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(54) Title: ANTIBODIES SELECTIVE FOR CELLS PRESENTING EGFR AT HIGH DENSITY

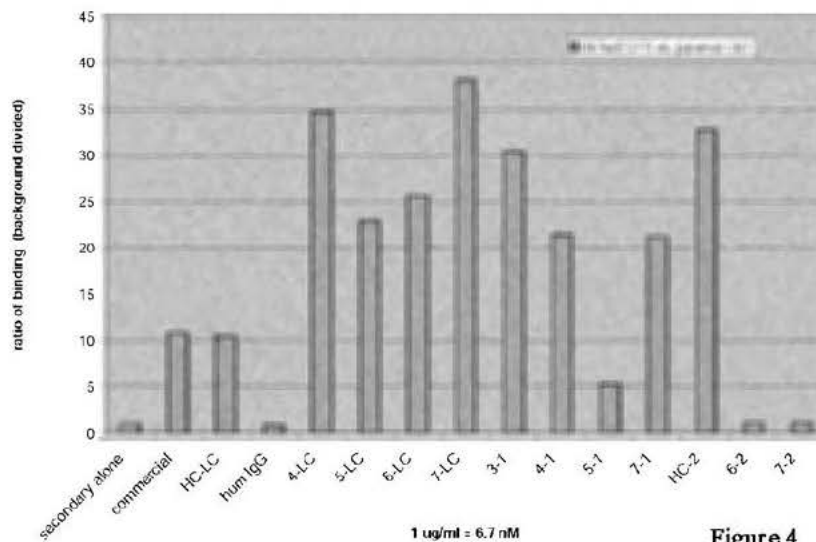


Figure 4

(57) Abstract: Herein described are antibodies to epidermal growth factor receptor (EGFR) having an EGFR binding affinity that is sufficient to kill disease cells presenting EGFR at high density, but is insufficient for binding to normal cells. A therapeutic effect is thus achieved while avoiding adverse events that result from unintended binding to normal cells.

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ANTIBODIES SELECTIVE FOR CELLS PRESENTING EGFR AT HIGH DENSITY

Field of the Invention

5 This invention relates to antibodies having therapeutic and diagnostic utility. More particularly, the present invention relates to antibodies that bind selectively to cells that present EGFR (epidermal growth factor receptor) at abnormally high density. The antibodies are useful therapeutically and diagnostically in the fields of oncology and other diseases.

10

Background to the Invention

15 Drugs for the treatment of cancer and other diseases have a so-called “therapeutic window”. In the case of cancer, the therapeutic window defines the drug dosage that can kill cancer cells preferentially to normal cells, thereby establishing a safety range for the use of the drug. The therapeutic window for conventional chemotherapeutics is narrow with, in many cases, significant adverse effects coinciding with marginal slowing of tumour growth. Targeted treatments that spare normal cells are urgently needed.

20 Therapeutic antibodies form a newer class of cancer therapies that specifically target an antigen presented on the surface of cancer cells. When the target surface protein is unique to the cancer cell, adverse antibody effects on normal cells can be avoided. However, for the majority of antigens, target expression is not restricted completely to tumour cells, with some normal cells also expressing the antigen. In these cases, the antibody may have an effect on normal cells as well as tumor cells, leading to “on-target, off-tissue” adverse events. In the case of the EGFR antigen, because of its ubiquitous presence on the surface of normal cells such as keratinocytes as well as on cancer cells, the clinical use of EGFR-targeting therapeutics is associated with adverse events that include severe rash.

25 30 Considering the efficacy of anti-EGFR therapies in treating patients that overexpress EGFR, the risk associated with severe skin reaction is currently considered acceptable when managed properly. The risk of anti-EGFR therapy-associated toxicity can be reduced by prior administration of anti-histamine, or by administering anti-EGFR antibody at a reduced and less effective dose.

35

40 Efforts to improve upon EGFR antibodies are aimed at generating antibodies having even greater affinity for the target antigen. In WO 2006/009694 published 26 January 2006, Kussie et al describe the crystal structure of the interaction between EGFR and cetuximab Fab fragment, and identify residues that may be modified to improve the effectiveness of cetuximab as an EGFR antagonist.

45 It would be desirable to provide an EGFR antibody that is useful to treat subjects presenting with EGFR over-expressing disease cells, while avoiding significant interaction with tissues including skin and particularly keratinocytes and other cells that also present the EGFR antigen at normal levels.

It is an object of the present invention to provide therapeutic antibodies, and fragments and conjugates thereof that bind effectively to a given target only when that target is presented at a relatively higher density characteristic of a disease state.

- 5 It is a further object of the present invention to provide such antibodies, fragments and conjugates in pharmaceutical compositions, particularly for therapeutic and diagnostic use.

- 10 It is a further object of the present invention to provide a method useful, in a subject in need thereof, to control the growth of disease cells that present EGFR at a density greater than normal EGFR density, while avoiding or minimizing adverse effects on normal cells.

Summary of the Invention

- 15 In one aspect, the present invention provides an isolated, EGFR antibody or bivalent fragment thereof that binds preferentially to target cells that present EGFR at a density above a normal EGFR density. Cells that present EGFR at a density greater than normal EGFR density are disease cells, including cancer cells such as colorectal and other cancer cells, that over-express the her-1 gene, and manifest on their surface a greater number of
20 EGFR proteins than cells that express the her-1 gene at normal levels.

- The antibodies of the present invention, and their bivalent fragments, display a preference for binding to disease cells having the higher EGFR density, and show reduced and desirably minimal or negligible, i.e., insignificant, binding to normal cells having a
25 normal EGFR density. The present antibodies and their bivalent binding fragments thus are well suited for use in reducing or eradicating high density EGFR disease cells while minimizing or avoiding effects on normal cells, thereby reducing the number or severity of adverse events in subjects receiving EGFR antibody therapy.

- 30 In one aspect, the EGFR antibody comprises a heavy chain and a light chain, each chain having a constant region and a variable region, each variable region comprising framework regions and complementarity determining regions (CDRs), wherein the CDRs have an amino acid sequence set forth below:

- 35 For the heavy chain:

CDR1	NYGVH	(SEQ ID No. 1)
CDR2	VIWSSGNTD ⁵⁸ YNTPF ^{TS}	(SEQ ID No. 2)
CDR3	ALTY ¹⁰¹ Y ¹⁰² D ¹⁰³ YE ¹⁰⁵ FAY	(SEQ ID No. 3)

- 40 For the light chain:

CDR1	RASQSIGTNIH	(SEQ ID No. 4)
CDR2	ASE ⁵³ SIS	(SEQ ID No. 5)
CDR3	QQNNNW ⁹⁴ PTT	(SEQ ID No. 6)

- 45 wherein at least one of E⁵³, D⁵⁸, W⁹⁴, Y¹⁰¹, Y¹⁰², D¹⁰³, and E¹⁰⁵ is replaced by a substituting amino acid that reduces the EGFR binding affinity of said antibody. In

embodiments, the substituting amino acid(s) are selected to confer on the antibody a binding affinity (Kd) for EGFR that is about 10 fold or more weaker than the EGFR binding affinity of cetuximab.

5 In embodiments, the present invention provides an EGFR antibody comprising a heavy chain and a light chain, each chain having a constant region and a variable region, wherein the heavy chain variable region comprises the sequence of SEQ ID No. 7 and the light chain variable region comprises the sequence of SEQ ID No. 8, wherein at least one of E⁵³, D⁵⁸, W⁹⁴, Y¹⁰¹, Y¹⁰², D¹⁰³, and E¹⁰⁵ is replaced by a substituting amino acid that
10 reduces the EGFR binding affinity of said antibody.

In other embodiments, the substituting amino acid is selected to reduce EGFR binding affinity of the antibody or bivalent fragment to a level that substantially eliminates binding to cells presenting EGFR at a normal EGFR density, and retains effective binding
15 at targeted disease cells that present EGFR at a greater density relative to normal cell EGFR density.

In still other embodiments, the antibody or bivalent fragment is a variant of cetuximab having one or more substitutions at the residues identified herein. In particular
20 embodiments, the substitutions are non-conservative amino acid substitutions.

In another of its aspects, the present invention provides conjugates, i.e., immunoconjugates, comprising an antibody or bivalent fragment thereof according to the present invention and, conjugated therewith, an agent useful to treat or detect cells
25 presenting EGFR at a density characteristic of disease cells.

In a further aspect, the present invention provides medically useful compositions comprising an antibody, bivalent fragment thereof or immunoconjugate thereof according to the present invention, in combination with a medically acceptable carrier, such as a
30 pharmaceutically acceptable carrier or a diagnostically useful carrier.

In a related aspect, the present invention provides a method for treating a subject having disease cells that present EGFR at a density greater than the EGFR density on normal cells, comprising the step of administering to the subject an effective amount of an
35 antibody, bivalent fragment thereof, or an immunoconjugate of the present invention. Subjects so treated will manifest adverse events that are fewer in number and/or severity given the reduced affinity of the present antibodies for normal cells and tissue.

These and other aspects of the present invention are now described in greater detail with reference to the accompanying drawings, in which:
40

Reference to the Figures

45 Figure 1 is a graph showing binding of antibodies to cell surface EGFR present on the surface of (A) parental U87MG cells, (B) U87MGwtEGFR, U87 cells engineered to overexpress wt EGFR, (C) U87MG-EGFRvIII, U87 cells engineered to overexpress

EGFR vIII and (D) primary human epidermal keratinocytes (HEK), at 1 and 10 µg/ml mAb (A-C) or 0.1 and 1 µg/ml mAb (D). These can be compared to wt mAb (HC/LC) which was set arbitrarily to 100%. Similarly, Figure 1-1 (A-B) shows results from these same experiments plus additional experiments, but using a different data presentation approach, i.e. all binding is divided by background binding, (that is, is expressed as a fold change over background binding) rather than background binding being subtracted from all binding values (as was done in Figure 1). These results demonstrate a greater reduction in binding of some anti-EGFR mAb variants to cells expressing lower EGFR levels (parental U87 or HEK cells) as compared to the reduction observed on U87 cells overexpressing EGFR.

Figure 2 is a graph representing binding selectivity of antibodies. The ratio of antibody binding (with background subtracted) to EGFR overexpressing cells [U87MGwtEGFR or A431 cells (which naturally overexpress wt EGFR)] relative to antibody binding to normal HEK cells was calculated and compared to that seen with wild type antibody (ratio set arbitrarily to 1 for wt antibody). In Figure 2-1, the same results as in Figure 2 are shown using a different data presentation approach, i.e. all binding is divided by background. Note - all additional figures present data analysed in this manner. These results clearly show that some of the EGFR mAbs exhibit better binding to tumor cells that overexpress EGFR relative to normal HEK cells (e.g. mutant HC-2 exhibits a 20-fold (Fig. 2) or 6-fold (Fig. 2-1) better ratio of binding, and mutant 3-1 exhibits a 40-fold (Fig. 2) or 9-fold (Fig. 2-1) better ratio of binding to tumor than normal cells). The pattern of binding specificity was similar amongst the tumor cell lines analyzed (U87MGwt EGFR and A431) suggesting that the selectivity of binding is universally high for tumor cells overexpressing EGFR (~2 million receptors per cell or more).

Figure 3 depicts graphs showing binding of mutated antibodies at 1µg/ml (6.7nM) to (A) U87MGwtEGFR, U87 cells overexpressing wt EGFR, (B) parental U87MG cells;

Figure 4 is a graph illustrating the binding selectivity of antibodies, based on data from Figure 3. The ratio of antibody binding to EGFR overexpressing cells (U87MGwtEGFR) relative to antibody binding to parental U87MG cells was calculated. This results in a ratio of 11 for wild type antibody binding to U87MGwtEGFR cells versus parental cells; and in ratios of up to 35 for certain mutated antibodies, e.g. mutant 7-LC and 4-LC. In other words, these mutant antibodies show a 3-4 fold better ratio (selectivity) of binding.

Figure 5 illustrates the ability of the EGFR mAbs to bind cells and deliver a protein toxin, saporin. Specifically, 1nM EGFR mAbs were incubated with 2nM anti-human secondary antibody that was chemically conjugated with saporin toxin (Advanced Targeting Systems, San Diego, CA), a ribosome inactivating enzyme that needs to be internalized to cause cell death. The antibody complex was then added to the cell types indicated (plated in triplicate) and their effects on cell viability were measured after 72hr incubation at 37°C. EGFR directed cytotoxicity can be quantitated following evaluation with controls for non-specific cytotoxicity (no primary or an irrelevant primary antibody (control human IgG) were used).

Detailed Description of the Invention and Preferred Embodiments

As used herein, the term “EGFR” refers to any protein that comprises the expressed and processed product of the her-1 gene, wherein the protein is designated as
5 UniProtKB/Swiss-Prot P04626-1, including antibody-binding variants thereof.

The present invention relates to EGFR antibodies and bivalent fragments thereof that display a preference for binding to disease cells presenting EGFR at a density greater than normal cells. On cells that present EGFR, the normal density of EGFR is generally
10 less than about 10,000 EGFR molecules per cell, and is usually less than about 1,000 EGFR molecules per cell. EGFR-presenting disease cells, on the other hand, present EGFR at a density generally greater than 10,000 EGFR molecules per cell, and usually greater than about 100,000 EGFR molecules per cell. Generally, the EGFR density is thus about 10^3 or less on normal cells, and about 10^5 or more on disease cells. The actual
15 number of EGFR molecules on any given cell can be determined by established methods, including the antibody based radiolabeled binding or flow cytometry binding to live cells herein exemplified. The binding avidity of the present antibodies is greater for the higher EGFR density disease cells than for the lower EGFR density normal cells. This greater avidity is revealed conveniently using techniques established for determining affinity
20 constants for antibody-target interactions, also as exemplified herein.

In embodiments, the present EGFR antibodies having a binding affinity for EGFR that is about 10 fold or more weaker than the EGFR binding affinity of cetuximab. Desirably, the binding affinity of the antibody for EGFR is about 15-fold, 20-fold, 25-fold, and
25 preferably 30-fold or more weaker than the EGFR binding affinity of cetuximab. In absolute terms, and given an EGFR binding affinity of about 0.3 nM for cetuximab, the present antibodies incorporate amino acid substitution(s) that reduce their EGFR binding affinity (Kd) to about 1.0 nM and weaker, more desirably about 10 nM and weaker, e.g., to an EGFR binding affinity that is in the range from 1 nM to 1 μ M, more desirably 2 nM
30 to 500 nM, such as 10 nM to 500 nM or 10 nM to 100 nM.

In embodiments, the antibody is an intact antibody comprising features common to all natural antibodies, and thus comprises a heavy chain and a light chain, each chain having a constant region and a variable region, each variable region comprising framework
35 regions (FRs) and complementarity determining regions (CDRs). In the alternative, the antibody is provided as a bivalent fragment, i.e., an antibody fragment comprising both “arms” of an intact antibody, joined through a linker that can be represented by the hinge region of the antibody or any equivalent. Such bivalent fragments include F(ab)₂ fragments and any other bivalent fragment that retains preference for high density EGFR.
40 In particular embodiments, the bivalent fragment is a F(ab')₂ fragment, generated for instance by papain-based digestion of the parent antibody using standard procedures for digestion and subsequent fragment isolation. In the alternative, the bivalent fragment can be a so-called single chain Fv (scFv), consisting of the variable light and variable heavy antibody domains joined by an amino acid linker, or a bivalent form of a so-called
45 diabody prepared using a 5 amino acid linker such as SGGGG between the light and heavy chain variable domains and a C-terminal cysteine modification to GGC to give a

final diabody product as VL-SGGG-VH-GGC. Still other bivalent fragments can be prepared by coupling the light and heavy chain variable domains through thioether linkages such as bis-maleimidomethyl ether (BMME), N,N'-p-phenylene dimaleimide (PDM and N,N'-bismaleimido-hexane BMH), to stabilize the F(ab')₂ fragments.

5

In the intact antibody or bivalent fragment, the CDRs comprise or consist of the following amino acid sequences:

For the heavy chain:

10 CDR1 NYGVH (SEQ ID No. 1)
 CDR2 VIWSSGNTD⁵⁸YNTPF⁵⁸TS (SEQ ID No. 2)
 CDR3 ALTY¹⁰¹Y¹⁰²D¹⁰³YE¹⁰⁵FAY (SEQ ID No. 3)

For the light chain:

15 CDR1 RASQSIGTNIH (SEQ ID No. 4)
 CDR2 ASE⁵³SIS (SEQ ID No. 5)
 CDR3 QQNNNW⁹⁴PTT (SEQ ID No. 6)

20 wherein at least one of E⁵³, D⁵⁸, W⁹⁴, Y¹⁰¹, Y¹⁰², D¹⁰³, and E¹⁰⁵ is replaced by a substituting amino acid that reduces the EGFR binding affinity of said antibody or bivalent fragment.

The substituting amino acids are most suitably genetically encoded amino acids that are selected desirably, but not essentially, from an amino acid class that is different from the amino acid class to which the parent amino acid belongs. For instance, in the case of Y¹⁰¹ and Y¹⁰², suitable substituting amino acids are those that are not polar/neutral/large amino acids. The selection process can be conducted by applying computer aided tools that couple saturation virtual mutagenesis engines with algorithms for *in silico* scoring of binding affinities and/or association rates. Amino acid selections can also be made based on the following Table 1:

30

Amino Acid	3 letter 1 letter		Polarity (side chain)	Charge (pH 7.4)	Size*
Alanine	Ala	A	nonpolar	neutral	tiny
5 Arginine	Arg	R	polar	positive	large
Asparagine	Asn	N	polar	neutral	small
Aspartic acid	Asp	D	polar	negative	small
Cysteine	Cys	C	nonpolar	neutral	small
Glutamic acid	Glu	E	polar	negative	small
10 Glutamine	Gln	Q	polar	neutral	small
Glycine	Gly	G	nonpolar	neutral	tiny
Histidine	His	H	polar	neutral (90%)	large
Isoleucine	Ile	I	nonpolar	neutral	large
Leucine	Leu	L	nonpolar	neutral	large
15 Lysine	Lys	K	polar	positive	large
Methionine	Met	M	nonpolar	neutral	large
Phenylalanine	Phe	F	nonpolar	neutral	large
Proline	Pro	P	non-polar	neutral	small
Serine	Ser	S	polar	neutral	tiny
20 Threonine	Thr	T	polar	neutral	small
Tryptophan	Trp	W	nonpolar	neutral	bulky
Tyrosine	Tyr	Y	polar	neutral	large
Valine	Val	V	nonpolar	neutral	small

25 * based on volume in Å³, where 50-100 is tiny, 100-150 is small, 150-200 is large and >200 is bulky

It will be appreciated that the conservative amino acid families include (i) G, A, V, L and I; (ii) D and E; (iii) A, S and T; (iv) H, K and R; (v) N and Q; and (vi) F, Y and W.

30 In embodiments, the heavy chain variable region of the antibody or bivalent fragment incorporates at least one substitution at D⁵⁸, Y¹⁰¹, Y¹⁰², D¹⁰³, or E¹⁰⁵. In other
embodiments, the heavy chain variable region incorporates substitutions at least two such
residues, such as at D⁵⁸ and D¹⁰³, or three such residues, such as at D⁵⁸, D¹⁰³ and E¹⁰⁵. In
35 an alternative embodiment, the heavy chain variable region is wild type and incorporates
no such substitutions, provided there is at least one substitution in the light chain variable
region.

In embodiments, in the heavy chain CDRs, Y¹⁰¹ and/or Y¹⁰², independently, is replaced
by a substituting amino acid having a side chain that is nonpolar and/or a side chain that
40 is non-neutral and/or a side chain that is not large. Desirably, Y¹⁰¹ and/or Y¹⁰² is replaced
by an amino acid selected independently from A, C, G, I, L, M, F, W and V; preferably
from A, G, I, L and V; and more preferably from A, V, I and L. In a specific
embodiment, the tyrosine occurring at one or both of positions 101 and 102 is replaced by
alanine, thus yielding the substitutions designated Y¹⁰¹A and Y¹⁰²A.

45 In other embodiments, D⁵⁸ in the heavy chain CDR2 and/or D103 in the heavy chain
CDR3 is replaced, independently, by a substituting amino acid having a side chain that is

nonpolar and/or is charge neutral or positive and/or is not small. Desirably, D⁵⁸ and/or D¹⁰³ is replaced by an amino acid having a side chain that is charge neutral or positive, as well as polar, as well as small, and is selected desirably from N and Q. In a specific embodiment, D⁵⁸ is replaced by N⁵⁸, thus yielding the substitution designated D⁵⁸N. In another specific embodiment D¹⁰³ is replaced by N¹⁰³, thus yielding the substitution designated D¹⁰³N.

In other embodiments, E¹⁰⁵ in the heavy chain CDR3 is replaced by a substituting amino acid having a side chain that is nonpolar and/or is charge neutral or positive and/or is not small. Desirably, E¹⁰⁵ is replaced by an amino acid having a side chain that is charge neutral or positive, as well as polar, as well as small, and is selected desirably from N and Q. In a specific embodiment, E¹⁰⁵ is replaced by Q¹⁰⁵, thus yielding the substitution designated E¹⁰⁵Q.

In embodiments, the light chain variable region of the antibody or bivalent fragment incorporates at least one substitution at E⁵³ or at W⁹⁴. In a specific embodiment, the light chain variable region comprises substitutions at both E⁵³ or at W⁹⁴. In another specific embodiment, the light chain variable region incorporates substitution only at E⁵³, or only at W⁹⁴. In an alternative embodiment, the light chain variable region is wild type and incorporates no such substitutions, provided there is at least one substitution in the heavy chain variable region.

When substituted, E⁵³ is replaced by a substituting amino acid having a side chain that is either nonpolar and/or is neutral or positive in charge and/or may not be small. In embodiments, E⁵³ is substituted by an amino acid selected from R, D, E, H, or K. In a preferred embodiment, E⁵³ is substituted by K, yielding the substitution designated E⁵³K.

When substituted, W⁹⁴ is replaced by a substituting amino acid having a side chain that is either polar and/or is charge positive or negative and/or is not bulky. In embodiments, W⁹⁴ is replaced by R, N, D, E, Q, H, K, A, S, T or Y. In particular embodiments, W⁹⁴ is replaced by N, Q, H, S, T, A or Y. In a preferred embodiment, W⁹⁴ is replaced by A, yielding the substitution designated W⁹⁴A.

The antibody or bivalent fragment thereof comprises at least one substitution at a location noted above. The at least one substitution can occur in either the light chain variable region or the heavy chain variable region. In specific embodiments, antibodies comprising single site substitutions include:

An antibody comprising an E⁵³K substitution in CDR2 of the light chain, wherein the CDRs are otherwise the wild type versions specified above; or wherein the light chain is otherwise the wild type version as set out in SEQ ID No. 8, or wherein the antibody is otherwise cetuximab, i.e. [E⁵³K]cetuximab.

An antibody comprising a W⁹⁴A substitution in CDR3 of the light chain, wherein the CDRs are otherwise the wild type versions specified above; or wherein the light chain is

otherwise the wild type version as set out in SEQ ID No. 8, or wherein the antibody is otherwise cetuximab, i.e., [W⁹⁴A]cetuximab.

5 An antibody comprising a D⁵⁸N substitution in CDR2 of the heavy chain, wherein the CDRs are otherwise the wild type versions specified above; or wherein the heavy chain is otherwise the wild type version as set out in SEQ ID No. 7, or wherein the antibody is otherwise cetuximab, i.e., [D⁵⁸N]cetuximab

10 An antibody comprising a Y¹⁰¹A substitution in CDR3 of the heavy chain, wherein the CDRs are otherwise the wild type versions specified above; or wherein the heavy chain is otherwise the wild type version as set out in SEQ ID No. 7, or wherein the antibody is otherwise cetuximab, i.e., [Y¹⁰¹A]cetuximab.

15 An antibody comprising a Y¹⁰²A substitution in CDR3 of the heavy chain, wherein the CDRs are otherwise the wild type versions specified above; or wherein the heavy chain is otherwise the wild type version as set out in SEQ ID No. 7, or wherein the antibody is otherwise cetuximab, i.e., [Y¹⁰²A]cetuximab.

20 An antibody comprising a D¹⁰³N substitution in CDR3 of the heavy chain, wherein the CDRs are otherwise the wild type versions specified above; or wherein the heavy chain is otherwise the wild type version as set out in SEQ ID No.7, or wherein the antibody is otherwise cetuximab, i.e., [D¹⁰³N]cetuximab.

25 An antibody comprising an E¹⁰⁵Q substitution in CDR3 of the heavy chain, wherein the CDRs are otherwise the wild type versions specified above; or wherein the heavy chain is otherwise the wild type version as set out in SEQ ID No. 7, or wherein the antibody is otherwise cetuximab, i.e., [E¹⁰⁵Q]cetuximab.

30 In other embodiments, the antibody or binding fragment thereof comprises at least two such substitutions, either in the light chain variable region, in the heavy chain variable region, or at least one substitution in each of the light and heavy chain variable regions. In specific embodiments, antibodies including at least two such substitutions include:

35 An antibody comprising both a E⁵³K substitution in CDR2 of the light chain and a Y¹⁰¹A substitution in CDR3 of the heavy chain, wherein the CDRs are otherwise the wild type versions specified above; or wherein the heavy chain is otherwise the wild type version as set out in SEQ ID No. , or wherein the antibody is otherwise cetuximab, i.e., [E⁵³K, Y¹⁰¹A]cetuximab.

40 An antibody comprising both a E53K substitution in CDR2 of the light chain and a Y102A substitution in CDR3 of the heavy chain, wherein the CDRs are otherwise the wild type versions specified above; or wherein the heavy chain is otherwise the wild type version as set out in SEQ ID No. , or wherein the antibody is otherwise cetuximab, i.e., [E53K, Y102A]cetuximab.

45

5 An antibody comprising both a D⁵⁸N substitution in CDR2 of the heavy chain, and a D¹⁰³N substitution in CDR3 of the heavy chain, wherein the CDRs are otherwise the wild type versions specified above; or wherein the heavy chain is otherwise the wild type version as set out in SEQ ID No. , or wherein the antibody is otherwise cetuximab, i.e., [D⁵⁸N, D¹⁰³N]cetuximab.

10 An antibody comprising at least three substitutions, including a D⁵⁸N substitution in CDR2 of the heavy chain, a D¹⁰³N substitution in CDR3 of the heavy chain, and an E¹⁰⁵Q substitution in CDR3 of the heavy chain, wherein the CDRs are otherwise the wild type versions specified above; or wherein the heavy chain is otherwise the wild type version as set out in SEQ ID No. , or wherein the antibody is otherwise cetuximab, i.e., [D⁵⁸N, D¹⁰³N, E¹⁰⁵Q]cetuximab.

15 In preferred embodiments, the antibody is one of [E53K, Y102A]cetuximab, [D58N, D103N]cetuximab, or [D58N, D103N, E105Q]cetuximab.

20 In addition to the recited three CDRs present in each of the light and heavy chain variable regions, the heavy and light chains of the intact antibody comprise four intervening framework regions that present the CDRs in a conformation suitable for EGFR binding, and constant regions that confer antibody effector function. The CDRs can be integrated into any suitable acceptor antibody, by grafting the present CDRs into the acceptor antibody, in accordance with practices and techniques well established for the production of chimeric, humanized and human antibodies.

25 Particularly suitable acceptor antibodies are antibodies already known to have EGFR binding affinity. Such donor antibodies are most desirably of human origin, but they can also derive from acceptor antibodies of non-human origin, including mouse, rat, rabbit, goat, sheep, primate and the like. It will be appreciated that human antibody acceptor sequences different from those exemplified herein can be identified and used to
30 accommodate the presently desired CDRs. This is achieved by modeling the structure of a preferred antibody using for instance the Swiss-Model [<http://swissmodel.expasy.org/repository>] or similar software and selecting, from among the numerous human antibody sequences available in public databases, a human acceptor antibody sequence that, with CDR sequences altered as herein preferred, approximates
35 the same structural conformation as the preferred antibodies. In embodiments, the acceptor antibodies, and the resulting present antibodies, are of the IgG1 isotype, but they may also be IgG2 or IgG4. Moreover, the isotype of the antibody, as dictated by the constant region, can be manipulated to alter or eliminate the effector function of the resulting antibody. That is, the constant region of the present antibodies is either wild
40 type human antibody constant region, or a variant thereof that incorporates amino acid modifications, i.e., amino acid additions, substitutions or deletions that alter the effector function of the constant region, such as to enhance serum half-life, reduce complement fixation, reduce antigen-dependent cellular cytotoxicity and improve antibody stability. The number of amino acid modifications in the constant region is usually not more than
45 20, such as 1-10 e.g., 1-5 modifications, including conservative amino acid substitutions.

In embodiments, the half life of the antibody is improved by incorporating one more amino acid modification, usually in the form of amino acid substitutions, for instance at residue 252, e.g., to introduce Thr, at residue 254, e.g., to introduce Ser, and/or at residue 256 e.g., to introduce Phe. Still other modifications can be made to improve half-life,
 5 such as by altering the CH1 or CL region to introduce a salvage receptor motif, such as that found in the two loops of a CH2 domain of an Fc region of an IgG. Such alterations are described for instance in US 5869046 and US 6121022.

10 Altered C1q binding, or reduced complement dependent cytotoxicity, can be introduced by altering constant region amino acids at locations 329, 331 and 322, as described in US 6194551. The ability of the antibody to fix complement can further be altered by introducing substitutions at positions 231 and 239 of the constant region, as described in WO94/029351.

15 The framework regions of the light and heavy chains of the present antibodies and fragments also desirably have the sequence of a human antibody variable region, but incorporating the CDRs herein specified. In embodiments, the heavy chain variable region is human IgG4 in origin. In specific embodiments, the heavy chain variable region is that of human IgG, such as the human IgG1 antibody variant having the
 20 sequence designated Genbank gi 2414502. Alternatively, and preferably, the heavy chain variable region is that of human IgG4 antibody species designated Genbank gi 2414502.

The framework regions of the heavy and light chains of the present antibodies may also incorporate amino acid modifications, i.e., amino acid deletions, additions or
 25 substitutions, which further improve upon the properties of the antibody or fragment, in accordance with techniques established for antibody humanization. Such framework modifications can be modeled on the framework regions of antibody sequences provided in public databases, and on framework regions of antibodies known to bind EGFR, such as those antibodies referenced in the background section hereof. Preferred framework
 30 substitutions are those which yield antibodies having a greater preference for binding EGFR at the higher density associated with disease cells, relative to normal cells.

35 Framework modifications can also be made to reduce immunogenicity of the antibody or to reduce or remove T cell epitopes that reside therein, as described for instance by Carr et al in US2003/0153043.

In accordance with embodiments of the present invention, the heavy and light chain variable regions are modeled on the antibody cetuximab, and comprise a heavy chain variable region of SEQ ID No.7, and/or a light chain variable region having SEQ ID
 40 No.8, as follows:

Light chain variable region (VL):

45 DILLTQSPVILSVSPGERVFSFSCRASQSIGTNIHWYQORTNGSPRLLIKYASE⁵³SISGIPSRFSGSGSGTD
 FTLSINSVESEDIADYYCQQNNW⁹⁴PTTFGAGTKLELK [SEQ ID No. 7]; wherein E⁵³ or W⁹⁴
 are as defined hereinabove;

Heavy chain variable region (VH):

5 QVQLKQSGPGLVQPSQSLITCTVSGFSLTNYGVHWVRQSPGKGLEWLGVIWSSGNTD⁵⁸YNTPFTRSLSIN
 KDNSKSVQFFKMNLSQNDTAIYYCARALTY¹⁰¹Y¹⁰²D¹⁰³YE¹⁰⁵FAYWGQGLTIVTSA [SEQ ID No.
 8]; wherein D⁵⁸, Y¹⁰¹, Y¹⁰², D¹⁰³, or E¹⁰⁵ are as defined hereinabove.

In more specific and preferred embodiments, the entire light and heavy chains of the intact antibody are set out below as SEQ ID Nos. 9 and 10, respectively:

10 Entire Light chain:

15 DILLTQSPVILSVSPGERVSFSCRASQSIGTNIHWYQORTNGSPRLLIKYASE⁵³SISGIPSRFSGSGSGTD
 FTLSINSVESEDIADYYCQQNNW⁹⁴PTTFGAGTKLELKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFY
 PREAKVQWKVDNALQSGNSQESVTEQDSKDSYSLSSLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRG
 E [SEQ ID No. 9]; wherein E⁵³ and W⁹⁴ are as defined hereinabove;

Entire Heavy chain:

20 QVQLKQSGPGLVQPSQSLITCTVSGFSLTNYGVHWVRQSPGKGLEWLGVIWSSGNTD⁵⁸YNTPFTRSLSIN
 KDNSKSVQFFKMNLSQNDTAIYYCARALTY¹⁰¹Y¹⁰²D¹⁰³YE¹⁰⁵FAYWGQGLTIVTSAASTKGPSVFPPLAPSS
 KSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSVHTFPAVLQSSGLYSLSSVTVTPSSSLGTQTYICNVNH
 KPSNTKVKDKRVEPKSCDKTHTCPPCPAPPELLGGPSVFLFPPKPKDTLMI SRTPEVTCVVVDVSHEDPEVKFN
 WYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQV
 YTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQG
 25 NVFSCSVMH EALHNHYTQKSLSLSPGK [SEQ ID No. 10]; wherein D⁵⁸, Y¹⁰¹, Y¹⁰², D¹⁰³, or
 E¹⁰⁵ are as defined hereinabove.

30 As noted, final selection of an antibody or binding fragment is made based on the binding
 preference displayed by the desired antibody or bivalent fragment for cells that present
 EGFR at a density greater than normal. The target cells are thus disease cells presenting
 greater than normal EGFR density, as a hallmark. Screening can be performed *in vitro*,
 as exemplified herein, using as reference cells a first disease cell known from analysis to
 present EGFR at a density greater than normal, such as the U87wtEGFR or related lines
 35 that incorporate an altered EGFR such as U87EGFRvIII or the line A431, and a second,
 normal cell known from analysis to present EGFR at a normal density, such as primary
 human epidermal keratinocytes (~ 20,000 EGFR/cell). The choice of epidermal
 keratinocytes as the reference, normal cell is prudent, given that marketed EGFR
 antibodies, such as cetuximab, are known to elicit severe skin rash side effects through
 their interaction with these cells. Any other human cell line that presents EGFR at
 40 normal density can be used, in the alternative.

45 The cell-based assay can use flow cytometry with appropriate EGFR antibody and
 labeled secondary antibody to report and measure binding affinity and avidity, as
 exemplified herein. In the alternative, selection of the desired antibody can be performed
 based on absolute binding affinities obtained for instance using surface plasmon
 resonance, also as exemplified herein.

For purposes of identifying disease cells that can be targeted by the present EGFR
 antibodies and bivalent fragments, the commercial test EGFRpharmDX (DAKO) can

conveniently be used. This is a semi-quantitative immunohistochemical assay for determination of her-1 protein overexpression in colorectal tissues. Positive or negative results aid in the classification of abnormal cells/tissues and provide a basis for treatment with EGFR antibody.

5

The antibodies and binding fragments thus are useful both for diagnostic purposes, including sample testing and in vivo imaging, and for therapeutic purposes to treat diseases in which EGFR density is increased on disease cells.

- 10 For either purpose, the antibody or binding fragment can be conjugated to an appropriate agent, to form an immunoconjugate. Agents appropriate for treating disease include cytotoxic agents include chemotherapeutics and radiotherapeutics. For diagnostic purposes, appropriate agents are detectable labels that include radioisotopes, for whole body imaging, and radioisotopes, enzymes, fluorescent labels and the like for sample testing.
- 15

For therapy, the cytotoxin may be conjugated with the antibody or bivalent binding fragment through non-covalent interaction, but more desirably, are coupled by covalent linkage either directly or, more preferably, through a suitable linker. In a preferred embodiment, the conjugate comprises a cytotoxin and an antibody. Immunoconjugates of the antibody and cytotoxin are made using a variety of bifunctional protein coupling agents such as N-succinimidyl-3-(2-pyridyldithiol) propionate, iminothiolane, bifunctional derivatives of imidoesters such as dimethyl adipimidate HCL, active esters such as disuccinimidyl suberate, aldehydes such as glutaraldehyde, bis-azido compounds such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates such as toluene 2,6-diisocyanate, and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). Carbon-14-labeled 1-isothiocyanobenzyl-3-methyldiethylene triaminepentaacetic acid (MX-DTPA) is a chelating agent suitable for conjugation of radio nucleotide to the antibody.

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The cytotoxin component of the immunoconjugate can be a chemotherapeutic agent, a toxin such as an enzymatically active toxin of bacterial, fungal, plant or animal origin, or fragments thereof, or a small molecule toxin, or a radioactive isotope such as ^{212}Bi , ^{131}I , ^{131}In , ^{111}In , ^{90}Y , and ^{186}Re , or any other agent that acts to inhibit the growth or proliferation of a cancer cell.

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Chemotherapeutic agents useful in the generation of such immunoconjugates include adriamycin, doxorubicin, epirubicin, 5-fluorouracil, cytosine arabinoside ("Ara-C"), cyclophosphamide, thiotepa, busulfan, cytosine, taxoids, e.g. paclitaxel, and docetaxel, taxotere, methotrexate, cisplatin, melphalan, vinblastine, bleomycin, etoposide, ifosgamide, mitomycin C, mitoxantrone, vincristine, vinorelbine, carboplatin, teniposide, daunomycin, carminomycin, aminopterin, dactinomycin, mitomycins, esperamicins, 5-FU, 6-thioguanine, 6-mercaptopurine, actinomycin D, VP-16, chlorambucil, melphalan, and other related nitrogen mustards. Also included are hormonal agents that act to regulate or inhibit hormone action on tumors such as tamoxifen and onapristone.

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Toxins and fragments thereof which can be used include diphtheria A chain, nonbonding

active fragments of diphtheria toxin, cholera toxin, botulinus toxin, exotoxin A chain (from *Pseudomonas aeruginosa*), ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, *Aleurites fordii* proteins, dianthin proteins, *Phytolacca Americana* proteins (PAPI, PAPII, and PAP-S), momordica charantia inhibitor, curcin, crotin, sapaonaria, officinalis inhibitor, gelonin, saporin, mitogellin, restrictocin, phenomycin, enomycin, and the tricothcenes. Small molecule toxins include, for example, calicheamicins, maytansinoids, palytoxin and CC1065.

Therapeutic formulations of the antibody, bivalent fragment or the conjugate are prepared for storage by mixing the antibody or conjugate having the desired degree of purity with optional pharmaceutically acceptable carriers, excipients or stabilizers (Remington's Pharmaceutical Sciences, 16th edition, Osol, A. Ed. [1980]), in the form of lyophilized formulations or aqueous solutions. Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl, or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins such as serum, albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagines, histidine, arginine or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrans; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (e.g., Zn-protein complexes); and/or non-ionic surfactants such as TWEEN, PLURONICS or polyethylene glycol (PEG).

The active ingredients to be used for *in vivo* administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes.

Sustained-release preparations may be prepared. Suitable examples of sustained-release include semipermeable matrices of solid hydrophobic polymers containing the antibody, which matrices are in the form of shapes articles, e.g., films or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly (2-hydroxyethyl-methacrylate), polylactides (U.S. Pat. No. 3,773,919), copolymers of L-glutamic acid and ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate, and poly-D-(-)-3-hydroxybutyric acid. While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods. When encapsulated antibodies remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37° C., resulting in a loss of biological activity and possible changes in immunogenicity. Rational strategies can be devised for stabilization depending on the mechanism involved. For example, if the aggregation mechanism is discovered to be intermolecular S-S bond formation through thio-disulfide interchange, stabilization may be achieved by modifying sulfhydryl

residues, lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions.

Administration "in combination with" one or more further therapeutic agents includes
5 simultaneous (concurrent) and consecutive administration in any order.
Other therapeutic regimens may be combined with the administration of the anti-cancer
agents, e.g., antibodies or conjugates, of the instant invention. For example, the patient to
be treated with such anti-cancer agents may also receive radiation therapy, such as
10 external beam radiation. Alternatively, or in addition, a chemotherapeutic agent may be
administered to the patient. Preparation and dosing schedules for such chemotherapeutic
agents may be used according to manufacturers' instructions or as determined empirically
by the skilled practitioner. Preparation and dosing schedules for such chemotherapy are
also described in *Chemotherapy Service Ed.*, M. C. Perry, Williams & Wilkins,
15 Baltimore, Md. (1992). The chemotherapeutic agent may precede, or follow
administration or the anti-tumor agent, e.g., antibody, or may be given simultaneously
therewith. The antibody may be combined with any of the toxins described above with
reference to the conjugates, or any other suitable drug particularly include irinotecan
(CPT-11), cisplatin, cyclophosphamide, melphalan, dacarbazine, doxorubicin,
20 daunorubicin, and topotecan, as well as tyrosine kinase inhibitors. .

It may be desirable to also administer antibodies or conjugates against other tumor
associated antigens or their ligands, such as antibodies which bind to the ErbB2, ErbB3,
ErbB4, or vascular endothelial factor (VEGF), and/or antibodies that bind to EGF or
TGF α . Alternatively, or in addition, two or more antibodies binding that same or two or
25 more different antigens disclosed herein may be co-administered to the patient.
Sometimes it may be beneficial to also administer one or more cytokines to the patient. In
a preferred embodiment, the antibodies herein are co-administered with a growth
inhibitory agent. For example, the growth inhibitory agent may be administered first,
followed by an antibody of the present invention. However, simultaneous administration
30 or administration of the antibody of the present invention first is also contemplated.
Suitable dosages for the growth inhibitory agent are those presently used and may be
lowered due to combined action (synergy) of the growth inhibitory agent and the
antibody herein.

35 In another embodiment of the invention, an article of manufacture containing materials
useful for the diagnosis or treatment of the disorders described herein is provided. The
article of manufacture comprises a container and a label. Suitable containers include, for
example, bottles, vials, syringes, and test tubes. The containers may be formed from a
variety of materials such as glass or plastic. The container holds a composition which is
40 effective for treating the condition and may have a sterile access port (for example the
container may be an intravenous solution bag or vial having a stopper pierceable by a
hypodermic injection needle). The label on, or associated with, the container indicates
that the composition is used for treating a cancer condition. The article of manufacture
may further comprise a second container comprising a pharmaceutically-acceptable
45 buffer, such as phosphate-buffered saline, Ringer's solution and dextrose solution. It may
further include other matters desirable from a commercial and use standpoint, including

other buffers, diluents, filters, needles, syringes, and package inserts with instructions for use.

5 An anti-cancer therapeutic according to the invention may be administered with a pharmaceutically-acceptable diluent, carrier, or excipient, in unit dosage form.

Any appropriate route of administration can be employed, for example, parenteral, intravenous, subcutaneous, intramuscular, intracranial, intraorbital, ophthalmic, intraventricular, intracapsular, intraspinal, intracisternal, intraperitoneal, intranasal, aerosol, or oral administration.

10

For the treatment of subjects presenting with cancer cells presenting EGFR at greater density than normal cells, the appropriate dosage of an anti-tumor agent, e.g., an antibody, fragment or conjugate, will depend on the type of disease to be treated, as defined above, the severity and course of the disease, whether the agent is administered

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for preventative or therapeutic purposes, previous therapy, the patient's clinical history and response to the agent, and the discretion of the attending physician. The agent is suitably administered to the patient at one time or over a series of treatments. For example, depending on the type and severity of the disease, about 1 µg/kg to 15 mg/kg (e.g., 0.1-20 mg/kg) of antibody or conjugate is a candidate dosage for

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administration to the patient, whether, for example, by one or more separate administrations, or by continuous infusion. A typical daily dosage might range from about 1 µg/kg to 100 mg/kg or more, depending on the factors mentioned above. For repeated administrations over several days or longer, depending on the condition, the treatment is sustained until a desired suppression of disease symptoms occurs. However,

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It will thus be appreciated that an effective amount of the antibody, fragment or immunoconjugate is an amount effective alone or as part of a treatment regimen that

30

retards or inhibits the growth or proliferation of disease cells presenting with higher than normal EGFR density.

In embodiments, the present antibodies are administered by intravenous infusion, such as at an initial dose of 4mg/kg over 90 minutes, then 2 mg/kg over 30 minutes, once weekly

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for 52 weeks, with follow up as required.

The antibody and bivalent fragments are useful in the treatment of a variety of cancers, to inhibit the growth or proliferation of cancer cells and tumours comprising them, including hematopoietic cell cancers and solid tumours. Conditions or disorders to be treated

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include benign or malignant tumors (e.g., renal, liver, kidney, bladder, breast, gastric, ovarian, colorectal, prostate, pancreatic, lung, vulva, and thyroid); hepatic carcinomas; sarcomas; glioblastomas; and various head and neck tumors; leukemias and lymphoid malignancies. In particular embodiments, the antibody or bivalent fragment are used in the treatment of such cancer cells that express high density EGFR, as determined by the

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screening assays herein described. In particular embodiments, the cancer cells are EGFR-presenting cancer cells that include head and neck cancers and especially

squamous cell carcinoma of the head and neck, colorectal cancers, gastrointestinal cancers, brain tumours including glioblastomas, and tumours of the lung including non-small-cell lung carcinoma, and of the breast, pancreas, esophagus, kidney, ovary, cervix and prostate.

5

It will be appreciated that subjects who could benefit from the present method include mammals including humans as well as livestock, and pets.

10 Antibodies and bivalent fragments thereof that bind selectively to the target antigen, e.g. EGFR, are used, in accordance with an aspect of the invention, to screen cancer cells to detect those which present the EGFR antigen at high density. In a preferred embodiment, screening is applied to a sample of cancer cells taken from a subject that is a candidate for EGFR antibody therapy. Subjects testing positive for cancer cells that present the EGFR antigen at high density can then be scheduled for therapy with the present antibody or
15 fragment, or an immunoconjugate thereof. Standard techniques, combined with the antibodies or other binding agents herein described, can be used to screen cancer cells. Desirably, the antibodies incorporate a detectable label. The label may be detectable by itself. (e.g., radio-isotope labels or fluorescent labels) or, in the case of an enzymatic label, may catalyze chemical alteration of a substrate compound or composition which is
20 detectable. Radionuclides that can serve as detectable labels include, for example, I-131, I-123, I-125, Y-90, Re-188, Re-186, At-211, Cu-67, Bi-212, and Pd-109.

In situ detection of the binding to cancer cells bearing high density EGFR can be
25 performed, using the present antibody or fragment, by immunofluorescence or immunoelectron microscopy. For this purpose, a histological specimen is removed from the patient, and a labeled form of the present antibody is applied to it, preferably by overlaying the antibody on a biological sample. This procedure also allows for distribution of the EGFR antigen to be examined within biopsied tumour tissue, to reveal only those sites at which the antigen is presented at a density higher than normal. It will
30 be apparent for those skilled in the art that a wide variety of histological methods are readily available for *in situ* detection.

More particularly, EGFR antibodies or binding fragments of the present invention may be
35 used to monitor the presence or absence of antibody reactivity in a biological sample (e.g., a tissue biopsy, a cell, or fluid) using standard detection assays. Immunological assays may involve direct detection, and are particularly suited for screening large amounts of samples for the presence of cancer cells that overexpress EGFR. For example, antibodies may be used in any standard immunoassay format (e.g., ELISA, Western blot, immunoprecipitation, flow cytometry or RIA assay) to measure complex
40 formation. Any appropriate label which may be directly or indirectly visualized may be utilized in these detection assays including, without limitation, any radioactive, fluorescent, chromogenic (e.g., alkaline phosphatase or horseradish peroxidase), or chemiluminescent label, or hapten (for example, digoxigenin or biotin) which may be visualized using a labeled, hapten-specific antibody or other binding partner (e.g.,
45 avidin). Exemplary immunoassays are described, e.g., in Ausubel et al., supra, Harlow and Lane, *Antibodies: A Laboratory Approach*, Cold Spring Harbor Laboratory, New

York (1988), and Moynagh and Schimmel, Nature 400:105, 1999. For example, using the antibodies described herein, high density EGFR is readily detected at the cell surface using standard flow cytometry methods. Samples found to contain labeled complex compared to appropriate control samples are taken as indicating the presence of high
5 density EGFR, and are thus indicative of a cancer or other disease amenable to treatment with the present antibodies.

The present antibody is produced suitably by recombinant DNA means, as exemplified herein. For production, there is provided a DNA molecule that encodes the heavy chain
10 of the present antibody, and a DNA molecule that encodes the light chain thereof. The DNA further encodes any suitable signal peptide suitable for expression of a secretable chain precursor that enables proper externalization with folding and disulfide formation to elaborate the desired antibody as a secreted, dimerized and processed protein. To this end, the present invention provides, in one embodiment, a polynucleotide comprising a
15 sequence that encodes the variable region of the light chain of a presently preferred EGFR antibody, as set out in SEQ ID No. 9 appearing at the end of the disclosure. Also provided, in another embodiment, is a polynucleotide comprising a sequence that encodes the variable region of the heavy chain of a presently preferred EGFR antibody, as set out in SEQ ID No. 10 also appearing at the end of the disclosure.

In more specific embodiments, the present invention provides a polynucleotide that encodes the entire light chain (SEQ ID No. 11) and the entire heavy chain (SEQ ID No. 14) of a preferred EGFR antibody of the present invention. These sequences also are
20 provided at the end of this disclosure.

It will be appreciated that polynucleotide equivalents also can be used, in which synonymous codons are replaced within the sequences provided, to produce the present
25 antibodies.

In embodiments, there are also provided vectors that comprise polynucleotides that encode the heavy chain or the variable region thereof and that encode the light chain or the variable region thereof. To express the antibodies, the polynucleotides are incorporated operably within expression vectors, i.e., operatively linked to
30 transcriptional and translational control sequences. Expression vectors include plasmids, retroviruses, cosmids, and the like. The expression vector and expression control sequences are chosen to be compatible with the expression host cell used. The antibody light chain gene and the antibody heavy gene can be inserted into separate vectors. In a preferred embodiment, both genes are inserted into the same expression vector. The antibody genes are inserted into the expression vector by standard methods (e.g., ligation
35 of complementary restriction sites on the antibody gene fragment and vector, or blunt end ligation if no restriction sites are present).

A convenient vector is one that encodes a functionally complete human CH or CL immunoglobulin sequence, with appropriate restriction sites engineered so that any VH or
40 VL sequence can be easily inserted and expressed, as described above. In such vectors, splicing usually occurs between the splice donor site in the inserted J region, and the

- splice acceptor site preceding the human C region, and also at the splice regions that occur within the human CH exons. Polyadenylation and transcription termination occur at native chromosomal sites downstream of the coding regions. The recombinant expression vector can also encode a signal peptide that facilitates secretion of the antibody chain from a host cell. The antibody chain gene may be cloned into the vector such that the signal peptide is linked in-frame to the amino terminus of the antibody chain gene. The signal peptide can be an immunoglobulin signal peptide or a heterologous signal peptide (i.e., a signal peptide from a non-immunoglobulin protein).
- Polynucleotides encoding the heavy chain and/or the light chain, and vectors comprising these can be used for transformation of a suitable mammalian host cell. Methods for introduction of heterologous polynucleotides into mammalian cells include dextran-mediated transfection, calcium phosphate precipitation, polybrene-mediated transfection, protoplast fusion, electroporation, encapsulation of the polynucleotide(s) in liposomes, biolistic injection and direct microinjection of the DNA into nuclei. In addition, polynucleotides may be introduced into mammalian cells by viral vectors. Mammalian cell lines useful as hosts for expression of the antibody-encoding polynucleotides include many immortalized cell lines available from the American Type Culture Collection (ATCC). These include, inter alia, Chinese hamster ovary (CHO) cells, NSO, SP2 cells, HeLa cells, baby hamster kidney (BHK) cells, monkey kidney cells (COS, human hepatocellular carcinoma cells (e.g., Hep G2), A549 cells, 3T3 cells, and a number of other cell lines. Mammalian host cells include human, mouse, rat, dog, monkey, pig, goat, bovine, horse, and hamster cells. Cell lines of particular preference are selected through determining which cell lines have high expression levels. Other cell lines that may be used are insect cell lines, such as S19 cells, amphibian cells, bacterial cells, plant cells and fungal cells. When recombinant expression vectors encoding the heavy chain or antigen-binding portion thereof are introduced into mammalian host cells, the antibodies are produced by culturing the host cells for a period of time sufficient to allow for expression of the antibody in the host cells or, more preferably, secretion of the antibody into the culture medium in which the host cells are grown. Antibodies can be recovered from the culture medium using standard protein purification methods. It is likely that antibodies expressed by different cell lines or in transgenic animals will have different glycosylation from each other. However, all antibodies encoded by the polynucleotides provided herein, or comprising the amino acid sequences provided herein are part of the instant invention.

Embodiments are now described in the following examples.

Examples

- The structure of cetuximab bound to EGFR [1] was used as starting point for mutant design. Mutations were introduced only in the CDR regions of the light and heavy chain. First, single-point mutations were generated and evaluated computationally. Virtual mutagenesis was carried out with optional conformational relaxation upon mutation by means of conformational sampling algorithms, such as Monte Carlo minimization [2]. Prediction of antigen-antibody relative binding affinities between parent and mutant

antibodies was carried out with binding affinity scoring functions, such as the solvated interaction energy (SIE) function [3]. Prediction of relative antigen-antibody association rates (k_{on}) between parent and mutant antibodies was carried out with methods that evaluate long-range electrostatic interactions, such as HyPARE [4]. Candidate single-point mutants were assembled into multiple-point mutants and re-scored for relative binding affinity.

Multiple-point mutants were generated by combining single-point mutants between light and heavy chains to achieve the targeted change in affinity. A requirement was to use as few single-point mutants as possible and to maximize the number of generated assembled antibodies. Another desirable feature was to generate a pool of mutants with reduced affinities due to either increased dissociation rates (k_{off}) or to decreased association rates (k_{on}). Among suitable candidate single-point mutations, those targeting distinct locations within the antibody-antigen interface, preferably at its periphery, were given higher priority.

Preparation of Plasmids

All the cDNAs encoding the heavy and light chains of the antibodies were ordered from GeneArt (Regensburg Germany). The cDNAs were removed from the plasmid provided by GeneArt by digestion with *Hind*III and cloned into the *Hind*III site of plasmid pKCR5 previously dephosphorylated with calf intestinal phosphatase (NEB) to prevent recircularization. In pKCR5, transcription of the cDNA is under the control of the strong CR5 promoter, part of the cumate gene switch. The plasmid pKCR5 is available from the Biotechnology Research Institute, Montreal, Canada and is described by Mullick et al [6]. This 3.9kb plasmid incorporates a *Hind*III in proper context with the CR5 promoter and a rabbit b-globin polyA, together with a B-lactamase gene for selection, and *colE1* and *f1* origins of replication. For transfection of CHO cells, all plasmids were isolated from large culture of *E. coli* using the Plasmid Maxi kit (Qiagen Inc, Mississauga, ON) according to the manufacturer's recommendation. Briefly, 200 ml of LB medium containing 100 µg/ml ampicillin were inoculated with a single fresh colony of *E. coli* and incubated overnight at 37°C with vigorous shaking (250 rpm). The bacteria were pelleted by centrifugation at 6000 x *g*, for 15 min, at 4°C and the plasmid was isolated using the protocols, buffers and columns provided by the kit. The pure plasmids was resuspended in sterile 50 mM TRIS, pH 8 and quantified by measuring the optical density at 260 nm.

Cell line (CHO-cTA; clone 5F1) and growth conditions

The CHO-cTA cell line (Gaillet et al [5]; Mullick et al. [6]) used for transient transfection is a Chinese Hamster Ovary cell line (CHO) adapted to grow in suspension and in protein-free medium. The cell line stably expresses the cumate transactivator (cTA) which activates transcription by binding to the CR5 promoter. The CHO-cTA are maintained in CD-CHO medium (Invitrogen, CDCHO 10743), supplemented with 4 mM glutamine, 50 µg/mL and dextran sulfate (Amersham Pharmacia Biotech) at 37°C under an atmosphere of 5% CO₂. When the cells reach a concentration of 1.0 X 10⁶ cells/ml (on average three times a week) they are passaged by diluting them to a concentration of 5.0 x 10⁴ cells/ml using fresh medium.

Transient transfection of CHO-cTA

Before transfection, the cells were washed with PBS and resuspended at a concentration of 2.5×10^6 cell/ml in growth medium without dextran sulfate for 3 hrs in suspension culture. 50 ml of cells were transfected by adding slowly 2.5 ml of a CDCHO medium supplemented with 1 μ g/ml of plasmid and 5 μ g/ml. polyethylenimine (PEI Max; Polysciences). After 2 hrs, the cells were transferred at 30°C. The next days, 50 μ g/mL of dextran sulfate was added to the cells and they were incubated at 30°C for a total of 4 days. The supernatant was clarified by centrifugation and filtered through a 0.22 μ M filter and transferred at -80°C until further analysis.

Polyacrylamide gel electrophoresis (SDS-PAGE)

Known amounts of supernatant were resuspended into an equal volume of Laemmli 2X and heated at 95°C for 5 min and chilled on ice. The samples were then separated on a polyacrylamide Novex 10% Tris-Glycine gel (Invitrogen Canada Inc., Burlington, ON). A standard curve was made by adding known amount of purified human IgG. The gel was then stained using a solution of Coomassie FluorTM-Orange (Molecular Probes, Eugene OR) according to the manufacturer's recommendations. The signal was visualized and quantified using the Typhoon Scanner.

Western blot analysis

Known amounts of supernatant were separated on a SDS-PAGE as described above and then transferred onto a Hybond-N nitrocellulose membrane (Amersham Bioscience Corp., Baie d'Urfée, QC) for 1 h at 275 mA. The membrane was blocked for 1 h in 0.15% Tween 20, 5% skimmed milk in PBS and incubated for 1 h with an anti-human IgG conjugated to Cy5 (Jackson, Cat# 109-176-099). The signal was revealed by scanning with the Typhoon Trio+ (Amersham Biosciences, GE Healthcare).

ELISA

96 wells/plates were coated with 50 μ l of affiniPure Goat Anti-Human IgG, (H+L) (Jackson Immuno Research) and incubated overnight at 4°C. The wells were washed with PBS and incubated for 30 min at 37°C with 100 μ l of 1% BSA in PBS at 37°C. 25 μ l of samples diluted with 1% BSA in PBS were added to the wells, which were incubated for 2 hrs at 37°C. The wells were washed with 0.05% Tween 20 in PBS and incubated with an alkaline Phosphatase-conjugated AffiniPure Goat Anti-Human IgG (H+L) (Jackson Immuno Research) for 1 hr at 37°C. The wells were washed with 0.05% Tween 20 in PBS, followed by PBS. The signal was revealed by incubation with PNPP for 30 min at 37°C. The signal intensity was measure at 405 nm. A standard curve was made using known amount of purified antibody (IgG1, kappa from myeloma plasma (Athens Research Technology).

Purification of antibody

The supernatant was concentrated with an Amicon Ultra (Ultracel-50K) at 1500 rpm to a volume of 500 μ l. The wild type and mutants, antibodies were purified using the ProPur protein A mini spin columns (Nunc) according to the manufacture's recommendations. The purified antibodies were then desalted and resuspended in PBS using the desalting column PD-10 (GE Healthcare). The antibodies were then concentrated by centrifugation

on an Amica Ultra 100,000 MWCO membrane. The purified antibodies were quantified by reading the optical density at 280nm using the Nanodrop spectrophotometer. The purified antibodies were kept frozen at -20°C in 50% glycerol.

5 **In vitro binding by Surface Plasmon Resonance**

Kinetic and affinity analysis was carried out using a BioRad Proteon surface plasmon resonance instrument. The running buffer for all steps was 10 mM HEPES, 150 mM NaCl, 3.5 mM EDTA and 0.05% Tween20 at pH 7.4. An antibody capture sensorchip was prepared by injecting 6.5 µg/mL of anti-human Fc (Jackson Immunochemicals Inc.) in 10 mM sodium acetate pH 4.5 at flow rate 25 µL/min over a GLM sensorchip (BioRad Inc.) that had previously been activated with a 1/10 dilution of sNHS/EDC (BioRad Inc.) until the surface was saturated (approximately 5000 RUs). This procedure was carried out in the analyte direction to ensure all of the interspots for referencing have immobilized anti-mouse Fc. Wild-type cetuximab and variants were captured in the ligand direction by injecting 100 µL of 4% culture supernatants or purified samples in running buffer at flow rate of 25 µL/min until 400 to 800 resonance units have been captured. This was immediately followed by two pulses of running buffer in the analyte direction, 50 µL each at flow rate 100 µL/min to stabilize the baseline. Next, the simultaneous injection of 100 µL of five EGFR ectodomain (EGFRed) concentrations (3-fold dilutions of 20 nM to 1000 nM EGFR depending on the affinity of the cetuximab variant) and buffer blank at a flow rate of 50 µL/min with a 600 s dissociation was carried out to analyse the EGFRed-antibody interaction. Kinetic rate constants (on- and off-rates) and affinity constants were generated from the aligned and double referenced sensorgrams with the Langmuir binding model using BioRad Proteon Manager software v3.1. Mutants with fast on- and off-rates had their affinity constants determined using the equilibrium fit model which uses plateau values from the sensorgrams to generate a binding isotherms for KD constant determination.

Cell culture

The U87MG glioblastoma cell line was obtained from ATCC (HTB-14). A stably transfected full length wt EGFR or a deleted version of EGFR (variant 3_ overexpressing cell line variants were gifts from W. Cavanee, Ludwig Institute for Cancer Research, University of California at San Diego). The human epidermoid A431 cell line was obtained from ATCC (CRL-1555). Cell lines were maintained in DMEM (Gibco) containing 10% fetal bovine serum (Gibco). Primary adult human epithelial keratinocytes were obtained from ScienCell (Catalog # 2110) and cultured using manufacturer's recommended Keratinocyte Medium (KM, Cat. No. 2101). Generally cells were passaged once or twice a week and used within 4-6 weeks for all experiments.

40 **Detection of antibody binding to surface EGFR level by flow cytometry**

Prior to analysis, cells were plated such that they were not more than 80% confluent on the day of analysis. Tumor (U87 MG derivatives, A431) or normal (human epidermal keratinocytes) cell were washed in PBS and harvested by the addition of cell dissociation buffer (Sigma.). A cell suspension containing 2.5×10^5 cells (in 500 µl corresponding cell culture media) was incubated with various concentrations (0.01-100 µg/ml) of anti-EGFR antibodies for 2 h at 4°C (to prevent internalization). Following 1 wash with cell

culture media, primary antibody was incubated with 2 ug Dylight 488 conjugated AffiniPure goat anti-human IgG Alexa 488 secondary antibody (Jackson Immuno Research 109-487-003) in 100 ul of media for 1h at 4°C. Cells were then pelleted and stored on ice until ready to be analyzed by flow cytometry. Prior to analysis, cell pellets were resuspended in 300-500 ul media and filtered through a 50 um nylon mesh filter to remove cell aggregates. Flow cytometry analyses were performed on 10,000 viable cells gated on forward scattering, side scattering parameters and propidium iodide dye exclusion using a BD LSRII flow Cytometer (Becton-Dickinson Biosciences, CA, USA) and a standard filter set using BD FACSDiva™ acquisition software, according to manufacturer's instructions.

Specific antibody binding was calculated as the mean fluorescent intensity of binding to each antibody after background level subtraction of the mean fluorescent intensity of binding in the absence of primary antibody (but containing secondary detection antibody). An alternative approach was used to calculate specific antibody binding on cells, i.e. it was calculated as fold-binding over background by dividing the mean fluorescent intensity in the presence of primary antibody by the mean fluorescent intensity obtained in the absence of primary antibody (but containing secondary antibody). To examine the binding selectivity of the antibodies, the value of antibody binding to tumor (overexpressing EGFR) was divided by the binding observed with cells not overexpressing EGFR. This parameter, named the ratio of binding, was calculated and compared to that seen with wild type antibody. A commercial source of Cetuximab (Merck kGA) was used as a benchmark for comparison purposes.

25 **Evaluation of antibody-mediated cytotoxicity as antibody-drug conjugates**

In this set of experiments, primary antibodies (typically 1nM in concentration) were incubated with 2nM anti-human secondary antibody that was chemically conjugated with saporin toxin (from Advanced Targeting Systems, San Diego, CA), a ribosome inactivating enzyme that needs to be internalized to cause cell death. The antibody complex was then added to the cell types indicated (plated in triplicate) and their effects on cell viability measured after 72 hr incubation at 37°C. EGFR directed cytotoxicity can be quantitated following evaluation with controls for non-specific cytotoxicity (no primary antibody or an irrelevant primary antibody (control human IgG) were used to assess non-specific cytotoxicity). Cell viability can be measured using standard techniques, including the use of sulforhodamine B.

35 **Results:**

40 **1. Production and Purification of EGFR Antibodies**

Nine cDNAs corresponding to the coding sequence of the EGFR antibodies were synthesized (GeneArt). All the cDNAs were cloned into the HindIII site of pKCR5, an expression vector regulated by the cumate-switch (pKCR5 vector (see map)). For each antibody, 50 ml of CHOcTA (expressing the cumate transactivator, cTA) were transfected with various combinations of heavy and light chain. Four days after transfection the supernatant was analyzed by SDS-PAGE, Western Blot and ELISA.

Table 3 below summarizes quantification of the antibodies produced by transient transfection in CHOcTA cells, done by ELISA and by western blot using a purified human IgG1 as standard.

5

Table 3

Mutants		Quantification by Western blot (mg/L)	Quantification by E.L.I.S.A (mg/L)
HC_LC	wt_HC + wt_LC	13.72	10.09
HC_1	wt_HC + LC_E53K	6.02	3.44
HC_2	wt_HC + LC_W94A	5.34	5.97
3_LC	HC_Y101A + wt_LC	10.73	6.57
3_1	HC_Y101A + LC_E53K	1.72	3.62
3_2	HC_Y101A + LC_W94A	6.24	5.13
4_LC	HC_Y102A + wt_LC	6.77	6.64
4_1	HC_Y102A + LC_E53K	6.59	6.19
4_2	HC_Y102A + LC_W94A	9.17	7.16
5_LC	HC_D103N + wt_LC	18.46	9.58
5_1	HC_D103N + LC_E53K	2.52	6.59
5_2	HC_D103N + LC_W94A	21.05	13.59
6_LC	HC_D58N_D103N + wt_LC	18.55	15.71
6_1	HC_D58N_D103N + LC_E53K	6.47	7.03
6_2	HC_D58N_D103N + LC_W94A	29.13	20.03
7_LC	HC_D58N_D103N_E105Q + wt_LC	16.36	11.04
7_1	HC_D58N_D103N_E105Q + LC_E53K	9.86	5.82
7_2	HC_D58N_D103N_E105Q + LC_W94A	17.38	12.41

The 2 wild type chains and 7 mutant chains were purified by chromatography using protein A. The purified proteins were quantified by OD₂₈₀ (NanoDrop). The purified antibodies were analyzed by non-denaturing and denaturing SDS-PAGE.

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2. Binding affinity determination of EGFR antibodies by SPR

The SPR results are provided in Table 4 and Table 4-1 below:

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Antibody	K _a (1/Ms) Table 4	K _d (1/s)	K _D (nM) k _d /k _a	K _D (nM) steady state
Cetuximab (purchased)	4.09E+6	1.15E-3	0.28	nd
	4.82E+6	1.42E-3	0.30	nd
HC/LC = wt_HC + wt_LC	4.47E+6	1.55E-3	0.35	nd
HC/1 = wt_HC + LC_E53K	2.48E+6	3.29E-3	1.33	nd
	2.31E+6	3.71E-3	1.61	nd
	2.36E+6	2.86E-3	1.21	nd
3/LC = HC_Y101A + wt_LC	2.32E+6	0.06	27.3	15.4
4/LC = HC_Y102A + wt_LC	1.36E+6	0.13	98.9	50.3
	2.17E+6	0.09	43.3	44.5
5/LC = HC_D103N + wt_LC	1.29E+6	0.1	80.3	44.8
3/1 = HC_Y101A + LC_E53K	1.8E+6	0.1	57.3	62.9
HC/2 = wt_HC + LC_W94A	2.36E+6	0.18	75.1	72.3
7/LC = HC_D58N/D103N/E105Q + wt_LC	1.08E+6	0.07	67.3	67.0
	1.27E+6	0.08	61.9	89.0
6/LC = HC_D58N/D103N + wt_LC	nd	nd		74.4

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Table 4-1

Antibody name	Description	N	KD ± SD (nM)
Cetuximab	commercial mAb	4	0.3 ± 0.2
HC_LC	wt_HC + wt_LC	3	0.3 ± 0.2
HC_1	wt_HC + LC_E53K	4	1.2 ± 0.5
HC_2	wt_HC + LC_W94A	2	71 ± 3
3_LC	HC_Y101A + wt_LC	2	1.2 ± 0.5
4_LC	HC_Y102A + wt_LC	3	46 ± 4
5_LC	HC_D103N + wt_LC	2	41 ± 5
6_LC	HC_D58N_D103N + wt_LC	1	250
7_LC	HC_D58N_D103N_E105Q + wt_LC	3	60 ± 8
3_1	HC_Y101A + LC_E53K	2	66 ± 4
4_1	HC_Y102A + LC_E53K	2	200 ± 100
5_1	HC_D103N + LC_E53K	1	840
6_1	HC_D58N_D103N + LC_E53K	1	>> 100
7_1	HC_D58N_D103N_E105Q + LC_E53K	1	1400
3_2	HC_Y101A + LC_W94A	1	Too weak to be detected
4_2	HC_Y102A + LC_W94A	1	Too weak to be detected
5_2	HC_D103N + LC_W94A	1	Too weak to be detected
6_2	HC_D58N_D103N + LC_W94A	1	>>1000
7_2	HC_D58N_D103N_E105Q + LC_W94A	1	>>1000

5 Table 4-1 provides SPR-based affinity determinations that have either been refined or are additional to those provided in Table 4.

10 The results indicated that approximately 50% of the cetuximab variants did not have any detectable activity at the 100 nM EGFR tested (data not shown). Of those that showed binding activity (Table 4), only the wild-type (HC/LC) and mutant HC/1 had a moderately-slow off rate. All of the other variants with activity (3/LC, 4/LC, 4/1, 5/LC, 15 6/LC, 7/LC, 3/1 and HC/2) had both a fast association and dissociation from the flowing EGFRred. Affinity constants (KDs) were determined from the ratio of the kinetic rates (kd s-1 / ka s-1M-1) using a 1:1 langmuir binding model where amenable, otherwise affinity constants were determined from an equilibrium fit using plateau binding values only. Mutants 6_2 and 7_2 showed very weak binding at 1000 nM EGFR but were not quantifiable.

3. Binding of EGFR antibodies to various cell lines as determined by indirect flow cytometry

Figure 1 depicts graphs showing binding of antibodies to cell surface EGFR present on the surface of (A) parental U87MG cells, (B) U87 cells overexpressing wt EGFR, (C) U87 cells overexpressing EGFR vIII and (D) primary human epidermal keratinocytes (HEK), at 1 and 10 µg/ml mAb (A-C) or 0.1 and 1 µg/ml mAb (D). These were compared to wt mAb (HC/LC, set arbitrarily to 100%). In Figure 1-1 (A and B), the same plus additional results are presented differently, i.e. all binding is divided by background binding (that is, is expressed as a fold change over background binding) rather than background binding being subtracted from all binding values (as in Figure 1). This data analysis approach de-emphasizes variations caused by small changes in background binding. As expected, these results demonstrate less binding of anti-EGFR mAbs to parental U87 cells or HEK cells compared to tumor cells which overexpress EGFR. Importantly, these results demonstrate a greater reduction in binding of some anti-EGFR mAb variants to cells expressing lower EGFR levels (parental U87 or HEK cells) as compared to U87 cells overexpressing EGFR.

4. Evaluation of antibody binding to tumor and normal cell lines

Figure 2 is a graph representing binding selectivity of antibodies. The ratio of antibody binding (with background subtracted) to EGFR overexpressing cells [U87MGwtEGFR or A431 cells (which naturally overexpress wt EGFR)] relative to antibody binding to normal HEK cells was calculated and compared to that seen with wild type antibody (ratio set arbitrarily to 1 for wt antibody). This result clearly shows that some of the EGFR mAbs exhibit a better ratio of binding to tumor relative to normal HEK cells (e.g. mutant HC-2 exhibits a 20-fold better ratio, and mutant 3-1 exhibits a 40-50-fold better ratio of binding to tumor versus normal cells). In Figure 2-1, the same results as in Figure 2 are shown using a different data presentation approach, i.e. all binding is divided by background. These results also clearly show that some of the EGFR mAbs exhibit a better ratio of binding to tumor cells that overexpress EGFR relative to normal HEK cells (e.g. mutant HC-2 exhibits 80-100 fold differential binding, and mutant 3-1 exhibits 120-140 fold differential binding to tumour cells versus normal cells, whereas wt antibody (HC-LC) exhibits 12-15 fold differential binding to tumour cells versus normal cells. In other words, HC-2 exhibits an ~6-fold better ratio of binding, and mutant 3-1 exhibits an ~9-fold better ratio of binding to tumor than normal cells. The pattern of binding specificity was similar amongst the tumor cell lines analyzed (U87MGwt EGFR and A431) suggesting that the selectivity of binding is universally high for tumor cells overexpressing EGFR (~2 million receptors per cell or more).

It will further be appreciated from the results shown in Figure 3 that there is a greater reduction in binding of some anti-EGFR mAb variants to cells expressing (A) lower EGFR levels (parental U87) as compared to (B) U87 cells overexpressing EGFR.

Also, as shown in Figure 4, it is clear that the ratio of antibody binding to EGFR overexpressing cells (U87MGwtEGFR) relative to antibody binding to parental U87MG cells was improved in most cases by 2-4 fold. That is, a ratio of 11 for wild type antibody

binding to U87MGwtEGFR cells versus parental cells, and ratios up to 35 for certain mutated antibodies, e.g. mutant 7-LC and 4-LC, were observed. Antibody 6-2 and 7-2 exhibited no detectable binding to EGFR on either cell type at the concentrations used (1 ug/ml).

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Finally, in Figure 5 it is shown and confirmed that some mutant antibodies can bind to EGFR and deliver a protein toxin, in this case saporin. Mutant antibodies 6-2 and 7-2 exhibited cytotoxicity similar to that seen with the non-specific controls, which is not unexpected since they do not detectably bind EGFR on the surface of these cells (Figure 3). Notably, in comparison to the cytotoxicity profile seen for the wt EGFR MAb (HC/LC), antibodies 6-LC, 7-LC and 4-1 exhibited decreased cytotoxicity on cells with low levels of EGFR (human epidermal keratinocytes (HEK) and parental U87 cells) with little decrease in cytotoxicity on U87 cells overexpressing wild type EGFR.

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In summary, this data indicates that mutant antibodies can be generated that bind highly selectively to cells that present EGFR at abnormally high density, and that these antibodies may be useful in oncology and other diseases as antibody-drug conjugates with broad therapeutic windows, and/or as diagnostic agents for the detection of EGFR overexpressing cells.

20

All references cited herein, including all database references and the sequence information referenced therein, are hereby incorporated herein in their entirety.

REFERENCES

25

1. Li S, Schmitz KR, Jeffrey PD, Wiltzius JJ, Kussie P, Ferguson KM (2005) *Cancer Cell* 7:301-311.
2. Li Z, Scheraga HA (1987) *Proc. Natl. Acad. Sci. U.S.A.* 84:6611-6615.
3. Naim M, Bhat S, Rankin KN, Dennis S, Chowdhury SF, Siddiqi I, Drabik P, Sulea T, Bayly CI, Jakalian A, Purisima EO (2007) *J. Chem. Inf. Model.* 47:122-133.
4. Selzer T, Albeck S, Schreiber G (2000) *Nat. Struct. Biol.* 7:537-541.
5. Gaillet, B., R.Gilbert, R.Amziani, C.Guilbault, C.Gadoury, A.W.Caron, A.Mullick, A.Garnier, and B.Massie. 2007. High-Level Recombinant Protein Production in CHO Cells Using an Adenoviral Vector and the Cumate Gene-Switch. *Biotechnol. Prog.* 23:200-209.
6. Mullick, A., Y.Xu, R.Warren, M.Koutroumanis, C.Guilbault, S.Broussau, F.Malenfant, L.Bourget, L.Lamoureux, R.Lo, A.W.Caron, A.Pilotte, and B.Massie. 2006. The cumate gene-switch: a system for regulated expression in mammalian cells. *BMC Biotechnol.* 6:43.

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Polynucleotides encoding the various mutant antibody chains are provided below. Substituted codons are shaded, and HindIII sites are highlighted:

5 **Light chain wild-type (shown here with the signal peptide) [SEQ ID No. 11]:**

GTTTAAACGAATTCGCCCTTGAGGTACCAAGCTTGCCACCATGGTGCTGCAGACCCAGGT
 GTTCATCTCCCTGCTGCTGTGGATCTCTGGCGCCTACGGCGACATCCTGCTGACCCAGTC
 CCCC GTGATCCTGTCCGTGTCCCCTGGCGAGCGGGTGTCTTCTCTTGCCGGGCCTCCCA
 10 GTCCATCGGCACCAACATCCACTGGTATCAGCAGCGGACCAACGGCTCCCCTCGGCTGCT
 GATCAAGTACGCCTCCGAGTCTATCTCCGGCATCCCTTCCC GGTCTCCGGCTCTGGCTC
 CGGCACCGACTTCACCCTGTCCATCAACTCCGTGGAGTCCGAGGATATCGCCGACTACTA
 CTGCCAGCAGAACAACA AACTGGCCTACCACCTTCGGCGCTGGCACCAAGCTGGA AACTGAA
 GCGGACCGTGGCCGCTCCTTCCGTGTTTCATCTTCCCACCTTCCGACGAGCAGCTGAAGTC
 15 CGGCACCGCCTCTGTGGTGTGCCTGCTGAACA AACTTCTACCCTCGGGAGGCCAAGGTGCA
 GTGGAAGGTGGACAACGCCCTGCAGTCCGGCAACTCCCAGGAATCCGTCACCGAGCAGGA
 CTCCAAGGACTCTACCTACTCCCTGTCTCCACCCTGACCCTGTCCAAGGCCGACTACGA
 GAAGCACAAGGTGTACGCCTGCGAAGTGACCCACCAGGGCCTGTCCAGCCCTGTGACCAA
 GTCCTTCAACCGGGCGAGTGCTGAAAGCTTGAGCTCAGTAAGGGCGAATTCGCGGCCG

20 **Light chain E58K mutant (shown here with the signal peptide) [SEQ ID No. 12]:**

CGGAAGGCCATGAGGCCAGTTAATTAAGAGGTACCAAGCTTGCCACCATGGTGCTGCAG
 ACCCAGGTGTTTCATCTCCCTGCTGCTGTGGATCTCTGGCGCCTACGGCGACATCCTGCTG
 ACCCAGTCCCCCGTGATCCTGTCCGTGTCCCCTGGCGAGCGGGTGTCTTCTCTTGCCGG
 GCCTCCCAGTCCATCGGCACCAACATCCACTGGTATCAGCAGCGGACCAACGGCTCCCCT
 25 CGGCTGCTGATCAAGTACGCCTCC [shaded] TCTATCTCCGGCATCCCTTCCC GGTCTCCGGC
 TCTGGCTCCGGCACC GACTTCACCCTGTCCATCAACTCCGTGGAGTCCGAGGATATCGCC
 GACTACTACTGCCAGCAGAACAACA AACTGGCCTACCACCTTCGGCGCTGGCACCAAGCTG
 GAACTGAAGCGGACCGTGGCCGCTCCTTCCGTGTTTCATCTTCCCACCTTCCGACGAGCAG
 CTGAAGTCCGGCACC GCCTCTGTGGTGTGCCTGCTGAACA AACTTCTACCCTCGGGAGGCC
 30 AAGGTGCAGTGGAAGGTGGACAACGCCCTGCAGTCCGGCAACTCCCAGGAATCCGTCACC
 GAGCAGGACTCCAAGGACTCTACCTACTCCCTGTCTCCACCCTGACCCTGTCCAAGGCC
 GACTACGAGAAGCACAAGGTGTACGCCTGCGAAGTGACCCACCAGGGCCTGTCCAGCCCT
 GTGACCAAGTCCTTCAACCGGGGCGAGTGCTGAAAGCTTGAGCTCATGGCGCGCCTAGGC
 CTTGACGGCCTTCCG

35 **Light chain W94A mutant (shown here with the signal peptide):[SEQ ID No.13]:**

CGGAAGGCCATGAGGCCAGTTAATTAAGAGGTACCAAGCTTGCCACCATGGTGCTGCAG
 ACCCAGGTGTTTCATCTCCCTGCTGCTGTGGATCTCTGGCGCCTACGGCGACATCCTGCTG
 ACCCAGTCCCCCGTGATCCTGTCCGTGTCCCCTGGCGAGCGGGTGTCTTCTCTTGCCGG
 40 GCCTCCCAGTCCATCGGCACCAACATCCACTGGTATCAGCAGCGGACCAACGGCTCCCCT
 CGGCTGCTGATCAAGTACGCCTCCGAGTCTATCTCCGGCATCCCTTCCC GGTCTCCGGC
 TCTGGCTCCGGCACC GACTTCACCCTGTCCATCAACTCCGTGGAGTCCGAGGATATCGCC
 GACTACTACTGCCAGCAGAACAACA AACT [shaded] CCTACCACCTTCGGCGCTGGCACCAAGCTG
 GAACTGAAGCGGACCGTGGCCGCTCCTTCCGTGTTTCATCTTCCCACCTTCCGACGAGCAG
 45 CTGAAGTCCGGCACC GCCTCTGTGGTGTGCCTGCTGAACA AACTTCTACCCTCGGGAGGCC
 AAGGTGCAGTGGAAGGTGGACAACGCCCTGCAGTCCGGCAACTCCCAGGAATCCGTCACC
 GAGCAGGACTCCAAGGACTCTACCTACTCCCTGTCTCCACCCTGACCCTGTCCAAGGCC
 GACTACGAGAAGCACAAGGTGTACGCCTGCGAAGTGACCCACCAGGGCCTGTCCAGCCCT

GTGACCAAGTCCTTCAACCGGGGCGAGTGCTGAAAGCTTGAGCTCATGGCGCGCCTAGGC
CTTGACGGCCTTCCG

Heavy chain wild-type (shown here with the signal peptide) [SEQ ID No. 14]:

5 CGAATTGAAGGAAGGCCGTCAAGGCCGCATGGTACCAAGCTTGCCACCATGGACTGGACC
TGGCGGATCCTGTTTCTGGTGGCCGCTGCTACCGGCACACACGCCCAGGTGCAGCTGAAG
CAGTCTGGCCCTGGCCTGGTGCAGCCTTCCCAGTCCCTGTCCATCACCTGTACCGTGTCC
10 GGCTTCTCCCTGACCAACTACGGCGTGCAGTGGGTGCGCCAGTCTCCAGGCAAGGGCCTG
GAATGGCTGGGAGTGATTTGGTCCGGCGGCAACACCGACTACAACACCCCTTTCACCTCC
CGGCTGTCCATCAACAAGGACAACCTCCAAGTCCCAGGTGTTCTTCAAGATGAACTCCCTG
CAGTCCAACGACACCGCCATCTACTACTGCGCCAGGGCTCTGACCTACTACGACTACGAG
15 TTCGCCTACTGGGGCCAGGGCACCCTGGTGACCGTGTCCGCCGCTTCCACCAAGGGCCCT
AGCGTGTTCCTCTGGCCCCCTTCCAGCAAGTCTACCTCTGGCGGCACCGCTGCTCTGGGC
TGCCTGGTGAAGGACTACTTCCCTGAGCCTGTGACAGTGTCTGGAAGTCTGGCGCCCTG
ACCTCCGGAGTGCACACCTTCCCTGCTGTGCTGCAGTCTCCGGCCTGTACTCCCTGTCC
TCCGTGGTGCAGTGCCTTCCCTCCAGCCTGGGCACACAGACCTACATCTGCAACGTGAAC
CACAAGCCTTCCAACACCAAGGTGGACAAGCGGGTGGAGCCTAAGTCTGCGACAAGACC
CACACCTGTCTCCATGCCCTGCCCTGAGCTGCTGGGCGGACCCTCCGTGTTCTCTGTTT
20 CCTCCAAAGCCTAAGGACACCCTGATGATCTCCCGGACCCCTGAAGTGACCTGCGTGGT
GTGGACGTGTCCACGAGGATCCTGAAGTGAAGTTC AATTGGTACGTGGACGGCGTGGAG
GTGCACAACGCCAAGACCAAGCCTCGGGAGGAACAGTACAACCTCCACCTACCGGGTGGT
TCCGTGCTGACCGTGTGCAACAGGACTGGCTGAACGGCAAAGAGTACAAGTGAAGGTC
TCCAACAAGGCCCTGCCTGCCCTATCGAAAAGACCATCTCCAAGGCCAAGGGCCAGCCT
25 CGGGAACCTCAGGTGTACACACTGCCTCCCAGCAGGGACGAGCTGACCAAGAACCAGGT
TCCCTGACCTGTCTGGTGAAGGGCTTCTACCCTTCCGATATCGCCGTGGAGTGGGAGTCT
AACGGCCAGCCTGAGAACAACCTACAAGACCACCCCTCCTGTGCTGGACTCCGACGGCTCC
TTCTTCTGTACTCCAAACTGACCGTGGACAAGTCCCGGTGGCAGCAGGGCAACGTGTTT
TCCCTGCTCCGTGATGCACGAGGCCCTGCACAACCACTACACCCAGAAGTCCCTGTCTCTG
30 TCCCTGGCAAGTGAAGCTTGAGCTCCTGGGCCTCATGGGCCTTCCCTTCACTGCC

Heavy chain Y101A mutant (shown here with the signal peptide) [SEQ ID No.15]:

35 CGGAAGGCCCATGAGGCCAGTTAATTAAGAGGTACCAAGCTTGCCACCATGGACTGGACC
TGGCGGATCCTGTTTCTGGTGGCCGCTGCTACCGGCACACACGCCCAGGTGCAGCTGAAG
CAGTCTGGCCCTGGCCTGGTGCAGCCTTCCCAGTCCCTGTCCATCACCTGTACCGTGTCC
GGCTTCTCCCTGACCAACTACGGCGTGCAGTGGGTGCGCCAGTCTCCAGGCAAGGGCCTG
GAATGGCTGGGAGTGATTTGGTCCGGCGGCAACACCGACTACAACACCCCTTTCACCTCC
40 CGGCTGTCCATCAACAAGGACAACCTCCAAGTCCCAGGTGTTCTTCAAGATGAACTCCCTG
CAGTCCAACGACACCGCCATCTACTACTGCGCCAGGGCTCTGACC [REDACTED] TACGACTACGAG
TTCGCCTACTGGGGCCAGGGCACCCTGGTGACCGTGTCCGCCGCTTCCACCAAGGGCCCT
AGCGTGTTCCTCTGGCCCCCTTCCAGCAAGTCTACCTCTGGCGGCACCGCTGCTCTGGGC
TGCCTGGTGAAGGACTACTTCCCTGAGCCTGTGACAGTGTCTGGAAGTCTGGCGCCCTG
ACCTCCGGAGTGCACACCTTCCCTGCTGTGCTGCAGTCTCCGGCCTGTACTCCCTGTCC
45 TCCGTGGTGCAGTGCCTTCCCTCCAGCCTGGGCACACAGACCTACATCTGCAACGTGAAC
CACAAGCCTTCCAACACCAAGGTGGACAAGCGGGTGGAGCCTAAGTCTGCGACAAGACC
CACACCTGTCTCCATGCCCTGCCCTGAGCTGCTGGGCGGACCCTCCGTGTTCTCTGTTT

CCTCCAAAGCCTAAGGACACCCTGATGATCTCCCGGACCCCTGAAGTGACCTGCGTGGTG
 GTGGACGTGTCCCACGAGGATCCTGAAGTGAAGTTCAATTGGTACGTGGACGGCGTGGAG
 GTGCACAACGCCAAGACCAAGCCTCGGGAGGAACAGTACAACCTCCACCTACCGGGTGGTG
 TCCGTGCTGACCGTGTGCACCAGGACTGGCTGAACGGCAAAGAGTACAAGTGAAGGTC
 5 TCCAACAAGGCCCTGCCTGCCCTATCGAAAAGACCATCTCCAAGGCCAAGGGCCAGCCT
 CGGGAACCTCAGGTGTACACACTGCCTCCCAGCAGGGACGAGCTGACCAAGAACCAGGTG
 TCCCTGACCTGTCTGGTGAAGGGCTTCTACCCTTCCGATATCGCCGTGGAGTGGGAGTCT
 AACGGCCAGCCTGAGAACAACACTACAAGACCACCCCTCCTGTGCTGGACTCCGACGGCTCC
 TTCTTCTGTACTCCAAACTGACCGTGGACAAGTCCCAGTGGCAGCAGGGCAACGTGTTG
 10 TCCTGCTCCGTGATGCACGAGGCCCTGCACAACCCTACACCCAGAAGTCCCTGTCTCTG
 TCCCTGGCAAGTGAAAGCTTGAGTTCATGGCGCGCCTAGGCCTTGACGGCCTTCCG

Heavy chain Y102A mutant (shown here with the signal peptide) [SEQ ID No.16]:

CGGAAGGCCCATGAGGCCAGTTAATTAAGAGGTACCAAGCTTGCCACCATGGACTGGACC
 15 TGGCGGATCCTGTTTCTGGTGGCCGCTGCTACCGGCACACACGCCCAGGTGCAGCTGAAG
 CAGTCTGGCCCTGGCTGGTGCAGCCTTCCCAGTCCCTGTCCATCACCTGTACCGTGTCC
 GGCTTCTCCCTGACCAACTACGGCGTGCAGTGGGTGCGCCAGTCTCCAGGCAAGGGCCTG
 GAATGGCTGGGAGTGATTTGGTCCGGCGGCAACACCGACTACAACACCCCTTTCACCTCC
 CGGCTGTCCATCAACAAGGACAACCTCCAAGTCCCAGGTGTTCTTCAAGATGAACTCCCTG
 20 CAGTCCAACGACACCGCCATCTACTACTGCGCCAGGGCTCTGACCTACTGACTACGAG
 TTCGCCTACTGGGGCCAGGGCACCCTGGTGACCGTGTCCGCCGCTTCCACCAAGGGCCCT
 AGCGTGTTCCTCTGGCCCTTCCAGCAAGTCTACCTCTGGCGGCACCGCTGCTCTGGGC
 TGCCTGGTGAAGGACTACTTCCCTGAGCCTGTGACAGTGTCCCTGGAACCTGGCGCCCTG
 ACCTCCGGAGTGCACACCTTCCCTGCTGTGCTGCAGTCCCTCCGGCCTGACTCCCTGTCC
 25 TCCGTGGTGCAGTGCCTTCCCTCCAGCCTGGGCACACAGACCTACATCTGCAACGTGAAC
 CACAAGCCTTCCAACACCAAGGTGGACAAGCGGGTGGAGCCTAAGTCCCTGCGACAAGACC
 CACACCTGTCTCCATGCCCTGCCCTGAGCTGCTGGGCGGACCCTCCGTGTTCTGTTC
 CCTCCAAAGCCTAAGGACACCCTGATGATCTCCCGACCCCTGAAGTGACCTGCGTGGTG
 GTGGACGTGTCCCACGAGGATCCTGAAGTGAAGTTCAATTGGTACGTGGACGGCGTGGAG
 30 GTGCACAACGCCAAGACCAAGCCTCGGGAGGAACAGTACAACCTCCACCTACCGGGTGGTG
 TCCGTGCTGACCGTGTGCACCAGGACTGGCTGAACGGCAAAGAGTACAAGTGAAGGTC
 TCCAACAAGGCCCTGCCTGCCCTATCGAAAAGACCATCTCCAAGGCCAAGGGCCAGCCT
 CGGGAACCTCAGGTGTACACACTGCCTCCCAGCAGGGACGAGCTGACCAAGAACCAGGTG
 TCCCTGACCTGTCTGGTGAAGGGCTTCTACCCTTCCGATATCGCCGTGGAGTGGGAGTCT
 35 AACGGCCAGCCTGAGAACAACACTACAAGACCACCCCTCCTGTGCTGGACTCCGACGGCTCC
 TTCTTCTGTACTCCAAACTGACCGTGGACAAGTCCCAGTGGCAGCAGGGCAACGTGTTG
 TCCTGCTCCGTGATGCACGAGGCCCTGCACAACCCTACACCCAGAAGTCCCTGTCTCTG
 TCCCTGGCAAGTGAAAGCTTGAGTTCATGGCGCGCCTAGGCCTTGACGGCCTTCCG

Heavy chain D103N mutant (shown here with the signal peptide) [SEQ ID No.17]:

CGGAAGGCCCATGAGGCCAGTTAATTAAGAGGTACCAAGCTTGCCACCATGGACTGGACC
 40 TGGCGGATCCTGTTTCTGGTGGCCGCTGCTACCGGCACACACGCCCAGGTGCAGCTGAAG
 CAGTCTGGCCCTGGCTGGTGCAGCCTTCCCAGTCCCTGTCCATCACCTGTACCGTGTCC
 GGCTTCTCCCTGACCAACTACGGCGTGCAGTGGGTGCGCCAGTCTCCAGGCAAGGGCCTG
 GAATGGCTGGGAGTGATTTGGTCCGGCGGCAACACCGACTACAACACCCCTTTCACCTCC
 45 CGGCTGTCCATCAACAAGGACAACCTCCAAGTCCCAGGTGTTCTTCAAGATGAACTCCCTG
 CAGTCCAACGACACCGCCATCTACTACTGCGCCAGGGCTCTGACCTACTACTGACTACGAG

TTCGCCTACTGGGGCCAGGGCACCCCTGGTGACCGTGTCCGCCGCTTCCACCAAGGGCCCT
 AGCGTGTTCCTCTGGCCCCCTCCAGCAAGTCTACCTCTGGCGGCACCGCTGCTCTGGGC
 TGCCTGGTGAAGGACTACTTCCCTGAGCCTGTGACAGTGTCTGGAACCTCTGGCGCCCTG
 ACCTCCGGAGTGCACACCTTCCCTGCTGTGCTGCAGTCCTCCGGCCTGTACTCCCTGTCC
 5 TCCGTGGTGACAGTGCCTTCCCTCCAGCCTGGGCACACAGACCTACATCTGCAACGTGAAC
 CACAAGCCTTCCAACACCAAGGTGGACAAGCGGGTGGAGCCTAAGTCCTGCGACAAGACC
 CACACCTGTCTCCATGCCCTGCCCCTGAGCTGCTGGGCGGACCCTCCGTGTTCTCTGTTT
 CCTCCAAAGCCTAAGGACACCCTGATGATCTCCCGGACCCCTGAAGTGACCTGCGTGGTG
 GTGGACGTGTCCCACGAGGATCCTGAAGTGAAGTTCAATTGGTACGTGGACGGCGTGGAG
 10 GTGCACAACGCCAAGACCAAGCCTCGGGAGGAACAGTACAACCTCCACCTACCGGGTGGTG
 TCCGTGCTGACCGTGTGACACCAGGACTGGCTGAACGGCAAAGAGTACAAGTGCAAGGTC
 TCCAACAAGGCCCTGCCTGCCCCTATCGAAAAGACCATCTCCAAGGCCAAGGGCCAGCCT
 CGGGAACCTCAGGTGTACACACTGCCTCCCAGCAGGGACGAGCTGACCAAGAACCAGGTG
 TCCCTGACCTGTCTGGTGAAGGGCTTCTACCCTTCCGATATCGCCGTGGAGTGGGAGTCT
 15 AACGGCCAGCCTGAGAACAACACTACAAGACCACCCCTCCTGTGCTGGACTCCGACGGCTCC
 TTCTTCTGTACTCCAAACTGACCGTGGACAAGTCCCGGTGGCAGCAGGGCAACGTGTTT
 TCCTGCTCCGTGATGCACGAGGCCCTGCACAACCACTACACCAGAAAGTCCCTGTCTCTG
 TCCCCTGGCAAGTGAAAGCTTGAGCTCATGGCGCGCCTAGGCCCTTGACGGCCTTCCG

20 **Heavy chain D58N/D103N mutant (shown here with the signal peptide) [SEQ ID
 No.18]:**

CGGAAGGCCCATGAGGCCAGTTAATTAAGAGGTACCAAGCTTGCCACCATGGACTGGACC
 TGGCGGATCCTGTTTCTGGTGGCCGCTGCTACCGGCACACACGCCCAGGTGCAGCTGAAG
 CAGTCTGGCCCTGGCCTGGTGCAGCCTTCCCAGTCCCTGTCCATCACCTGTACCGTGTCC
 25 GGCTTCTCCCTGACCAACTACGGCGTGCAGTGGGTGCGCCAGTCTCCAGGCAAGGGCCTG
 GAATGGCTGGGAGTGATTTGGTCCGGCGGCAACACC[REDACTED]TACAACACCCCTTTACCTCC
 CGGCTGTCCATCAACAAGGACAACCTCCAAGTCCCAGGTGTTCTTCAAGATGAACTCCCTG
 CAGTCCAACGACACCGCCATCTACTACTGCGCCAGGGCTCTGACCTACTAC[REDACTED]TACGAG
 TTCGCCTACTGGGGCCAGGGCACCCCTGGTGACCGTGTCCGCCGCTTCCACCAAGGGCCCT
 30 AGCGTGTTCCTCTGGCCCCCTTCCAGCAAGTCTACCTCTGGCGGCACCGCTGCTCTGGGC
 TGCCTGGTGAAGGACTACTTCCCTGAGCCTGTGACAGTGTCTGGAACCTCTGGCGCCCTG
 ACCTCCGGAGTGCACACCTTCCCTGCTGTGCTGCAGTCCTCCGGCCTGTACTCCCTGTCC
 TCCGTGGTGACAGTGCCTTCCCTCCAGCCTGGGCACACAGACCTACATCTGCAACGTGAAC
 CACAAGCCTTCCAACACCAAGGTGGACAAGCGGGTGGAGCCTAAGTCCTGCGACAAGACC
 35 CACACCTGTCTCCATGCCCTGCCCCTGAGCTGCTGGGCGGACCCTCCGTGTTCTCTGTTT
 CCTCCAAAGCCTAAGGACACCCTGATGATCTCCCGGACCCCTGAAGTGACCTGCGTGGTG
 GTGGACGTGTCCCACGAGGATCCTGAAGTGAAGTTCAATTGGTACGTGGACGGCGTGGAG
 GTGCACAACGCCAAGACCAAGCCTCGGGAGGAACAGTACAACCTCCACCTACCGGGTGGTG
 TCCGTGCTGACCGTGTGACACCAGGACTGGCTGAACGGCAAAGAGTACAAGTGCAAGGTC
 40 TCCAACAAGGCCCTGCCTGCCCCTATCGAAAAGACCATCTCCAAGGCCAAGGGCCAGCCT
 CGGGAACCTCAGGTGTACACACTGCCTCCCAGCAGGGACGAGCTGACCAAGAACCAGGTG
 TCCCTGACCTGTCTGGTGAAGGGCTTCTACCCTTCCGATATCGCCGTGGAGTGGGAGTCT
 AACGGCCAGCCTGAGAACAACACTACAAGACCACCCCTCCTGTGCTGGACTCCGACGGCTCC
 TTCTTCTGTACTCCAAACTGACCGTGGACAAGTCCCGGTGGCAGCAGGGCAACGTGTTT
 45 TCCTGCTCCGTGATGCACGAGGCCCTGCACAACCACTACACCAGAAAGTCCCTGTCTCTG
 TCCCCTGGCAAGTGAAAGCTTGAGCTCATGGCGCGCCTAGGCCCTTGACGGCCTTCCG

Heavy chain D58N/D103N/E105Q mutant (shown here with the signal peptide)

[SEQ ID No. 19]:

CGGAAGGCCCATGAGGCCAGTTAATTAAGAGGTACCAAGCTTGCCACCATGGACTGGACC
 TGGCGGATCCTGTTTCTGGTGGCCGCTGCTACCGGCACACACGCCAGGTGCAGCTGAAG
 5 CAGTCTGGCCCTGGCCTGGTGCAGCCTTCCCAGTCCCTGTCCATCACCTGTACCGTGTCC
 GGCTTCTCCCTGACCAACTACGGCGTGCAGTGGGTGCGCCAGTCTCCAGGCAAGGGCCTG
 GAATGGCTGGGAGTGATTTGGTCCGGCGGCAACACC **■■■■** TACAACACCCCTTTCACCTCC
 CGGCTGTCCATCAACAAGGACAACCTCCAAGTCCCAGGTGTTCTTCAAGATGAACTCCCTG
 CAGTCCAACGACACCGCCATCTACTACTGCGCCAGGGCTCTGACCTACTAC **■■■■** TAC **■■■■**
 10 TTCGCCTACTGGGGCCAGGGCACCCTGGTGACCGTGTCCGCCGCTTCCACCAAGGGCCCT
 AGCGTGTTCCTCTGGCCCCCTTCCAGCAAGTCTACCTCTGGCGGCACCGCTGCTCTGGGC
 TGCCTGGTGAAGGACTACTTCCCTGAGCCTGTGACAGTGTCTTGGAACTCTGGCGCCCTG
 ACCTCCGGAGTGCACACCTTCCCTGCTGTGCTGCAGTCCCTCCGGCCTGTACTCCCTGTCC
 TCCGTGGTGCAGTGCCTTCCCTCCAGCCTGGGCACACAGACCTACATCTGCAACGTGAAC
 15 CACAAGCCTTCCAACACCAAGGTGGACAAGCGGGTGGAGCCTAAGTCTGCGACAAGACC
 CACACCTGTCTCCATGCCCTGCCCTGAGCTGCTGGGCGGACCCTCCGTGTTCTCTGTTT
 CCTCCAAAGCCTAAGGACACCCTGATGATCTCCCGGACCCCTGAAGTGACCTGCGTGGTG
 GTGGACGTGTCCACGAGGATCCTGAAGTGAAGTTCAATTGGTACGTGGACGGCGTGGAG
 GTGCACAACGCCAAGACCAAGCCTCGGGAGGAACAGTACAACCTCCACCTACCGGGTGGTG
 20 TCCGTGCTGACCGTGTGACACCAGGACTGGCTGAACGGCAAAGAGTACAAGTGCAAGGTC
 TCCAACAAGGCCCTGCCCTGCCCTATCGAAAAGACCATCTCCAAGGCCAAGGGCCAGCCT
 CGGGAACCTCAGGTGTACACACTGCCTCCCAGCAGGGACGAGCTGACCAAGAACCAGGTG
 TCCCTGACCTGTCTGGTGAAGGGCTTCTACCCCTCCGATATCGCCGTGGAGTGGGAGTCT
 AACGGCCAGCCTGAGAACAACCTACAAGACCACCCCTCCTGTGCTGGACTCCGACGGCTCC
 25 TTCTTCTGTACTCCAAACTGACCGTGGACAAGTCCCGGTGGCAGCAGGGCAACGTGTTT
 TCCTGCTCCGTGATGCACGAGGCCCTGCACAACCACTACACCCAGAAGTCCCTGTCTCTG
 TCCCTGGCAAGTCAAAGCTTGAGCTCATGGCGCGCCTAGGCCTTGACGGCCTTCCG

30 Amino acid sequences constituting the antibody wild type and mutant chains are provided below. The signal peptide is indicated using lower case letters, and is not included in the residue numbering. Mutated positions are bolded in mutant sequences.

Light chain wild-type [SEQ ID No. 20]:

35 mvlqtqvvislllwisgaygDILLTQSPVILSVSPGERVSVFSCRASQSIGTNIHWYQQRT
 NGSPRLLIKYASESISGIPSRFSGSGSGTDFTLINSVESEDIADYYCQQNNNWPTTFGA
 GTKLELKRITVAAPSVFI FPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQ
 ESVTEQDSKDYSLSSSTLTLKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

Light chain E58K mutant [SEQ ID No. 21]:

40 mvlqtqvvislllwisgaygDILLTQSPVILSVSPGERVSVFSCRASQSIGTNIHWYQQRT
 NGSPRLLIKYASKSISGIPSRFSGSGSGTDFTLINSVESEDIADYYCQQNNNWPTTFGA
 GTKLELKRITVAAPSVFI FPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQ
 ESVTEQDSKDYSLSSSTLTLKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

Light chain W94A mutant [SEQ ID No. 22]:

45 mvlqtqvvislllwisgaygDILLTQSPVILSVSPGERVSVFSCRASQSIGTNIHWYQQRTN
 GSPRLLIKYASESISGIPSRFSGSGSGTDFTLINSVESEDIADYYCQQNNNAPTTFGAGT
 KLELKRITVAAPSVFI FPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESV
 TEQDSKDYSLSSSTLTLKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
 50

Heavy chain wild-type [SEQ ID No. 23]:

mdwtwri~~l~~flvaaatgthaQVQLKQSGPGLVQPSQSL SITCTVSGFSLTNYGVHWVRQSP
 GKLEWLGVIWGGNTDYNT~~P~~FTSRLSINKDNSKSKVFFKMNSLQSNDAIYYCARALTY
 YDYEFAYWGQGLTLVTVSAASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWN
 5 SGALTS~~G~~VHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVPEPKS
 CDKTH~~T~~CP~~P~~CPAPELLGGPSVFLFPPKPKDTLMI SRTPEVTCVVVDVSHEDPEVKFNWYV
 DGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTI SKA
 KGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTT~~P~~PVLD
 SDGSFFLYSKLTVDKSRWQQGNV~~F~~SCSVMHEALHNHYTQKSLSLSPGK

10

Heavy chain Y101A mutant [SEQ ID No. 24]:

mdwtwri~~l~~flvaaatgthaQVQLKQSGPGLVQPSQSL SITCTVSGFSLTNYGVHWVRQSPG
 KLEWLGVIWGGNTDYNT~~P~~FTSRLSINKDNSKSKVFFKMNSLQSNDAIYYCARALTY~~A~~YD
 YEFAYWGQGLTLVTVSAASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGA
 15 LTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVPEPKSCDKT
 HTCP~~P~~CPAPELLGGPSVFLFPPKPKDTLMI SRTPEVTCVVVDVSHEDPEVKFNWYVDGVEV
 HNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTI SKAKGQPRE
 PQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTT~~P~~PVLDSDGSFFL
 YSKLTVDKSRWQQGNV~~F~~SCSVMHEALHNHYTQKSLSLSPGK

20

Heavy chain Y102A mutant [SEQ ID No. 25]:

mdwtwri~~l~~flvaaatgthaQVQLKQSGPGLVQPSQSL SITCTVSGFSLTNYGVHWVRQSPGK
 LEWLGVIWGGNTDYNT~~P~~FTSRLSINKDNSKSKVFFKMNSLQSNDAIYYCARALTY~~A~~DY
 FAYWGQGLTLVTVSAASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTS
 25 VHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVPEPKSCDKTH~~T~~CP~~P~~CP
 PAPELLGGPSVFLFPPKPKDTLMI SRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKP
 EEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTI SKAKGQPREPQVYTLPPSR
 DELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTT~~P~~PVLDSDGSFFLYSKLTVDKSRWQ
 QGNV~~F~~SCSVMHEALHNHYTQKSLSLSPGK

30

Heavy chain D103N mutant [SEQ ID No. 26]:

mdwtwri~~l~~flvaaatgthaQVQLKQSGPGLVQPSQSL SITCTVSGFSLTNYGVHWVRQSPGKGL
 EWL~~G~~VIWGGNTDYNT~~P~~FTSRLSINKDNSKSKVFFKMNSLQSNDAIYYCARALTY~~Y~~NYEFAYW
 GQGLTLVTVSAASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTS~~G~~VHTF
 35 AVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVPEPKSCDKTH~~T~~CP~~P~~CPAPELL
 GGPSVFLFPPKPKDTLMI SRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNST
 YRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTI SKAKGQPREPQVYTLPPSRDELTKNQV
 SLTCLVKGFYPSDIAVEWESNGQPENNYKTT~~P~~PVLDSDGSFFLYSKLTVDKSRWQQGNV~~F~~SCSV
 MHEALHNHYTQKSLSLSPGK

40

Heavy chain D58N/D103N mutant [SEQ ID No. 27]:

mdwtwri~~l~~flvaaatgthaQVQLKQSGPGLVQPSQSL SITCTVSGFSLTNYGVHWVRQSPGKGL
 WLGVIWGGNT~~N~~YNT~~P~~FTSRLSINKDNSKSKVFFKMNSLQSNDAIYYCARALTY~~Y~~NYEFAYWG
 45 GTLVTVSAASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTS~~G~~VHTFPAVL
 QSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVPEPKSCDKTH~~T~~CP~~P~~CPAPELLGGPS
 VFLFPPKPKDTLMI SRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVV
 SVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTI SKAKGQPREPQVYTLPPSRDELTKNQVSLTCLV
 KGFYPSDIAVEWESNGQPENNYKTT~~P~~PVLDSDGSFFLYSKLTVDKSRWQQGNV~~F~~SCSVMHEALHN
 HYTQKSLSLSPGK

50

Heavy chain D58N/D103N/E105Q mutant [SEQ ID No. 28]:

mdwtwri~~l~~flvaaatgthaQVQLKQSGPGLVQPSQSL SITCTVSGFSLTNYGVHWVRQSPGKGL
 WLGVIWGGNT~~N~~YNT~~P~~FTSRLSINKDNSKSKVFFKMNSLQSNDAIYYCARALTY~~Y~~NYQFAYWG
 55 GTLVTVSAASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTS~~G~~VHTFPAVL
 QSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVPEPKSCDKTH~~T~~CP~~P~~CPAPELLGGPS

VFLFPPKPKDTLMI SRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVS
VLTVLHQDWLNGKEYKCKVSNKALPAPI EKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLV
KGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVFSVCSVMHEALHN
HYTQKSLSLSPGK

WE CLAIM:

1. An EGFR antibody that binds preferentially to disease cells having an EGFR density greater than a normal EGFR density, the EGFR antibody comprising a heavy chain and a light chain, each chain having a constant region and a variable region, each variable region comprising framework regions and complementarity determining regions (CDRs), wherein the CDRs have an amino acid sequence set forth below:
- 5
- For the heavy chain:
- 10 CDR1 NYGVH (SEQ ID No. 1)
 CDR2 VIWSGGNTD⁵⁸YNTPF⁵⁸TS (SEQ ID No. 2)
 CDR3 ALTY¹⁰¹Y¹⁰²D¹⁰³YE¹⁰⁵FAY (SEQ ID No. 3)
- For the light chain:
- 15 CDR1 RASQSIGTNIH (SEQ ID No. 4)
 CDR2 ASE⁵³SIS (SEQ ID No. 5)
 CDR3 QQNNNW⁹⁴PTT (SEQ ID No. 6)
- 20 wherein at least one of E⁵³, D⁵⁸, W⁹⁴, Y¹⁰¹, Y¹⁰², D¹⁰³, and E¹⁰⁵ is substituted by an amino acid that confers on said antibody a reduced EGFR binding affinity (Kd) that is 1.0 nM or weaker.
2. The antibody according to claim 1, wherein Kd is 10 nM or weaker.
- 25 3. The antibody according to claim 1, wherein Kd is from 10 nM to 500 nM.
4. The antibody according to any one of claims 1 to 3, wherein at least one of said substitutions is in the heavy chain.
- 30 5. The antibody according to claim 4, wherein Y¹⁰¹ is substituted by A¹⁰¹.
6. The antibody according to claim 4, wherein Y¹⁰² is substituted by A¹⁰².
- 35 7. The antibody according to claim 4, wherein D¹⁰³ is replaced by N¹⁰³.
8. The antibody according to claim 4, wherein at least one of said substitutions is in the heavy chain and at least one of said substitutions is in the light chain.
- 40 9. The antibody according to claim 8, wherein E⁵³ and Y¹⁰² are both substituted.
10. The antibody according to claim 9, wherein E⁵³ is substituted by K⁵³ and Y¹⁰² is substituted by A¹⁰².
- 45 11. The antibody according to claim 10, wherein said heavy chain comprises at least two of said substitutions.

12. The antibody according to claim 11, wherein D⁵⁸ and D¹⁰³ are both substituted.
- 5 13. The antibody according to claim 9, wherein D⁵⁸ is substituted by N⁵⁸ and D¹⁰³ is substituted by N¹⁰³.
14. The antibody according to claim 12, further wherein E¹⁰⁵ is substituted.
- 10 15. The antibody according to claim 14, wherein E¹⁰⁵ is substituted by Q¹⁰⁵.
16. The antibody according to any one of claims 1 to 3, wherein at least one of said substitutions is in the light chain.
- 15 17. The antibody according to claim 16, wherein E⁵³ is substituted by K⁵³.
18. The antibody according to claim 16, wherein W⁹⁴ is substituted by A⁹⁴.
19. An antibody according to any preceding claim, the antibody having the
20 framework region sequences of cetuximab.
20. An antibody according to any preceding claim, the antibody having the
framework region sequences and the constant region sequence of cetuximab.
- 25 21. A bivalent fragment of an antibody according to any one of claims 1-20.
22. A conjugate comprising a cytotoxin or a detectable label and, conjugated thereto,
an antibody or bivalent fragment thereof as defined according to any one of claims 1-21.
- 30 23. A pharmaceutical composition comprising a pharmaceutically acceptable carrier
and an EGFR antibody in an amount useful to control the growth of cells presenting
EGFR at a density greater than the normal EGFR density, wherein the EGFR antibody
has an affinity (Kd) for EGFR that is within the range from about 1.0 nM to about 1 uM,
said antibody having insignificant binding affinity for a cell presenting EGFR at a normal
35 EGFR density.
24. A pharmaceutical composition according to claim 24, wherein the antibody is
defined according to any of claims 1-20.
- 40 25. A method for treating a subject presenting with disease cells having an EGFR
density greater than normal, comprising treating the subject with a pharmaceutical
composition comprising a pharmaceutically acceptable carrier and an amount of an
EGFR antibody, bivalent fragment thereof or conjugate thereof effective to control the
growth of said disease cells while minimizing adverse effects on cells presenting EGFR
45 at a normal density, wherein the EGFR antibody has an affinity (Kd) for EGFR that is

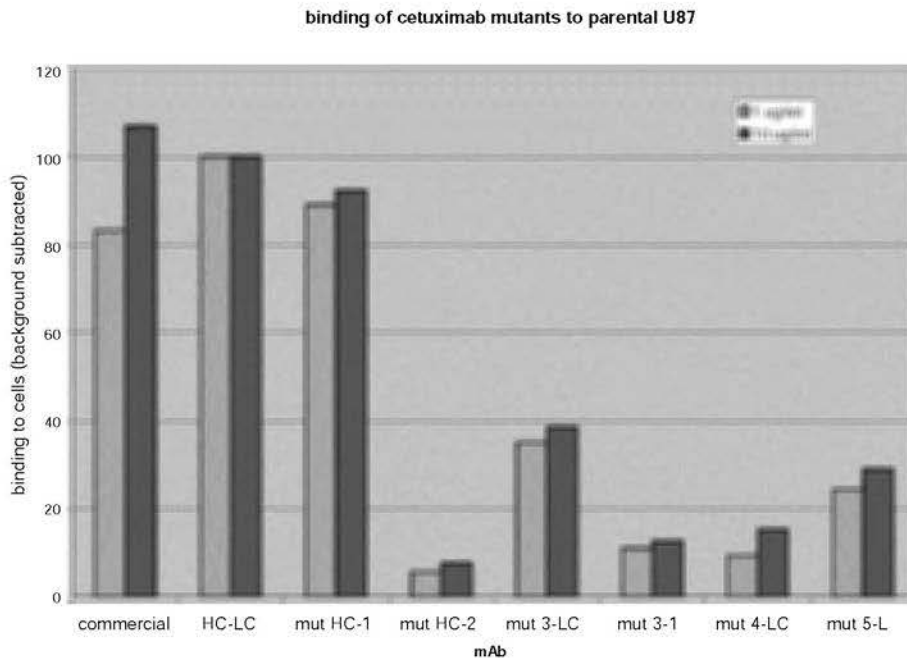
within the range from about 1.0 nM to about 1 uM, said antibody having insignificant binding affinity for a cell presenting EGFR at a normal EGFR density.

5 26. The method according to claim 25, wherein the antibody is defined according to any of claims 1-20.

27. The method according to claim 26, wherein the disease cells are cancer cells.

10 28. The method according to claim 27, wherein the cancer cells are colorectal cancer cells.

A)



B)

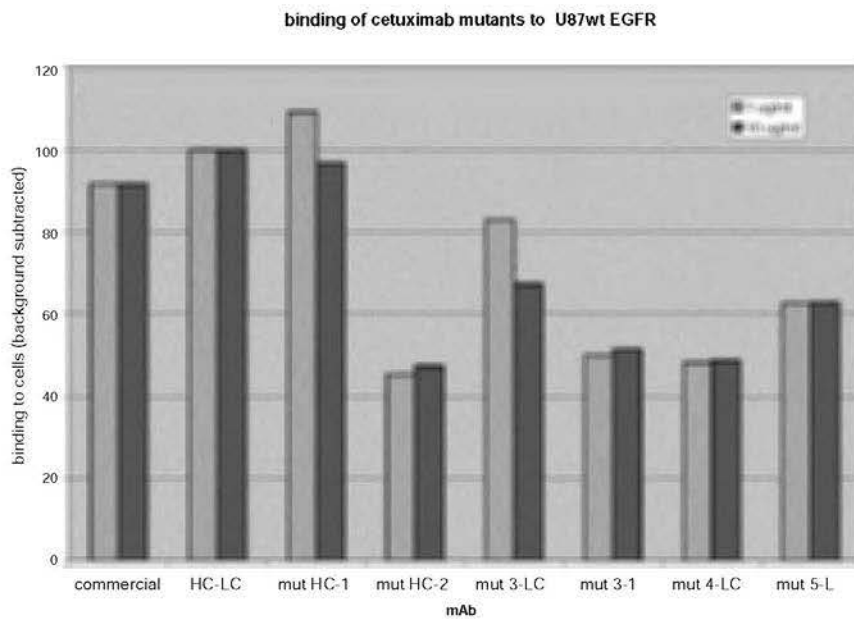
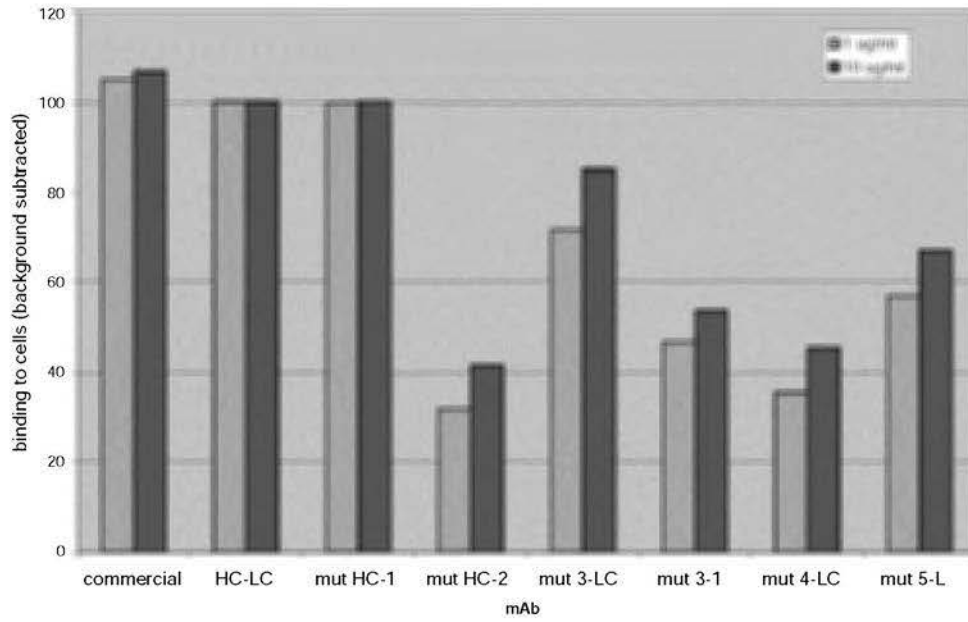


Figure 1 (A&B)

C)

binding of cetuximab mutants to U8 EGFRvIII



D)

binding of cetuximab mutants to HEK cells

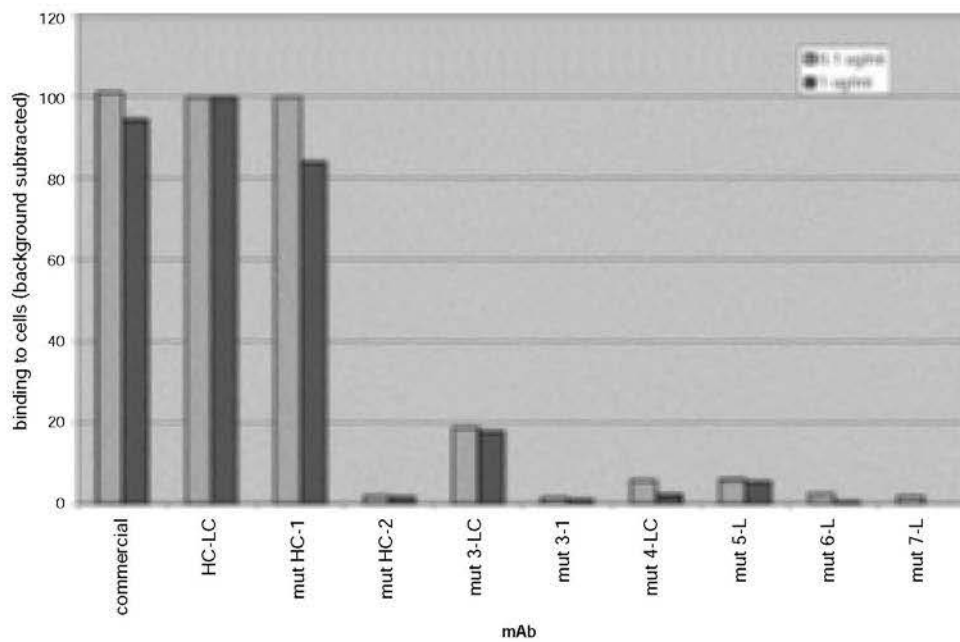


Figure 1(C&D)

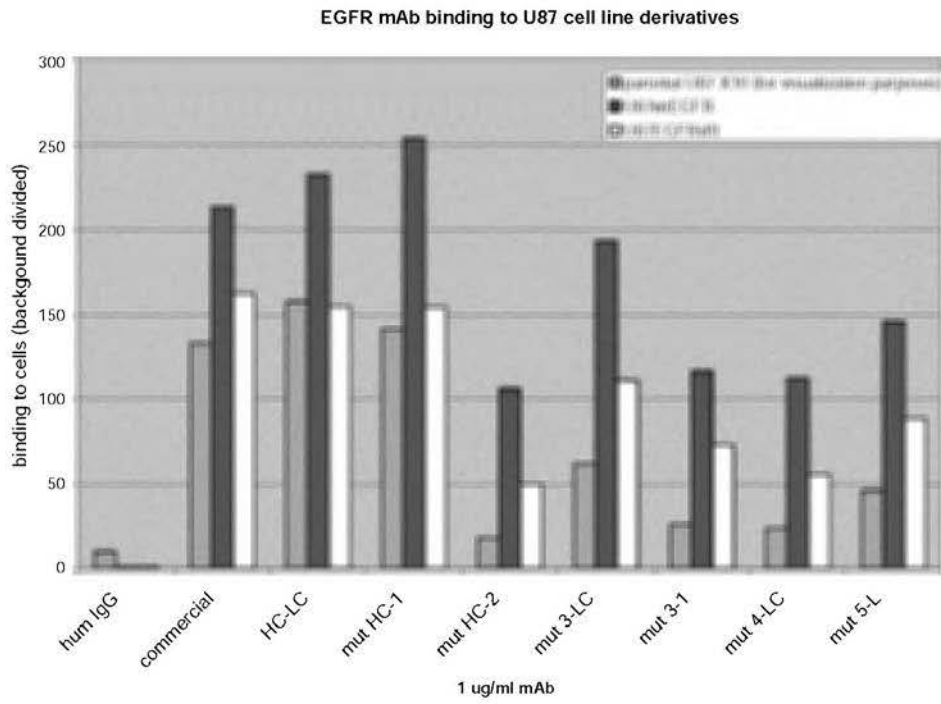


Figure 1-1A

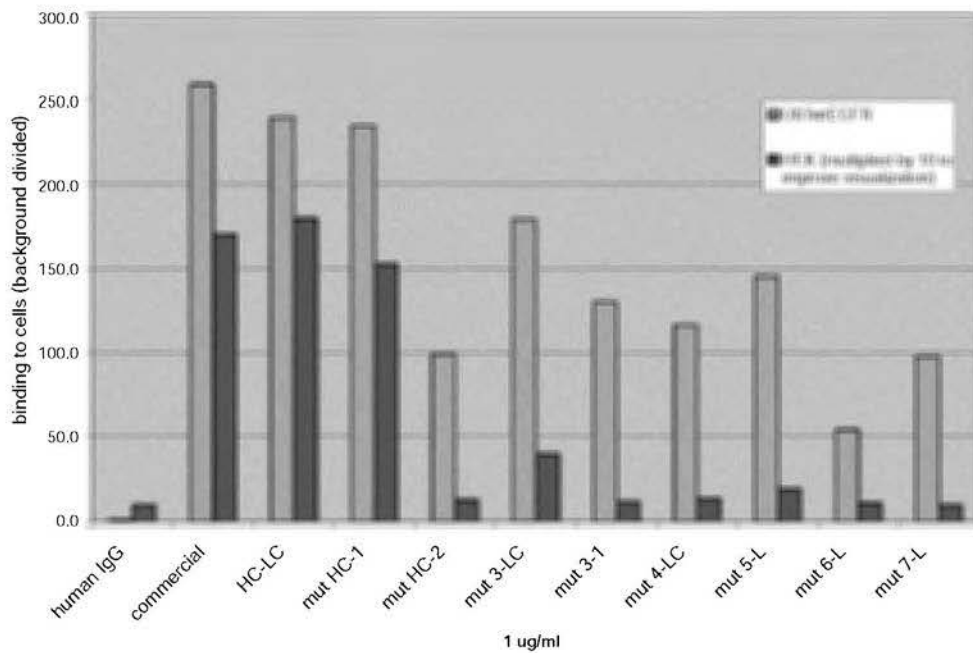


Figure 1-1B

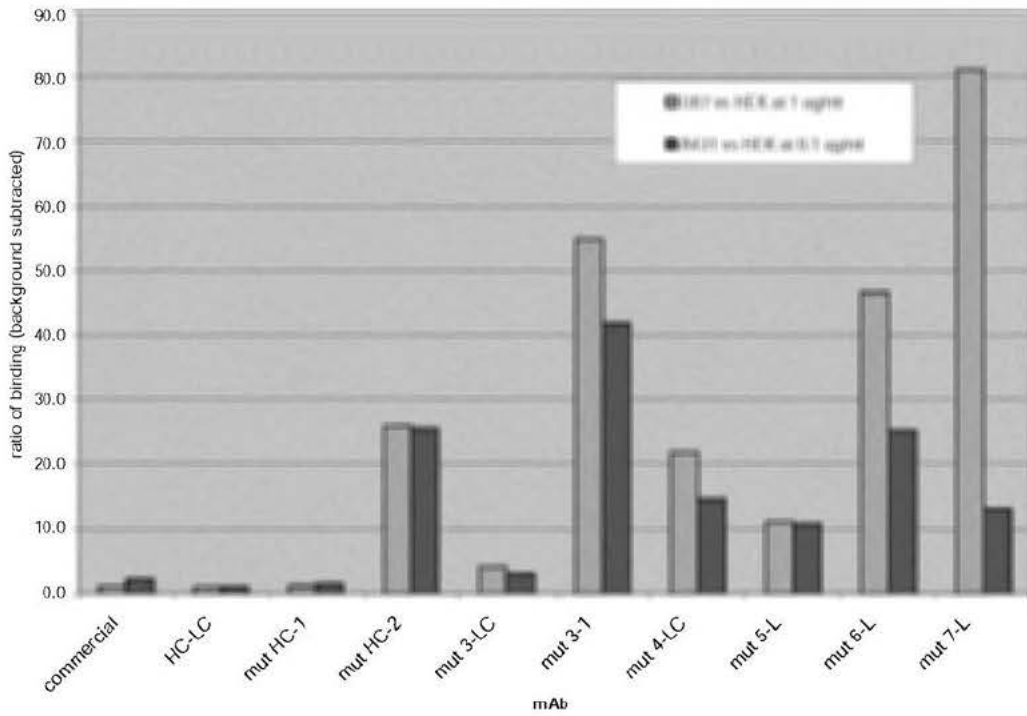


Figure 2

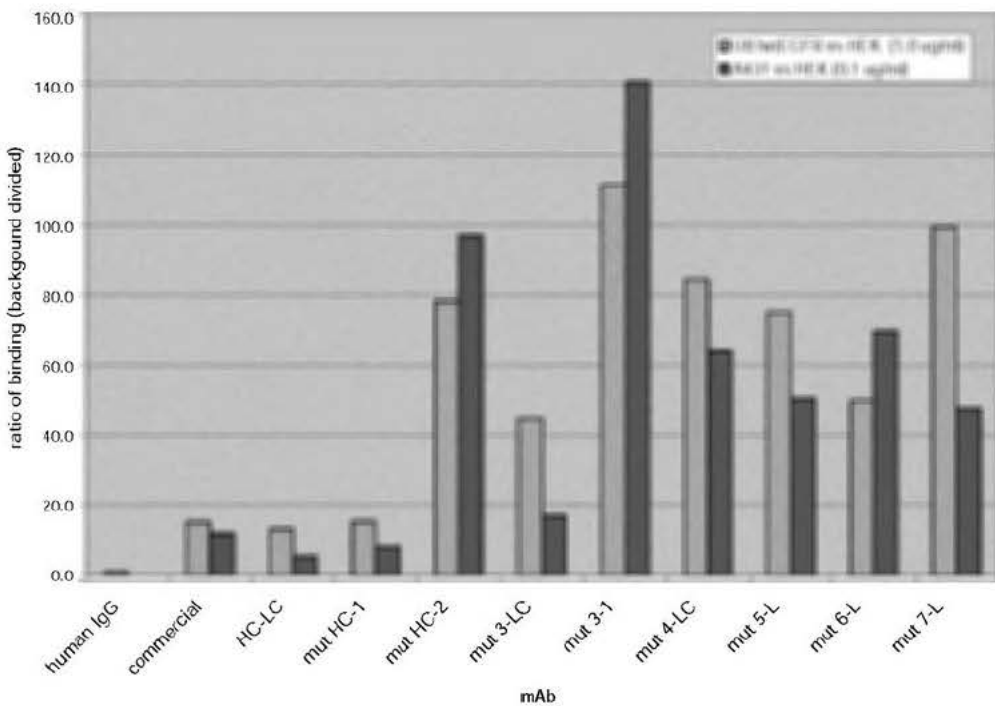


Figure 2-1

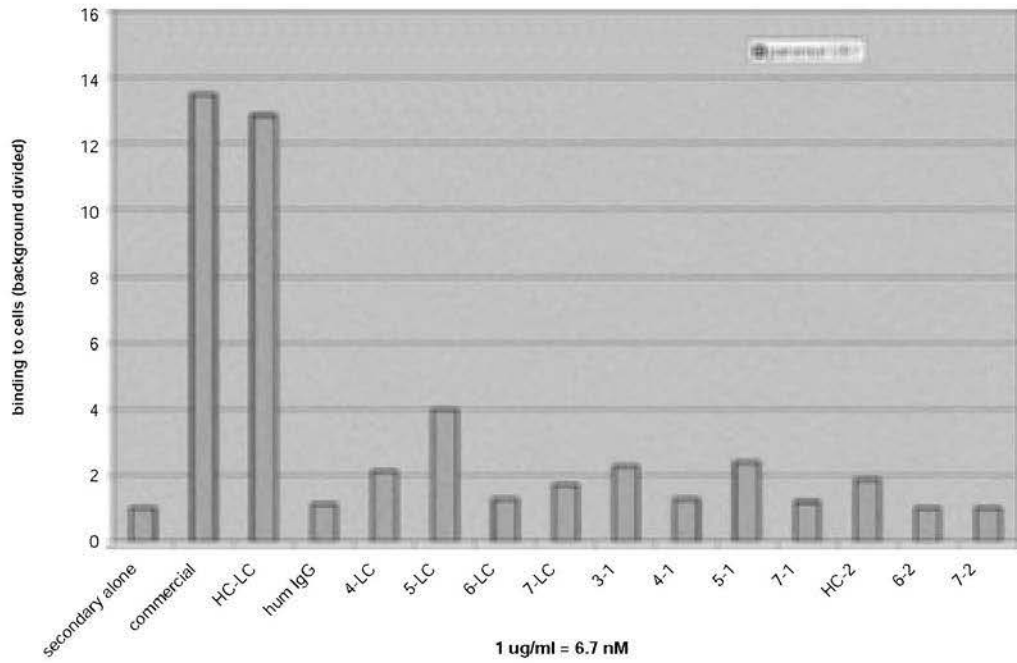
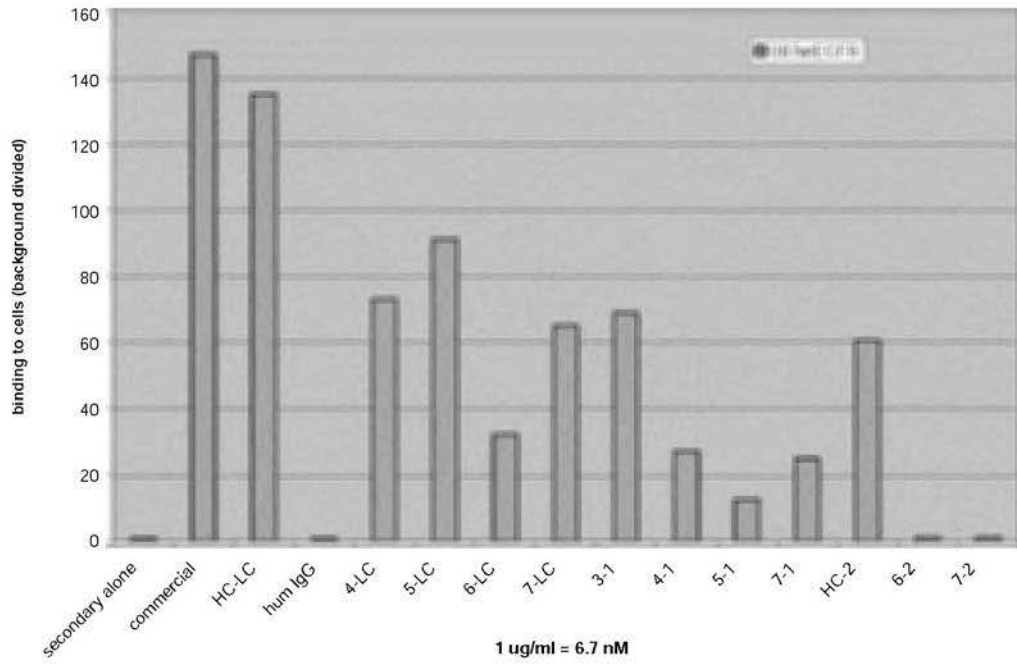


Figure 3

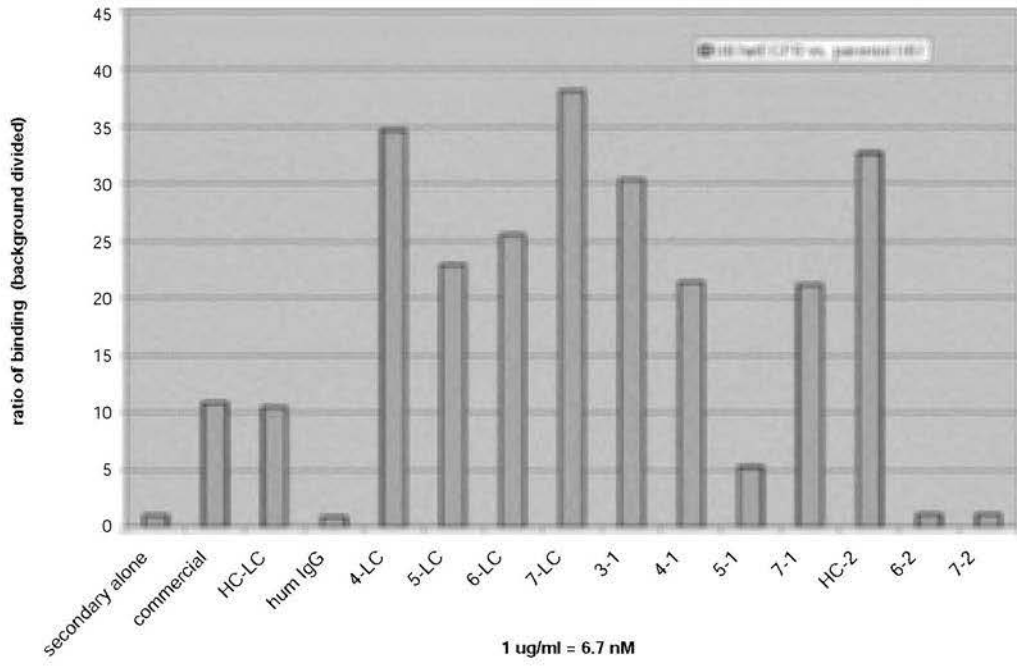


Figure 4

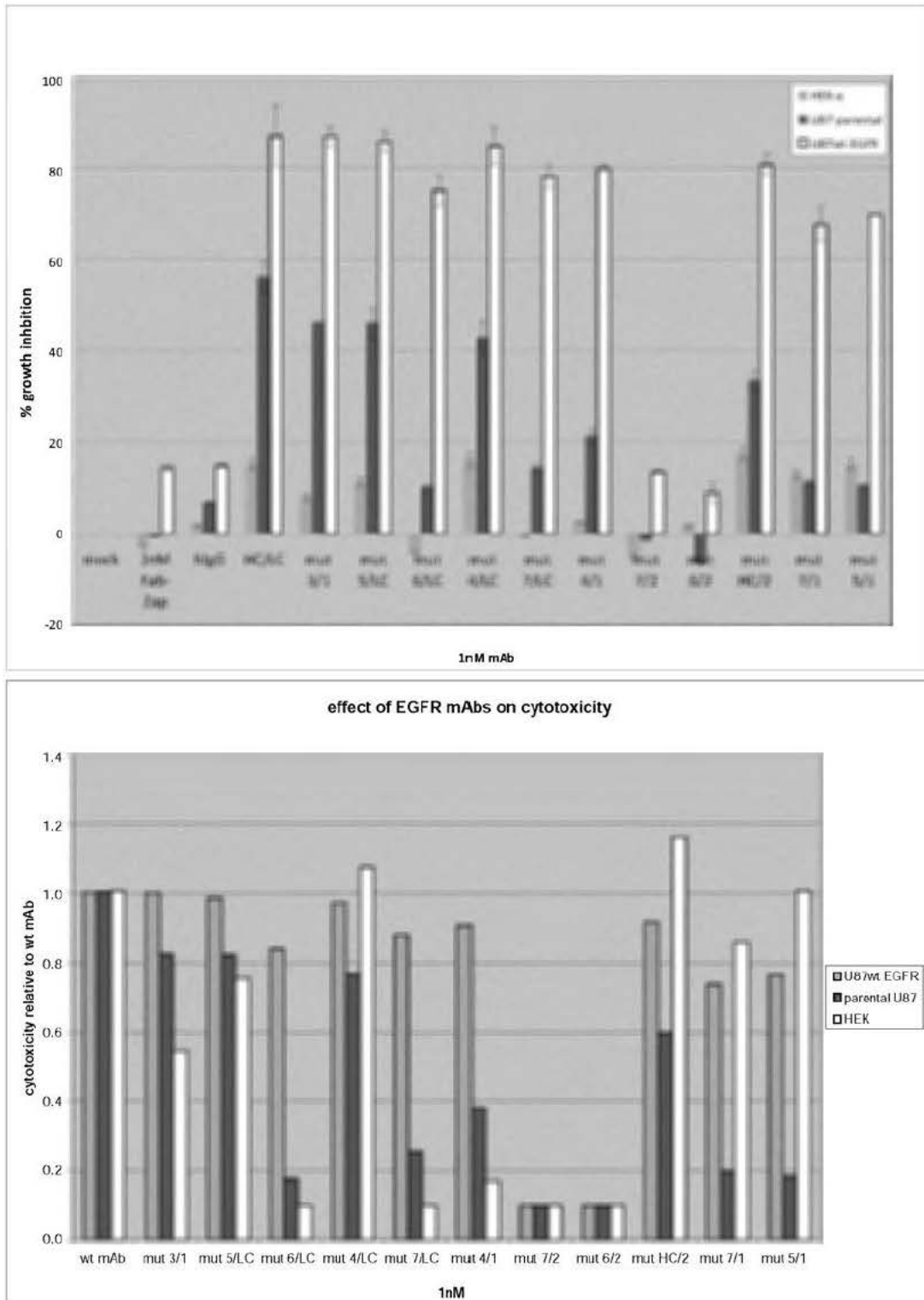


Figure 5

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2012/050034

<p>A. CLASSIFICATION OF SUBJECT MATTER IPC: <i>C07K 16/28</i> (2006.01), <i>A61K 39/395</i> (2006.01), <i>A61K 47/48</i> (2006.01), <i>A61P 35/00</i> (2006.01), <i>C07K 16/46</i> (2006.01) According to International Patent Classification (IPC) or to both national classification and IPC</p>		
<p>B. FIELDS SEARCHED</p>		
<p>Minimum documentation searched (classification system followed by classification symbols) IPC: <i>C07K 16/28</i> (2006.01), <i>A61K 39/395</i> (2006.01), <i>A61K 47/48</i> (2006.01), <i>A61P 35/00</i> (2006.01), <i>C07K 16/46</i> (2006.01)</p>		
<p>Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched</p>		
<p>Electronic database(s) consulted during the international search (name of database(s) and, where practicable, search terms used) Databases: Canadian Patent Database, EspaceNet, CAPlus, Genome Quest, Scopus and Pubmed. Keywords: Cetuximab, Erbitux, 225, C225, reduced, binding, affinity, constant, Kd, mutation, humani*, alteration, modification, YM Bioscience, Jaramillo and Tikhomirov.</p>		
<p>C. DOCUMENTS CONSIDERED TO BE RELEVANT</p>		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BOLAND, W & BEBB, G. The Emerging Role Of Nimotuzumab In The Treatment Of Non-Small Cell Lung Cancer. BIOLOGICS	23, 25, 27 and 28
Y	9 November 2010 (09-11-2010) Vol. 4, pages 289 - 298 ISSN 1177-5475 (page 291, left column, first paragraph; paragraph bridging pages 293 and 294; and Table 1 on page 292)	1 - 22, 24 and 26
<p><input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C.</p>		<p><input checked="" type="checkbox"/> See patent family annex.</p>
* Special categories of cited documents :		
"A" document defining the general state of the art which is not considered to be of particular relevance		"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"E" earlier application or patent but published on or after the international filing date		"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)		"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O" document referring to an oral disclosure, use, exhibition or other means		"&" document member of the same patent family
"P" document published prior to the international filing date but later than the priority date claimed		
Date of the actual completion of the international search	Date of mailing of the international search report	
11 April 2012 (11-04-21012)	14 May 2012 (14-05-2012)	
Name and mailing address of the ISA/CA Canadian Intellectual Property Office Place du Portage I, C114 - 1st Floor, Box PCT 50 Victoria Street Gatineau, Quebec K1A 0C9 Facsimile No.: 001-819-953-2476	Authorized officer Jacinth Abraham (819) 934-7598	

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2012/050034

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DONALDSON, J. M. et al. Design And Development Of Masked Therapeutic Antibodies To Limit Off-Target Effects: Application To Anti-EGFR Antibodies. CANCER BIOL THER November 2009 (11-2009) Vol. 8, pages 2147 - 2152 ISSN 1538-4047 (page 2146, right column, third paragraph; and Table 1 on page 2147)	23, 25, 27 and 28
X	WO 2008/104183 A2 (PEDERSEN, M. W. et al.) 4 September 2008 (04-09-2008) (Table 8 on page 92; Examples 5- 8 and corresponding figures 15 - 21)	23, 25, 27 and 28
X	WO 96/40210 A1 (GOLDSTEIN, N. I. et al.) 19 December 1996 (19-12-1996) (paragraph bridging pages 2 and 3; page 12, line 9 - page 13, line 19; page 23, lines 1 - 9; Table 1 on page 28; page 33; and Examples III and IV)	23, 25, 27 and 28
Y		1 - 22, 24 and 26

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing filed or furnished:
- a. (means)
- on paper
- in electronic form
- b. (time)
- in the international application as filed
- together with the international application in electronic form
- subsequently to this Authority for the purposes of search
2. In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments :

Sequence listing received on 13 March 2012 (13-03-2012).

INTERNATIONAL SEARCH REPORTInternational application No.
PCT/CA2012/050034**Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of the first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons :

1. Claim Nos. : 25 - 28

because they relate to subject matter not required to be searched by this Authority, namely :

Claims 25 - 28 are directed to methods for treatment of the human or animal body which the International Search Authority is not required to search. However, this Authority has carried out a search based on the alleged effects or purposes/uses of the antibody defined in claims 25 - 28.

2. Claim Nos. :

because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically :

3. Claim Nos. :

because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows :

1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claim Nos. :

4. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim Nos. :

Remark on Protest The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/CA2012/050034

Patent Document Cited in Search Report	Publication Date	Patent Family Member(s)	Publication Date
WO 2008/104183 (A2)	04 September 2008 (04-09-2008)	AU2008221118A1	04 September 2008 (04-09-2008)
		CA2676049A1	04 September 2008 (04-09-2008)
		CN101675075A	17 March 2010 (17-03-2010)
		EP2132229A2	16 December 2009 (16-12-2009)
		IL199725D0	15 April 2010 (15-04-2010)
		IL216850D0	31 January 2012 (31-01-2012)
		JP2010535012A	18 November 2010 (18-11-2010)
		KR20100014722A	10 February 2010 (10-02-2010)
		MX2009008909A	28 August 2009 (28-08-2009)
		RU2009136340A	10 April 2011 (10-04-2011)
		TW200902552A	16 January 2009 (16-01-2009)
		US2009004192A1	01 January 2009 (01-01-2009)
		US7887805B2	15 February 2011 (15-02-2011)
		US2011129855A1	02 June 2011 (02-06-2011)
		US2011135636A1	09 June 2011 (09-06-2011)
		WO2008104183A3	13 November 2008 (13-11-2008)
		ZA200905341A	26 May 2010 (26-05-2010)
WO 96/40210 (A1)	19 December 1996 (19-12-1996)	AU6267896A	30 December 1996 (30-12-1996)
		CA2222231A1	19 December 1996 (19-12-1996)
		EP0831880A1	01 April 1998 (01-04-1998)
		EP0831880A4	01 December 2004 (01-12-2004)
		JPH11507535A	06 July 1999 (06-07-1999)
		JP2006246896A	21 September 2006 (21-09-2006)
		JP2009062375A	26 March 2009 (26-03-2009)
		US7060808B1	13 June 2006 (13-06-2006)
		US2003224001A1	04 December 2003 (04-12-2003)
		US2004006212A1	08 January 2004 (08-01-2004)
		US2007116707A1	24 May 2007 (24-05-2007)
		US2009099339A1	16 April 2009 (16-04-2009)