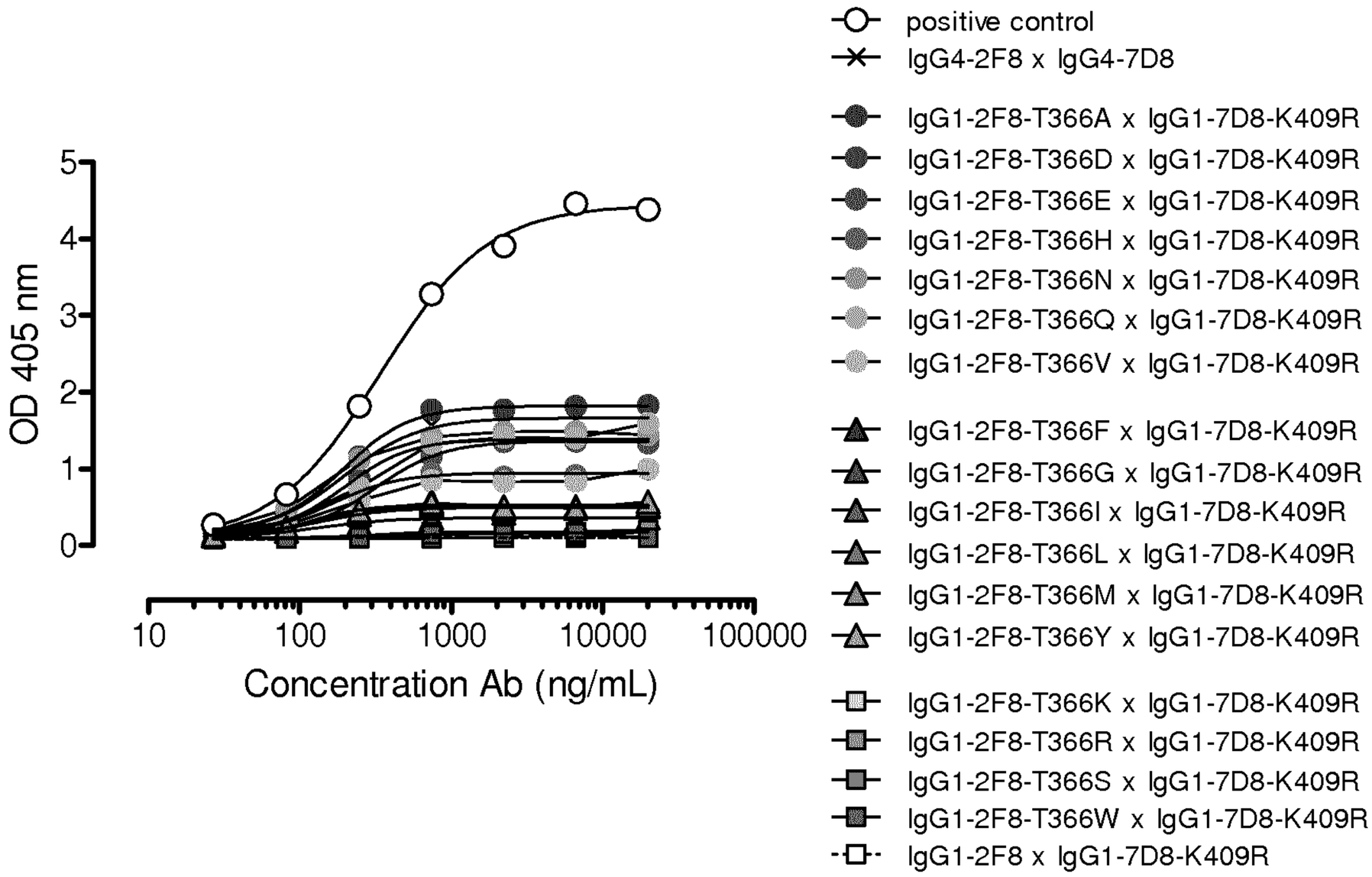


Figure 32B

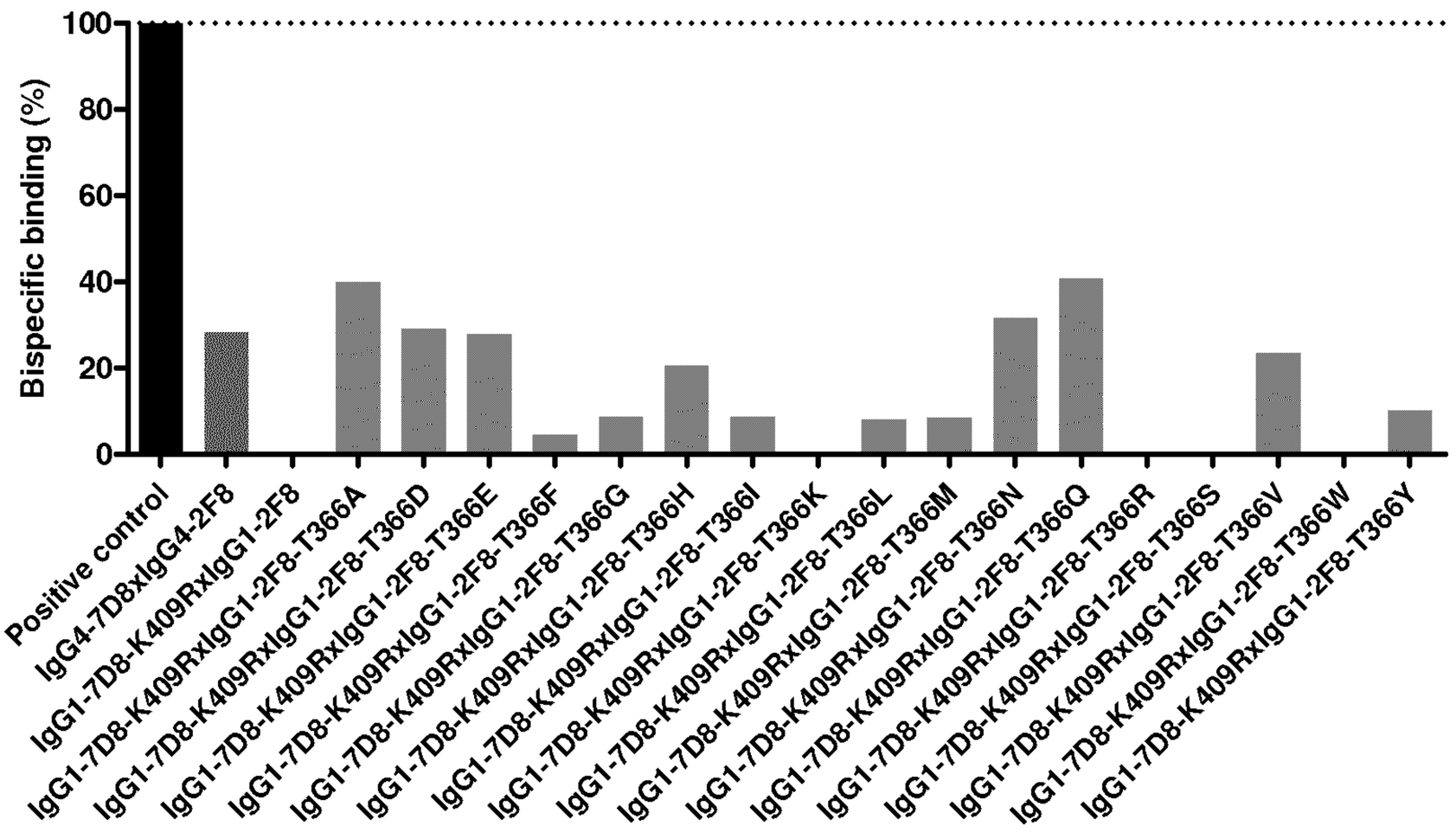


Figure 33A

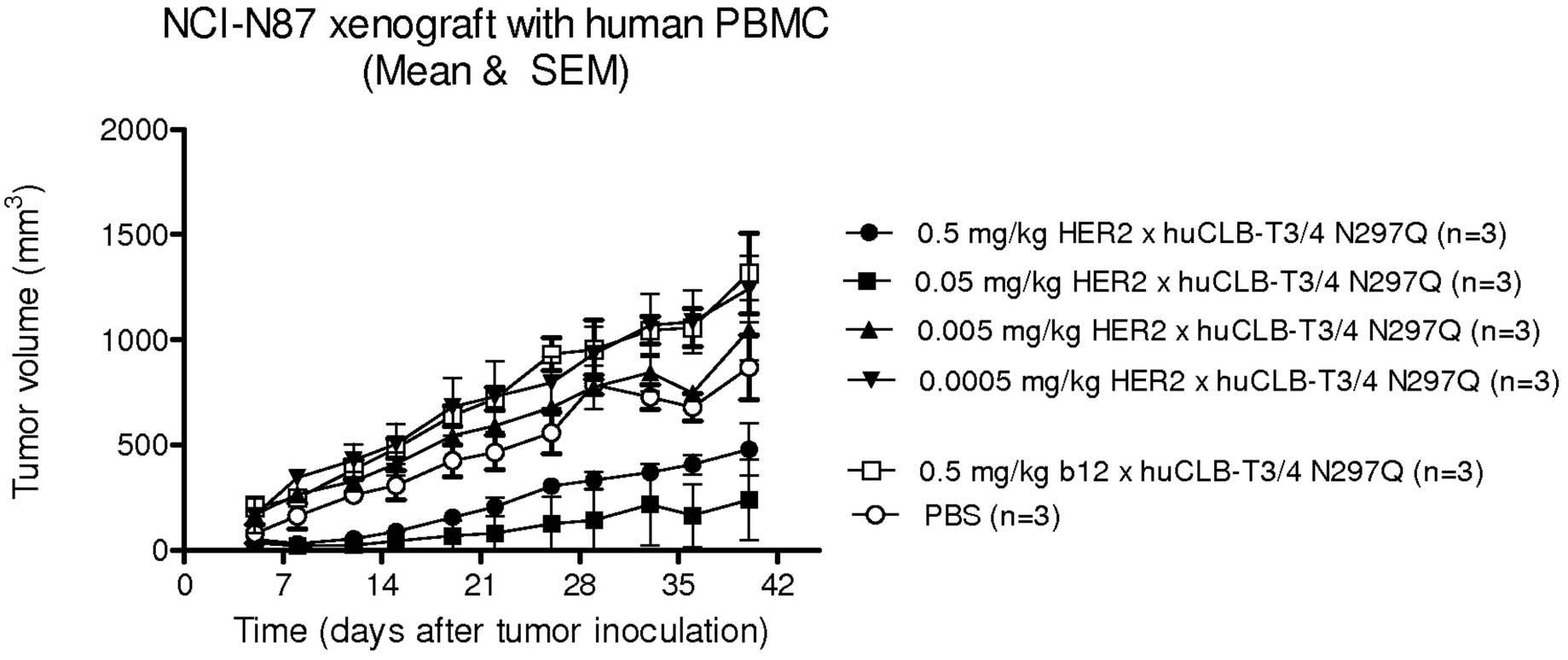


Figure 33B

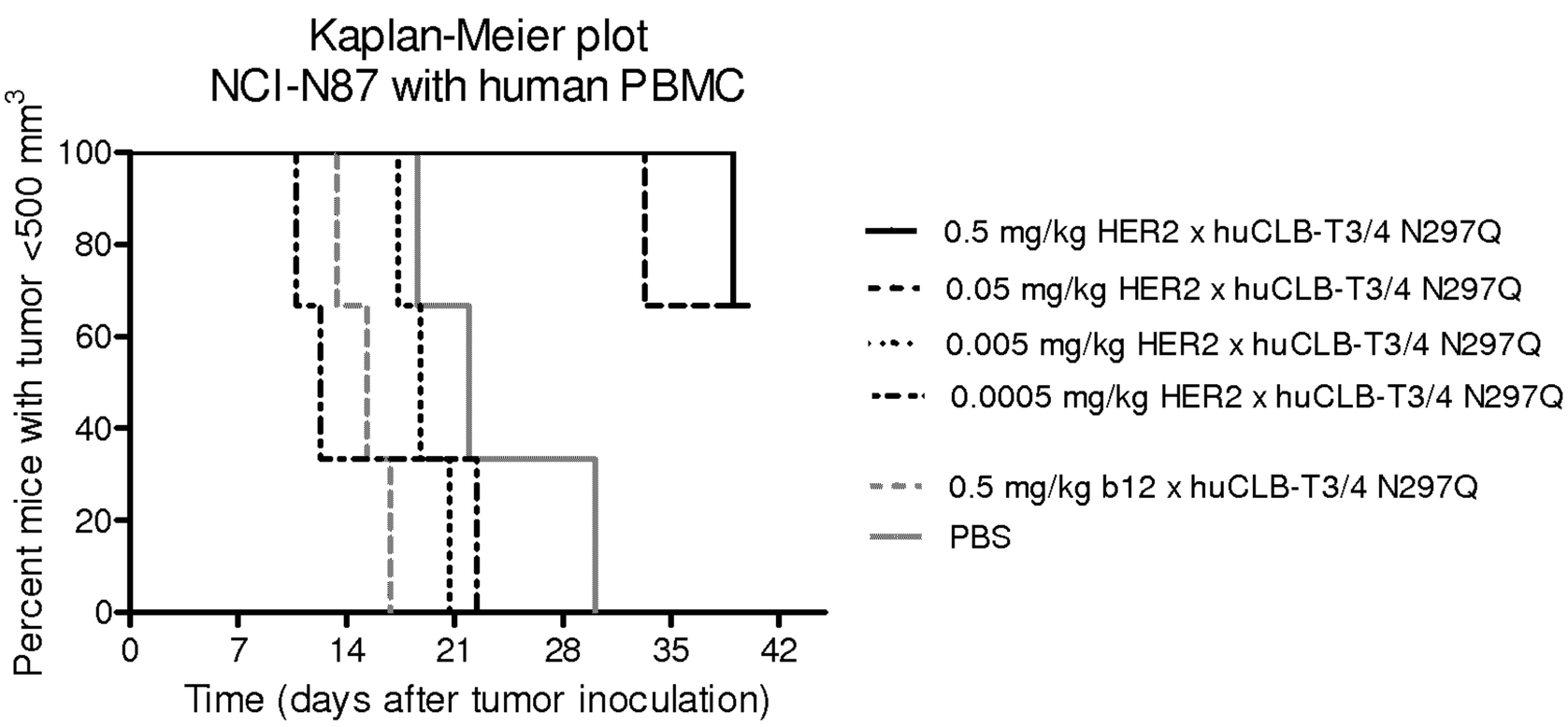
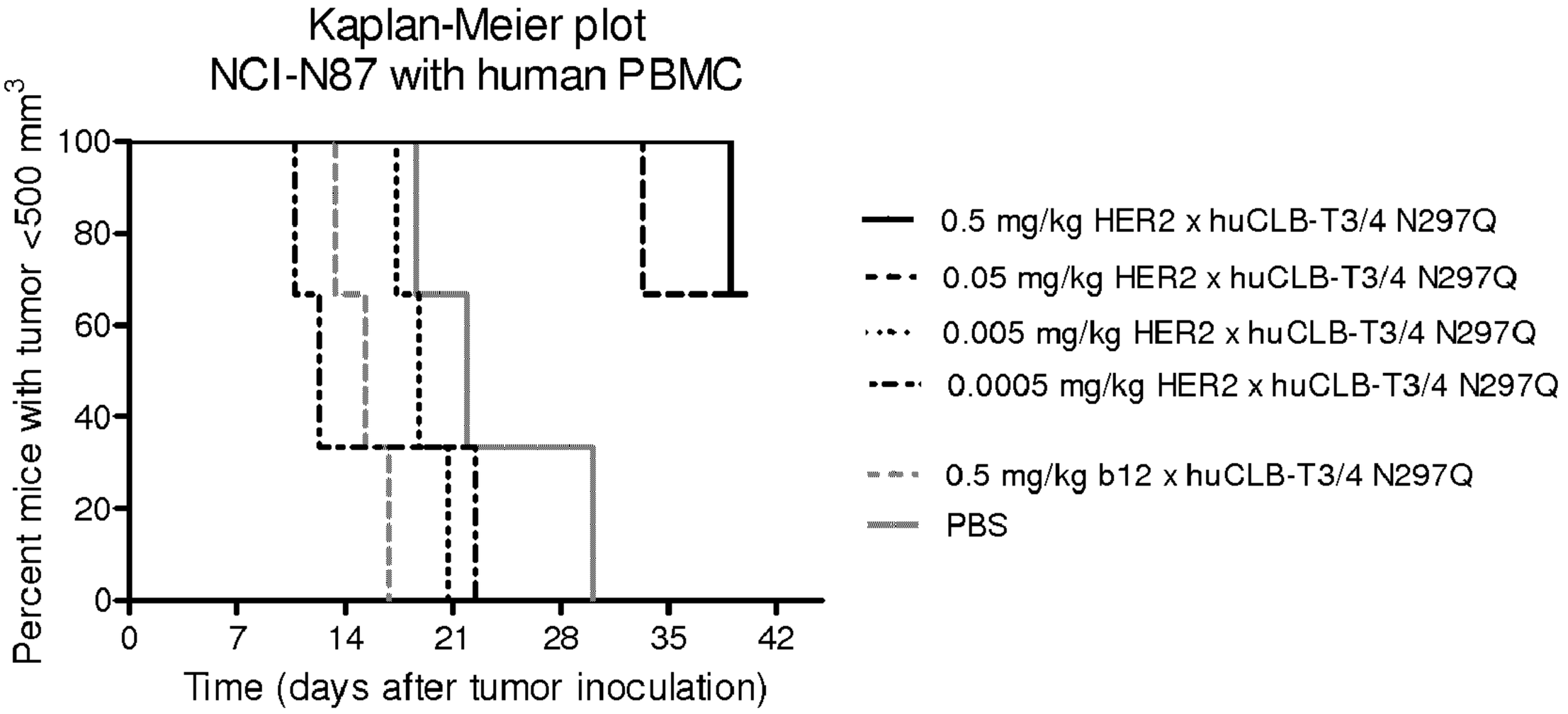


Figure 33B


Kaplan-Meier plot
NCI-N87 with human PBMC
Percent mice with tumor <500 mm3
100
80
60
40
20
0
0
7
14
21
28
35
42
Time (days after tumor inoculation)
0.5 mg/kg HER2 x huCLB-T3/4 N297Q
0.05 mg/kg HER2 x huCLB-T3/4 N297Q
0.005 mg/kg HER2 x huCLB-T3/4 N297Q
0.0005 mg/kg HER2 x huCLB-T3/4 N297Q
0.5 mg/kg b12 x huCLB-T3/4 N297Q
PBS