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(54) Title: METHOD OF IDENTIFYING DIAGNOSTIC REAGENTS

(57) Abstract: Disclosed herein are methods for identifying diagnostic reagents (e.g., antigen-binding molecules, such as antibodies) that are useful, for instance, as primary diagnostic, prognostic, and/or predictive (e.g., companion diagnostic) reagents in a disease state, such as cancer.



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METHOD OF IDENTIFYING DIAGNOSTIC REAGENTS

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims priority to U.S. Provisional Application Nos.
5 60/949,792 filed July 13, 2007 and 60/988,196 filed November 15, 2007, herein incorporated by reference.

FIELD OF THE DISCLOSURE

This disclosure concerns a method for identifying diagnostic reagents
10 (*e.g.*, antigen-binding molecules, such as antibodies) that are useful, for instance, as primary diagnostic, prognostic, and/or predictive (*e.g.*, companion diagnostic) reagents of a disease state, such as cancer.

BACKGROUND

15 Cancer is a generic name for a wide range of cellular malignancies characterized by unregulated growth, lack of differentiation, and the ability to invade local tissues and metastasize. These neoplastic malignancies affect, with various degrees of prevalence, every tissue and organ in the body. Historically, cancers have been diagnosed using conventional histological and clinical features of the
20 affected tissue or organ. However, it is now apparent that tumors, even from the same tissue or organ, are heterogeneous on the cellular and/or molecular level. As one consequence, the prognosis and/or responsiveness to therapy of each patient may differ. This unpredictability confounds treatment selection and may expose patients to the risks and discomforts of unneeded therapies or may lead to failure to
25 treat a patient with a beneficial therapy.

EGFR-positive cancers offer a case in point. EGFR and its downstream signaling effectors, including members of the Ras/Raf/MAP kinase pathway, play an important role in both normal and malignant epithelial cell biology (Normanno *et al.*, *Gene*, 366:2-16, 2006). Amplification and/or mutation of the EGFR gene and/or
30 EGFR protein overexpression have been associated with various malignancies, including breast cancer, lung cancer, colorectal cancer, ovarian cancer, renal cell cancer, bladder cancer, head and neck cancer, glioblastoma, and/or astrocytoma.

Increased EGFR activity (whether as a result of abnormally high protein expression, dysregulation of receptor activity, or other mechanism) is believed to contribute to carcinogenesis. Consequently, EGFR is one established target for therapeutic development.

5 Several EGFR inhibitors are available for clinical treatment. These include EGFR-specific antibodies (*e.g.*, cetuximab (ERBITUX™) and panitumumab (VECTIBIX™)) and small molecular tyrosine kinase inhibitors (*e.g.*, gefitinib (IRESSA™) and erlotinib (TARCEVA™)). While these treatments have benefited subsets of cancer patients, responses to the drugs are variable. For example, three
10 clinical studies of patients with advanced colorectal cancer using cetuximab in a monotherapy setting and/or in combination with irinotecan (a chemotherapeutic agent) demonstrated response rates of 10.5% or 10.8% for cetuximab alone and 22.5% or 22.9% for the combined therapy (reviewed by Iqbal and Lenz, *Cancer Chemother. Pharmacol.*, 54(Suppl. 1):S32-39, 2004). Similarly, about 10% or about
15 20% of non-small cell lung cancer (“NSCLC”) patients treated with 250 or 500 gefitinib per day, respectively, responded to the drug and exhibited improved symptoms (Birnbau and Ready, *Curr. Treat. Options Oncol.*, 6(1):75-81, 2005).

 Patient responses to EGFR inhibitors have been correlated with various EGFR metrics. For example, EGFR expression (as measured by
20 immunohistochemistry) was associated with an objective response to erlotinib treatment in NSCLC patients (Tsao *et al.*, *N. Engl. J. Med.*, 353:133-144, 2005). However, survival after treatment in these patients was not influenced by EGFR expression, the number of EGFR copies, or EGFR mutation (Tsao *et al.*, *N. Engl. J. Med.*, 353:133-144, 2005). In both preclinical and clinical settings, somatic
25 mutations in the EGFR tyrosine kinase domain were found to correlate with sensitivity of NSCLC patients to gefitinib and erlotinib but not to cetuximab (Janne *et al.*, *J. Clin. Oncol.*, 23:3227-3234, 2005). Clinical studies of gefitinib demonstrated an association between increased EGFR copy number, mutational status, and clinical response in advanced NSCLC (Cappuzzo *et al.*, *J. Natl. Cancer*
30 *Inst.*, 97:643-655, 2005).

 EGFR is only one of many cancer-related biomarkers that is used and/or considered for use as a prognostic marker and for which targeted cancer therapies

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have been and/or are being developed. Any cancer-related biomarker that plays a meaningful role in cancer onset, growth (*e.g.*, proliferation and/or inhibition of apoptosis), metastasis, vascularization or the like is a candidate for a prognostic marker and/or for targeted therapy. Many of these biomarkers, the biological pathways in which they function, and the corresponding molecular pathogenesis(es) resulting in cancer are known. Now, a challenge is to use this information to identify useful diagnostic and therapeutic reagents to provide cancer patients with an accurate prediction of disease outcome and to administer “the appropriate drug, at the appropriate dose, at the appropriate time.”

SUMMARY OF THE DISCLOSURE

Disclosed herein are methods of identifying specific binding molecules (such as antibodies or fragments thereof) that interrogate the activity state of a neoplasm (*e.g.*, cancer)-related biomarker in biological samples (such as formalin-fixed, paraffin-embedded (“FFPE”) tissue sections). In particular examples, the biomarker is a receptor tyrosine kinase (RTK), such as an oncogenic RTK. Activation status of such biomarker predicts, among other things, expression of the biomarker by the tumor, the aggressiveness of the neoplasm comprising such biomarker and/or the potential efficacy of therapies targeted to such biomarker. In particular examples the method of identifying a diagnostic reagent includes identifying amino acid residues that form a protein-protein interface between an RTK (*e.g.*, a region of the intracellular domain of RTK) and an RTK regulatory protein. A specific binding reagent is produced that specifically binds at least some of the amino acid residues that form the protein-protein interface, such as amino acids of the RTK intracellular domain that form a part of the interface, wherein the binding occurs in the absence of the protein-protein interaction (*e.g.*, the binding is detectable, for example above background) but fails to specifically bind amino acid residues that form the protein-protein interface in the presence of the protein-protein interaction (*e.g.*, binding is not detectable, for example at a background level).

Also provided are diagnostic reagents identified using these methods, as well as methods of using such reagents to diagnose a subject with cancer, for example to estimate life expectancy (*e.g.*, prognosis), determine the likely outcome of a

particular anti-cancer therapy (*e.g.*, predictive), and to develop a treatment protocol for a subject with cancer.

The foregoing and other features will become more apparent from the following detailed description of several embodiments, which proceeds with
5 reference to the accompanying figures.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 is a flow chart schematically showing the steps of exemplary immunostaining methods.

10 FIG. 2 is a schematic representation of the EGFR regulatory domain showing the locations of autophosphorylation sites (white boxes with number indicating position of autophosphorylated amino acid residue) and known binding sites for exemplary EGFR regulatory proteins, including Signal Transducer and Activator of Transcription (STAT) proteins (*e.g.*, STAT1 and/or STAT3; OMIM Accession
15 Nos. 600555 or 102582), Growth Factor Receptor-Bound Protein 2 (Grb2; OMIM Accession No. 108355), Signaling and Transforming Protein Containing Src Homology 2 and 3 Domains (Shc) (*e.g.*, OMIM Accession Nos. 600560, 605217, or 605263), Int-P, and Suppressor of Cytokine Signaling proteins (*e.g.*, SOCS1 and/or SOCS3).

20 FIGS. 3A and 3B are a series of schematic representations involving an RTK molecule which, in its wild-type state, has an extracellular ligand-binding domain (LBD) and intracellular tyrosine kinase (TKD) and regulatory (RD) domains. In each schematic, as applicable, EDBM represents an extracellular-domain-specific binding molecule (such as an antibody); RDBM represents a
25 regulatory-domain-specific binding molecule (such as an antibody); and RDIM represents a regulatory domain inhibitory molecule (such as a SOCS protein like SOCS1 or SOCS3 or other negative regulatory protein listed in Table 3) and RDSM represents a regulatory domain stimulatory molecule (such as a positive regulatory protein listed in Table 3). Each of the three schematics (from left to right) represents
30 a different molecular setting. The left-most panel shows a full-length RTK not associated with a RDIM or RDSM. The center panel shows a constitutively active N-terminal truncated RTK mutant. The right-most panel shows a full-length RTK

associated with a RDIM or RDSM. In each case, predictions based on the binding (or not) of the EDBM and RDBM or RDSM are listed below the molecular schematic.

FIG. 4 shows the results of two Western blots in which three total protein concentrations (as indicated) of A431 (EGFR-positive) and BT474 (EGFR-negative) cell lysates were run. The left and right representations show clone 5B7 and clone 3C6, respectively, binding to a single protein band (appropriate in size for EGFR) in A431 cell lysates. Molecular weights (in kD) are shown at the far left.

FIG. 5 shows the results of staining the indicated tissues with EGFR extracellular-domain-specific clone 3C6 and EGFR regulatory-domain-specific clone 5B7 in tabular (FIG. 5A) and image (FIG. 5B) formats.

FIG. 6 shows images demonstrating the differences in the binding of EGFR regulatory-domain-specific (RDBM) clone 5B7 and EGFR external-domain-specific (EDBM) clone 3C6 in two representative non-small cell lung cancer (NSCLC) tissue sections.

FIG. 7 shows the results of peptide inhibition studies mapping the epitope of the EGFR regulatory-domain-specific clone 5B7. The boxed region in FIG. 7B represents an exemplary clone 5B7 epitope, the C-terminus of which may be a few amino acids longer or shorter.

FIG. 8 shows images demonstrating the differences in the binding of EGFR regulatory-domain-specific (RDBM) clone 5B7 and EGFR external-domain-specific (EDBM) clone 3C6 in two normal mouse livers (left-most two panels) and the livers of SOCS3-knock-out mice (right-most two panels).

FIG. 9 shows two Kaplan Meier plots demonstrating overall survival of NSCLC patients as a function of clone 3C6 (panel A) or 5B7 (panel B) status. In panel A, 3C6-positive staining is shown by a black line and 3C6-negative staining is shown by a gray line. In panel B, 5B7-positive staining is shown by a black line and 5B7-negative staining is shown by a gray line.

FIG. 10 shows two Kaplan Meier plots demonstrating disease-free survival of NSCLC patients as a function of clone 3C6 (panel A) or 5B7 (panel B) status. In panel A, 3C6-positive staining is shown by a black line and 3C6-negative staining is

shown by a gray line. In panel B, 5B7-positive staining is shown by a black line and 5B7-negative staining is shown by a gray line.

SEQUENCE LISTING

5 The nucleic and amino acid sequences listed in the accompanying sequence listing are shown using standard letter abbreviations for nucleotide bases, and three letter code for amino acids, as defined in 37 C.F.R. 1.822. Only one strand of each nucleic acid sequence is shown, but the complementary strand is understood as included by any reference to the displayed strand. All sequence database accession
10 numbers referenced herein are understood to refer to the version of the sequence identified by that accession number as it was available on the filing date of this application. In the accompanying sequence listing:

 SEQ ID NO: 1 is a reference amino acid sequence (REFSEQ) of human EGFR (isoform a) as set forth in GENBANK™ Accession No. NM_005228. A
15 nucleic acid sequence encoding this polypeptide also is set forth in GENBANK™ Accession No. NM_005228.

 SEQ ID NO: 2 is the amino acid sequence of a peptide corresponding to residues 1167-1185 of SEQ ID NO: 1.

 SEQ ID NO: 3 is the curated reference amino acid sequence (REFSEQ) of
20 human Suppressor of Cytokine Signaling 3 (SOCS3) as set forth in GENBANK™ Accession No. NM_003955. A nucleic acid sequence encoding this polypeptide also is set forth in GENBANK™ Accession No. NM_003955.

 SEQ ID NO: 4 is a reference amino acid sequence (REFSEQ) of human c-kit (isoform 1) as set forth in GENBANK™ Accession No. NM_000222. A nucleic
25 acid sequence encoding this polypeptide also is set forth in GENBANK™ Accession No. NM_000222.

 SEQ ID NO: 5 is a reference amino acid sequence (REFSEQ) of human HER2 (isoform a) as set forth in GENBANK™ Accession No. NM_004448. A
30 nucleic acid sequence encoding this polypeptide also is set forth in GENBANK™ Accession No. NM_004448.

 SEQ ID NO: 6 is a reference amino acid sequence (REFSEQ) of human IGF1R as set forth in GENBANK™ Accession No. NM_000875. A nucleic acid

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sequence encoding this polypeptide also is set forth in GENBANK™ Accession No. NM_000875.

SEQ ID NO: 7 is a reference amino acid sequence (REFSEQ) of human c-Met (isoform b) as set forth in GENBANK™ Accession No. NM_000245. A
5 nucleic acid sequence encoding this polypeptide also is set forth in GENBANK™ Accession No. NM_000245.

SEQ ID NO: 8 is a reference amino acid sequence (REFSEQ) of human FGFR1 (isoform 1) as set forth in GENBANK™ Accession No. NM_023110. A
10 nucleic acid sequence encoding this polypeptide also is set forth in GENBANK™ Accession No. NM_023110.

DETAILED DESCRIPTION

I. Introduction

The present disclosure provides methods for identifying one or more
15 diagnostic reagents, such as a reagent that can be used to as a diagnostic (*e.g.*, predictive, prognostic, or companion diagnostic) in a disease, for example a neoplasm, such as a tumor, for example cancer. In some examples, the identified diagnostic reagent can be used as a companion diagnostic to determine if a particular receptor tyrosine kinase (RTK) is present in a sample (*e.g.*, to determine if a subject
20 has a neoplasm (*e.g.*, cancer) expressing a particular RTK, such as HER2), determine the activity state of a cancer biomarker, for example the activity state of a RTK (*e.g.*, to determine the prognosis of a subject having a neoplasm (*e.g.*, cancer) or to predict the likelihood that a subject will respond to a particular therapy) or to identify treatments for a subject with a neoplasm (*e.g.*, cancer), or combinations
25 thereof.

In particular examples the disclosed methods of identifying a diagnostic reagent include identifying an RTK that is a component of a cellular pathway that confers a growth advantage on a cancer cell (*e.g.*, is oncogenic). In some examples, such an RTK is referred to herein as a target RTK. When the RTK is inhibited in a
30 cancer cell (*e.g.*, the biological activity or presence of the protein is substantially decreased or even eliminated), the cancer cell growth advantage is decreased or eliminated (such as a decrease of at least 20%, at least 40%, at least 50%, at least

75%, at least 90%, or even at least 99%). As a result, for example, the cancer cell may undergo apoptosis or growth of the cancer cell may substantially slow, stop, or decrease. In contrast, when the RTK is stimulated or activated in a cancer cell (*e.g.*, the biological activity or presence of the protein is substantially increased), the cancer cell growth advantage is increased (such as an increase of at least 20%, at least 40%, at least 50%, at least 75%, at least 90%, or even at least 100%), thereby permitting or even enhancing growth of the cancer cell.

RTKs useful for the disclosed methods are positively or negatively regulated by a regulatory protein when the RTK and the regulatory protein specifically bind to one another to form a protein-protein interaction. RTK-regulatory protein interactions are known in the art, and additional ones can be identified using routine methods (*e.g.*, immunoprecipitation, yeast two-hybrid system). The resulting protein-protein interface results in the interaction between RTK amino acids and regulatory protein amino acids. For example, at least 3, at least 5, at least 10, at least 12, at least 15, at least 18, at least 20, at least 25, at least 30 or even at least 50 amino acids (such as 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45 or 50 amino acids) form a protein-protein interface between an RTK and its regulatory protein. In specific examples, a portion or segment of the RTK intracellular domain (ID, such as a regulatory domain, RD) specifically binds to a regulatory protein, creating an interaction between amino acids of both proteins.

In particular examples, the method further includes identifying three or more amino acid residues that form the protein-protein interface between the RTK and its bound regulatory protein (*e.g.*, a positive or negative regulatory protein). Methods for identifying amino acids that comprise a protein-protein interface are known in the art. In some examples, at least 3, at least 5, at least 10, or at least 20 amino acid residues are identified, such as 3 to 100, 5 to 100, 5 to 50, 5 to 30, 5 to 20, 10 to 15, or 4 to 15 amino acids, for example 5 to 50 or 5 to 100 contiguous amino acids of the RTK or regulatory protein that forms the interface, such as 5 to 30, 5 to 25, 6 to 25, or 7 to 15 contiguous amino acids. In some examples, at least some of such identified amino acids are contiguous; however, one skilled in the art will appreciate that the interface may not solely include contiguous amino acids.

A specific binding reagent can be generated to at least a portion of the amino acid residues that form the RTK protein-regulatory protein interface using methods known in the art. In some examples, amino acids from an RTK intracellular domain (ID), such as from the regulatory domain (RD), are used to produce a specific binding reagent that can disrupt interaction between an RTK and its regulatory protein. In some examples, amino acid residues that form the protein-protein interface between the RTK and its bound regulatory protein are identified, and a specific binding reagent generated to at least a portion of the amino acid residues of the interface using methods known in the art. Such an interface specific binding reagent can be used as a diagnostic (*e.g.*, companion diagnostic, prognostic, or predictive) reagent in the disclosed methods. In some examples the specific binding reagent is specific for amino acids of the RTK (such as the ID or RD of the RTK). The resulting specific binding reagent specifically binds (*e.g.*, detectable binding is observed or is significantly above background levels) at least one amino acid residue of the protein-protein interface in the absence of the protein-protein interaction and fails to specifically bind (*e.g.*, detectable binding is not observed or is at background levels) at least one amino acid residue of the protein-protein interface in the presence of the protein-protein interaction.

One skilled in the art will appreciate that not all amino acid residues of the protein-protein interface or of an RTK ID or RTK RD need be identified or used to generate a specific binding reagent. In some examples, only a portion of the amino acids forming the protein-protein interface (or of the RTK RD or ID) are identified, such as at least 95%, at least 90%, at least 75%, at least 50%, or at least 25% of the amino acids of the interface can be identified (such as 20 to 99%, for example 50-95% of the amino acids). For example, if 20 amino acids form a protein-protein interface between RTK and a regulatory protein, all 20 amino acids need not be identified. Instead, for example, fewer than 20 amino acids may be identified, such as 5 to 15 or 6 to 12 of such amino acids. Similarly, not all identified amino acid residues of the protein-protein interface or of an RTK ID need be used to generate a specific binding reagent. As the regulatory protein need only sterically hinder the binding of the specific binding agent to the protein-protein interface (*e.g.*, the RTK ID or RD), the epitope of the specific binding agent may be entirely within the

protein-protein interface or just partially (*e.g.*, as little as one amino acid) in the interface. So long as the specific binding agent does not significantly bind the protein-protein interface when the regulatory protein is bound to the RTK, but does specifically bind the interface when the regulatory protein is not bound, it is
5 sufficient. For example, an antigenic region within the identified amino acid residues of the protein-protein interface (*e.g.*, an epitope) can be identified and used to generate a specific binding reagent. For example, if 20 amino acids of the protein-protein interface or an RTK RD are identified, all 20 amino acids need not
10 95%, at least 90%, at least 75%, at least 50%, or at least 25% of the identified amino acids can be used (such as 20 to 99%, for example 50-95% of the amino acids) to generate a specific binding agent.

Methods of producing specific binding reagents are known in the art. In any embodiment involving a specific binding molecule (whether composition or
15 method), a specific binding molecule can be (but is not necessarily) an aptamer, an antibody (*e.g.*, a monoclonal antibody, such as a rabbit or mouse monoclonal antibody) or an antigen-binding fragment thereof. In one example the method includes producing antibodies, such as an antibody specific for amino acids of the RTK that form a protein-protein interface with a regulatory protein. In particular
20 examples, the method includes producing antibodies specific for amino acids of the RTK (*e.g.*, amino acids of the ID or RD) that form all or part of the protein-protein interface between the proteins. Some method embodiments involve immunizing a non-human mammal with an immunogen that includes a carrier protein and amino acid residues that form the protein-protein interface between RTK and its bound
25 regulatory protein (or an immunogenic fragment of such an interface, such as an epitope of the interface). In some examples, an adjuvant is also administered to the non-human mammal. Some such methods include a further step of isolating serum from the non-human mammal and isolating polyclonal antibody specific for the immunogen. Other such methods include a further step of fusing spleen cells from
30 the non-human animals with a fusion cell partner to make antibody-producing hybridomas.

Also disclosed are diagnostic reagents identified using the disclosed methods, as well as compositions that include such reagents. For example, such detection reagents can be in a purified or isolated form. In some examples the identified reagents are present with other agents such as water, saline, or other liquid (e.g., buffered liquid) that permits suspension of the detection reagents. Such diagnostic reagents can be present in a separate container, and part of a kit, such as a kit that includes one or more other reagents for immunohistochemistry (IHC), such as labeled secondary antibodies (wherein the labeled antibodies are appropriately matched to the detection reagent, e.g., if the diagnostic reagent is a mouse monoclonal antibody, the secondary antibody is a labeled anti-mouse antibody), buffers, agents that permit development or detection of the label, and the like.

Methods are provided for using the disclosed diagnostic reagents as companion diagnostics, for example to determine whether a sample contains (e.g., is positive for) an RTK. For example, such methods can be used to determine if a cancer expresses a particular RTK. Such information can be useful in identifying treatment modalities for the subject. For example, diagnostic reagents specific for the protein-protein interface formed between an RTK intracellular domain (ID) and a regulatory protein can be used to determine if a sample containing cancer cells expresses a target RTK. In some examples, a positive-control specific binding reagent (e.g., an antibody specific for an extracellular domain (ED) of the target RTK) is used in combination with the disclosed diagnostic reagents. As discussed above, diagnostic specific binding reagents identified using the disclosed methods can bind to a protein-protein interface in the absence of binding of a regulatory protein to the RTK, but cannot bind to the interface in the presence of binding of the regulatory protein to the RTK. Detection of specific binding of such a diagnostic reagent to cancer cells in a sample indicates that the cells express the RTK. Subject's having cancer cells that express the target RTK may benefit from an inhibitory RTK anti-cancer therapy (e.g., a tyrosine kinase inhibitor (TKI)). In contrast, substantially no detectable binding of the specific binding agent to the cells indicates (but does not alone confirm) that the cells do not express the RTK. If substantially no specific binding is detected, the cells in the sample may express the target RTK, but the epitope recognized by the specific binding reagent may be

masked by an interaction of the RTK with one or more regulatory proteins with which the RTK forms protein-protein interaction(s). In some method embodiments, it may be advantageous to confirm whether or not the cells express the RTK by using a second diagnostic reagent that recognizes an ED of the target RTK (such as

5 in the same tissue section or another tissue section in a series of sections). If both specific binding agents are used, and substantially no specific binding is detected using a disclosed diagnostic specific binding reagent (*e.g.*, which recognizes a target RTK ID), and substantially no specific binding is detected using the specific binding reagent for the RTK ED, this indicates that the cell does not express the target RTK.

10 In contrast, if substantially no specific binding is detected using the disclosed diagnostic reagent (*e.g.*, one that recognizes the target RTK ID), and specific binding is detected using the specific binding reagent for the RTK ED, this indicates the regulated status of the target RTK (*e.g.*, the target RTK is down-regulated by a negative regulatory protein or the target RTK is up-regulated by a positive

15 regulatory protein). In some embodiments, the regulated status of the target RTK further indicates whether or not a subject may benefit from therapies that are intended to inhibit the target RTK. As such, the disclosed diagnostic reagents can be used as prognostics, for example to predict the outcome of a subject having cancer in the absence of administering additional therapy to the subject. This is discussed in

20 more detail below.

Disclosed methods also include using a diagnostic specific binding reagent as a prognostic, for example to determine the prognosis of a subject by determining the activity state of an RTK, such as a subject having a neoplasm (*e.g.*, cancer). Thus, the disclosed diagnostic specific binding reagents can be used to predict a

25 subject's disease outcome in the absence of administering additional therapies to the subject. For example, such methods can be used to predict the prognosis of a neoplastic disease (such as a tumor or a cancer, for example lung cancer, colorectal cancer, head and neck cancer, gastric cancer, or glioblastoma). In some examples, the method includes detecting in a biological sample from a patient having a

30 neoplastic disease the specific binding of a disclosed diagnostic specific binding reagent that is specific for the interface between an RTK and a negative regulator to one or more RTK-positive neoplastic cells in the biological sample; wherein the

specific binding of the reagent in the one or more RTK-positive neoplastic cells predicts a worse or poor prognosis of the neoplastic disease in the patient. In some such method embodiments, if the specific binding reagent (*e.g.*, antibody or aptamer) specifically binds to at least 10% (such as at least 15%, at least 20%, at least 30%, at least 50%, or at least 75%) of the RTK-positive neoplastic cells in the biological sample, this indicates a positive result (*e.g.*, positive for binding). In contrast, in other examples the specific binding of a disclosed diagnostic specific binding reagent that is specific for the interface between an RTK and a positive regulator to one or more RTK-positive neoplastic cells in the biological sample is determined; wherein the absence of specific binding of the diagnostic specific binding reagent in the one or more RTK-positive neoplastic cells predicts a worse or poor prognosis of the neoplastic disease in the patient. In some method embodiments, a poor prognosis is less than 5-year survival (such as less than 1-year survival or less than 2-year survival) of the patient after initial diagnosis of the neoplastic disease.

Other prognostic method embodiments involve detecting in a biological sample from a patient having a neoplastic disease (such as lung cancer, colorectal cancer, head and neck cancer, gastric cancer, or glioblastoma) the specific binding of a disclosed diagnostic specific binding reagent that is specific for the interface between an RTK and a negative regulator to one or more RTK-positive neoplastic cells in the biological sample is determined; wherein substantially no specific binding of the diagnostic specific binding reagent in the one or more RTK-positive neoplastic cells predicts a good prognosis of the neoplastic disease in the patient. For example, this result indicates that the subject has a cancer in which a target RTK that is “naturally” inhibited by its protein-protein interaction with a negative regulatory protein, and is thus likely to have a better outcome than a cancer in which such negative regulation of the oncogenic target RTK is lacking (and vice versa). The presence of binding of a negative regulator to the target RTK indicates that the RTK is marked for degradation as a consequence of its protein-protein interaction with a negative regulatory protein (*e.g.*, in the ubiquitin-dependent proteasome pathway, in a ubiquitin-independent proteasome pathways, or in a 20S or 26S proteasome-dependent pathway) and thus cannot confer a continuing growth

advantage on a cancer cell. As a result, prognosis for a subject with such a cancer is likely to be better than a patient with an opposite result (*i.e.*, oncogenic target RTK not marked for degradation). In contrast, in other examples the specific binding of a disclosed diagnostic specific binding reagent that is specific for the interface
5 between an RTK and a positive regulator to one or more RTK-positive neoplastic cells in the biological sample is determined; wherein specific binding of the diagnostic specific binding reagent in the one or more RTK-positive neoplastic cells predicts a good prognosis of the neoplastic disease in the patient. In some method embodiments, a good prognosis is greater than 2-year survival (such as greater than
10 3-year survival, greater than 5-year survival, or greater than 7-year survival) of the patient after initial diagnosis of the neoplastic disease.

In addition to using the disclosed diagnostic specific binding reagents to determine the aggressiveness of a neoplasm (*e.g.*, prognosis of the neoplasm), the disclosed diagnostic specific binding reagents can also be used to predict the
15 response of a neoplasm to RTK inhibitor therapy. Thus, the disclosed diagnostic specific binding reagents can be used as predictors of the likelihood that a particular tumor will respond to an RTK inhibitor therapy. In some examples, the methods include detecting in a biological sample, which includes one or more neoplastic cells (such as cancer cells), the specific binding of a disclosed diagnostic specific binding
20 reagent that is specific for the protein-protein interface between an RTK and a negative regulator (*e.g.*, a binding reagent specific for an RTK ID) to the one or more of the neoplastic cells; wherein specific binding of the specific binding reagent to one or more of the neoplastic cells indicates that the neoplastic cells will respond to an inhibitor of the RTK. For example, the detection of binding of a disclosed
25 specific binding reagent to a sample indicates that a negative regulatory protein is not bound to the RTK, and thus the RTK is “available” for binding to an administered RTK inhibitor (*e.g.*, an inhibitor that recognizes an RTK ID, such as a TKI). In some method embodiments, the specific binding of the specific binding reagent to at least 10% (such as at least 15%, at least 20%, at least 30%, at least
30 50%, or at least 75%) of the neoplastic cells in the biological sample indicates that the candidate is likely to respond to treatment with an RTK inhibitor. In some method embodiments, the neoplastic cell response to the RTK inhibitor is slowed

growth (such as, net zero growth or net negative growth). In other method
embodiments, the slowed growth is at least 10% (such as at least 15%, at least 20%,
at least 30%, at least 50%, or at least 75%) less than the neoplastic cell growth prior
to treatment with the RTK inhibitor. In some method embodiments, the neoplastic
5 cell response to the RTK inhibitor is apoptosis, and, in some such embodiments, at
least 10% (such as at least 15%, at least 20%, at least 30%, at least 50%, or at least
75%) of the neoplastic cells undergo apoptosis.

Other disclosed methods involve predicting the response of a neoplasm to an
RTK inhibitor by detecting in a biological sample comprising one or more
10 RTK-positive neoplastic cells substantially no specific binding of a disclosed
specific binding reagent that is specific for the protein-protein interface between an
RTK and a negative regulator (*e.g.*, a RTK ID-binding antibody) to the one or more
RTK-positive neoplastic cells; wherein substantially no specific binding of the
specific binding reagent to the RTK-positive neoplastic cells indicates that the
15 neoplastic cells will not substantially respond to an RTK inhibitor that is specific for
the RTK ID. Without wishing to be bound to a particular theory, it is proposed that
the absence of such binding indicates that the RTK is being “naturally” inhibited (by
the negative regulator to which it is bound) and thus the RTK inhibitory therapy has
less (or nothing) left to inhibit. Thus, in the absence of any therapy the target RTK
20 is unlikely to be further inhibited by a therapy designed to inhibit the target RTK;
and a patient receiving such RTK inhibitory therapy is unlikely to respond to it. In a
more particular example, an target RTK that has been marked for degradation as a
consequence of its protein-protein interaction with a negative regulatory protein
(*e.g.*, in the ubiquitin-dependent proteasome pathway, in a ubiquitin-independent
25 proteasome pathways, or in a 20S or 26S proteasome-dependent pathway) is
unlikely to be further inhibited by a therapy designed to inhibit the target RTK; thus,
again, a patient receiving such therapy is unlikely to respond to it. Some such
methods further involve detecting in a control biological material (such as normal
skin, normal testis, or normal tonsil) the specific binding to RTK of the specific
30 binding reagent. Other such methods further involve detecting in the biological
sample specific binding of a second specific binding reagent (*e.g.*, antibody) specific
for the RTK external domain (ED).

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In some examples, the methods include detecting in a biological sample, which includes one or more neoplastic cells (such as cancer cells), the specific binding of a disclosed diagnostic specific binding reagent that is specific for the protein-protein interface between an RTK and a positive regulator (*e.g.*, a binding reagent specific for an RTK ID) to the one or more of the neoplastic cells; wherein the absence of specific binding of the specific binding reagent to one or more of the neoplastic cells indicates that the neoplastic cells will respond to an inhibitor of the RTK. Without wishing to be bound to a particular theory, it is proposed that the absence of binding of a disclosed specific binding reagent to a sample indicates that the oncogenic RTK is very active, and thus there is a significant amount of RTK activity to be inhibited. In some method embodiments, the neoplastic cell response to the RTK inhibitor is slowed growth (such as, net zero growth or net negative growth). In other method embodiments, the slowed growth is at least 10% (such as at least 15%, at least 20%, at least 30%, at least 50%, or at least 75%) less than the neoplastic cell growth prior to treatment with the RTK inhibitor. In some method embodiments, the neoplastic cell response to the RTK inhibitor is apoptosis, and, in some such embodiments, at least 10% (such as at least 15%, at least 20%, at least 30%, at least 50%, or at least 75%) of the neoplastic cells undergo apoptosis.

Other disclosed methods involve predicting the response of a neoplasm to an RTK inhibitor by detecting in a biological sample comprising one or more RTK-positive neoplastic cells significant specific binding of a disclosed specific binding reagent that is specific for the protein-protein interface between an RTK and a positive regulator (*e.g.*, a RTK ID-binding antibody) to the one or more RTK-positive neoplastic cells; wherein substantial specific binding of the specific binding reagent to the RTK-positive neoplastic cells indicates that the neoplastic cells will not substantially respond to an RTK inhibitor that is specific for the RTK ID. In some method embodiments, the specific binding of the specific binding reagent to at least 10% (such as at least 15%, at least 20%, at least 30%, at least 50%, or at least 75%) of the neoplastic cells in the biological sample indicates that the candidate is not likely to respond to treatment with an RTK inhibitor. Without wishing to be bound to a particular theory, it is proposed that the presence of such binding indicates that the oncogenic RTK is less activity than if the positive

regulator were present, and thus the RTK inhibitory therapy has less to inhibit. Some such methods further involve detecting in a control biological material (such as normal skin, normal testis, or normal tonsil) the specific binding to RTK of the specific binding reagent. Other such methods further involve detecting in the
5 biological sample specific binding of a second specific binding reagent (*e.g.*, antibody) specific for the RTK external domain (ED).

Still other disclosed methods involve predicting the response of a neoplasm to RTK inhibitor (*e.g.*, a TKI) administration, by detecting RTK expression in a first sample of a biological material comprising one or more neoplastic cells; and
10 detecting in a second sample of the biological material substantially no specific binding to RTK of a disclosed specific binding reagent; wherein detecting RTK expression in the first sample and substantially no specific binding to RTK of a disclosed specific binding reagent indicates that the neoplasm is likely to respond to RTK inhibitor (*e.g.*, a TKI) administration. In some such methods, the first sample
15 and the second sample are serial sections of the biological material. Other such methods further involve detecting in a control biological material (such as, normal skin, normal testis, or normal tonsil) the specific binding to RTK of the disclosed specific binding reagent (*e.g.*, RKT ID-binding antibody).

Immunostaining methods also are disclosed. Such methods can involve
20 contacting a biological sample containing one or more cells with a disclosed specific binding reagent (*e.g.*, RKT ID-binding antibody or aptamer), and detecting the specific binding of the specific binding reagent to an antigen (*e.g.*, an RTK ID) in the one or more cells. In some examples, a labeled secondary antibody is used to detect the specific binding reagent (*e.g.*, RKT ID-binding antibody). However, one
25 skilled in the art will appreciate that other routine immunostaining methods can be used.

Other disclosed methods involve detecting a direct interaction between an RTK and an RTK regulatory protein (such as a positive or negative regulatory protein), by contacting a biological sample, comprising one or more RTK-positive
30 cells, with a disclosed specific binding reagent (*e.g.*, RKT ID-binding antibody), and detecting the specific binding of the specific binding reagent to the one or more RTK-positive cells, wherein the specific binding of the specific binding reagent to

the one or more RTK-positive cells detects that the RTK is not significantly interacting with an RTK regulatory protein, wherein an interaction between RTK and the RTK regulatory protein masks the epitope of the specific binding reagent (*e.g.*, RKT ID-binding antibody).

5 Other methods of detecting a direct interaction between an RTK and an RTK regulatory protein (such as a positive or negative regulatory protein) are disclosed. Such methods can include contacting a biological sample that includes one or more RTK-positive cells, with a disclosed specific binding reagent (*e.g.*, RKT ID-binding antibody), and detecting the specific binding of the specific binding reagent to the
10 one or more RTK-positive cells, wherein substantially no specific binding of the specific binding reagent to the one or more RTK-positive cells detects that the RTK is interacting with an RTK regulatory protein, wherein an interaction between RTK and the RTK regulatory protein masks the epitope of the specific binding reagent (*e.g.*, RKT ID-binding antibody).

15 In any disclosed method embodiment involving a biological sample, such biological sample can be (but is not necessarily) mounted on a microscope slide, is a tissue section (such as a formalin-fixed and paraffin-embedded tissue section), and/or is a neoplastic tissue (such as a cancer, for example a lung cancer, colorectal cancer, head and neck cancer, gastric cancer, or glioblastoma).

20 Provided herein is a particular example of identifying a diagnostic specific binding agent for EGFR. Using the disclosed methods, an antibody specific for the EGFR regulatory domain (EGFR-RD) was identified. It was confirmed that this antibody can be used to diagnose and prognose a subject having an EGFR-expressing neoplasm. One skilled in the art will appreciate that similar methods can
25 be used for other RTKs, and for other RTK-regulatory binding proteins, using the methods provided herein.

 Disclosed herein are EGFR regulatory domain peptides ("RDPs"), which include, *e.g.*, isolated peptides consisting of amino acid residues 1167-1185 of SEQ ID NO: 1 or an immunogenic fragment of said peptide. Also disclosed are (EGFR)
30 regulatory domain (RD)-binding molecules that specifically binds to such peptides. Some embodiments include a RD-binding molecule that specifically binds to residues 1138-1196 of SEQ ID NO: 1 or a SOCS-protein-binding fragment thereof

(*e.g.*, a SOCS3-binding fragment). Also disclosed are compositions including an EGFR RD-binding molecule the binding of which to EGFR is competitively inhibited by a disclosed RDP (such as an isolated peptides consisting of amino acid residues 1167-1185 of SEQ ID NO: 1 or an immunogenic fragment of said peptide).

5

II. Abbreviations and Terms

ED	extracellular (or external) domain of RTK
EGFR	epidermal growth factor receptor (<i>e.g.</i> , OMIM Accession No. 131550)
FFPE	formalin-fixed, paraffin-embedded
FGFR	fibroblast growth factor receptor (<i>e.g.</i> , OMIM Accession No. 136350 for FGFR1, 176943 for FGFR2, 134934 for FGFR3, and 134935 for FGFR4)
HER	human epidermal growth factor receptor (<i>e.g.</i> , OMIM Accession No. 164870 for HER2 (ErbB2), 190151 for HER3 (ErbB3), and 600543 for HER4 (ErbB4))
HGFR (Met)	hepatocyte growth factor receptor (<i>e.g.</i> , OMIM Accession No. 164860)
ID	intracellular (or internal) domain of RTK
IGFR	insulin-like growth factor receptor (<i>e.g.</i> , OMIM Accession No. 147370 for IGF1R and 147280 for IGF2R)
IHC	Immunohistochemistry
PDGFR	Platelet derived growth factor receptor (<i>e.g.</i> , OMIM Accession No. 173490 and 173410)
NSCLC	non-small cell lung cancer
RDP	regulatory domain peptide
RD	regulatory domain of RTK
RET	rearranged following transfection (<i>e.g.</i> , OMIM Accession No. 164761)
RON receptor	recepteur d'origine nantais receptor (<i>e.g.</i> , OMIM Accession No. 600168)
RTK	receptor tyrosine kinase
SH2 domain	Src homology 2 domain
SOCS protein	suppressor of cytokine signaling protein (<i>e.g.</i> , OMIM Accession Nos. 604176 or 603597)
STAT	signal transducer and activator of transcription
VEGF	vascular endothelial growth factor receptor (<i>e.g.</i> , OMIM Accession No. 165070 for VEGFR1, 191306 for VEGFR2, and 136352 for VEGFR3)

Unless otherwise noted, technical terms are used according to conventional usage. Definitions of common terms in cell and molecular biology may be found in

- Benjamin Lewin, *Genes*, published by Oxford University Press, 1994 (ISBN 0-19-854287-9); Kendrew *et al.* (eds.), *The Encyclopedia of Molecular Biology*, published by Blackwell Science Ltd., 1994 (ISBN 0-632-02182-9); and Robert A. Meyers (ed.), *Molecular Biology and Biotechnology: a Comprehensive Desk Reference*, published by VCH Publishers, Inc., 1995 (ISBN 1-56081-569-8).

In order to facilitate review of various embodiments of a disclosed invention, the following explanations of specific terms are provided:

Antigen-binding molecule: A molecule that specifically binds to an epitope in a target molecule (*e.g.*, an antigen, such as a protein or nucleic acid molecule). Exemplary antigen-binding molecules are provided elsewhere in this disclosure, and include, for example, antibodies and aptamers.

Species of antigen-binding molecules described herein include, without limitation, interface-specific binding molecules, RTK-binding molecules, RTK ID-binding molecules, RTK RD-binding molecules, regulatory protein-binding molecules, and control antigen-binding molecules. These species of antigen-binding molecules are characterized by the nature of the target molecule and/or the location in the target molecule of the epitope to which the species specifically binds as more particularly defined elsewhere in this disclosure. In some examples, the target molecule is an intracellular domain of RTK that specifically binds to a regulatory protein.

Cancer: Malignant neoplasm, for example one that has undergone characteristic anaplasia with loss of differentiation, increased rate of growth, invasion of surrounding tissue, and is capable of metastasis.

Contact: To bring one agent into close proximity to another agent, thereby permitting the agents to interact. For example, an antibody (or other specific binding agent) can be applied to a microscope slide or other surface containing a biological sample, thereby permitting detection of proteins (or protein-protein interactions or protein-nucleic acid interactions) in the sample that are specific for the antibody.

Detect: To determine if an agent or interaction (*e.g.*, binding between two proteins or a protein and a nucleic acid) is present or absent. In some examples this can further include quantification. For example, use of an antibody specific for a

particular protein (*e.g.*, an RTK) or a particular protein-protein interface, permits detection of the of the protein or protein-protein interaction in a sample, such as a sample containing cancer tissue. In particular examples, an emission signal from a label is detected. Detection can be in bulk, so that a macroscopic number of
5 molecules can be observed simultaneously. Detection can also include identification of signals from single molecules using microscopy and such techniques as total internal reflection to reduce background noise.

Diagnose: The process of identifying a medical condition or disease, for example from the results of one or more diagnostic procedures. Diagnostic reagents
10 can be used as companion diagnostics, as prognostics, and as predictors. In a specific example, a neoplasm, such as a cancer, is diagnosed in a subject by detecting the activation status of an RTK protein associated with the neoplasm.

In some examples, diagnosis includes determining whether or not a subject has a particular disease, such as whether the subject has a cancer that expresses a
15 particular RTK. In particular examples, diagnosis includes determining the prognosis of a subject, such as determining the likely outcome of a subject having a disease in the absence of additional therapy (*e.g.*, life expectancy). In yet other examples, diagnosis includes predicting a subject's response to a particular therapy, such as predicting the likely outcome of treating a subject's tumor with an RTK
20 inhibitor.

Epidermal growth factor receptor (EGFR): The cell-surface receptor for members of the epidermal growth factor family of extracellular protein ligands. EGFR is a member of the ErbB family of receptors, a subfamily of four closely related receptor tyrosine kinases: EGFR (ErbB-1), HER2/c-neu (ErbB-2), Her 3
25 (ErbB-3) and Her 4 (ErbB-4). Mutations affecting EGFR expression or activity can result in cancer, and thus EGFR can have oncogenic activity. Exemplary sequences are provided herein.

Epitope: A site on a target molecule (*e.g.*, an antigen, such as a protein or nucleic acid molecule) to which an antigen-binding molecule (*e.g.*, an antibody,
30 antibody fragment, scaffold protein containing antibody binding regions, or aptamer) binds. Epitopes can be formed both from contiguous or juxtaposed noncontiguous residues (*e.g.*, amino acids or nucleotides) of the target molecule

(e.g., a protein-protein interface). Epitopes formed from contiguous residues (e.g., amino acids or nucleotides) typically are retained on exposure to denaturing solvents whereas epitopes formed by tertiary folding typically are lost on treatment with denaturing solvents. An epitope typically includes at least 3, and more usually, at least 5 or 8-10 residues (e.g., amino acids or nucleotides). Typically, an epitope also is less than 20 residues (e.g., amino acids or nucleotides) in length, such as less than 15 residues or less than 12 residues.

Human epidermal growth factor receptor 2 (HER2): A member of the ErbB protein family, which is a proto-oncogene located at the long arm of human chromosome 17(17q11.2-q12). Approximately 25-30% of breast cancers have an amplification of the HER2/neu gene or overexpression of its protein product, referred to as "HER2 positive" (HER2+). HER2+ patients may receive the monoclonal antibody trastuzumab (Herceptin), which binds the HER2 ED, as a therapy for breast cancer. Overexpression of HER2 in breast cancer has been associated with increased disease recurrence and worse prognosis.

Immunogen: A molecule (also called an antigen) capable of provoking an immune response (e.g., the production of antibodies) when introduced into an animal with a functioning immune system. Exemplary immunogens including, for instance, proteins (or protein fragments) such as an RTK or portion thereof, polysaccharides, and small molecules (haptens) or peptides coupled to a carrier molecule (e.g., a protein such as bovine serum albumin ("BSA"), keyhole limpet hemocyanin ("KLH") or polylysine). An "**immunogenic fragment**" is a portion of a polypeptide or other immunogen that is capable of provoking an immune response either by itself or when conjugated to a carrier molecule. Immunogens and immunogenic fragments include one or more epitopes within their sequences.

Isolated: An "isolated" biological component (e.g., a nucleic acid molecule, chemical compound, protein or organelle) has been substantially separated or purified away from other biological components (e.g., nucleic acid molecules, chemical compounds, proteins or organelles) with which the component is commingled (e.g., in the cell of an organism or in a plant cell extract). Nucleic acids, proteins and chemical compounds that have been "isolated" include nucleic acids, proteins and chemical compounds purified by standard purification methods.

The term “isolated” also embraces nucleic acids and proteins prepared by recombinant expression in a host cell as well as chemically synthesized nucleic acids and chemical compounds.

Label: An agent capable of detection, for example by spectrophotometry, flow cytometry, or microscopy. For example, one or more labels can be attached to an antibody, thereby permitting detection of a target protein or a target protein-protein interaction. Exemplary labels include radioactive isotopes, fluorophores, ligands, chemiluminescent agents, enzymes, and combinations thereof.

Neoplasm: Abnormal growth of cells.

Normal cells or tissue: Non-tumor, non-malignant cells and tissue.

Peptide: Two or more amino acids joined by a peptide bond. Typically, a peptide consists of fewer than fifty amino acids; for example, consisting of approximately 7 to approximately 40 amino acids, consisting of approximately 7 to approximately 30 amino acids, consisting of approximately 7 to approximately 20 amino acids.

Sample: A biological specimen, such as one that includes detectable proteins and/or nucleic acids, obtained from a subject. Examples include, but are not limited to, peripheral blood, urine, saliva, tissue biopsy, surgical specimen, bone marrow, amniocentesis samples and autopsy material. In one example, a sample includes proteins. In some examples, the sample is a tissue sample obtained from a subject known to have, or suspected to have, cancer. Samples, such as tissue samples, can be placed on microscope slides. In particular examples, samples are used directly, or can be manipulated prior to use, for example, by fixing (*e.g.*, using formalin) or embedding (*e.g.*, in plastic or paraffin).

Specific binding (or obvious derivations of such phrase, such as specifically binds, specific for, *etc.*) refers to the particular interaction between one binding partner (such as an RTK, for example an ID of the RTK) and another binding partner (such as a regulatory protein of the RTK, for example a positive or negative regulator, or an antibody or aptamer specific for an RTK protein-regulatory protein interface). Such interaction is mediated by one or, typically, more noncovalent bonds between the binding partners (or, often, between a specific region or portion of each binding partner). In contrast to non-specific binding sites, specific binding

sites are saturable. Accordingly, one exemplary way to characterize specific binding is by a specific binding curve. A specific binding curve shows, for example, the amount of one binding partner (the first binding partner) bound to a fixed amount of the other binding partner as a function of the first binding partner concentration. As the first binding partner concentration increases under these conditions, the amount of the first binding partner bound will saturate. In another contrast to non-specific binding sites, specific binding partners involved in a direct association with each other (*e.g.*, a protein-protein interaction) can be competitively removed (or displaced) from such association (*e.g.*, protein complex) by excess amounts of either specific binding partner. Such competition assays (or displacement assays) are very well known in the art.

Subject: Includes any multi-cellular vertebrate organism, such as human and non-human mammals (*e.g.*, veterinary subjects). In some examples, a subject is one who has cancer, or is suspected of having cancer. In some examples veterinary subjects are used to produce antibodies, such as in mice, rabbits, cows, or chickens.

Tumor: A neoplasm. Includes solid and hematological (or liquid) tumors.

Unless otherwise explained, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which a disclosed invention belongs. The singular terms “a,” “an,” and “the” include plural referents unless context clearly indicates otherwise. Similarly, the word “or” is intended to include “and” unless the context clearly indicates otherwise. “Comprising” means “including.” Hence “comprising A or B” means “including A” or “including B” or “including A and B.”

Suitable methods and materials for the practice and/or testing of embodiments of the disclosure are described below. Such methods and materials are illustrative only and are not intended to be limiting. Other methods and materials similar or equivalent to those described herein can be used. For example, conventional methods well known in the art to which a disclosure pertains are described in various general and more specific references, including, for example, Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, 2d ed., Cold Spring Harbor Laboratory Press, 1989; Sambrook *et al.*, *Molecular Cloning: A Laboratory*

Manual, 3d ed., Cold Spring Harbor Press, 2001; Ausubel *et al.*, Current Protocols in Molecular Biology, Greene Publishing Associates, 1992 (and Supplements to 2000); Ausubel *et al.*, Short Protocols in Molecular Biology: A Compendium of Methods from Current Protocols in Molecular Biology, 4th ed., Wiley & Sons, 1999; Harlow and Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, 1990; and Harlow and Lane, Using Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, 1999.

III. Receptor Tyrosine Kinases

Receptor tyrosine kinases (RTKs) are cell surface receptor proteins that include an extracellular ligand-binding domain (ED) (*e.g.*, capable of binding a growth factor, cytokine, or hormone), a transmembrane spanning domain, and an intracellular domain (ID) responsible for kinase activity. Most RTKs are single subunit receptors but some (*e.g.*, the insulin receptor) are multimeric complexes. Ligand binding to the ED induces RTK dimerization and autophosphorylation. The resulting activated phosphorylated RTK can then function to recruit intracellular signaling proteins via its ID (such as a Src homology 2 (SH2) domain). Thus, ligand binding to the ED generates a signal inside the cell via the ID. For example, phosphorylation of specific tyrosine residues within the activated RTK creates binding sites for SH2- and phosphotyrosine binding (PTB) domain-containing regulatory proteins. Binding of regulatory proteins to the RTK ID initiates signal transduction pathways. Other regulatory proteins that interact with the activated RTK may function as adaptor proteins and have no intrinsic enzymatic activity of their own, but can link RTK activation to downstream signal transduction pathways, such as the MAP kinase signaling cascade.

There are several RTK families, wherein each generally has related ligands and similar extracellular domains. Exemplary families useful for the methods provided herein include those shown in Table 1.

Table 1. Exemplary RTK families

Family	Exemplary members
Platelet derived growth factor receptor (PDGFR)	colony stimulating factor 1 (CSF-1), PDGFRA, PDGFRB, c-Kit

Fibroblast growth factor receptor (FGFR)	FGFR1, FGFR2, FGFR3, FGFR4
Insulin receptor	insulin receptor, insulin like growth factor-1 (IGF1R), IGF2R
Epidermal growth factor receptor (EGFR)	EGFR, HER2 (ErbB2), HER3 (ErbB3), HER4 (ErbB4)
Vascular endothelial growth factor receptor (VEGFR)	VEGFR 1 (Flt1) VEGFR 2 (KDR/Flk-1), VEGFR3 (Flt4), Flt3
Trk receptors	trkA, trkB and trkC
Hepatocyte growth factor receptor (HGFR)	c-Met, RON
RET	Ret
EPH	EphA1-8,10; EphB1-4, 6
UFO/Axl/Tyro-3	Axl, Mer, Tyro3

RTKs that can be used in the methods provided herein include those that are oncogenic, that is, that can cause or induce the development of tumors, such as cancer. Generally, RTK oncogenes can cause unregulated cell growth of a cancer cell, for example by activating signaling proteins involved in cellular proliferation as well as activating phosphoinositide 3-kinase (PI3-K)-dependent pathways which can also contribute to oncogenesis (for example by producing an anti-apoptotic signal). For example, oncogenic RTKs can confer a growth advantage to a cancer cell in which the RTK is expressed, such as when a positive regulatory protein is specifically bound to the RTK ID. For example, a positive RTK regulator may increase RTK biological activity (such as downstream effects) or expression, or prevent or reduce RTK degradation (or combinations thereof). In contrast, inhibition of an oncogenic RTK can reduce or eliminate the growth advantage to a cancer cell, such as when a negative regulatory protein is specifically bound to the RTK ID. For example, a negative RTK regulator may target the RTK for degradation, decrease biological activity (such as downstream effects) or decrease RTK expression (or combinations thereof). Therefore, use of the diagnostic reagents obtained using the methods herein can be used to determine the activation status of a target RTK, and thus allow one to predict or determine the aggressiveness of a neoplasm (*e.g.*, cancer) expressing the RTK, the potential efficacy of therapies targeted to the RTK, and determine if a subject has a neoplasm that expresses the RTK.

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In some examples, an oncogenic RTK is one whose amino acid sequence differs from the native RTK sequence (*e.g.*, RET, Kit), resulting in a protein that is not regulated in the same manner as the native RTK sequence (*e.g.*, unregulated cell proliferation). In some examples, an oncogenic RTK has the same sequences as a native or wild-type sequence (*e.g.*, EGFR, MET) but which becomes significantly upregulated (*e.g.*, due to the binding of a positive regulator or loss of binding of a negative regulator).

Exemplary RTKs that can be used in the methods provided herein include, but are not limited to, those listed in Table 2. RTK protein sequences are publicly available, for example on GenBank or EMBL websites. Exemplary non-limiting examples include those shown in Table 2. One skilled in the art will appreciate that other sequences can be used in the methods provided herein, such as those from other mammals besides humans, as well as polymorphic and mutant variants thereof.

Table 2: Exemplary GenBank Accession Numbers for RTK Protein Sequences

RTK	GenBank Accession Nos.*
EGFR	NM_005228; NM_201282; NM_201283; NM_201284
FGFR1	NM_023105; NM_023106; NM_023110; NM_023111; NM_015850
FGFR2	NM_000141; NM_022970
FGFR3	NM_000142; NM_022965
FGFR4	NM_213647; NM_002011; NM_022963
Flt3	NM_004119
HER2 (ErbB2)	NM_004448; NM_001005862
HER3 (ErbB3)	NM_001982; NM_001005915
HER4 (ErbB4)	NM_005235; NM_001042599
HGFR (Met)	NM_000245; NM_001127500
IGF1R	NM_000875
Kit	NM_000222; NM_001093772
PDGFR (PDGFRA and PDGFRB)	NM_006206; NM_002609
Ret	NM_020975; NM_020630
RON (MST1R)	NM_002447
VEGFR1 (Flt1)	NM_002019
VEGFR2 (KDR/flk-1)	NM_002253
VEGFR3 (Flt4)	NM_182925; NM_002020

*sequences associated with GenBank Accession Numbers as of July 11, 2008 are herein incorporated by reference.

As described above, activated RTKs can form specific protein-protein interactions with positive and negative regulatory proteins. Exemplary positive and

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negative regulatory proteins for particular RTKs are listed in Table 3. For example, the ID of an oncogenic RTK can bind with high specificity to a regulatory protein. Such an interaction can form a protein-protein interface, resulting in positive or negative regulation of the RTK. For example binding of a positive regulatory

5 protein to the RTK ID can increase the biological activity of the RTK, increase the biological activity of downstream activators, decrease degradation of RTK, or combinations thereof. Binding of a positive regulator to RTK can therefore confer a growth advantage to a cancer cell expressing the RTK. In contrast, binding of a

10 negative regulatory protein to the RTK ID can decrease the biological activity of the RTK, decrease the biological activity of downstream activators, increase degradation of RTK, or combinations thereof. Binding of a negative regulator to RTK can therefore decrease or eliminate a growth advantage to a cancer cell expressing the RTK.

15 **Table 3: Exemplary Positive and Negative Regulators of RTKs**

RTK	Positive Regulators	Negative Regulators
EGFR	STAT1, STAT5B, GRB7, HER2, MUC1	SOCS1, SOCS3, SOCS5, CBL, LRIG1, SHP1, PKC
FGFR1	GRB2, GRB4, GRB14, SOS1, SHC1	SEF
FGFR2		CBL, SEF
FGFR3	GRB2, STAT1, STAT3, SHP2	CBL
FGFR4	STAT1, STAT3	
Flt3	GRB2, SHC1	SOCS1
HER2 (ErbB2)	GRB2, GRB7, SOS1, SHC1, SHP2	CAV1, LRIG1, CHIP
HER3 (ErbB3)	GRB2, GRB7, SHC1	CBL, LRIG1
HER4 (ErbB4)	GRB2, STAT5, SHC1, SHP2	CBL, LRIG1, PKC
HGFR (Met)	GRB2, SHC1, STAT3, SRC, SHP2	CBL, PKC, LRIG
IGF1R	IRS1-4, GRB10, SHC1	SOCS1, SOCS2, SOCS3
Kit	GRB2, GRB7, GRB10, STAT1, STAT5, SHP2,	SHP1, SOCS1, SOCS6, CBL, PKC
PDGFR (PDGFRA and PDGFRB)	GRB2, GRB10, GRB14, SHP2, SHC1	CAV1, CAV3, CBL
Ret	IRS1, GRB2, GRB7, GRB10,	DOK4

	SOS1, SHC, SHANK3, SH2B1	
RON	GRB2, SHC1, SRC	CBL
VEGFR1 (Flt1)	GRB2, STAT1, STAT3, SHC1, SHP2	CBL
VEGFR2 (KDR/flk-1)	GRB2, GRB10, SHC1, SHC2, STAT1, SHP2	SHP1,CBL, PKC
VEGFR3 (Flt4)	GRB2, SHC1, STAT3, SRC, SHP2	CBL

Although particular regulatory proteins are provided in Table 3, one skilled in the art will appreciate that others are publicly known. In addition, additional regulatory proteins can be identified using routine methods. For example, regulatory proteins that interact with an RTK (or portion thereof, such as an ID or RD thereof) can be identified using immunoprecipitation, a two-hybrid system (*e.g.*, by using the RTK ID or RD as the “bait”), or pull-down assays. For example, an RTK of interest can be expressed in a cell, and isolated from the cell using an antibody to the RTK (*e.g.*, an antibody that recognizes an ED or non RD of the RTK). In another example, an RTK of interest can be expressed in a cell along with a marker, thereby resulting in expression of an RTK-fusion protein (such as a 6X-HIS-RTK protein) which can be isolated from the cell using a molecule specific for the marker (*e.g.*, Ni beads). Other proteins that are isolated with the RTK complex (*e.g.*, regulatory proteins) can be then identified, for example by using western blotting or mass spectrometry methods.

Using the methods provided herein, a diagnostic binding reagent can be identified or generated that specifically binds to at least a portion of the RTK protein - regulatory protein interface in the absence of the RTK protein-regulatory protein interaction but does not specifically bind the protein-protein interface in the presence of the RTK protein-regulatory protein interaction. Positive and negative RTK regulatory proteins are known (*e.g.*, see Table 3). In such examples, a particular RTK/regulatory protein combination may be selected based on the desired diagnostic reagent desired. For example, if diagnosis of a HER2-expressing tumor is desired, for example to determine the prognosis of a subject having a HER2-expressing tumor, a diagnostic binding reagent that specifically binds to at least a portion of the amino acids that form a HER2-negative regulator protein-protein interface (*e.g.*, an interface between HER2 and one of CAV1, CBL or LRIG1) or a

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HER2-positive regulator protein-protein interface (*e.g.*, an interface between HER2 and one of GRB2, GRB7, SOS1, SHP2, or SHC1) but does not specifically bind the protein-protein interface in the presence of the protein- protein interaction, can be generated. However, one skilled in the art will appreciate that a diagnostic binding reagent can be identified without knowing the identity of the regulatory protein. For example, all that may be known is that the regulatory protein is a negative or positive regulator. In specific examples, amino acids (*e.g.*, at least 5) that make up a least a portion of a protein-protein interface between an RTK and a regulatory protein listed in Table 3 are identified and used to produce a diagnostic specific binding agent.

RTKs associated with oncogenesis are known in the art. Some RTKs, such as EGFR, are associated with many different cancer types. However, some RTKs are associated with particular cancers, such as those listed in Table 4. When generating a diagnostic specific binding agent using the methods provided herein, an RTK can be identified or selected based on the neoplasm or cancer for which a diagnostic specific binding agent is desired. For example, if a general cancer diagnostic reagent is desired, an RTK that is oncogenic for several cancers can be selected, such as EGFR or HGFR. If a diagnostic reagent for a particular cancer type is desired, an RTK associated with the cancer can be selected, for example as shown in Table 4. For example, if a diagnostic reagent for prostate cancers that express VEGFR1 is desired, the VEGFR1 RTK can be selected as the RTK for which a diagnostic reagent can be generated.

Table 4: Exemplary cancers associated with RTKs

RTK	Cancer
EGFR	non-small cell lung, bladder, cervical, ovarian, kidney and pancreatic cancer, squamous head and neck carcinoma, glioblastoma multiforme
FGFR1	Glioblastoma
FGFR2	Breast cancer
FGFR3	Bladder cancer, multiple myeloma
FGFR4	Breast cancer, prostate cancer, rhabdomyosarcoma
Flt3	Acute myeloid leukemia, acute lymphoblastic leukemia
HER2 (ErbB2)	breast cancer, colon cancer, ovarian cancer, non-small cell lung cancer
HER3 (ErbB3)	Non-small cell lung cancer, breast cancer

HER4 (ErbB4)	Prostate cancer, breast cancer, colorectal cancer
HGFR (MET)	Thyroid papillary carcinoma, multiple papillary renal cell carcinoma, colorectal cancer, breast cancer, non-small cell lung cancer, ovarian cancer, pancreatic cancer
IGF1R	gastrointestinal neuroendocrine tumor, breast cancer, prostate cancer
Kit	gastrointestinal stromal tumors, mast cell disease, acute myelogenous leukemia, melanoma, thyroid carcinoma
PDGFR	gastrointestinal stromal tumor, glioblastoma
Ret	Neuroblastoma, medullary thyroid carcinoma, multiple endocrine neoplasias type II and III, pheochromocytoma and parathyroid tumors
RON	breast cancer, colon cancer, pancreatic cancer
VEGFR1 (Flt1)	non-small cell lung cancer, colorectal cancer, breast cancer, bladder cancer, prostate cancer
VEGFR2 (KDR/flk-1)	breast cancer, prostate cancer, ovarian cancer
VEGFR3 (Flt4)	breast cancer, non-small cell lung cancer

IV. Identifying Amino Acids that Form a Protein-Protein Interface

This disclosure provides, among other things, methods for producing a diagnostic binding reagent that specifically binds to at least a portion of the RTK protein - regulatory protein interface in the absence of the RTK protein-regulatory protein interaction but does not specifically bind the protein-protein interface in the presence of the RTK protein-regulatory protein interaction. In some examples, prior to generating such a diagnostic specific binding reagent, at least a portion of the amino acids that form the RTK protein - regulatory protein interface are identified.

For example, if diagnosis of a VEGF-expressing tumor is desired, for example to determine the prognosis of a subject having a VEGF-expressing tumor, at least three amino acids that form a VEGF-negative regulator protein-protein interface (*e.g.*, an interface between VEGF1 and one of GRB2, STAT1, STAT3, SHC1 or SHP2) or a VEGF1-positive regulator protein-protein interface (*e.g.*, an interface between VEGF1 and CBL) can be identified, if not already known in the art. In some examples, only amino acids of the RTK ID or RD that are part of the interface are identified (*e.g.*, regulatory protein amino acids are not identified).

Methods are provided for identifying protein-protein interactions, for example in biological samples (*e.g.*, isolated cells or tissues). Such methods can be used to identify amino acids that form a protein-protein interface between an RTK

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ID and a regulatory protein to permit production of diagnostic reagents for use in determining the activity state of an RTK. Upon specific binding of a regulatory protein to the ID of an RTK, certain amino acids of the RTK ID will interact with certain amino acids of the regulatory protein. By identifying at least a subset of the amino acids that form the resulting protein-protein interface, diagnostic specific binding reagents can be generated that can be used to determine the activity state of an RTK.

Amino acid residues that form an interface between an RTK and one of its regulatory proteins (such as a positive or negative regulator, *e.g.*, see Table 3) are identified. Although not all amino acids that form the interface need be identified, a number of amino acids is identified that permits generation of a diagnostic specific binding reagent that specifically binds to at least a subset (*e.g.*, at least one) of the amino acid residues of the protein-protein interface in the absence of the RTK protein-regulatory protein interaction and fails to specifically bind amino acid residues of the RTK protein-regulatory protein interface in the presence of the RTK protein-regulatory protein interaction. One skilled in the art will appreciate that the specific binding reagent need not bind all amino acids that comprise the protein-protein interface. So long as the specific binding reagent binds to the interface in the absence of regulatory protein binding to the RTK, and does not bind the interface when regulatory protein is bound to the RTK, it can be used in the disclosed methods. Thus, in some examples the specific binding reagent need only bind at least 1 amino acid of the interface, such as at least 2, for example 1 to 5, 1 to 10 or 5 to 20 amino acids of the interface. In particular examples, a sufficient number of amino acids are identified that permit generation of a specific binding reagent, such as identification of an immunogenic or antigenic peptide. For example, immunogenic peptides can produce a significant antibody response in an animal (*e.g.*, rabbit, mouse, or chicken) immunized with the peptide under routine conditions for forming antibodies.

In particular examples, amino acids of the RTK ID that form part of the protein-protein interface are identified, such amino acids of the regulatory domain that form part of the protein-protein interface. For example, at least 3, at least 5, at least 10, or at least 20 amino acids of the protein-protein interface can be identified,

such as 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 40, 50, or 100 amino acids, for example 5 to 8, 5 to 12, 8 to 10, 8 to 12, 8 to 15, or 8 to 20 amino acids. In one example, at least 3, at least 5, at least 10, or at least 20 amino acids of an RTK ID (or RD) that forms a protein-protein interface with a regulatory protein is
5 identified, such as 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 40, 50, or 100 amino acids of an RTK ID (or RD), for example 5 to 8, 5 to 12, 8 to 10, 8 to 12, 8 to 15, or 8 to 20 amino acids of an RTK ID (or RD).

One skilled in the art will recognize that a protein-protein interface formed by binding of a RTK ID to a regulatory protein may include non-consecutive amino
10 acid residues from each protein. That is, although a protein-protein interface may span numerous amino acids (*e.g.*, may span 100 amino acids of an RTK), not all amino acids in the span may form or be part of the protein-protein interface (*e.g.*, maybe only 5 to 20 of the 100 RTK amino acids are part of the protein-protein interface). Therefore, the amino acids identified and used to generate a specific
15 binding reagent may or may not be contiguous, or may include a combination of contiguous and non-contiguous amino acids. For example, amino acids 20-22, 30, 32 and 40-50 of an RTK ID may bind to amino acids 5-10 and 30-40 of a regulatory protein. In such an example, at least three or at least five of such amino acids, such as amino acids 20-22 and 40-43 of the RTK ID, can be identified.

20 In some examples, a positive or a negative regulatory protein is selected. That is, if a diagnostic reagent for determining whether an RTK is bound to a positive regulator, amino acids that form an interface between such two proteins can be identified, while if a diagnostic reagent for determining whether an RTK is bound to a negative regulator, amino acids that form an interface between such two
25 proteins can be identified. Table 3 above provides exemplary known positive and negative regulators for particular RTKs. Thus for example, if a diagnostic reagent for determining if Kit is bound to one of its negative regulators (*e.g.*, SHP1, SHP2, SOCS1, SOCS6, CBL) or for determining if Ret is bound to the positive regulator IRS1, GRB2, GRB7, GRB10, SOS1, or SHC, then amino acids forming at least a
30 portion of the protein-protein interface between such proteins can be identified.

In some examples, the identity of the regulator is not known prior to identifying amino acids of the protein-protein interface (*e.g.*, not known whether it is

a positive or negative regulator, or it is known that it is a positive or negative regulatory protein, but the specific identity of the protein is not known). Once the specific binding agent is generated, it can subsequently be determined if the bound regulator is a positive or negative regulatory protein (*e.g.*, by determining the effect of the regulator on cancer cell growth, wherein a positive regulatory protein is one that enhances such growth while a negative regulatory protein is one that decreases such growth).

Biological components (*e.g.*, proteins) that form direct interactions (such as protein-protein interactions) are known to those of ordinary skill in the art. Various exemplary protein-protein interactions, including RTK-regulatory protein interactions, can be identified as shown in Table 3, as well as in one or more of the following publicly available databases: AllFuse (European Bioinformatics Institute), Alanine Scanning Energetics DataBase (ASEdb; Harvard University), Binding Interface Database (BID; A & M University Texas); The General Repository for Interaction Datasets (BioGRID; Samuel Lunenfeld Research Institute); Biomolecular Object Network Databank (BOND; Thomson Corp.); Database of Interacting Proteins (DIP; UCLA); Genomic Knowledge Database (RIKEN, Institute of Physical and Chemical Research); HIV-1/Human Protein Interaction Database (NCBI); Human Protein Interaction Database (HPID; Indiana University); Human Protein Reference Database (Johns Hopkins University and The Institute of Bioinformatics, India); Inter-Chain Beta-Sheets database (ICBS; University of California); Kinetic Data of Bio-molecular Interactions (KDBI; National University of Singapore); Biomolecular Relations in Information Transmission and Expression (KEGG BRITE; Kyoto University); Molecular Interactions database (MINT; CBM, Rome); Mammalian Protein-Protein Interaction database (MPPI; MIPS); PDZBase (Weill Medical College of Cornell University); POINT (National Health Research Institutes & National Taiwan University); PRotein Interactions and Molecular Information database (PRIME; Human Genome Center, University of Tokyo); Protein Interaction Database (Protein Lounge); SNAPPiView (University of Dundee).

Some of the foregoing databases further identify the residues or regions of the applicable proteins involved in the protein-protein interface. Therefore, in some

examples the amino acids of the protein-protein interface are known in the art. Alternatively, residues or regions involved in a protein-protein interaction can be determined using any technique known to the ordinarily skilled artisan; for example, peptide competition studies (where a peptide having a sequence corresponding to
5 residues believed to be involved in a protein-protein interface is used to competitively inhibit the protein-protein interaction; successful inhibition by the peptide of the interaction indicates that the subject sequence likely is involved in the protein-protein interaction), mutational analysis of one or both components of the protein-protein interaction, or crystallography of the RTK-regulatory protein (or a
10 portion thereof; for example an RTK ID) complex.

Methods of identifying amino acids that form a protein-protein interface between two proteins are routine. In some examples an activated RTK is incubated with a regulatory protein in solution (*e.g.*, using purified proteins) under conditions that permit the two proteins to bind and form a protein-protein interface. In other
15 examples, a cell, such as a cancer cell, that expresses the RTK and the regulatory protein is under conditions that permit the two proteins to bind and form a protein-protein interaction, resulting in a protein-protein interface. The resulting protein-protein interaction can then be analyzed, for example identifying at least three or at least five amino acids that form the protein-protein interface (such as identifying
20 RTC ID amino acids that form a protein interface with the regulatory protein).

A. Competition Studies

In one example, peptide competition studies are performed to identify amino
25 acid residues that form a protein-protein interface between an RTK and a regulatory protein. Such methods are routine in the art. For example, peptide fragments of a target RTK ID (*e.g.*, RTK RD, see Table 5) can be generated. Such fragments can include contiguous amino acids of the RTK ID, but can also include non-contiguous amino acids. Exemplary peptide fragments are at least 3 amino acids in length, such
30 as at least 5, at least 10, or at least 20 amino acids in length, such as 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 40, 50, or 100 amino acids in length, for example 5 to 8, 5 to 12, 8 to 10, 8 to 12, 8 to 15, or 8 to 20 amino acids in length,

such as this number of amino acids of an RTK RD. Peptide fragments can be generated randomly (*e.g.*, by forming a library of molecules that span an entire RTK ID) or can be generated to specific sequence(s) corresponding to residues believed to be involved in a protein-protein interface between an RTK and a regulatory protein.

- 5 In some examples, peptide fragments are generated (for example using a computer program) that are suspected of not only of being involved in a protein-protein interface, but are also suspected to be immunogenic.

The resulting protein fragments are incubated with a regulatory protein and an RTK protein (or portions thereof) to competitively inhibit the protein-protein
10 interaction that would normally result between the RTK and the regulatory protein. For example, purified regulatory and RTK proteins (or portions thereof) can be incubated *in vitro* or *in vivo* (for example the fragment can be incubated with or introduced into a cancer cell that expresses the activated RTK and regulatory proteins) with different concentrations of the fragment peptide (*e.g.*, serial dilutions,
15 for example in the range of 0.01 μM to 10,000 μM , 0.01 μM to 10,000 μM , or 1 μM to 1000 μM) and effects on binding between the RTK and its regulatory protein determined using routine protein detection methods, such as Western blotting, immunoprecipitation, and immunostaining. If the peptide fragment significantly reduces or inhibits the protein-protein interaction, this indicates that the subject
20 sequence of the fragment likely is involved in the protein-protein interaction. Such identified sequences can be used to generate diagnostic specific binding reagents. Similarly, functional studies (*e.g.*, cancer cell growth) can be performed in the presence and absence of the competing peptides, to identify peptides that interfere with the binding of a regulatory peptide to an RTK. For example, identification of
25 peptides that target an RTK-positive regulator can be selected by identifying those peptides that decrease the growth of cancer cells in culture relative to the absence of the peptide (*e.g.*, a decrease of at least 20%, such as at least 50%, or at least 80%) (decreased cancer cell growth in the presence of the peptide indicates that the positive regulator is not bound to the RTK, and thus the growth advantage provided
30 by the RTK is reduced). Alternatively, identification of peptides that target a RTK-negative regulator can be selected by identifying those peptides that increase the growth of cancer cells in culture relative to the absence of the peptide (*e.g.*, an

increase of at least 20%, such as at least 50%, or at least 80%) (increased growth in the presence of the peptide indicates that the negative regulator is not bound to the RTK, and thus the growth advantage provided by the RTK is increased, as the RTK is no longer targeted for degradation).

5 **B. Mutagenesis Studies**

In another example, mutagenesis studies are performed to identify amino acid residues that form a protein-protein interface between an RTK and a regulatory protein. Such methods are routine in the art. For example, deletion mutants of either the target RTK ID (*e.g.*, RTK RD, see Table 5) or deletion mutants of the regulatory protein can be generated (*e.g.*, using recombinant molecular biology methods). In one example, a plurality of deletion mutants of a target RTK ID (or RD) are generated, and can include deletion of contiguous or non-contiguous amino acids (or combinations thereof). Exemplary deletions include deletion of at least 3 amino acids, such as at least 5, at least 10, or at least 20 amino acids, such as 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 40, 50, or 100 amino acids, for example 5 to 8, 5 to 12, 8 to 10, 8 to 12, 8 to 15, or 8 to 20 amino acids, such as this number of amino acids deleted from an RTK RD or regulatory protein.

Mutagenized RTK or regulatory proteins can be generated randomly (*e.g.*, by forming a library of deletion mutants that span an entire RTK ID or RD) or can be generated to specific sequence(s) corresponding to residues believed to be involved in a protein-protein interface between an RTK and a regulatory protein.

The resulting mutant proteins (*e.g.*, mutant RTK + mutant regulatory protein; mutant RTK + native regulatory protein, or native RTK + mutant regulatory protein) are incubated under conditions that would normally permit the native RTK protein and native regulatory protein to interact. For example, purified regulatory and RTK proteins (*e.g.*, mutant RTK + mutant regulatory protein; mutant RTK + native regulatory protein, or native RTK + mutant regulatory protein) can be incubated *in vitro* or *in vivo* (for example the proteins can be incubated with or introduced into a cancer cell that expresses the proteins) and effects on binding between the RTK and its regulatory protein determined using routine protein detection methods, such as Western blotting, immunoprecipitation, and immunostaining. For example, if a mutant RTK is unable to bind to its native regulatory protein (and thus the mutation

significantly reduces or inhibits the protein-protein interaction), this indicates that the subject deleted sequence is likely involved in the protein-protein interaction. The sequence of the deletion can be used to generate diagnostic specific binding reagents.

5 Similarly, functional studies (*e.g.*, cancer cell growth) can be performed with various combinations of the native and mutant peptides (*e.g.*, mutant RTK + mutant regulatory protein; mutant RTK + native regulatory protein, or native RTK + mutant regulatory protein), to identify mutants that do not permit binding between a regulatory peptide and an RTK. For example, identification of deletion mutants that
10 target an RTK-positive regulator can be selected by identifying those deletion mutants that decrease the growth of cancer cells (*e.g.*, a decrease of at least 20%, such as at least 50%, or at least 80%) (decreased cancer cell growth in the presence of the deletion mutant indicates that the positive regulator is not bound to the RTK, and thus the growth advantage provided by the RTK is reduced). Alternatively,
15 identification of deletion mutants that target an RTK-negative regulator can be selected by identifying those deletion mutants that increase the growth of cancer cells (*e.g.*, an increase of at least 20%, such as at least 50%, or at least 80%) (increased cancer cell growth in the presence of the deletion mutant indicates that the negative regulator is not bound to the RTK, and thus the growth advantage
20 provided by the RTK is increased, as the RTK is no longer targeted for degradation). Further refinements to an identified mutant sequence that results in decreased binding between an RTK and a regulatory protein can be made by site directed mutagenesis. For example if a region of 20 amino acids is identified as playing a role in the protein-protein interaction, each of the 20 amino acids can be mutated
25 individually or in various combinations and tested as described above, to permit identification of the particular residues that comprise the protein-protein interface. Such identified residues can be used to generate a specific binding agent.

C. Crystallography

30 In another example, crystallography is used to identify amino acids of an RTK protein-regulatory protein interface. For example, purified RTK and regulatory protein (or fragments thereof, such as an RTK ID or RD) can be incubated under appropriate conditions to allow the proteins to bind and crystallize,

and an X-ray diffraction pattern obtained and used to build an electron density map using tools well known to those skilled in the art of crystallography and X-ray diffraction techniques. For an overview of the procedures of collecting, analyzing, and utilizing X-ray diffraction data for the construction of electron densities see, for example, Campbell *et al.*, *Biological Spectroscopy*, The Benjamin/Cummings Publishing Co., Inc., Menlo Park, Calif., 1984; Cantor *et al.*, *Biophysical Chemistry*, Part II: Techniques for the study of biological structure and function, W.H. Freeman and Co., San Francisco, Calif. 1980; A. T. Brunger, X-plor Version 3.1: A system for X-ray crystallography and NMR, Yale Univ. Pr., New Haven, Conn. 1993; M. Woolfson, *An Introduction to X-ray Crystallography*, Cambridge Univ. Pr., Cambridge, UK, 1997; J. Drenth, *Principles of Protein X-ray Crystallography* (Springer Advanced Texts in Chemistry), Springer Verlag; Berlin, 1999; and Tsirelson *et al.*, *Electron Density and Bonding in Crystals: Principles, Theory and X-ray Diffraction Experiments in Solid State Physics and Chemistry*, *Inst. of Physics Pub.*, 1996. Information on molecular modeling can be found for example in, M. Schlecht, *Molecular Modeling on the PC*, 1998, John Wiley & Sons; Gans *et al.*, *Fundamental Principles of Molecular Modeling*, Plenum Pub. Corp., 1996; N. C. Cohen (editor), *Guidebook on Molecular Modeling in Drug Design*, Academic Press, 1996; and W. B. Smith, *Introduction to Theoretical Organic Chemistry and Molecular Modeling*, 1996.

Typically, a well-ordered crystal that will diffract x-rays strongly is used to solve the three-dimensional structure of a RTK protein bound to a regulatory protein by x-ray crystallography. The crystallographic method directs a beam of x-rays onto a regular, repeating array of many identical molecules. The x-rays are diffracted from it in a pattern from which the atomic positions of the atoms that make up the protein-protein interface of interest can be determined.

Substantially pure and homogeneous protein samples are usually used for crystallization. Typically, crystals form when molecules are precipitated very slowly from supersaturated solutions. A typical procedure for making protein crystals is the hanging-drop method, in which a drop of protein solution (*e.g.*, that includes RTK protein, regulatory protein, and RTK protein bound to regulatory protein) is brought very gradually to supersaturation by loss of water from the

droplet to the larger reservoir that contains salt, polyethylene glycol, or other solution that functions as a hydroattractant, although any other method that generates diffraction quality crystals can be used. In some examples diffraction quality crystals are obtained by seeding the supersaturated solution with smaller
5 crystals that serve as templates.

Powerful x-ray beams can be produced from synchrotron storage rings where electrons (or positrons) travel close to the speed of light. These particles emit very strong radiation at all wavelengths from short gamma rays to visible light. When used as an x-ray source, only radiation within a window of suitable wavelengths is
10 channeled from the storage ring.

In diffraction experiments a narrow and parallel beam of x-rays is taken out from the x-ray source and directed onto the crystal to produce diffracted beams. The incident x-ray beam causes damage to both protein and solvent molecules. The crystal is, therefore, usually cooled to prolong its lifetime (for example to -220° to -
15 50° C). In some examples, single crystals are used to obtain a data set, while in other examples; multiple crystals are used to obtain a data set. The x-ray beam must strike the crystal from many different directions to produce all possible diffraction spots, thereby creating a complete data set. Therefore, the crystal is rotated relative to the beam during data collection. The diffracted spots are recorded either on a
20 film, or by an electronic detector, both of which are commercially available.

When the primary beam from an x-ray source strikes the crystal, x-rays interact with the electrons on each atom in the crystal and cause them to oscillate. The oscillating electrons serve as a new source of x-rays, which are emitted in almost all directions in a process referred to as scattering. When atoms (and hence
25 their electrons) are arranged in a regular three-dimensional array, as in a crystal, the x-rays emitted from the oscillating electrons interfere with one another. In most cases, these x-rays, colliding from different directions, cancel each other out; those from certain directions, however, will add together to produce diffracted beams of radiation that can be recorded as a pattern on a photographic plate or detector.

30 The diffraction pattern obtained in an x-ray experiment is related to the crystal that caused the diffraction. X-rays that are reflected from adjacent planes travel different distances, and diffraction only occurs when the difference in distance

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is equal to the wavelength of the x-ray beam. This distance is dependent on the reflection angle, which is equal to the angle between the primary beam and the planes.

Each atom in a crystal scatters x-rays in all directions, and only those that
5 positively interfere with one another, according to Bragg's law ($2d \sin \theta = \lambda$), give rise to diffracted beams that can be recorded as a distinct diffraction spot above background. Each diffraction spot is the result of interference of all x-rays with the same diffraction angle emerging from all atoms. To extract information about individual atoms from such a system requires considerable computation. The
10 mathematical tool that is used to handle such problems is called the Fourier transform.

Each diffracted beam, which is recorded as a spot on the film, is defined by three properties: the amplitude, which is measured as the intensity of the spot; the wavelength, which is determined by the x-ray source; and the phase information,
15 which is lost in x-ray experiments and must be calculated. All three properties are used for all of the diffracted beams, in order to determine the position of the atoms giving rise to the diffracted beams. Methods of determining the phases are well known in the art. For example, phase differences between diffracted spots can be determined from intensity changes following heavy atom derivatization. Another
20 example would be determining the phases by molecular replacement.

The amplitudes and the phases of the diffraction data from the protein crystals are used to calculate an electron-density map of the repeating unit of the crystal. A model of the particular amino acid sequence is built to approximate the electron density map. Such information can be used to identify amino acids that
25 form a protein-protein interface that results when an RTK protein binds to a regulatory protein.

The initial model may contain some errors. Provided the protein crystals diffract to high enough resolution (*e.g.*, better than 3.5 Å), most or substantially all of the errors can be removed by crystallographic refinement of the model using
30 computer algorithms. In this process, the model is changed to minimize the difference between the experimentally observed diffraction amplitudes and those calculated for a hypothetical crystal containing the model. This difference is

expressed as an R factor (residual disagreement) which is 0.0 for exact agreement and about 0.59 for total disagreement.

Typically, the R factor of a refined model is between 0.15 and 0.35 (such as less than about 0.24-0.28) for a well-determined protein structure. The residual
5 difference is a consequence of errors and imperfections in the data. These derive from various sources, including slight variations in the conformation of the protein molecules, as well as inaccurate corrections both for the presence of solvent and for differences in the orientation of the microcrystals from which the crystal is built. Thus, the final model represents an average of molecules that are slightly different,
10 both in conformation and orientation.

In refined structures at high resolution, there are usually no major errors in the orientation of individual residues, and the estimated errors in atomic positions are usually around 0.1-0.2 Å, provided the amino acid sequence is known.

Most x-ray structures are determined to a resolution between 1.7 Å. and 3.5
15 Å. Electron-density maps with this resolution range are preferably interpreted by fitting the known amino acid sequences into regions of electron density in which individual atoms are not resolved.

Upon identification of amino acids that form the protein-protein interface, diagnostic specific binding reagents can be generated that recognize at least a
20 portion of this sequence.

V. RTK Intracellular Domains

In some examples, as an alternative to (or in addition to) identifying amino acids of the protein-protein interface formed upon binding of a regulatory protein to
25 an RTK to generate diagnostic specific binding reagents, amino acids of the RTK ID, such as the RTK RD, are used to generate diagnostic specific binding reagents in accordance with the methods provided herein. In some examples such RTK ID or RD amino acids are identified and used to generate diagnostic specific binding reagents, or if already known (*e.g.*, see Table 5), can be simply used to generate
30 diagnostic specific binding reagents.

Table 5 provides exemplary known RTK IDs as well as portions thereof, such as RDs and for some inhibitory domains. For example, the RTK EGFR has an

EGFR regulatory domain (corresponding to residues 980-1210 of SEQ ID NO: 1), which includes an inhibitory subdomain (corresponds to residues 1138-1196 of SEQ ID NO: 1). With this information, specific binding reagents can be generated that specifically bind to an RTK-regulatory protein interface (for example by binding to the RTK ID, RD, or inhibitory domains) in the absence of RTK-regulatory protein binding, but fail to specifically bind to an RTK-regulatory protein interface (*e.g.*, fail to bind the RTK ID, RD or inhibitory domains) in the presence of RTK-regulatory protein binding. For example, using the methods provided in Section VI below and the information in Table 5, specific binding reagents can be generated to the RTK ID, RD or inhibitory domain listed in Table 5 (*e.g.*, by identifying epitopes within or that include these regions), and screened using routine methods. In some examples, the information in Table 5 is used to identify a protein-protein interface as described in Section IV above. For example, peptide fragments of the RTK ID and RD regions or RTK proteins with mutations in the ID or RD regions can be generated and tested as described above to identify amino acids comprising the protein-protein interface.

Table 5. Exemplary RTK intracellular domains

RTK	Intracellular domain	Regulatory domain	Inhibitory domain	Tyrosine Kinase domain
EGFR	aa 669-1210 of SEQ ID NO 1 (NM_005228)	aa 980-1210 of SEQ ID NO 1 (NM_005228)	aa 1138-1196 of SEQ ID NO 1 (NM_005228)	aa 712-968 of SEQ ID NO 1 (NM_005228)
FGFR1	aa 398-822 of NM_023110	aa 755-822 of NM_023110		aa 478-754 of NM_023110
FGFR2	aa 399-822 of NM_022970	aa 759-822 of NM_022970		aa 482-758 of NM_022970
FGFR3	aa 396-806 of NM_000142	aa 749-806 of NM_000142		aa 472-748 of NM_000142
FGFR4	aa 389-798 of NM_002011	aa 744-798 of NM_002011		aa 467-743 of NM_002011
Flt3	aa 565-993 of NM_004119	aa 944-993 of NM_004119		aa 610-943 of NM_004119
HER2	aa 676-1255 of NM_004448	aa 977-1255 of NM_004448		aa 720-976 of NM_004448
HER3	aa 667-1342 of NM_001982	aa 966-1342 of NM_001982		aa 709-965 of NM_001982
HER4	aa 676-1292 of	aa 719-1292 of		aa 718-974 of

	NM_001042599	NM_001042599		NM_001042599
HGFR (MET)	aa 956-1390 of NM_000245	aa 1338-1390 of NM_000245		aa 1078-1337 of NM_000245
IGF-1R	aa 956-1367 of NM_000875	aa 1267-1367 of NM_000875	aa 1275-1367 of NM_000875	aa 999-1266 of NM_000875
Kit	aa 544-976 of NM_000222	aa 925-976 of NM_000222		aa 589-924 of NM_000222
PDGFRB	aa 557-1106 of NM_002609	aa 959-1106 of NM_002609		aa 600-958 of NM_002609
Ret	aa 658-1114 of NM_020975	aa 1006-1114 of NM_020975		aa 724-1005 of NM_020975
RON	aa 983-1400 of NM_002447	aa 1342-1400 of NM_002447		aa 1082-1341 of NM_002447
VEGFR1 (Flt1)	aa 780-1338 of NM_002019	aa 1155-1338 of NM_002019		aa 827-1154 of NM_002019
VEGFR2 (KDR/flk-1)	aa 766-1356 of NM_002253	aa 1161-1356 of NM_002253	aa 1161-1199 of NM_002253	aa 834-1160 of NM_002253
VEGFR3 (Flt4)	aa 799-1363 of NM_182925	aa 846-1363 of NM_182925		aa 845-1169 of NM_182925

For example, if a specific binding agent for assessing the activity state of Ret was desired, amino acids 658-1114 or 1006-1114 of GenBank Accession No. NM_020975 could be used to generate antibodies or aptamers, for example by

5 identifying one or more epitopes within or that include these residues and using such epitopes to generate antibodies (see Section VI below). Alternatively, peptides that spanned the regions that included amino acids 658-1114 or 1006-1114 of GenBank Accession No. NM_020975, or peptides that included deletions or other mutations in these regions, could be generated and tested as described above to identify amino

10 acids of the protein-protein interface (see Section IV above). Crystallography with a peptide that included amino acids 658-1114 or 1006-1114 of GenBank Accession No. NM_020975 and a Ret regulatory protein (see Table 3) could be performed to identify amino acids of the protein-protein interface (see Section IV above).

A. EGFR Regulatory Domain Peptides

15 This disclosure concerns, among other things, the discovery of a 19-amino acid region of EGFR that can be used, *e.g.*, to interrogate the structural and/or functional state of the receptor. For example, this region can be used to generate diagnostic specific binding reagents. This region has the sequence:

LDNPDYQQDFFPKEAKPNG (SEQ ID NO: 2; "L2G Peptide"). It is found in the C-terminal, intracellular (or cytoplasmic) domain of EGFR (for exemplary EGFR sequences, see, *e.g.*, GENBANK™ Accession Nos. XP_001156546.1; XP_001156495.1; XP_519102.2; XP_001156439.1; BAD92679.1; AAS07524.1; 5 AAX41033.1; NP_113695.1; AAT52212.1; NP_005219.2; and CAA25240.1).

The intracellular domain of EGFR, which corresponds to residues 669-1210 of SEQ ID NO: 1, includes a kinase domain (residues 712-979 of SEQ ID NO: 1) and a regulatory domain (residues 980-1210). The EGFR regulatory domain includes at least five tyrosine residues (Tyr1016, Tyr1092, Tyr1110, Tyr1172, and 10 Tyr1197 of SEQ ID NO: 1), which are believed to be autophosphorylation sites (Chattopadhyay *et al.*, *J. Biol. Chem.*, 274:26091-26097, 1999). Among all of the C-terminal tyrosine residues, there are three YXXL/V and four YXXP/D motifs, which, for many transmembrane receptors, serve as the docking sites for Src homology 2 (SH2) domain-containing proteins (Xia *et al.*, *J. Biol. Chem.*, 15 277(34):30716-30723, 2002). As a class, SH2 domain-containing proteins are accepted phosphorylation-dependent regulators of intracellular signal cascades.

The EGFR regulatory domain contains an inhibitory subdomain (Xia *et al.*, *J. Biol. Chem.*, 277(34):30716-30723, 2002), which corresponds to residues 1138-1196 of SEQ ID NO: 1. The L2G Peptide sequence is contained 20 within this inhibitory subdomain. The inhibitory subdomain is believed at least to mediate a protein-protein interaction between EGFR and SOCS proteins (*e.g.*, SOCS1 and SOCS3) (Xia *et al.*, *J. Biol. Chem.*, 277(34):30716-30723, 2002). The interaction between EGFR and SOCS proteins is further believed to stimulate the proteasomal degradation of the EGFR complex and/or induce degradation of 25 EGFR-associated STAT proteins and/or block EGFR from further recruitment and activation of STAT proteins (Xia *et al.*, *J. Biol. Chem.*, 277(34):30716-30723, 2002). In each instance, the SOCS protein (*e.g.*, SOCS1 and/or SOCS3) interaction directly or indirectly inhibits EGFR activity.

As demonstrated in this disclosure, epitopes present in the L2G Peptide 30 sequence of EGFR are inaccessible to cognate RD-binding molecules (*e.g.*, antibodies) in some normal or neoplastic tissues. The accessibility of such epitope is restored in tissues that lack proteins that normally bind the EGFR regulatory domain

and the EGFR inhibitory subdomain. Hence, the disclosed L2G Peptide and other RDPs derived therefrom are useful, at least, to make RD-binding molecules (such as antibodies, antibody fragments, scaffold polypeptides including antibody binding domains and aptamers) that expose the structural and corresponding functional states of EGFR.

In one embodiment, a disclosed RDP is the L2G Peptide, which has the sequence LDNPDYQQDFFPKAKEAKPNG (SEQ ID NO: 2). Also contemplated in some embodiments are immunogenic fragments of the L2G Peptide, which fragments can be useful for producing a disclosed RD-binding molecule. For example, as demonstrated in Example 4, at least the subsequence QQDFFPK (residues 7-13 of SEQ ID NO: 2) is sufficient to produce a disclosed RD-binding molecule (*e.g.*, monoclonal antibody). Thus, in some embodiments, an immunogenic fragment of SEQ ID NO: 2 is at least 7 contiguous residues of SEQ ID NO: 2 and includes the sequence QQDFFPK (residues 7-13 of SEQ ID NO: 2). In more specific embodiments an immunogenic fragment of SEQ ID NO: 2 is between 7 and 18 contiguous residues of SEQ ID NO: 2 and includes the sequence QQDFFPK (residues 7-13 of SEQ ID NO: 2). In other specific embodiments an immunogenic fragment of SEQ ID NO: 2 is between 10 and 18 contiguous residues of SEQ ID NO: 2 and includes the sequence QQDFFPK (residues 7-13 of SEQ ID NO: 2). In each instance an immunogenic fragment of SEQ ID NO: 2 has a function described herein (see, *e.g.*, Abbreviations and Terms) or otherwise known in the art.

Further, at least because the subsequence QQDFFPK (residues 7-13 of SEQ ID NO: 2) is sufficient to produce a disclosed antigen-binding molecule, other RDP embodiments have the consensus sequence X1-6QQDFFPKX7-12, where X1 through X12 are any amino acid. In more specific embodiments, an L2G peptide has the sequence X1-6QQDFFPKX7-12, where X1 through X12 are any conservative substitution (*e.g.*, very highly conserved substitution, highly conserved substitution or conserved substitution) of the corresponding amino acid residue in SEQ ID NO: 2. Exemplary conservative amino acid substitutions are set forth in Table 6.

Table 6. Exemplary conservative amino acid substitutions.

Original Residue	Very Highly - Conserved Substitutions	Highly Conserved Substitutions (from the Blosum90 Matrix)	Conserved Substitutions (from the Blosum65 Matrix)
Ala	Ser	Gly, Ser, Thr	Cys, Gly, Ser, Thr, Val
Arg	Lys	Gln, His, Lys	Asn, Gln, Glu, His, Lys
Asn	Gln; His	Asp, Gln, His, Lys, Ser, Thr	Arg, Asp, Gln, Glu, His, Lys, Ser, Thr
Asp	Glu	Asn, Glu	Asn, Gln, Glu, Ser
Cys	Ser	None	Ala
Gln	Asn	Arg, Asn, Glu, His, Lys, Met	Arg, Asn, Asp, Glu, His, Lys, Met, Ser
Glu	Asp	Asp, Gln, Lys	Arg, Asn, Asp, Gln, His, Lys, Ser
Gly	Pro	Ala	Ala, Ser
His	Asn; Gln	Arg, Asn, Gln, Tyr	Arg, Asn, Gln, Glu, Tyr
Ile	Leu; Val	Leu, Met, Val	Leu, Met, Phe, Val
Leu	Ile; Val	Ile, Met, Phe, Val	Ile, Met, Phe, Val
Lys	Arg; Gln; Glu	Arg, Asn, Gln, Glu	Arg, Asn, Gln, Glu, Ser,
Met	Leu; Ile	Gln, Ile, Leu, Val	Gln, Ile, Leu, Phe, Val
Phe	Met; Leu; Tyr	Leu, Trp, Tyr	Ile, Leu, Met, Trp, Tyr
Ser	Thr	Ala, Asn, Thr	Ala, Asn, Asp, Gln, Glu, Gly, Lys, Thr
Thr	Ser	Ala, Asn, Ser	Ala, Asn, Ser, Val
Trp	Tyr	Phe, Tyr	Phe, Tyr
Tyr	Trp; Phe	His, Phe, Trp	His, Phe, Trp
Val	Ile; Leu	Ile, Leu, Met	Ala, Ile, Leu, Met, Thr

Some exemplary RDPs having the consensus sequence

X1-6QQDFFPKX7-12, wherein any 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or 11 or all 12 of
 5 residues X1 through X12 will have conservative amino acid changes (such as, very highly conserved substitutions, highly conserved substitutions or conserved substitutions) as compared to SEQ ID NO: 2 and, as applicable, the remaining residues will have no change as compared to SEQ ID NO: 2.

In other embodiments, a RDP is a sequence variant of an L2G Peptide that
 10 has at least 99%, at least 98%, at least 95%, at least 92%, at least 90%, at least 85%, at least 80%, at least 75%, at least 70%, at least 65%, or at least 60% amino acid sequence identity to the amino acid sequence set forth in SEQ ID NO: 2. "Sequence identity" is a phrase commonly used to describe the similarity between two amino acid sequences (or between two nucleic acid sequences). Sequence identity
 15 typically is expressed in terms of percentage identity; the higher the percentage, the more similar the two sequences.

Methods for aligning sequences for comparison and determining sequence identity are well known in the art. Various programs and alignment algorithms are

described in: Smith and Waterman, *Adv. Appl. Math.*, 2:482, 1981; Needleman and Wunsch, *J. Mol. Biol.*, 48:443, 1970; Pearson and Lipman, *Proc. Natl. Acad. Sci. USA*, 85:2444, 1988; Higgins and Sharp, *Gene*, 73:237-244, 1988; Higgins and Sharp, *CABIOS*, 5:151-153, 1989; Corpet *et al.*, *Nucleic Acids Research*, 16:10881-10890, 1988; Huang, *et al.*, *Computer Applications in the Biosciences*, 8:155-165, 1992; Pearson *et al.*, *Methods in Molecular Biology*, 24:307-331, 1994; Tatiana *et al.*, *FEMS Microbiol. Lett.*, 174:247-250, 1999. Altschul *et al.* present a detailed consideration of sequence-alignment methods and homology calculations (*J. Mol. Biol.*, 215:403-410, 1990).

10 The National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST™, Altschul *et al.*, *J. Mol. Biol.*, 215:403-410, 1990) is publicly available from several sources, including the National Center for Biotechnology Information (NCBI, Bethesda, MD) and on the Internet, for use in connection with the sequence-analysis programs blastp, blastn, blastx, tblastn and 15 tblastx. A description of how to determine sequence identity using this program is available on the internet under the help section for BLAST™.

For comparisons of amino acid sequences of greater than about 15 amino acids, the “Blast 2 sequences” function of the BLAST™ (Blastp) program is employed using the default BLOSUM62 matrix set to default parameters (cost to 20 open a gap [default = 5]; cost to extend a gap [default = 2]; penalty for a mismatch [default = 3]; reward for a match [default = 1]; expectation value (E) [default = 10.0]; word size [default = 3]; and number of one-line descriptions (V) [default = 100]. When aligning short peptides (fewer than around 15 amino acids), the alignment should be performed using the Blast 2 sequences function “Search for 25 short nearly exact matches” employing the PAM30 matrix set to default parameters (expect threshold = 20000, word size = 2, gap costs: existence = 9 and extension = 1) using composition-based statistics.

Any disclosed sequence variant of a L2G Peptide (whether it is a variant having one or more conservative amino acid substitutions as compare to SEQ ID 30 NO: 2 or a variant having a disclosed percentage sequence identity to SEQ ID NO: 2), at least, is immunogenic (alone or when couple to a carrier molecule) and, e.g., capable of eliciting production of a RD-binding molecule (such as a

monoclonal antibody). Similarly, the protein sequences associated with the GenBank Nos. listed in Table 5 for the RTK ID, RD, and inhibitory domains can be engineered to include one or more conservative amino acid substitutions (such as 1 to 5 or 1 to 10 conservative amino acid substitutions), wherein the resulting variant
5 retains RTK biological activity, and in some examples is immunogenic. In other embodiments, the protein sequences associated with the RTK ID, RD, and inhibitory domains for the GenBank Nos. listed in Table 5 have at least 99%, at least 98%, at least 95%, at least 92%, at least 90%, at least 85%, at least 80%, at least 75%, at least 70%, at least 65%, or at least 60% amino acid sequence identity to the amino
10 acid sequence set forth in the GenBank Accession No. (which are herein all incorporated by reference), wherein the resulting variant retains RTK biological activity, and in some examples is immunogenic.

VI. Generation of Diagnostic Specific Binding Reagents

15 This disclosure also concerns the generation of diagnostic specific binding reagents, which are useful for determining the activity state of an oncogenic RTK in a cell. For example, with knowledge of a region or amino acid residues of an interface between directly interacting proteins (*e.g.*, between an RTK ID and a regulatory protein) or knowledge of the location of an RTK ID or portion thereof
20 (such as an RD or an inhibitory domain) a binding molecule that specifically recognizes the interface region (*e.g.*, a region of the RTK RD) or an epitope that includes at least a portion of the interface residues (*i.e.*, an interface-specific binding molecule) can be obtained from a commercially available source or prepared using techniques common in the art. For example, methods of preparing antibodies,
25 antibody fragments, aptamers and other antigen-binding molecules are described in detail elsewhere in this disclosure.

Such diagnostic specific binding molecules are a species of antigen-binding molecules that can specifically bind to amino acids that form a protein-protein interface between an RTK and a regulatory protein in the absence of the RTK
30 protein-regulatory protein interaction and fail to specifically bind amino acids that form a protein-protein interface between an RTK and a regulatory protein in the presence of the RTK protein-regulatory protein interaction. In a specific example,

such specific binding reagents specifically bind one or more epitopes in an RTK ID, such as an RD within the ID (see Table 5). It will be appreciated that a diagnostic specific binding molecule need not bind all amino acids of the protein-protein interface to achieve the desired result. For example, binding to even a single amino acid (or at least one amino acid, such as 1 to 20, 1 to 10, or 2 to 10) of the protein-protein interface (along with binding to other amino acids *e.g.*, of the RTK ID) may still permit the diagnostic specific binding molecule to specifically bind to the protein-protein interface in the absence of the RTK protein-regulatory protein interaction and fail to specifically bind the protein-protein interface in the presence of the RTK protein-regulatory protein interaction.

Exemplary diagnostic specific binding reagents include those specific for an EGFR L2G peptide, or any epitope contained therein, or diagnostic specific binding reagents specific for a L2G peptide sequence in EGFR (including specific binding molecules that are competitively inhibited from binding EGFR by a L2G peptide or fragment thereof), diagnostic specific binding reagents that recognize particular structural states of EGFR (for instance, diagnostic specific binding reagents specific for epitopes masked by EGFR protein-protein interactions), and/or diagnostic specific binding reagents that recognize particular regulated states of EGFR (for instance, diagnostic specific binding reagents specific for epitopes contained with an EGFR inhibitory subdomain and which recognize the binding of a negative regulatory molecule (*e.g.*, SOCS1 or SOCS2) to EGFR).

Diagnostic specific binding reagents include, for example, antibodies or functional fragments or recombinant derivatives thereof, aptamers, mirror-image aptamers, or engineered nonimmunoglobulin binding proteins based on any one or more of the following scaffolds: fibronectin (*e.g.*, ADNECTINS™ or monobodies), CTLA-4 (*e.g.*, EVIBODIES™), tendamistat (*e.g.*, McConnell and Hoess, *J. Mol. Biol.*, 250:460-470, 1995), neocarzinostatin (*e.g.*, Heyd *et al.*, *Biochem.*, 42:5674-5683, 2003), CBM4-2 (*e.g.*, Cicortas-Gunnarsson *et al.*, *Protein Eng. Des. Sel.*, 17:213-221, 2004), lipocalins (*e.g.*, ANTICALINS™; Schlehuber and Skerra, *Drug Discov. Today*, 10:23-33, 2005), T-cell receptors (*e.g.*, Chlewicki *et al.*, *J. Mol. Biol.*, 346:223-239, 2005), protein A domain (*e.g.*, AFFIBODIES™; Engfeldt *et al.*, *ChemBioChem*, 6:1043-1050, 2005), Im9 (*e.g.*, Bernath *et al.*, *J. Mol. Biol.*,

- 345:1015-1026, 2005), ankyrin repeat proteins (*e.g.*, DARPins; Amstutz *et al.*, *J. Biol. Chem.*, 280:24715-24722, 2005), tetratricopeptide repeat proteins (*e.g.*, Cortajarena *et al.*, *Protein Eng. Des. Sel.*, 17:399-409, 2004), zinc finger domains (*e.g.*, Bianchi *et al.*, *J. Mol. Biol.*, 247:154-60, 1995), pVIII (*e.g.*, Petrenko *et al.*,
5 *Protein Eng.*, 15:943-950, 2002), GCN4 (Sia and Kim, *Proc. Natl Acad. Sci. USA*, 100:9756-61, 2003), avian pancreatic polypeptide (APP) (*e.g.*, Chin *et al.*, *Bioorg. Med. Chem. Lett.*, 11:1501-5, 2001), WW domains, (*e.g.*, Dalby *et al.*, *Protein Sci.*, 9:2366-76, 2000), SH3 domains (*e.g.*, Hiipakka *et al.*, *J. Mol. Biol.*, 293:1097-106, 1999), SH2 domains (Malabarba *et al.*, *Oncogene*, 20:5186-5194, 2001), PDZ
10 domains (*e.g.*, TELOBODIES™; Schneider *et al.*, *Nat. Biotechnol.*, 17:170-5, 1999), TEM-1 β -lactamase (*e.g.*, Legendre *et al.*, *Protein Sci.*, 11:1506-18, 2002), green fluorescent protein (GFP) (*e.g.*, Zeytun *et al.*, *Nat. Biotechnol.*, 22:601, 2004), thioredoxin (*e.g.*, peptide aptamers; Lu *et al.*, *Biotechnol.*, 13:366-372, 1995), Staphylococcal nuclease (*e.g.*, Norman, *et al.*, *Science*, 285:591-5, 1999), PHD
15 fingers (*e.g.*, Kwan *et al.*, *Structure*, 11:803-13, 2003), chymotrypsin inhibitor 2 (CI2) (*e.g.*, Karlsson *et al.*, *Br. J. Cancer*, 91:1488-94, 2004), bovine pancreatic trypsin inhibitor (BPTI) (*e.g.*, Roberts, *Proc. Natl. Acad. Sci. USA*, 89:2429-33, 1992) and many others (see review by Binz *et al.*, *Nat. Biotechnol.*, 23(10):1257-68, 2005 and supplemental materials).
- 20 Disclosed diagnostic specific binding reagents also include aptamers. In one example, an aptamer is a single-stranded nucleic acid molecule (such as, DNA or RNA) that assumes a specific, sequence-dependent shape and binds to a target protein (*e.g.*, an RTK protein-regulatory protein interface, for example a region of an RTK ID or RD that binds a regulatory protein) with high affinity and specificity.
- 25 Aptamers generally comprise fewer than 100 nucleotides, fewer than 75 nucleotides, or fewer than 50 nucleotides (such as 10 to 95 nucleotides, 25 to 80 nucleotides, 30 to 75 nucleotides, or 25 to 50 nucleotides). In a specific embodiment, a disclosed diagnostic specific binding reagent is a mirror-image aptamer (also called a SPIEGELMER™). Mirror-image aptamers are high-affinity L-enantiomeric nucleic
30 acids (for example, L-ribose or L-2'-deoxyribose units) that display high resistance to enzymatic degradation compared with D-oligonucleotides (such as, aptamers). The target binding properties of aptamers and mirror-image aptamers are designed

by an *in vitro*-selection process starting from a random pool of oligonucleotides, as described for example, in Wlotzka *et al.*, *Proc. Natl. Acad. Sci.* 99(13):8898-8902, 2002. Methods of generating aptamers are known in the art (see *e.g.*, Fitzwater and Polisky (*Methods Enzymol.*, 267:275-301, 1996; Murphy *et al.*, *Nucl. Acids Res.* 31:e110, 2003).

In another example, an aptamer is a peptide aptamer that binds to a target protein (*e.g.*, an RTK protein-regulatory protein interface, for example a region of an RTK ID or RD that binds a regulatory protein) with high affinity and specificity. Peptide aptamers include a peptide loop (*e.g.*, which is specific for the RTK protein-regulatory protein interface) attached at both ends to a protein scaffold. This double structural constraint greatly increases the binding affinity of the peptide aptamer to levels comparable to an antibody's (nanomolar range). The variable loop length is typically 8 to 20 amino acids (*e.g.*, 8 to 12 amino acids), and the scaffold may be any protein which is stable, soluble, small, and non-toxic (*e.g.*, thioredoxin-A, stefin A triple mutant, green fluorescent protein, eglin C, and cellular transcription factor Sp1). Peptide aptamer selection can be made using different systems, such as the yeast two-hybrid system (*e.g.*, Gal4 yeast-two-hybrid system) or the LexA interaction trap system.

Disclosed diagnostic specific binding reagents also include antibodies. The term "antibody" refers to an immunoglobulin molecule (or combinations thereof) that specifically binds to, or is immunologically reactive with, a particular antigen, and includes polyclonal, monoclonal, genetically engineered and otherwise modified forms of antibodies, including but not limited to chimeric antibodies, humanized antibodies, heteroconjugate antibodies (*e.g.*, bispecific antibodies, diabodies, triabodies, and tetrabodies), single chain Fv antibodies (scFv), polypeptides that contain at least a portion of an immunoglobulin that is sufficient to confer specific antigen binding to the polypeptide, and antigen binding fragments of antibodies. Antibody fragments include proteolytic antibody fragments [such as F(ab')₂ fragments, Fab' fragments, Fab'-SH fragments, Fab fragments, Fv, and rIgG], recombinant antibody fragments (such as sFv fragments, dsFv fragments, bispecific sFv fragments, bispecific dsFv fragments, diabodies, and triabodies), complementarity determining region (CDR) fragments, camelid antibodies (see, for

example, U.S. Patent Nos. 6,015,695; 6,005,079; 5,874,541; 5,840,526; 5,800,988; and 5,759,808), and antibodies produced by cartilaginous and bony fishes and isolated binding domains thereof (see, for example, International Patent Application No. WO03014161).

5 A Fab fragment is a monovalent fragment consisting of the VL, VH, CL and CH1 domains; a F(ab')₂ fragment is a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; an Fd fragment consists of the VH and CHI domains; an Fv fragment consists of the VL and VH domains of a single arm of an antibody; and a dAb fragment consists of a VH domain (see, *e.g.*,
10 Ward *et al.*, *Nature* 341:544-546, 1989). A single-chain antibody (scFv) is an antibody in which a VL and VH region are paired to form a monovalent molecule via a synthetic linker that enables them to be made as a single protein chain (see, *e.g.*, Bird *et al.*, *Science*, 242: 423-426, 1988; Huston *et al.*, *Proc. Natl. Acad. Sci. USA*, 85:5879-5883, 1988). Diabodies are bivalent, bispecific antibodies in which
15 VH and VL domains are expressed on a single polypeptide chain, but using a linker that is too short to allow for pairing between the two domains on the same chain, thereby forcing the domains to pair with complementary domains of another chain and creating two antigen binding sites (see, *e.g.*, Holliger *et al.*, *Proc. Natl. Acad. Sci. USA*, 90:6444-6448, 1993; Poljak *et al.*, *Structure*, 2:1121-1123, 1994). A
20 chimeric antibody is an antibody that contains one or more regions from one antibody and one or more regions from one or more other antibodies. An antibody may have one or more binding sites. If there is more than one binding site, the binding sites may be identical to one another or may be different. For instance, a naturally occurring immunoglobulin has two identical binding sites, a single-chain
25 antibody or Fab fragment has one binding site, while a "bispecific" or "bifunctional" antibody has two different binding sites.

 As discussed above, exemplary diagnostic specific binding reagents recognize particular regulated or structural states of a target RTK. For example, a disclosed diagnostic specific binding reagent can detect the masking (or unmasking)
30 of an epitope in an RTK ID or RD (such as the EGFR regulatory domain; residues 980-1210 of SEQ ID NO: 1, or other ID or RD listed in Table 5). Such epitope masking (or unmasking) can result, for instance, from a protein-protein

interaction between the RTK and another cellular protein (*e.g.*, between EGFR and a SOCS protein such as SOCS1 or SOCS3); wherein the binding of the cellular protein to the RTK masks the epitope and the disassociation (or lack of association) of the two proteins unmasks the epitope. Other examples involve diagnostic specific binding reagents (such as antibodies (*e.g.*, monoclonal antibody) or fragments thereof) that specifically bind to an RTK ID, RD or inhibitory domain; wherein such specific binding is competitively inhibited by a positive or negative regulatory protein, such as those listed in Table 3 (or a fragment of such regulatory proteins that binds to the RTK ID, for example, a region of the RTK ID including a phosphorylated Tyr residue).

In some examples, diagnostic specific binding reagents, such as antibodies (*e.g.*, monoclonal antibody) or fragments thereof, are characterized by specific binding to an RTK ID or RD (such as those disclosed herein, see, *e.g.*, Table 5 and Section V). In other examples, diagnostic specific binding reagents, such as antibodies (*e.g.*, monoclonal antibody) or fragments thereof, specifically bind to amino acid residues of EGFR that correspond to the sequence(s) of disclosed RDPs (see, *e.g.*, Section V above). In still other examples, diagnostic specific binding reagents, such as antibodies (*e.g.*, monoclonal antibody) or fragments thereof, specifically bind to an RTK regulatory domain (*e.g.*, EGRF RD) or to an inhibitory subdomain (*e.g.*, the EGFR inhibitory subdomain) and such specific binding is competitively inhibited by any one or more RDP (*e.g.*, EGFR RDPs) disclosed herein (see, *e.g.*, Section V). Other specific examples involve diagnostic specific binding reagents (such as antibodies (*e.g.*, monoclonal antibody) or fragments thereof) that specifically bind to an RTK (*e.g.*, the EGFR) RD or to an RTK (*e.g.*, EGFR) inhibitory subdomain; wherein such specific binding is competitively inhibited by a regulatory protein (*e.g.*, for EGFR the SOCS protein, such as SOCS1 or SOCS3, or a fragment of a SOCS protein that binds to the regulatory domain of EGFR, for example, a region of the regulatory domain including a phosphorylated Tyr residue).

In one particular embodiment, a diagnostic specific binding reagent is a rabbit monoclonal antibody deposited at ATCC Accession No. _____. In another particular embodiment, a diagnostic specific binding reagent is rabbit monoclonal

antibody clone 5B7, which is commercially available from Ventana Medical Systems (Tucson, AZ; product number 790 4347).

In some examples, an antibody specifically binds to a target (such as a protein-protein interface, for example an RTK ID) with a binding constant that is at least 10^3 M^{-1} greater, 10^4 M^{-1} greater or 10^5 M^{-1} greater than a binding constant for other molecules in a sample. In some examples, a diagnostic specific binding reagent (such as an antibody (*e.g.*, monoclonal antibody) or fragments thereof) has an equilibrium constant (K_d) of 1 nM or less. For example, diagnostic specific binding reagents are provided that bind to an RTK ID (such as a regulatory domain or inhibitory subdomain) with a binding affinity of at least about $0.1 \times 10^{-8} \text{ M}$, at least about $0.3 \times 10^{-8} \text{ M}$, at least about $0.5 \times 10^{-8} \text{ M}$, at least about $0.75 \times 10^{-8} \text{ M}$, at least about $1.0 \times 10^{-8} \text{ M}$, at least about $1.3 \times 10^{-8} \text{ M}$ at least about $1.5 \times 10^{-8} \text{ M}$, or at least about $2.0 \times 10^{-8} \text{ M}$. K_d values can, for example, be determined by competitive ELISA (enzyme-linked immunosorbent assay) or using a surface-plasmon resonance device such as the Biacore T100, which is available from Biacore, Inc., Piscataway, NJ.

A disclosed diagnostic specific binding reagent, such as an antibody (*e.g.*, monoclonal antibody), aptamer, or fragments thereof, optionally can be directly labeled with a detectable moiety. Useful detection agents include fluorescent compounds (including fluorescein, fluorescein isothiocyanate, rhodamine, 5-dimethylamine-1-naphthalenesulfonyl chloride, phycoerythrin, lanthanide phosphors, or the cyanine family of dyes (such as Cy-3 or Cy-5) and the like); bioluminescent compounds (such as luciferase, green fluorescent protein (GFP), or yellow fluorescent protein); enzymes that can produce a detectable reaction product (such as horseradish peroxidase, β -galactosidase, luciferase, alkaline phosphatase, or glucose oxidase and the like), or radiolabels (such as ^3H , ^{14}C , ^{15}N , ^{35}S , ^{90}Y , ^{99}Tc , ^{111}In , ^{125}I , or ^{131}I).

A. Making of Exemplary Diagnostic Antibodies

Methods of making diagnostic specific binding molecules are well known in the art. The method used will depend upon the nature of the desired binding molecules; for instance peptide-based diagnostic specific binding molecules that are not necessarily immunoglobulin in origin can be made using methods that are

similar to phage display methods. One such method is described in Szardenings, *J. Recept. Signal Transduct. Res.*, 23:307-309, 2003.

Methods of generating antibodies (such as monoclonal or polyclonal antibodies) are well established in the art (for example see Harlow and Lane, 5 *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, New York, 1988). For example identified peptide fragments of an RTK protein-regulatory protein interface (such as a region(s) of an RTK ID or RD or inhibitory domain) (see Table 5 and Section V) can be conjugated to carrier molecules (or nucleic acids encoding such epitopes or conjugated RDPs) can be injected into non-human 10 mammals (such as mice or rabbits), followed by boost injections, to produce an antibody response. Serum isolated from immunized animals may be isolated for the polyclonal antibodies contained therein, or spleens from immunized animals may be used for the production of hybridomas and monoclonal antibodies. In some examples, antibodies are purified before use.

15 In one example, monoclonal antibody to an epitope of an RTK protein-regulatory protein interface (*e.g.*, an epitope of an RTK ID, RD, or inhibitory domain) can be prepared from murine hybridomas according to the classical method of Kohler and Milstein (*Nature*, 256:495, 1975) or derivative methods thereof. Briefly, a mouse (such as Balb/c) is repetitively inoculated with a few micrograms 20 of the selected peptide fragment (*e.g.*, epitope of an RTK ID, such as SEQ ID NO: 2) or carrier conjugate thereof over a period of a few weeks. The mouse is then sacrificed, and the antibody-producing cells of the spleen isolated. The spleen cells are fused by means of polyethylene glycol with mouse myeloma cells, and the excess unfused cells destroyed by growth of the system on selective media 25 comprising aminopterin (HAT media). The successfully fused cells are diluted and aliquots of the dilution placed in wells of a microtiter plate where growth of the culture is continued. Antibody-producing clones are identified by detection of antibody in the supernatant fluid of the wells by immunoassay procedures, such as ELISA, as originally described by Engvall (*Enzymol.*, 70:419, 1980), and derivative 30 methods thereof. Selected positive clones can be expanded and their monoclonal antibody product harvested for use.

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In another example, monoclonal antibody to epitopes of an RTK protein-regulatory protein interface (*e.g.*, an epitope of an RTK ID, RD, or inhibitory domain) can be prepared from rabbit hybridomas as described in U.S. Pat. Nos. 7,148,332, 5,675,063, or 4,859,595.

5 In yet another example, monoclonal antibodies to epitopes of an RTK protein-regulatory protein interface (*e.g.*, an epitope of an RTK ID, RD, or inhibitory domain) can be prepared by repetitively inoculating a non-human mammal (such as a mouse or rabbit) with one or more plasmids encoding a disclosed RDP (such as a plasmid encoding SEQ ID NO: 2). For example, pcDNA3 (Invitrogen, Carlsbad,
10 CA) or a vector derived there from, can be manipulated using standard molecular biology methods to include a coding sequence for a disclosed peptide fragment of an RTK protein-regulatory protein interface (*e.g.*, SEQ ID NO: 2). In one exemplary method, Balb/c mice (6–8 weeks old) are immunized three times with the appropriate plasmid (20 µg in phosphate-buffered saline), and one boost can be
15 given with cells before fusion. Mice can be injected three times intradermally into the base of the tail on days 0, 10, and 20 using an insulin syringe with a 28–gauge needle attached. Serum can be drawn on days 30 and 45 for evaluation of the anti-serum titer. To boost the immunized mice, cells expressing the desired plasmid are injected (for example on day at least 50). These injections can be intravenous
20 and intraperitoneal. Spleens are harvested about 80–90 hours after the last cell boost for cell fusion.

Cell fusions of the splenocytes can be performed according to the protocol of Oi and Herzenberg (*Selected Methods in Cellular Immunology*, Freeman Press, San Francisco, 1980). Splenocytes and SP2/0 cells are mixed, for example at a 4:1 ratio.
25 The mixed cells are centrifuged and the cell pellet resuspended in polyethylene glycol (such as 40% -50% (w/v) polyethylene glycol) and appropriate medium. The resulting suspension is centrifuged and the cell pellet resuspended in HAT medium, and seeded in 96-well plates at 100 µl/ well (2.5×10^5 cells/well) and cultured in a CO₂ incubator. On the day after fusion, 100 µl of fresh HAT medium containing
30 500 µg/ml geneticin (Invitrogen) is added. On days 4 and 7, half of the spent medium is replaced by fresh HAT medium containing 250 µg/ml geneticin. On day 8, the growth of the hybridoma in each well is checked under a microscope.

mAb production in culture supernatants can be assayed on day 10 by ELISA assay or days 9 and 10 by FACS sorter. Positive clones can be expanded and the specific hybridomas cloned by a limiting dilution method.

In addition, protocols for producing humanized forms of monoclonal antibodies and fragments of monoclonal antibodies are known in the art (see, e.g.,
5 U.S. Pat. Nos. 6,054,297, 6,407,213, 6,639,055, 6,800,738, and 6,719,971 and U.S. Pat. Appl. Pub. Nos. 2005/0033031, and 2004/0236078). Similarly, methods for producing single chain antibodies have been described and can be useful for the making of diagnostic specific binding molecules disclosed herein (see, Buchner *et al.*,
10 *Anal. Biochem.* 205:263-270, 1992; Pluckthun, *Biotechnology* 9:545, 1991; Huse *et al.*, *Science* 246:1275, 1989 and Ward *et al.*, *Nature* 341:544, 1989).

B. Making of Exemplary Diagnostic Aptamers

Methods of generating aptamers (e.g., DNA or RNA aptamers) are well established in the art. For example, with knowledge of an RTK ID sequence (such
15 as a RD or inhibitory domain sequence, see Section V), or upon identification of at least 3 or at least 5 amino acid residues that form a protein-protein interface when a regulatory protein is bound to an RTK ID (see Section IV), aptamers can be selected that bind to amino acids of such an interface (such as an RTK ID or RD) in the absence of a regulatory protein binding to the RTK, but do not specifically bind to
20 amino acids of such an interface (such as an RTK ID or RD) when a regulatory protein is bound to an RTK.

In one example, DNA or RNA aptamers are selected using the *in vitro* method SELEX (systematic evolution of ligands by exponential enrichment), for example using the method of Fitzwater and Polisky (*Methods Enzymol.*, 267:275-
25 301, 1996). Such a method can be used to identify aptamers that bind with high specificity to an RTK protein-regulatory protein interface, such as that bind specifically to a region of an RTK ID or RD that binds a regulatory protein. The SELEX procedure is usually initiated with an RNA or DNA library containing about 10^{14} - 10^{15} random oligonucleotide sequences. In a fully randomized oligonucleotide
30 library, each molecule will exhibit a unique tertiary structure that will be dependent on the nucleotide sequence of that molecule. The binding affinity of the oligonucleotide for a particular protein-protein interface will be determined by the fit

between moieties on the surface of the oligonucleotide and epitopes on the target protein-protein interface. By starting from a library of vast diversity, aptamers of nanomolar or subnanomolar affinity for the target protein-protein interface with selectivity for that target protein-protein interface over other protein-protein
5 interfaces with a high degree of structural homology can be identified. For example, peptides comprising the amino acids that form the target protein-protein interface (or portion thereof, such as 3 to 20 amino acids of a protein-protein interface, for example an epitope) can be attached to a surface (such as a 96-well or other multi-well microtiter plate). The library of nucleic acid molecules can be added to the
10 bound peptide under conditions that permit members of the library to bind to the peptide (*e.g.*, by incubating at 37°C for 30 minutes). Unbound members of the library are washed away, and then bound members of the library are eluted (*e.g.*, by incubating at 95°C for 10 minutes). Reverse transcription is performed (if the aptamers are RNA), followed by polymerase chain reaction and transcription to
15 generate nucleic acids for the next round of SELEX. The dissociation constant (Kd) for resulting selected aptamer can be determined using routine methods. Aptamers with high affinity for the desired protein-protein interface can be selected, such as a Kd of less than 100 nM, such as less than 50 nM, less than 10 nM, or less than 1 nM (for example 0.1 to 50 nM). Aptamers can be modified to increase their half-life, for
20 example modified with 2'-fluorine-substituted pyrimidines, 2'-ribo purines, polyethylene glycol (PEG) linkage, and the like.

In one example, peptide aptamers are selected using a yeast two hybrid system, for example using the method of de Chassey *et al.* (*Molecular & Cellular Proteomics* 6:451-9, 2007). Reviews are provided, for example, in Borghouts *et al.*
25 (*Comb. Chem. High Throughput Screen.* 11:135-45, 2008) and Buerger *et al.* (*J. Cancer Res. Clin. Oncol.* 129:669-75, 2003). Such a method can be used to identify peptide aptamers that bind with high specificity to an RTK protein-regulatory protein interface, such as that bind specifically to a region of an RTK ID or RD that binds a regulatory protein. A peptide aptamer library of high complexity is
30 screened, such as 20mer or 8-12mer libraries. The library may or may not be based on information known about the sequence of the RTK protein-regulatory protein interface. In one example, the library includes oligonucleotides encoding variant

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peptides based on the amino acid sequence of the RTK protein-regulatory protein interface. The library includes oligonucleotides encoding the variable peptides inserted into a vector encoding the scaffold protein (*e.g.*, thioredoxin). When expressed, “prey” peptide sequences are embedded in the scaffold protein. A
5 nucleic acid sequence or vector encoding the “bait” target protein (*e.g.*, sequence of the RTK protein-regulatory protein interface) fused to a transcription module (*e.g.*, Gal4 or LexA) is expressed in the cells (*e.g.*, yeast) along with the “prey” coding sequences.

If the yeast-two-hybrid system is used, the “prey” peptide aptamer can be
10 fused to Gal4-transactivation domain (Gal4-AD) and can also include a nuclear localization signal and an HA tag for detection. Exemplary vectors that can be used to express the peptide aptamer include pRS424, pAD-Trx, pGAD424; pGAD-T7, pACT2, and pAD-Gal4-2.1. A vector encoding the “bait” target protein fused to the Gal4 DNA binding domain is expressed in yeast along with the “prey” coding
15 sequences. Exemplary vectors that can be used to express the “bait” peptide include pPC97, pLex9, pGBK-T7, and pDB-Gal4Cam. In some examples, the reporter yeast strain into which prey and bait vectors are introduced include His3, Ade2, Ura3 and LacZ genes under the control of a Gal upstream activating sequences to permit selection of clones where the bait and prey specifically bind. To select for desired
20 peptide aptamers, transformed yeast cells are placed on media lacking histidine, adenine, or uracil. β -gal assays can be performed to quantify binding between identified aptamers and the target. To increase selection stringency, the amount of 3-AT inhibitor (*e.g.*, 10-100 mM) can be increased. Cells that grow indicate the presence of peptide aptamer binding to the target RTK protein-regulatory protein
25 interface.

If the LexA interaction trap system is used, a vector encoding the “prey” peptide aptamer fused to B42 or B112 acid transactivation domain can be used. Exemplary vectors that can be used to express the peptide aptamer include pWP1, pWP2, pJG4-5, pJM1, pHA3). A vector encoding the “bait” target protein fused to
30 the DNA binding domain of the LexA repressor is expressed in yeast along with the “prey” coding sequences. An exemplary vector that can be used to express the “bait” peptide includes pEG202. Expression of the prey vector is induced if

galactose is present in the growth medium. To select for desired peptide aptamers, transformed yeast cells are placed on media with galactose. Interactions between bait protein and peptide aptamer are detected on galactose plates that lack leucine. Cells that grow indicate the presence of peptide aptamer binding to the target RTK protein-regulatory protein interface.

Clones indicated to carry the desired protein aptamer that binds to the RTK protein-regulatory protein interface can be selected, and the vector encoding the aptamer isolated and cloned using standard recombinant technology.

C. Screening Diagnostic Agents

The diagnostic specific binding reagents generated (*e.g.*, antibodies or aptamers) can be screened for their ability to specifically bind to amino acids that form a protein-protein interface between an RTK and a regulatory protein in the absence of the RTK protein-regulatory protein interaction and fail to specifically bind amino acids that form a protein-protein interface between an RTK and a regulatory protein in the presence of the RTK protein-regulatory protein interaction. For example, a plurality of diagnostic specific binding reagents can be screened to identify those that are excluded from binding when the protein-protein interface is present, but bind with high specificity when the protein-protein interface is absent. Such methods of screening are routine in the art.

In one example, cells that express the target RTK but are functionally deleted for the target regulatory protein associated with the diagnostic reagent are incubated with one or more of the diagnostic specific binding reagents. For example, cells that do not express the regulatory protein due to a lack of transcription or translation of the gene encoding the regulatory protein (*e.g.*, the regulatory protein gene is knocked out or the expression of the gene is inhibited using RNAi technology, such as siRNA) can be used. Methods of making such cells are routine in the art. In one example, the diagnostic specific binding reagent is generated to determine if the negative regulator CBL was bound to RON, and the cell used to screen the diagnostic reagents expresses RON but does not express functional CBL protein. After incubation of the functionally deleted cells with the one or more diagnostic specific binding reagents under conditions that would permit binding of the regulatory protein to the RTK, the ability of the diagnostic specific binding reagents

to bind to the RTK is determined, for example using western blotting, immunoprecipitation, pull-down assays, IHC and the like. The diagnostic specific binding reagents can include a label to permit detection if desired. Diagnostic specific binding reagents that bind with high specificity to the RTK in cells that are functionally deleted for the target regulatory protein, but do not substantially bind the RTK in cells that express the target regulatory protein, can be selected for further analysis.

In another example, an ELISA (enzyme-linked immunosorbent assay) is used to screen the plurality of diagnostic specific binding reagents. For example, the target RTK-regulatory protein complex or the RTK protein alone (or a portion thereof, such as the ID or RD) can be affixed to a solid surface (*e.g.*, microtiter plate or nitrocellulose). In some examples the RTK-regulatory complex is fixed to avoid competition with the diagnostic specific binding reagents for the binding site on the RTK. After incubation of the protein-containing surfaces with the one or more diagnostic specific binding reagents under conditions that would permit binding of the regulatory protein to the RTK in the absence of the regulatory protein, the ability of the diagnostic specific binding reagents to bind to the RTK is determined, for example by detecting a label on the diagnostic specific binding reagents. Diagnostic specific binding reagents that bind to the RTK in the absence of the regulatory protein, but do not bind the RTK-regulatory complexes, can be selected for further analysis.

In some examples, the plurality of diagnostic specific binding reagents are screened to identify those having a low dissociation constant (K_d), and thus a high affinity, for the desired protein-protein interface (*e.g.*, the desired RTK ID or RD). K_d values can, for example, be determined by methods known in the art, such as competitive ELISA or using a surface-plasmon resonance device such as the Biacore T100, which is available from Biacore, Inc., Piscataway, NJ. In particular examples, diagnostic specific binding reagents such as antibodies or aptamers are selected if they have a K_d of less than 100 nM, such as less than 50 nM, less than 10 nM, or less than 1 nM (for example 0.1 to 50 nM). In some examples, a diagnostic specific binding reagent (such as an antibody (*e.g.*, monoclonal antibody) or fragments thereof) has K_d of 1 nM or less.

D. Determining Diagnostic Value of Identified Specific Binding Reagents

In some examples, diagnostic specific binding reagents selected using the methods described above are analyzed for their diagnostic ability, such as the ability to determine if a subject has a cancer expressing a target RTK, predict a disease outcome (such as disease-free survival), and to predict a subject's response to a therapy (such as an RTK inhibitory therapy). For example, diagnostic specific binding reagents selected for their ability to specifically bind to amino acids that form a protein-protein interface between an RTK and a regulatory protein in the absence of the RTK protein-regulatory protein interaction and fail to specifically bind amino acids that form a protein-protein interface between an RTK and a regulatory protein in the presence of the RTK protein-regulatory protein interaction can be further analyzed for their ability to diagnose a subject (*e.g.*, be used as a companion diagnostic, prognostic, or predictor).

In one example, a plurality of selected diagnostic specific binding reagents are analyzed for their ability to be used as a diagnostic. Biological samples (*e.g.*, tissue samples, such as fixed biopsy samples) from a cohort of subjects are subjected to IHC or other methods known in the art (see Examples below) to permit determination of the activation status of the RTK using each of the selected diagnostic specific binding reagents. For example, the subjects can be those known to have (or had) a tumor (*e.g.*, cancer) expressing the target RTK.

To determine if the selected diagnostic specific binding reagent can function as a companion diagnostic, subject samples with a known survival outcome can be analyzed. For example, such subjects can be those who had a cancer expressing the target RTK, or had a cancer that did not express target RTK,. A plurality of samples for each group (*e.g.*, +/- RTK expression) can be tested. In some examples, at least 50 or at least 100 samples are analyzed for each test group. Each sample stained with a selected diagnostic specific binding reagent can be scored, for example using the Tables provided in the Examples below (*e.g.*, see Example 12), and the results plotted (*e.g.*, +/- RTK expression). Diagnostic specific binding reagents that have the ability to correlate patients by their RTK expression status (*e.g.*, +/- RTK

expression), are indicated to be useful companion diagnostics and can be selected for clinical use.

To determine if the selected diagnostic specific binding reagent can function as a prognostic, subject samples with a known survival outcome can be analyzed.

- 5 For example, such subjects can be those who had a cancer expressing the target RTK, did not receive an RTK-based therapy, and their time of survival after diagnosis known. A plurality of samples for each survival range (*e.g.*, at least 6-months, at least 1-year, or at least 5-years) can be tested. In some examples, at least 50 or at least 100 samples are analyzed for each test group. Each sample stained
- 10 with a selected diagnostic specific binding reagent can be scored, for example using the Tables provided in the Examples below (*e.g.*, see Example 12), and the results plotted (*e.g.*, fraction survived versus time). Diagnostic specific binding reagents that have the ability to correlate patient overall survivability (*e.g.*, the ability to distinguish patients having poor survivability from those with greater survivability),
- 15 are indicated to be useful in prognosing a subject and can be selected for clinical use.

To determine if the selected diagnostic specific binding reagent can function as a predictor, subject samples with a known response to an RTK targeted therapy (such as an RTK inhibitory therapy, for example treated with a TKI) are analyzed.

- 20 For example, such subjects can be those who had a cancer expressing the target RTK, received an RTK-based therapy, and their time of survival after the therapy (or other response to therapy) known. A plurality of samples for each response (*e.g.*, survival of at least 6-months, at least 1-year, or at least 5-years, a decrease in tumor growth, volume or metastasis of particular relative amounts, such as decreases of at
- 25 least 20%, at least 50%, or at least 90%) can be tested. In some examples, at least 50 or at least 100 samples are analyzed for each test group. Each sample stained with a selected diagnostic specific binding reagent can be scored, for example using the Tables provided in the Examples below (*e.g.*, see Example 12), and the results plotted (*e.g.*, fraction survived versus time or decrease in tumor growth versus time).
- 30 Diagnostic specific binding reagents that have the ability to correlate patient overall survivability or other response to the RTK therapy (*e.g.*, the ability to distinguish patients that responded to the RTK-targeted therapy from those that that did not

respond to the RTK-targeted therapy), are indicated to be useful as a predictive agent can be selected for clinical use.

VII. Detecting Molecular Interactions in a Biological Sample

5 The identification and/or generation of diagnostic specific binding reagents permits use of such agents to determine if particular protein-protein interactions are present in a biological sample (*e.g.*, isolated cells or tissues). For example, the disclosed diagnostic specific binding reagents can be used to determine the activation status of an RTK, the status of which can be used to determine if a subject
10 has a particular RTK-expressing tumor, the prognosis of a subject having a tumor, as well as the likelihood that the subject having a tumor will respond to a particular RTK therapy. In some examples, the sample to be analyzed using the disclosed diagnostic specific binding reagents is mounted on a solid surface (*e.g.*, a microscope slide) and treated (*e.g.*, formalin-fixed and paraffin-embedded (“FFPE”))
15 to substantially maintain the positions of components (*e.g.*, proteins, RNAs and/or DNA) within the sample relative to one another.

 Molecular interactions (*e.g.*, protein-protein interactions) previously have been studied in solution and using *in vivo* techniques, such as
co-immunoprecipitation assays (where a protein of interest is captured with an
20 antibody and any interaction partners bound to the protein are subsequently identified by Western blot); pull-down assays (which are similar to co-immunoprecipitation assays, but use some ligand other than an antibody to capture the protein complex); label transfer (where a known protein is tagged with a detectable label and the label is then passed to an interacting protein); *in vivo*
25 crosslinking of protein complexes (where cells are grown under conditions that cause photoreactive diazirine amino acid analogs to be incorporated into cellular proteins, which diazirines can be activated and bind to interacting proteins); the yeast two-hybrid screen (which investigates the interaction between artificial fusion proteins inside the nucleus of yeast); and dual polarisation interferometry (“DPI”)
30 (which provides real-time, high-resolution measurements of molecular size, density and mass). Each of the foregoing methods requires means to isolate (whether

physically, chemically or otherwise) the components having a specific interaction with one another from other non-interacting components.

Non-specific crosslinking reactions (such as, chemical crosslinking) also may be useful to examine protein-protein interactions in settings where non-specific
5 interactions between reaction components can be controlled. However, biological samples (*e.g.*, isolated cells or tissues) mounted on a solid surface (*e.g.*, microscope slides or support membranes) do not offer such a setting. Under those conditions, non-specific crosslinkers bond together (permanently or semi-permanently) any components in the sample that in proximity of each other whether or not such
10 components interact under biological conditions.

Rather than view non-specific crosslinking as a hindrance to examining protein-protein interactions, the present disclosure actually exploits the non-specific crosslinking of biological components within a fixed biological sample (*e.g.*, FFPE cells or FFPE tissues). Such crosslinking substantially ensures that the structural
15 relationship between interacting components in the sample (*e.g.*, protein-protein or protein-nucleic acid) is permanently or semi-permanently maintained; thereby, masking some or all residues that form the interface between the components. For example, any epitope present in the interface would not be available to a cognate antigen-binding protein (*e.g.*, antibody) following fixation of the sample.

20 Accordingly, the accessibility (or not) of the residues within the interface to binding proteins (*e.g.*, antibodies) specific for such residues can be used to determine whether or not the particular components were interacting in the biological sample at the time it was fixed. Therefore, by using fixed samples, the relationship between the RTK and a regulatory protein can be “frozen” in time and space (*i.e.*, the
25 presence of absence of a protein-protein interface is fixed), and as a result, a disclosed diagnostic specific binding reagent cannot compete for its binding site in the protein-protein interface. In a non-fixed system, the binding of a regulatory protein to an RTK might not be detected as the diagnostic specific binding reagent could “knock-off” the regulator from the RTK due to competitive binding. Thus, the
30 ability to determine the activation status of the RTK would be lost.

Some disclosed methods involve identifying at least two biological components (*e.g.*, two proteins) that together form a direct interaction, determining

the residues (*e.g.*, amino acids) involved in the interface between the at least two components, identifying or making at least one binding molecule (such as a monoclonal antibody or fragment thereof) specific for some or all of the residues involved in the interface between the at least two components, detecting in a fixed biological sample (such as FFPE tissue sections or fixed cell samples) the binding (or absence of binding) of the at least one interface-specific antigen-binding molecule. In some methods, the interacting components and the particular residues (or regions) involved in the interface between the at least two components are known; hence, identifying such components and the nature of their interface would be optional steps of the disclosed method.

Because fixation of the interface(s) between the at least two interacting components (*e.g.*, proteins) leads to the exclusion of diagnostic specific binding reagents (*e.g.*, interface-specific antigen-binding molecules) from binding residues in the interface(s), some methods will involve a negative result (*i.e.*, no binding). In some such methods, it can be advantageous to further detect the presence of one or more (*e.g.*, one or two) components of the interaction complex; thus, showing that the failure of the at least one interface-specific antigen-binding molecule(s) to bind its target(s) is not due to absence of one or more of the components involved in the making of the interface(s) but rather is due to the masking of the target(s).

Biological samples useful in a disclosed method are isolated and include any cell preparation or tissue preparation that can be fixed and mounted on a solid surface. Exemplary samples include, without limitation, blood smears, cytocentrifuge preparations, cytology smears, core biopsies, fine-needle aspirates, and/or tissue sections (*e.g.*, cryostat tissue sections and/or paraffin-embedded tissue sections). Exemplary biological samples may be isolated from normal cells or tissues, or from neoplastic cells or tissues. Neoplasia is a biological condition in which one or more cells have undergone characteristic anaplasia with loss of differentiation, increased rate of growth, invasion of surrounding tissue, and which cells may be capable of metastasis. Exemplary neoplastic cells or tissues may be isolated from solid tumors, including breast carcinomas (*e.g.* lobular and duct carcinomas), sarcomas, carcinomas of the lung (*e.g.*, non-small cell carcinoma, large cell carcinoma, squamous carcinoma, and adenocarcinoma), mesothelioma of the

lung, colorectal adenocarcinoma, stomach carcinoma, prostatic adenocarcinoma, ovarian carcinoma (such as serous cystadenocarcinoma and mucinous cystadenocarcinoma), ovarian germ cell tumors, testicular carcinomas and germ cell tumors, pancreatic adenocarcinoma, biliary adenocarcinoma, hepatocellular carcinoma, bladder carcinoma (including, for instance, transitional cell carcinoma, adenocarcinoma, and squamous carcinoma), renal cell adenocarcinoma, endometrial carcinomas (including, *e.g.*, adenocarcinomas and mixed Mullerian tumors (carcinosarcomas)), carcinomas of the endocervix, ectocervix, and vagina (such as adenocarcinoma and squamous carcinoma of each of same), tumors of the skin (*e.g.*, squamous cell carcinoma, basal cell carcinoma, melanoma, and skin appendage tumors), esophageal carcinoma, carcinomas of the nasopharynx and oropharynx (including squamous carcinoma and adenocarcinomas of same), salivary gland carcinomas, brain and central nervous system tumors (including, for example, tumors of glial, neuronal, and meningeal origin), tumors of peripheral nerve, soft tissue sarcomas and sarcomas of bone and cartilage.

A solid support useful in a disclosed method need only bear the biological sample and, optionally, but advantageously, permit the convenient detection of components (*e.g.*, proteins and/or nucleic acid sequences) in the sample. Exemplary supports include microscope slides (*e.g.*, glass microscope slides or plastic microscope slides), coverslips (*e.g.*, glass coverslips or plastic coverslips), tissue culture dishes, multi-well plates, membranes (*e.g.*, nitrocellulose or polyvinylidene fluoride (PVDF)) or BIACORE™ chips.

Fixatives for mounted cell and tissue preparations are well known in the art and include, without limitation, 95% alcoholic Bouin's fixative; 95% alcohol fixative; B5 fixative, Bouin's fixative, formalin fixative, Karnovsky's fixative (glutaraldehyde), Hartman's fixative, Hollande's fixative, Orth's solution (dichromate fixative), and Zenker's fixative (see, *e.g.*, Carson, *Histotechnology: A Self-Instructional Text*, Chicago:ASCP Press, 1997).

In some examples, the method includes detecting in a fixed biological sample a protein complex that includes (or consists of) a target RTK and an RTK-interacting protein (*e.g.*, a regulatory protein). Specific examples of known RTK-regulatory protein protein-protein interactions are listed above in Table 3. In a

specific example, the RTK is EGFR, and the method includes detecting in a fixed biological sample a protein complex that includes (or consists of) EGFR and an EGFR-interacting protein (*e.g.*, a regulatory protein, such as a SOCS protein like SOCS1 or SOCS3). EGFR is known to form protein-protein interactions *in vivo* and

5 *in vitro* with numerous other proteins. Some such interactions are listed in Table 7.

Table 7. Exemplary EGFR Interaction Partners

PARTNER 1	PARTNER 2	SYSTEM	SOURCE	PUBMED ID
AREG	EGFR	<i>In vivo</i>	Wong L <i>et al.</i>	10085134
CD44	EGFR	<i>In vivo</i>	Tsatas D <i>et al.</i>	12093135
EGFR	GRB2	<i>In vivo</i>	Lowenstein EJ <i>et al.</i>	1322798
EGFR	GRB2	<i>In vivo</i>	Okutani T <i>et al.</i>	7527043
EGFR	GRB2	<i>In vitro</i>	Lowenstein EJ <i>et al.</i>	1322798
EGFR	GRB2	<i>In vitro</i>	Okutani T <i>et al.</i>	7527043
EGFR	CTNNB1	<i>In vivo</i>	Takahashi K <i>et al.</i>	9233779
EGFR	CDC25A	<i>In vivo</i>	Wang Z <i>et al.</i>	11912208
EGFR	CDC25A	<i>In vitro</i>	Wang Z <i>et al.</i>	11912208
DCN	EGFR	<i>In vivo</i>	Santra M <i>et al.</i>	12105206
DCN	EGFR	<i>In vivo</i>	Iozzo RV <i>et al.</i>	9988678
DCN	EGFR	<i>In vitro</i>	Santra M <i>et al.</i>	12105206
DCN	EGFR	<i>In vitro</i>	Iozzo RV <i>et al.</i>	9988678
DCN	EGFR	Two-hybrid	Santra M <i>et al.</i>	12105206
DCN	EGFR	Two-hybrid	Iozzo RV <i>et al.</i>	9988678
EGFR	HBEGF	<i>In vivo</i>	Shin SY <i>et al.</i>	12725245
EGF	EGFR	<i>In vitro</i>	Stortelers C <i>et al.</i>	12093292
EGFR	CAV1	<i>In vivo</i>	Couet J <i>et al.</i>	9374534
EGFR	CAV1	<i>In vitro</i>	Couet J <i>et al.</i>	9374534
EGFR	PRKACA	<i>In vivo</i>	Tortora G <i>et al.</i>	9050991
EGFR	ERBB3	<i>In vivo</i>	Marques MM <i>et al.</i>	10527633
EGFR	SHC1	<i>In vitro</i>	Sakaguchi K <i>et al.</i>	9544989
ITGA5	EGFR	<i>In vitro</i>	Kuwada SK <i>et al.</i>	10888683
ITGA5	EGFR	<i>In vivo</i>	Kuwada SK <i>et al.</i>	10888683
EGFR	ZNF259	<i>In vivo</i>	Moore SL <i>et al.</i>	10938113
EGFR	RASA1	<i>In vitro</i>	Serth J <i>et al.</i>	1633149
PTK2	EGFR	<i>In vivo</i>	Sieg DJ <i>et al.</i>	10806474
PTK2	EGFR	<i>In vitro</i>	Sieg DJ <i>et al.</i>	10806474
PLSCR1	EGFR	<i>In vivo</i>	Sun J <i>et al.</i>	12009895
PLSCR1	EGFR	<i>In vivo</i>	Nanjundan M <i>et al.</i>	12871937
PLSCR1	EGFR	<i>In vitro</i>	Sun J <i>et al.</i>	12009895
PLSCR1	EGFR	<i>In vitro</i>	Nanjundan M <i>et al.</i>	12871937
GRB14	EGFR	<i>In vivo</i>	Daly RJ <i>et al.</i>	8647858
GRB14	EGFR	<i>In vitro</i>	Daly RJ <i>et al.</i>	8647858
EGFR	DOK2	<i>In vitro</i>	Jones N <i>et al.</i>	10508618
KRT7	EGFR	<i>In vivo</i>	Blagoev B <i>et al.</i>	12577067
KRT7	EGFR	<i>In vitro</i>	Blagoev B <i>et al.</i>	12577067
TGFA	EGFR	<i>In vitro</i>	Garrett TP <i>et al.</i>	12297049

PARTNER 1	PARTNER 2	SYSTEM	SOURCE	PUBMED ID
EGFR	XRCC6	<i>In vivo</i>	Bandyopadhyay D <i>et al.</i>	9430697
EGFR	PLD2	<i>In vitro</i>	Slaaby R <i>et al.</i>	9837959
EGFR	PLD2	<i>In vivo</i>	Slaaby R <i>et al.</i>	9837959
CD82	EGFR	<i>In vivo</i>	Odintsova E <i>et al.</i>	10985391
CD82	EGFR	<i>In vitro</i>	Odintsova E <i>et al.</i>	10985391
SNX6	EGFR	<i>In vivo</i>	Parks WT <i>et al.</i>	11279102
SNX6	EGFR	<i>In vitro</i>	Parks WT <i>et al.</i>	11279102
EGFR	PTK2B	<i>In vivo</i>	Keely SJ <i>et al.</i>	10777553
GRB10	EGFR	<i>In vivo</i>	Frantz JD <i>et al.</i>	9006901
GRB10	EGFR	<i>In vivo</i>	He W <i>et al.</i>	9506989
GRB10	EGFR	<i>In vitro</i>	Frantz JD <i>et al.</i>	9006901
GRB10	EGFR	<i>In vitro</i>	He W <i>et al.</i>	9506989
PRKCA	EGFR	<i>In vivo</i>	Gauthier ML <i>et al.</i>	12878187
PRKCA	EGFR	<i>In vitro</i>	Gauthier ML <i>et al.</i>	12878187
EGFR	STAT1	<i>In vivo</i>	Xia L <i>et al.</i>	12070153
EGFR	STAT1	<i>In vitro</i>	Xia L <i>et al.</i>	12070153
KRT18	EGFR	<i>In vivo</i>	Blagoev B <i>et al.</i>	12577067
KRT18	EGFR	<i>In vitro</i>	Blagoev B <i>et al.</i>	12577067
EGFR	SOCS3	<i>In vivo</i>	Xia L <i>et al.</i>	12070153
EGFR	SOCS3	<i>In vitro</i>	Xia L <i>et al.</i>	12070153
EGFR	MIG-6	<i>In vivo</i>	Hackel PO <i>et al.</i>	11843178
EGFR	MIG-6	Two-hybrid	Hackel PO <i>et al.</i>	11843178
EGFR	SOCS1	<i>In vivo</i>	Xia L <i>et al.</i>	12070153
PLEC1	EGFR	<i>In vivo</i>	Blagoev B <i>et al.</i>	12577067
PLEC1	EGFR	<i>In vitro</i>	Blagoev B <i>et al.</i>	12577067
EGFR	SNX4	<i>In vivo</i>	Haft CR <i>et al.</i>	9819414
EGFR	PRKAR1A	<i>In vivo</i>	Tortora G <i>et al.</i>	9050991
AMH	EGFR	<i>In vitro</i>	Maggard MA <i>et al.</i>	8596488
EGFR	MAP4K1	<i>In vivo</i>	Anafi M <i>et al.</i>	9346925
PLCG1	EGFR	<i>In vivo</i>	Bedrin MS <i>et al.</i>	9207933
SNX1	EGFR	<i>In vivo</i>	Haft CR <i>et al.</i>	9819414
SNX1	EGFR	<i>In vivo</i>	Kurten RC <i>et al.</i>	8638121
SNX1	EGFR	Two-hybrid	Haft CR <i>et al.</i>	9819414
SNX1	EGFR	Two-hybrid	Kurten RC <i>et al.</i>	8638121
EGFR	STAT5B	<i>In vivo</i>	Runge DM <i>et al.</i>	10558875
DEGS1	EGFR	<i>In vivo</i>	Cadena DL <i>et al.</i>	9188692
DEGS1	EGFR	<i>In vitro</i>	Cadena DL <i>et al.</i>	9188692
DEGS1	EGFR	Two-hybrid	Cadena DL <i>et al.</i>	9188692
EGFR	PIK3C2B	<i>In vitro</i>	Wheeler M <i>et al.</i>	11533253
KRT17	EGFR	<i>In vivo</i>	Blagoev B <i>et al.</i>	12577067
KRT17	EGFR	<i>In vitro</i>	Blagoev B <i>et al.</i>	12577067
RGS16	EGFR	<i>In vivo</i>	Derrien A <i>et al.</i>	11602604
HD	EGFR	<i>In vivo</i>	Liu YF <i>et al.</i>	9079622
PITPNA	EGFR	<i>In vivo</i>	Kauffmann-Zeh A <i>et al.</i>	7761838
PITPNA	EGFR	<i>In vitro</i>	Kauffmann-Zeh A <i>et al.</i>	7761838
EGFR	CEBPB	<i>In vivo</i>	Harmon AW <i>et al.</i>	12095417

PARTNER 1	PARTNER 2	SYSTEM	SOURCE	PUBMED ID
EGFR	CEBPB	<i>In vitro</i>	Harmon AW <i>et al.</i>	12095417
MUC1	EGFR	<i>In vivo</i>	Li Y <i>et al.</i>	11483589
MUC1	EGFR	<i>In vivo</i>	Schroeder JA <i>et al.</i>	11278868
NCK2	EGFR	<i>In vivo</i>	Chen M <i>et al.</i>	9737977
NCK2	EGFR	<i>In vivo</i>	Li W <i>et al.</i>	1333047
EGFR	PTK6	<i>In vivo</i>	Kamalati T <i>et al.</i>	8940083
EGFR	CAV3	<i>In vivo</i>	Couet J <i>et al.</i>	9374534
EGFR	CAV3	<i>In vitro</i>	Couet J <i>et al.</i>	9374534
EGFR	CRK	<i>In vivo</i>	Hashimoto Y <i>et al.</i>	9642287
EGFR	CRK	<i>In vitro</i>	Hashimoto Y <i>et al.</i>	9642287
EGFR	GRB7	<i>In vivo</i>	Tanaka S <i>et al.</i>	9710451
EGFR	GRB7	<i>In vitro</i>	Tanaka S <i>et al.</i>	9710451
EGFR	SNRPD2	<i>In vivo</i>	Blagoev B <i>et al.</i>	12577067
EGFR	SNRPD2	<i>In vitro</i>	Blagoev B <i>et al.</i>	12577067
EGFR	SRC	<i>In vitro</i>	Sato K <i>et al.</i>	10971656
CBLB	EGFR	<i>In vivo</i>	Ettenberg SA <i>et al.</i>	10086340
CBLB	EGFR	<i>In vitro</i>	Ettenberg SA <i>et al.</i>	10086340
EGFR	CBLC	<i>In vivo</i>	Keane MM <i>et al.</i>	10362357
EGFR	CBLC	<i>In vitro</i>	Keane MM <i>et al.</i>	10362357
EGFR	SHC1	<i>In vitro</i>	Blagoev B <i>et al.</i>	12577067
EGFR	PTPN6	<i>In vivo</i>	Keilhack H <i>et al.</i>	9733788
EGFR	PTPN6	<i>In vitro</i>	Keilhack H <i>et al.</i>	9733788
EPS8	EGFR	<i>In vivo</i>	Castagnino P <i>et al.</i>	7532293
EPS8	EGFR	<i>In vivo</i>	Di Fiore PP <i>et al.</i>	12127568
SNX2	EGFR	<i>In vivo</i>	Haft CR <i>et al.</i>	9819414
SNX2	EGFR	<i>In vitro</i>	Haft CR <i>et al.</i>	9819414
TNC	EGFR	<i>In vivo</i>	Swindle CS <i>et al.</i>	11470832
TNC	EGFR	<i>In vitro</i>	Swindle CS <i>et al.</i>	11470832
EGFR	CAMLG	<i>In vivo</i>	Tran DD <i>et al.</i>	12919676
EPPK1	EGFR	<i>In vivo</i>	Blagoev B <i>et al.</i>	12577067
EPPK1	EGFR	<i>In vitro</i>	Blagoev B <i>et al.</i>	12577067
EGFR	NCK1	<i>In vitro</i>	Tang J <i>et al.</i>	9362449
KRT8	EGFR	<i>In vivo</i>	Blagoev B <i>et al.</i>	12577067
KRT8	EGFR	<i>In vitro</i>	Blagoev B <i>et al.</i>	12577067
EGFR	TJP1	<i>In vivo</i>	Kaihara T <i>et al.</i>	12708492
VAV1	EGFR	<i>In vivo</i>	Moore SL <i>et al.</i>	10938113
EGFR	STAT5A	<i>In vivo</i>	Olayioye MA <i>et al.</i>	10358079
EGFR	GAB1	<i>In vivo</i>	Kameda H <i>et al.</i>	11432805
EGFR	TNK2	<i>In vivo</i>	Manser E <i>et al.</i>	8497321
EGFR	TNK2	<i>In vivo</i>	Satoh T <i>et al.</i>	8647288
EGFR	VAV2	<i>In vivo</i>	Pandey A <i>et al.</i>	10618391
EGFR	VAV2	<i>In vivo</i>	Moore SL <i>et al.</i>	10938113
EGFR	VAV2	<i>In vitro</i>	Pandey A <i>et al.</i>	10618391
EGFR	VAV2	<i>In vitro</i>	Moore SL <i>et al.</i>	10938113
SOS1	EGFR	<i>In vivo</i>	Qian X <i>et al.</i>	9447973
EGFR	MAP3K14	<i>In vivo</i>	Habib AA <i>et al.</i>	11116146
EGFR	ESR1	<i>In vitro</i>	Marquez DC <i>et al.</i>	11887937
EGFR	ERBB2	<i>In vivo</i>	Brockhoff G <i>et al.</i>	11500850
EGFR	ERBB2	<i>In vitro</i>	Brockhoff G <i>et al.</i>	11500850

PARTNER 1	PARTNER 2	SYSTEM	SOURCE	PUBMED ID
SH2D3A	EGFR	<i>In vivo</i>	Lu Y <i>et al.</i>	10187783
CDH1	EGFR	<i>In vivo</i>	Pece S <i>et al.</i>	10969083
EGFR	PTPN1	<i>In vivo</i>	Zhang ZY <i>et al.</i>	8621392
EGFR	PTPN1	<i>In vivo</i>	Jia Z <i>et al.</i>	7540771
EGFR	PTPN1	<i>In vivo</i>	Sarmiento M <i>et al.</i>	10889023
EGFR	PTPN1	<i>In vivo</i>	Li S <i>et al.</i>	12573287
EGFR	PTPN1	<i>In vitro</i>	Zhang ZY <i>et al.</i>	8621392
EGFR	PTPN1	<i>In vitro</i>	Jia Z <i>et al.</i>	7540771
EGFR	PTPN1	<i>In vitro</i>	Sarmiento M <i>et al.</i>	10889023
EGFR	PTPN1	<i>In vitro</i>	Li S <i>et al.</i>	12573287
VAV3	EGFR	<i>In vivo</i>	Zeng L <i>et al.</i>	11094073
INPPL1	EGFR	<i>In vitro</i>	Pesesse X <i>et al.</i>	11349134
INPPL1	EGFR	<i>In vivo</i>	Pesesse X <i>et al.</i>	11349134
EPS15	EGFR	<i>In vivo</i>	van Delft S <i>et al.</i>	9049247
EGFR	ARF4	<i>In vitro</i>	Kim SW <i>et al.</i>	12446727
EGFR	ARF4	<i>In vivo</i>	Kim SW <i>et al.</i>	12446727
EGFR	ARF4	Two-hybrid	Kim SW <i>et al.</i>	12446727
EGFR	GNAI2	<i>In vivo</i>	Zhang BH <i>et al.</i>	11286993
EGFR	PDGFRB	<i>In vivo</i>	Habib AA <i>et al.</i>	9506992
SHC3	EGFR	<i>In vivo</i>	Nakamura T <i>et al.</i>	9507002
EGFR	BTC	<i>In vivo</i>	Mixan B <i>et al.</i>	9528863
EGFR	SOS2	<i>In vivo</i>	Qian X <i>et al.</i>	10675333
NRG1	EGFR	<i>In vitro</i>	Pinkas-Kramarski R <i>et al.</i>	8702572
EGFR	CASP1	<i>In vivo</i>	Bae SS <i>et al.</i>	11226410
EGFR	CASP1	<i>In vitro</i>	Bae SS <i>et al.</i>	11226410
EGFR	EREG	<i>In vivo</i>	Komurasaki T <i>et al.</i>	9419975
EGFR	PTPN11	<i>In vitro</i>	Tomic S <i>et al.</i>	7673163
EGFR	PTPN11	<i>In vivo</i>	Tomic S <i>et al.</i>	7673163
PTPRJ	EGFR	<i>In vivo</i>	Jallal B <i>et al.</i>	9115287
EGFR	RIPK1	<i>In vivo</i>	Habib AA <i>et al.</i>	11116146
FER	EGFR	<i>In vivo</i>	Kim L <i>et al.</i>	7623846

Protein-protein interactions typically influence the activity of one or both interacting partners. For example, a protein-protein interaction may result in the negative regulation (*e.g.*, inhibition) of one or both partners, or may result in the positive regulation (*e.g.* activation) of one or both partners. Other functional outcomes also are possible. Exemplary positive and negative regulators for specific RTKs are provided in Table 3. Exemplary negative regulators of EGFR that form protein-protein interactions with EGFR include, for instance, SOCS1, SOCS3, SOCS5, and C-CBL. Exemplary positive regulators of EGFR that form protein-protein interactions with EGFR include, for instance, STAT1, STAT5B, GRB7, HER2, and MUC1.

Methods useful for detection of a protein-protein interaction using a diagnostic specific binding reagent (*e.g.*, interface-specific binding molecule) identified using the methods provided herein (such as a diagnostic specific binding reagent, for example an antibody, antibody fragment, recombinant antibody, scaffold polypeptide with antibody binding sites, and/or an aptamer) are well known in the art. In some examples, a fixed biological sample is contacted with a diagnostic specific binding molecule under conditions that permit (or would permit if it was accessible) binding of the diagnostic specific binding molecule to its epitope in the interface between the interacting proteins. Optionally, a control reaction is performed (*e.g.*, simultaneous with, prior to, or following) to ensure that the conditions are suitable for the detection reaction to occur. For example, the biological sample (or a serial section or a parallel-prepared cell sample) also may be contacted with a control antigen-binding molecule (such as, an antibody, antibody fragment, recombinant antibody, scaffold polypeptide with antibody binding sites, and/or an aptamer). The control antigen-binding molecule specifically binds to a non-interacting component of the sample (i) that is not involved in the protein-protein complex of interest and (ii) the epitope of which is known to be present and detectable in the sample under the particular detection conditions.

In exemplary methods, a diagnostic specific binding molecule and an optional control antigen-binding molecule are antibodies (*e.g.*, monoclonal antibodies) or antibody fragments. Detection of such antibodies or antibody fragments is performed by immunostaining, such as illustrated in FIG. 1, which is a standard technique in the art. Detection may be direct or indirect. With direct detection, primary antibodies (*i.e.*, the antibodies that specifically bind to biological component(s) of interest in the sample) are directly labeled, for instance, with a detectable moiety or with an enzyme that catalyzes a reaction leading to a detectable product. With indirect detection, one or more secondary reagents (such as secondary, tertiary, *etc.* antibodies) are used to detect the primary antibody (and, as applicable, secondary or subsequent antibodies) and the last of such reagents is detectable, for instance by labeling with detectable moiety or with an enzyme that catalyzes a reaction leading to a detectable product. Representative immunostaining procedures are provided in the Examples.

Some disclosed methods involve dual detection of an extracellular domain (ED) epitope present on an RTK and an ID (or regulatory domain) epitope of the RTK. Some RTK ID or regulatory domains have multiple binding sites for regulatory proteins (*e.g.*, the EGFR regulatory domain, see FIG. 2). Accordingly, epitopes in the RTK ID may not be accessible in fixed biological samples where, at the time of fixation, the RTK was involved in a protein-protein interaction with one or more of its regulatory proteins (see Tables 3 and 5). In comparison, the ED of RTKs function primarily as a ligand-binding domain, and, typically, it is not masked from antibodies specific for ED epitopes. Accordingly, in some methods, an antigen-binding molecule specific for an RTK ED (*e.g.*, an anti-RTK ED antibody or fragment thereof) can serve as a control for an antigen-binding molecule specific for a (potentially masked) epitope in an RTK protein - regulatory protein interface. One caveat of interest is that some RTKs, such as EGFR, HER2, cKit, cMet, and Her4, may “shed” its external domain or may be mutant and lack its ED (Pedersen *et al.*, *Ann. Oncol.*, 6:745, 2001). Under those circumstances, an antigen-binding molecule specific for the RTK ED (*e.g.*, anti-RTK ED antibody or fragment thereof) would have no target to bind and, therefore, would not be detected. This circumstance (and useful information that can be gleaned from such circumstance) is discussed in detail elsewhere in this disclosure.

Some of the foregoing method embodiments and other method embodiments in this disclosure involve substantially no specific binding of a diagnostic specific-binding molecule (such as a monoclonal antibody) to its epitope (*e.g.*, which is located in the protein-protein interface between an RTK and its regulatory molecule(s)). Substantially no binding can be determined by any method available to those of ordinary skill in the art. For example, substantially no binding of a diagnostic specific-binding molecule may be relative to the binding of the same diagnostic specific-binding molecule under substantially the same conditions in another sample in which the epitope of the diagnostic specific-binding molecule is known to be accessible. In another example, substantially no binding of a diagnostic specific-binding molecule may mean that the detection means (*e.g.*, detectable label or colorimetric reagent) used to visualize the specific binding of the diagnostic specific-binding molecule can not be seen under ordinary circumstances for such

detection, *e.g.*, under a light or fluorescence microscope with 4x, 10x, or 40x magnification. In still another example, substantially no binding of a diagnostic specific-binding molecule means that the diagnostic specific-binding molecule has less than about 25% (such as less than about 20%, less than about 15%, less than about 10%, less than about 5%, or less than about 1%) its binding under control circumstances (*e.g.*, in a tissue or cell sample where its epitope is known to be accessible).

VIII. Prognostic and Predictive Methods

10 The present disclosure provides methods than can be used to determine the activation status of a RTK in a biological sample (such as a sample from a subject having or suspected of having a neoplasm, for example cancer), and thus permit predictions about the subject. For example, using diagnostic specific binding reagents identified using the provided methods permits determination of the

15 prognosis of the subject (such as the disease outcome without further therapy, for example the likelihood of survival after initial diagnosis (such as a 1 year or 5 year survival time)), as well as predictions about the likelihood that a subject (such as a subject with a cancer thought or known to express the target RTK) will respond successfully to a particular RTK therapy, such as an RTK therapy designed to target

20 an ED or ID of the RTK. Therefore, methods are provided to detect RTK (*e.g.*, EGFR or HER2 or others shown in Table 2) molecular interactions in biological samples (such as a fixed biological sample) which opens the way to predicting RTK status and important corollaries in such samples or in subjects from which such samples are collected; for example, in neoplastic tissues and/or cells where RTK

25 expression or overexpression is believed to play an important role in tumorigenesis (*e.g.*, Arnold *et al.*, *Oncologist*, 6:602, 2006) and/or in cancer patients.

 The disclosed diagnostic methods are applicable to any type of neoplasm or cancer or to a subject with any type of neoplasm or cancer, for instance RTK-expressing (or -overexpressing) cancers. Exemplary neoplasms useful in all

30 disclosed methods (including predictive methods) are described elsewhere in this disclosure (*e.g.*, Section VI and the Examples). Particular predictive method embodiments involve lung cancer (*e.g.*, non-small cell lung cancer), ovarian cancer,

colorectal cancer, liver cancer, head and neck, prostate, and/or glioblastoma and/or subjects having any of such cancers.

Examples of staining intensity scores which indicate detectable binding (*e.g.*, a positive binding result) of a diagnostic specific binding reagent with a protein-
5 protein interface or no (or insignificant) detectable binding of a diagnostic specific binding reagent with a protein-protein interface (*e.g.*, negative binding result) are provided in the Examples below (*e.g.*, see Example 6).

A. Predicting Aggressiveness of RTK-Positive Neoplasms

Determining the prognosis of a subject having a neoplasm, such as a cancer,
10 can be achieved by using the diagnostic specific binding reagents identified using the disclosed methods. Determining the prognosis of a subject allows for a determination or estimation of the disease (*e.g.*, cancer) outcome in the absence of additional therapy (*e.g.*, estimated time of survival). For example, detection of a direct (*e.g.*, protein-protein) interaction between an RTK and a negative regulator of
15 RTK function or a positive regulator of RTK function (*e.g.*, see Table 3) can be used to predict inhibition of RTK function (if the diagnostic specific binding reagent is one that recognizes an interface between an RTK-negative regulatory protein interaction) or predict enhanced RTK function (if the diagnostic specific binding reagent is one that recognizes an interface between an RTK-positive regulatory
20 protein interaction), respectively, in a biological sample.

In one example, the diagnostic specific binding reagent is one that recognizes an interface between an RTK protein-negative regulatory protein interaction (such as the RTK RD amino acids involved in such an interaction). In such an example, detection of a direct (*e.g.*, protein-protein) interaction between an RTK and a
25 negative regulator of RTK indicates that the RTK protein is being downregulated or inhibited. Such a direct interaction between an RTK and a negative regulator of RTK is indicated by the absence of binding (or significantly reduced binding) by the diagnostic specific binding reagent to the biological sample. This is because when the inhibitor is bound to the RTK in the sample, the diagnostic specific binding
30 reagent is unable to specifically bind in any substantial amount to the RTK-negative regulatory protein interface. Inhibition of RTK function has important consequences in many cells and tissues. For example, in neoplastic cells and tissues where RTK

overexpression or increased biological activity is believed to play a role in tumorigenesis (*e.g.*, Arnold *et al.*, *Oncologist*, 6:602, 2006), detection of a direct interaction between RTK and a negative regulator of RTK function (*e.g.*, see Table 3) further predicts that a neoplasm may be less aggressive (*e.g.*, less rapidly growing, and/or less likely to metastasize). A better prognosis (independent of therapy) for a subject with such a neoplasm also may be predicted. However, if there is no significant detection of a direct (*e.g.*, protein-protein) interaction between an RTK and a negative regulator of RTK, this indicates that the RTK protein is not being downregulated or inhibited. An absence of direct interaction between an RTK and a negative regulator of RTK is indicated by the presence of detectable binding by the diagnostic specific binding reagent to the biological sample. This is because when the inhibitor is not bound to the RTK in the sample, the interface that forms between an RTK and a negative regulator is available for binding to the diagnostic specific binding reagent, and thus binding of the diagnostic specific binding reagent to the biological sample can be detected. This then indicates that the RTK is not being negatively regulated, and may predict that a neoplasm may be more aggressive (*e.g.*, rapidly growing, and/or more likely to metastasize). A worse prognosis (independent of therapy) for a subject with such a neoplasm also may be predicted.

In a specific example, the RTK is EGFR, wherein detection of a direct (*e.g.*, protein-protein) interaction between EGFR and a negative regulator of EGFR function (*e.g.*, a SOCS protein, such as SOCS1 or SOCS3, or SOCS5) predicts inhibition of EGFR function in that biological sample, and may further predict that a neoplasm may be less aggressive (*e.g.*, less rapidly growing, and/or less likely to metastasize). A better prognosis (independent of therapy) for a subject with such a neoplasm also may be predicted.

In another example, the diagnostic specific binding reagent is one that recognizes an interface between an RTK protein-positive regulatory protein interaction (such as the RTK RD amino acids involved in such an interaction). In such an example, detection of a direct (*e.g.*, protein-protein) interaction between an RTK and a positive regulator of RTK indicates that the RTK protein is being activated or upregulated (*e.g.*, increased RTK expression or biological activity). Such a direct interaction between an RTK and a positive regulator of RTK is

indicated by the absence of binding (or significantly reduced binding) by the diagnostic specific binding reagent to the biological sample. This is because when the positive regulator is bound to the RTK in the sample, the diagnostic specific binding reagent is unable to specifically bind in any substantial amount to the RTK-
5 positive regulatory protein interface. For the opposite of reasons discussed above, a worse prognosis (independent of therapy) for a subject with such a neoplasm also may be predicted. However, if there is no significant detection of a direct (*e.g.*, protein-protein) interaction between an RTK and a positive regulator of RTK, this indicates that the RTK protein is not being activated or upregulated. An absence of
10 direct interaction between an RTK and a positive regulator of RTK is indicated by the presence of detectable binding by the diagnostic specific binding reagent to the biological sample. This is because when the positive regulatory protein is not bound to the RTK in the sample, the interface that forms between an RTK and a positive regulatory protein is available for binding to the diagnostic specific binding reagent,
15 and thus binding of the diagnostic specific binding reagent to the biological sample can be detected. This then indicates that the RTK is not being activated or upregulated, and may predict that a neoplasm may be less aggressive (*e.g.*, less rapidly growing, and/or less likely to metastasize). A better prognosis (independent of therapy) for a subject with such a neoplasm also may be predicted.

20 In a specific example, the RTK is EGFR, wherein detection of a direct interaction between EGFR and a positive regulator of EGFR function (*e.g.*, STAT1, STAT5B, GRB7, HER2, and/or MUC1) predicts activation of EGFR function in that biological sample (*e.g.*, neoplastic tissue or cells). For the opposite of reasons discussed above, a worse prognosis (independent of therapy) for a subject with such
25 a neoplasm also may be predicted.

A less-aggressive tumor can be characterized by any parameters known in the art, including, for instance, decreased growth rate (*e.g.*, increased rate of apoptosis and/or decreased rate of cell division), decreased rate of metastasis, and/or increased sensitivity to chemotherapy.

30 Prognosis for a subject can be characterized by any parameter known in the art, including, for instance, actual survival after initial diagnosis (such as 1-year survival, 2-year survival, or 5-year survival), and/or actual survival relative to the

average survival for similarly situated patients. A better (or good) prognosis entails, *e.g.*, survival of a patient for more than 1 year after initial diagnosis (such as more than 2 years or more than 5 years), or survival of a patient for more than 6 months longer (*e.g.*, more than 1 year longer, more than 2 years longer, more than 5 years longer) than the average survival for similarly situated. A worse (or bad) prognosis entails, *e.g.*, survival of a patient for less than 5 years after initial diagnosis (such as less than 2 years or less than 1 years), or survival of patient less than the average survival for similarly situated patients (such as, about 3 months less than average survive, about 6 months less than average survive, or about 1 year less than average survival).

Exemplary prognoses based on detecting an interaction (or lack of interaction) between an RTK and, *e.g.*, a positive or negative regulator that binds the RTK ID (such as those listed in Table 3) are shown schematically in FIGS. 3A and 3B.

B. Predicting Responsiveness of a Cancer Patient to RTK-inhibitor Therapy

Methods of detecting an interaction (or lack of interaction) between a target RTK and its regulator(s) (such as those shown in Table 3) enables a variety of predictions with respect to the outcome of RTK inhibitor therapy in a cancer patient. RTK inhibitor therapies include at least two drug classes: (1) RTK antibody therapies, which are typically are directed to the RTK ED and block binding of an RTK ligand (such as EGF for the EGFR) to the receptor; thereby, inhibiting RTK activation, and (2) tyrosine kinase inhibitors (TKIs), which inhibit the intracellular kinase domain of a RTK, which also inhibits RTK activation. Exemplary inhibitory therapies for exemplary target RTKs are provided in Table 8.

Table 8. Exemplary RTK inhibitory therapies

RTK	Antibody Therapy	Tyrosine Kinase Inhibitor
EGFR	cetuximab (Erbix TM), panitumumab (Vectibix TM), IMC-11F8 (Imclone), matuzumab (Merck_KGA), zalutumumab,	gefitinib (Iressa TM), erlotinib (Tarceva TM), lapatinib ditosylate (GlaxoSmithKline), HKI-272 (Wyeth), AEE788 (Novartis), vandetanib (Zactima TM ; Astrazeneca), XL647 (Exelixis), BMS-599626 (Bristol-Myers Squibb), BIBW 2992 (Boehringer

		Ingelheim), ARRY-543, MP-412, JNJ-26483327, PF-299804, Neratinib
FGFR1		
FGFR2		
FGFR3		Sunitinib, CHIR258
FGFR4		
Flt3		ENMD-981693, KW-2449, ABT-869, AC220, SU014813, CHIR258
HER2 (ErbB2)	Trastuzumab (Herceptin®), bevacizumab (Avastin®), pertuzumab (OmniTarg™), ZM105180 (Zemab®), ertumaxomab (Rexonum)	Lapatinib (Tykerb®), XL647, ARRY-543, BMS-599626, MP-412, BIBW 2992, JNJ-26483327, PF-299804, ARRY-380, Neratinib, INSM-18
HER3 (ErbB3)		PF-299804
HER4 (ErbB4)		PF-299804
HGFR (MET)	AMG 102 (Amgen), MetMab (Genentech)	XL880, XL184, PF-02341066, SGX523, JNJ-38877605, ARQ197, PHA665752, MP470, ARQ-650RP, MGCD265, MK-2461, PHA665752
IGF-1R	AMG 479 (Amgen), CP-751,871, R1507, RAV12, IMC-A12 (ImClone), BIIB022	OSI-906, XL228, BMS-754807, AMG479, INSM-18
Kit		Imatinib (Gleevec™), OSI-930, OSI-817, sunitinib, XL820, motesanib diphosphate (Amgen), ENMD-981693, KW-2449, MP470, ABT-869, AC220, Telatinib, SU014813, Cediranib, CHIR258, MP470, STI571, Dasatinib
PDGFR		CP-547,632, sunitinib, motesanib diphosphate (Amgen), ABT-869, AC220, Telatinib, SU014813, CHIR258, XL820, Dasatinib
Ret		XL184, MP470, AC220, Vandetanib (Zactima™)
RON		MGCD265
VEGFR1		Sunitinib, OSI-930, OSI-817, XL647, XL820, XL880, XL184, motesanib diphosphate (Amgen), cediranib, CHIR258, MGCD265
VEGFR2 (KDR/flk-1)		CP-868,596, sunitinib, XL184, XL820, XL880, vandetanib (Zactima™), motesanib diphosphate (Amgen), ENMD-981693, Telatinib, SU014813, MGCD265
VEGFR3 (Flt4)		Sunitinib, XL228, motesanib diphosphate (Amgen), JNJ-26483327, Telatinib, MGCD265

Some method embodiments involve one or both of the foregoing classes of RTK inhibitors, and thus can be used to predict the response of a cancer patient to any of the inhibitors listed in Table 7. Particular method embodiments involve predicting the response of cancer patients to the following EGFR inhibitors:

- 5 cetuximab (Erbix™), panitumumab (Vectibix™), gefitinib (Iressa™), or erlotinib (Tarceva™), or any combination thereof (such as, cetuximab (Erbix™) or panitumumab (Vectibix™), gefitinib (Iressa™) or erlotinib (Tarceva™), or cetuximab (Erbix™), panitumumab (Vectibix™), gefitinib (Iressa™) or erlotinib (Tarceva™)), or the HER2 inhibitors Herceptin® and Avastin®.

- 10 Exemplary predictions based on detecting an interaction (or lack of interaction) between an RTK and, *e.g.*, a positive or negative regulator that binds the RTK ID (such as those listed in Table 3) are shown schematically in FIGS. 3A (negative regulator) and 3B (positive regulator). Exemplary predictions based on detecting an interaction (or lack of interaction) between an RTK and a negative
15 regulator that binds the RTK ID (such as those listed in Table 3) are shown in Table 9.

Table 9. Exemplary Predicted Therapeutic Response

	ID-Binding Molecule	ED-Binding Molecule	RTK Implication	Therapeutic	Predicted Therapeutic Response
1	Positive	Positive	RTK is present RDIM (<i>e.g.</i> SOCS3) is absent	ED-based therapy (<i>e.g.</i> , EGFR Ab)	Sensitive
				ID-based therapy (<i>e.g.</i> , TKI)	Sensitive
2	Negative	Positive	RTK is present RDIM (<i>e.g.</i> SOCS3) is present	ED-based therapy (<i>e.g.</i> , EGFR Ab)	Resistant
				ID-based therapy (<i>e.g.</i> , TKI)	Resistant
3	Positive	Negative	Mutant/Cleaved RTK is present RDIM (<i>e.g.</i> SOCS3) is absent	ED-based therapy (<i>e.g.</i> , EGFR Ab)	Resistant
				ID-based therapy (<i>e.g.</i> , TKI)	Sensitive
4	Negative	Negative	RTK is absent	ED-based therapy (<i>e.g.</i> , EGFR Ab)	No Response
				ID-based therapy (<i>e.g.</i> , TKI)	No Response

- 20 ID = RTK intracellular (or regulatory) domain; ED = RTK extracellular domain;
RDIM = regulatory domain inhibitory molecule; Ab = antibody (*e.g.*, monoclonal or otherwise engineered antibody)

In one method embodiment, an interaction between the ID (*e.g.*, regulatory domain) of RTK and at least one of its negative regulators (*e.g.*, see Table 3) is detected (*e.g.*, by masking of the epitope of a diagnostic specific binding molecule (such as a monoclonal antibody, including clone 5B7 (see, *e.g.*, Examples)).
5 Optionally, but advantageously, the presence of full-length (or substantially full-length) RTK also is detected using an antigen-binding molecule (*e.g.*, monoclonal antibody, such as clone 3C6 for EGFR) specific for the RTK ED. In this example, the diagnostic specific binding molecule (*e.g.*, clone 5B7 for EGFR)
10 is excluded from its binding site and, therefore, is not detected, while the external-domain antigen-binding molecule (*e.g.*, clone 3C6 for EGFR) binds to its epitope and is detected (right panel of FIG. 3A). These circumstances support a prediction that a therapy designed to inhibit RTK function likely would not be effective or would be less effective than in the absence of the negative regulator.
15 That is (solely for illustration purposes (and not to be limited by mechanism or implication of a mechanism)): Providing an RTK inhibitor to a subject in which RTK function already was inhibited maybe analogous to applying the brakes in a car that is already at a stop.

In another method embodiment, an interaction between the RTK ID and its
20 negative regulator (*e.g.*, see Table 3) is lacking (*e.g.*, as demonstrated by the binding of a diagnostic specific binding molecule (such as a monoclonal antibody, including clone 5B7 (see, *e.g.*, Examples)) to its epitope on the RTK, which would otherwise be masked by the RTK-negative regulator interaction). Optionally, but
advantageously, the presence of the RTK ED (*i.e.*, full-length or substantially
25 full-length RTK) also is detected using an antigen-binding molecule (*e.g.*, monoclonal antibody, including clone 3C6 for EGFR) specific for that domain. In this example, the diagnostic specific binding molecule (*e.g.*, clone 5B7 for EGFR) specifically binds its epitope in the RTK regulatory domain and, therefore, is detected, and the external-domain antigen-binding molecule (*e.g.*, clone 3C6) also
30 binds to its epitope and is detected (left panel of FIG. 3A). These circumstances support a prediction that a therapy designed to inhibit RTK function (*e.g.*, see Table 8) likely would be effective or would be more effective than in the presence of the

negative regulator, as there are fewer “brakes” on oncogenic RTK function and the therapy has something to inhibit.

In still another method embodiment, an interaction between the ID of an RTK and its negative regulator (*e.g.*, see Table 3) is lacking (*e.g.*, as demonstrated by the binding to the RTK of a diagnostic specific binding molecule (such as a monoclonal antibody, including clone 5B7 (see, *e.g.*, Examples)) to its epitope, which would otherwise be masked by the RTK-negative regulator interaction). Optionally, but advantageously, the presence or absence of the RTK ED (*i.e.*, full-length or substantially full-length RTK) also is detected using an antigen-binding molecule (*e.g.*, monoclonal antibody, including clone 3C6 for EGFR) specific for that domain. In this example, the diagnostic specific binding molecule (*e.g.*, clone 5B7 for EGFR) specifically binds its epitope in the RTK ID and, therefore, is detected; however, it also is determined that the RTK ED is lacking (*e.g.*, a mutant or N-terminal truncated RTK) by failure to bind of an antigen-binding molecule specific for that domain (*e.g.*, monoclonal antibody, including clone 3C6 for EGFR) (middle panel of FIG. 3A). These circumstances support a prediction that an antibody therapy designed to inhibit RTK function by blocking ligand binding to the RTK external domain (*e.g.*, those listed in Table 6) likely would be not effective because such domain is lacking. On the other hand, these circumstances further support a prediction that a TKI therapy (*e.g.*, those listed in Table 6), which inhibits the tyrosine kinase activity localized in the RTK ID, likely would be effective or would be more effective than in the presence of the negative regulator.

In one method embodiment, an interaction between the ID (*e.g.*, regulatory domain) of RTK and at least one of its positive regulators (*e.g.*, see Table 3) is detected (*e.g.*, by masking of the epitope of a diagnostic specific binding molecule. Optionally, but advantageously, the presence of full-length (or substantially full-length) RTK also is detected using an antigen-binding molecule (*e.g.*, monoclonal antibody, such as clone 3C6 for EGFR) specific for the RTK ED. In this example, the diagnostic specific binding molecule is excluded from its binding site and, therefore, is not detected, while the external-domain antigen-binding molecule (*e.g.*, clone 3C6 for EGFR) binds to its epitope and is

detected (right panel of FIG. 3B). These circumstances support a prediction that a therapy designed to inhibit RTK function likely would be effective. That is (solely for illustration purposes (and not to be limited by mechanism or implication of a mechanism)): Providing an RTK inhibitor to a subject in which RTK function is
5 being activated will be effective as the oncogenic RTK is very activity and thus there is significant RTK activity to be inhibited.

In another method embodiment, an interaction between the RTK ID and its positive regulator (*e.g.*, see Table 3) is lacking (*e.g.*, as demonstrated by the binding of a diagnostic specific binding molecule to its epitope on the RTK, which would
10 otherwise be masked by the RTK-positive regulator interaction). Optionally, but advantageously, the presence of the RTK ED (*i.e.*, full-length or substantially full-length RTK) also is detected using an antigen-binding molecule (*e.g.*, monoclonal antibody, including clone 3C6 for EGFR) specific for that domain. In this example, the diagnostic specific binding molecule specifically binds its
15 epitope in the RTK regulatory domain and, therefore, is detected, and the external-domain antigen-binding molecule (*e.g.*, clone 3C6) also binds to its epitope and is detected (left panel of FIG. 3B). These circumstances support a prediction that a therapy designed to inhibit RTK function (*e.g.*, see Table 8) likely would not be effective or would be less effective, for example, because the oncogenic RTK is
20 less activity than if the positive regulatory were present, thus the RTK inhibitory therapy has less to inhibit.

In still another method embodiment, an interaction between the ID of an RTK and its positive regulator (*e.g.*, see Table 3) is lacking (*e.g.*, as demonstrated by the binding to the RTK of a diagnostic specific binding molecule to its epitope,
25 which would otherwise be masked by the RTK-positive regulator interaction). Optionally, but advantageously, the presence or absence of the RTK ED (*i.e.*, full-length or substantially full-length RTK) also is detected using an antigen-binding molecule (*e.g.*, monoclonal antibody, including clone 3C6 for EGFR) specific for that domain. In this example, the diagnostic specific binding
30 molecule (*e.g.*, clone 5B7 for EGFR) specifically binds its epitope in the RTK ID and, therefore, is detected; however, it also is determined that the RTK ED is lacking (*e.g.*, a mutant or N-terminal truncated RTK) by failure to bind of an

antigen-binding molecule specific for that domain (*e.g.*, monoclonal antibody, including clone 3C6 for EGFR) (middle panel of FIG. 3B). These circumstances support a prediction that an antibody therapy designed to inhibit RTK function by blocking ligand binding to the RTK external domain (*e.g.*, those listed in Table 6) likely would be not effective because such domain is lacking, and also that therapy designed to inhibit RTK function (*e.g.*, see Table 8) likely would not be effective or would be less effective, for example, because the oncogenic RTK is less activity than if the positive regulatory protein were present, thus the RTK inhibitory therapy has less to inhibit.

10 The response of a subject to RTK inhibitor therapy can be measured by any relevant parameter known in the art. In some method embodiments, a subject response is cessation or slowing of tumor growth (as measured, for example, by tumor size), decrease in tumor cell proliferation, increase in tumor cell apoptosis, and/or decreased level of relevant tumor marker(s). In other method embodiments, a
15 subject response is at least a 50% slowing of tumor growth or tumor cell proliferation as compared to pre-treatment growth (such at least a 40% slowing, at least a 30% slowing, at least a 20% slowing, or at least a 10% slowing). In other method embodiments, a subject response is at least a 50% increase in tumor cell apoptosis as compared to pre-treatment levels (such at least a 40% increase, at least
20 a 30% increase, at least a 20% increase, or at least a 10% increase).

IX. Kits

Any of the diagnostic specific-binding molecules described in this disclosure can be supplied in the form of a kit useful, at least, for performing the methods
25 described herein. In one embodiment of such a kit, an appropriate amount of at least one diagnostic specific-binding molecule (*e.g.*, monoclonal antibody (such as clone 5B7) or fragment thereof) is provided in one or more containers. In other embodiments, at least one diagnostic specific-binding molecule (*e.g.*, monoclonal antibody (such as clone 5B7) or fragment thereof) may be provided suspended in an
30 aqueous solution or as a freeze-dried or lyophilized powder, for instance. The container(s) in which the at least one diagnostic specific-binding molecule (*e.g.*, monoclonal antibody (such as clone 5B7) or fragment thereof) is supplied can be

any conventional container that is capable of holding the supplied form, for instance, microfuge tubes, ampoules, or bottles. The amount of diagnostic specific-binding molecule (*e.g.*, monoclonal antibody (such as clone 5B7) or fragment thereof) supplied can be any appropriate amount, such as from about 1 to about 5 $\mu\text{g/ml}$.

- 5 In other embodiments, control slides upon which are mounted one or more tissue or cell preparations (*e.g.*, xenografts, cell pellets, or clotted cells) that may serve as positive and/or negative controls for a diagnostic specific-binding molecule (*e.g.*, monoclonal antibody (such as clone 5B7) or fragment thereof) may be provided in an appropriate and separate container. In some instances, A431,
10 DU145, and/or Caski cells (or xenografts prepared therewith) may serve as a positive control. In other instances, MCF-7 cells (or xenografts prepared therewith) may serve as a negative control.

- Other kit embodiments will include means for detection of the diagnostic specific-binding molecule, such as secondary antibodies (*e.g.*, goat anti-rabbit
15 antibodies or rabbit anti-mouse antibodies). In some such instances, the secondary antibody will be directly labeled with a detectable moiety (as described elsewhere in this disclosure). In other instances, the primary or secondary (or higher-order) antibody will be conjugated to a hapten (such as biotin, DNP, and/or FITC), which is detectable by a detectably labeled cognate hapten-binding molecule (*e.g.*,
20 streptavidin (SA)-horse radish peroxidase, SA-alkaline phosphatase, and/or SA-QDot™). Some kit embodiments may include colorimetric reagents (*e.g.*, DAB, and/or AEC) in suitable containers to be used in concert with primary or secondary (or higher-order) antibodies that are labeled with enzymes for the development of such colorimetric reagents.

- 25 In one embodiment, a kit includes instructional materials disclosing methods of use of the kit contents (*e.g.*, diagnostic specific-binding molecule) in a disclosed method. The instructional materials may be written, in an electronic form (*e.g.* computer diskette or compact disk) or may be visual (*e.g.* video files). The kits may also include additional components to facilitate the particular application for which
30 the kit is designed. Thus, for example, the kits may additionally include buffers and other reagents routinely used for the practice of a particular method. Such kits and appropriate contents are well known to those of skill in the art.

The following examples are provided to illustrate certain particular features and/or embodiments. These examples should not be construed to limit the invention to the particular features or embodiments described.

5

EXAMPLES

Example 1

AN EXEMPLARY MONOCLONAL ANTIBODY SPECIFIC FOR EGFR REGULATORY DOMAIN

10 This Example describes an exemplary RD-binding molecule; more particularly a monoclonal antibody that binds an epitope in the EGFR inhibitory subdomain. This antibody has the added advantage that it will identify not only full-length EGFR, but also truncated mutant forms of EGFR, which have been shown to be constitutively activated (Pedersen *et al.*, *Ann. Oncol.*, 12(6):745-60,
15 2001). Similar methods can be used to identify antibodies for other RTK intracellular domains.

A computer program (DNASTAR™, Madison, WI) was used for the selection of immunogenic peptide sequences within the EGFR intracellular domain. The program examined the input protein sequence for short (*e.g.*, less than 20
20 contiguous amino acids) sequences that likely had a high probability for producing an antibody response in animals immunized with immunogens including such short sequences.

One identified short sequence was LDNPDYQQDFFPKEAKPNG (L2G Peptide; SEQ ID NO: 2), which, by computer analysis, had high antigenicity, high
25 hydrophilic regions, and high surface probability regions. This amino acid sequence was selected and a corresponding peptide was synthesized using a commercially available service (Anaspec, San Jose, CA).

The synthesized peptide was conjugated to Keyhole Limpet Hemocyanin (KLH) using standard methods. Rabbits were immunized with the KLH-peptide
30 conjugate by a commercially available service (Strategic Diagnostics, Inc. Newark, DE).

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Rabbit sera containing antibodies specific for the L2G Peptide were identified by ELISA assay. The animal with the strongest serum titer was selected for a splenectomy. The viable spleen was shipped to Eptomics, Inc (Burlingame, CA) overnight where the immunized spleen cells were prepared for fusion with an immortalized cell line (240E-w) as described, *e.g.*, in U.S. Pat. No. 5675063 or European Pat. No. EP0815213B1.

Hybridoma supernatants were tested by ELISA assay for the presence of antibodies specific for the L2G Peptide. One hybridoma was selected based on a relatively high antibody titer in the corresponding supernatant. The specificity of antibodies produced by the selected hybridoma cell line was confirmed by immunohistochemistry (IHC) testing on known EGFR-positive tissues (including squamous cell carcinoma of the lung, colon adenocarcinomas, and normal skin). The hybridoma cell line delivered by the manufacturer was subcloned to homogeneity to isolate a high-producing hybridoma clone designated 5B7.

A Western blot analysis was performed to ensure the specificity of clone 5B7. Total protein lysates were prepared from A431 cells, which are known to express high levels of EGFR on their cell surface, and from BT474 cells, which are negative for EGFR, but which express related EGFR family members, EGFR2 and EGFR3. As shown in FIG. 4, clone 5B7 and a mouse monoclonal antibody specific for the EGFR external domain (clone 3C6) recognized the same 170 kDa band, which is consistent with the size of the EGFR protein. The lack of staining of the BT474 cell lysates for both antibodies indicated that neither antibody cross reacted with EGFR family members that have conserved homology. Thus, the 3C6 and 5B7 antibodies were specific for EGFR.

Example 2

EXEMPLARY METHODS FOR IMMUNOHISTOCHEMICAL STAINING OF TISSUE WITH EGFR-SPECIFIC ANTIBODIES

Immunohistochemistry is the well-known method and variations on such methods are readily determined with routine experimentation by those of ordinary skill in the art (see, *e.g.*, Dabbs, *Diagnostic Immunohistochemistry*, Churchill Livingstone, 2002). Exemplary methods for detecting in FFPE tissue by manual

- IHC an EGFR RD-binding molecule (*e.g.*, monoclonal antibody clone 5B7) or an antigen-binding molecule specific for the EGFR extracellular domain (*e.g.*, monoclonal antibody clone 3C6) are provided in Table 10. One skilled in the art will recognize that similar methods provide in the Tables below can be used for
- 5 diagnostic specific binding reagents for other RTKs.

Table 10: Exemplary IHC Methods

Step #	Manual IHC Assay
0	Fresh tissue is placed in a fixative (such as, 10% neutral buffered formalin) for approximately 12-48 hours at room temperature. Then, the tissue is dehydrated through graded alcohols (<i>e.g.</i> , 50% to 70% to 90% to 95% to 100% EtOH) for 1-2 hours at each grade, and infiltrated with a clearing reagent (such as, xylene) for 3-5 hours at room temperature. The cleared tissue is placed in melted (approximately 63 degrees C) paraffin for 3-6 hours. Samples are removed and embedded in paraffin blocks for subsequent microtome sectioning. 3-10 μ m sections are cut and placed on glass slides.
1	Deparaffinize tissue sections in xylene; then, rehydrate through graded alcohols to distilled water.
2	Place tissue sections in 0.5% v/v hydrogen peroxide/methanol for approximately 10 minutes.
3	Pretreat slides for antigen retrieval using an appropriate method (<i>e.g.</i> , high-temperature antigen unmasking, trypsin, <i>etc.</i>) if required.
4	Wash slides with distilled water for approximately 5 minutes.
5	Wash slides in saline buffer (<i>e.g.</i> , PBS, TBS) for 5 minutes.
6	Cover tissue sections with blocking reagent (<i>e.g.</i> , 10% v/v normal rabbit serum in buffer) for approximately 10 minutes.
7	Remove excess blocking reagent and replace with primary antibody (<i>e.g.</i> , rabbit monoclonal antibody or mouse monoclonal antibody) diluted in blocking reagent as required for approximately 60 minutes at 25°C or overnight at 4°C.
8	Wash twice in buffer for approximately 5 minutes per wash.
9	Remove excess buffer and incubate tissue sections with biotinylated secondary antibody (<i>e.g.</i> , biotinylated rabbit anti-mouse antibody or biotinylated goat anti-rabbit antibody as appropriate for the subject primary antibody) diluted in blocking reagent for 30 minutes at 25°C.
10	Wash twice in buffer for approximately 5 minutes per wash.
11	Remove excess buffer and incubate tissue sections with streptavidin-horse radish peroxidase (HRP) conjugate for 30 minutes at 25°C.
12	Wash twice in buffer for approximately 5 minutes per wash.
13	Develop detectable color with 3,3'-diaminobenzidine tetrahydrochloride (DAB) at room temperature for approximately 5-10 minutes.
14	Rinse slides in water.
15	If desired counterstain with hematoxylin (<i>e.g.</i> , Carson, <i>Histotechnology: A Self-Instructional Text</i> , Chicago:ASCP Press, 1997).
16	Dehydrate, clear and mount coverslip on slides.

IHC for the detection antibodies specific for the EGFR regulatory domain also can be performed on automated staining platforms, such as the BenchMark™

series instruments manufactured by Ventana Medical Systems (Tucson, AZ). An exemplary assay for the detection of a monoclonal antibody specific for the EGFR regulatory domain (*e.g.*, clone 5B7) on a BenchMark™ series automated tissue stainer is described in Table 11.

5

Table 11: Exemplary method of monoclonal antibody detection

Step #	Anti-EGFR Regulatory Domain Antibody Staining (Automated Assay)
1	***** Select EZ Prep *****
2	***** Start Timed Steps *****
3	***** Mixers Off *****
4	Warmup Slide to 75°C, and Incubate for 4 Minutes
5	Apply EZPrep Volume Adjust
6	Rinse Slide
7	Apply EZPrep Volume Adjust
8	Rinse Slide
9	Apply EZPrep Volume Adjust
10	Apply Coverslip
11	Warmup Slide to 76°C, and Incubate for 4 Minutes
12	Rinse Slide
13	Apply Depar Volume Adjust
14	Apply Coverslip
15	Disable Slide Heater
16	***** Mixers On *****
17	[Short - 8 Minute Conditioning]
18	Rinse Slide
19	Apply Long Cell Conditioner #1
20	Apply CC Coverslip Long
21	***** Select SSC Wash *****
22	Warmup Slide to 95°C, and Incubate for 8 Minutes
23	[Mild - 30 Minute Conditioning]
24	Apply Cell Conditioner #1
25	Apply CC Medium Coverslip No BB
26	Warmup Slide to 100°C, and Incubate for 4 Minutes
27	Apply CC Medium Coverslip No BB
28	Apply Cell Conditioner #1
29	Apply CC Medium Coverslip No BB
30	Apply Cell Conditioner #1
31	Apply CC Medium Coverslip No BB
32	Apply Cell Conditioner #1
33	Apply CC Medium Coverslip No BB
34	Apply Cell Conditioner #1
35	Apply CC Medium Coverslip No BB
36	Apply Cell Conditioner #1
37	Apply CC Medium Coverslip No BB
38	[Standard - 60 Minute Conditioning]
39	Apply Cell Conditioner #1
40	Apply CC Medium Coverslip No BB

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Step #	Anti-EGFR Regulatory Domain Antibody Staining (Automated Assay)
41	Apply Cell Conditioner #1
42	Apply CC Medium Coverslip No BB
43	Apply Cell Conditioner #1
44	Apply CC Medium Coverslip No BB
45	Apply Cell Conditioner #1
46	Apply CC Medium Coverslip No BB
47	Apply Cell Conditioner #1
48	Apply CC Medium Coverslip No BB
49	Apply Short Cell Conditioner #1
50	Apply CC Medium Coverslip No BB
51	Apply Cell Conditioner #1
52	Apply CC Medium Coverslip No BB
53	Disable Slide Heater
54	Incubate for 8 Minutes
55	Rinse Slide With Reaction Buffer
56	Adjust Slide Volume With Reaction Buffer
57	Apply Coverslip
58	Rinse Slide With Reaction Buffer
59	Adjust Slide Volume With Reaction Buffer
60	Apply Coverslip
61	***** Procedure Synchronization *****
62	Warmup Slide to 37°C, and Incubate for 4 Minutes
63	Rinse Slide With Reaction Buffer
64	Adjust Slide Volume With Reaction Buffer
65	Apply One Drop of I-VIEW INHIBITOR, Apply Coverslip, and Incubate for 4 Minutes
66	Rinse Slide With Reaction Buffer
67	Adjust Slide Volume With Reaction Buffer
68	Apply Coverslip
69	Warmup Slide to 37°C, and Incubate for 4 Minutes
70	Rinse Slide With Reaction Buffer
71	Adjust Slide Volume With Reaction Buffer
72	Apply Coverslip
73	Apply One Drop of Antibody (e.g., clone 5B7), and Incubate for [0 Hr 16 Min]
74	Rinse Slide With Reaction Buffer
75	Adjust Slide Volume With Reaction Buffer
76	Apply Coverslip
77	Warmup Slide to 37°C, and Incubate for 4 Minutes
78	Rinse Slide With Reaction Buffer
79	Adjust Slide Volume With Reaction Buffer
80	Apply One Drop of I-VIEW BIOTIN Ig, Apply Coverslip, and Incubate for 8 Minutes
81	Rinse Slide With Reaction Buffer
82	Adjust Slide Volume With Reaction Buffer
83	Apply One Drop of I-VIEW SA-HRP, Apply Coverslip, and Incubate for 8 Minutes
84	Rinse Slide With Reaction Buffer
85	Adjust Slide Volume With Reaction Buffer
86	Apply Coverslip

Step #	Anti-EGFR Regulatory Domain Antibody Staining (Automated Assay)
87	Rinse Slide With Reaction Buffer
88	Adjust Slide Volume With Reaction Buffer
89	Apply One Drop of I-VIEW DAB and One Drop of I-VIEW H ₂ O ₂ , Apply Coverslip, Incubate for 8 Minutes
90	Rinse Slide With Reaction Buffer
91	Adjust Slide Volume With Reaction Buffer
92	Apply One Drop of I-VIEW COPPER, Apply Coverslip, and Incubate for 4 Minutes
93	Rinse Slide With Reaction Buffer
94	Adjust Slide Volume With Reaction Buffer
95	Apply One Drop of [HEMATOXYLIN II] (Counterstain), Apply Coverslip, and Incubate for [4 Minutes]
96	Rinse Slide With Reaction Buffer
97	Adjust Slide Volume With Reaction Buffer
98	Apply Coverslip
99	Rinse Slide With Reaction Buffer
100	Adjust Slide Volume With Reaction Buffer
101	Apply One Drop of [BLUING REAGENT] (Post Counterstain), Apply Coverslip, and Incubate for [4 Minutes]
102	Rinse Slide With Reaction Buffer
103	Apply Coverslip
104	Disable Slide Heater
105	***** Select Optional Wash *****
106	***** Select SSC Wash *****
107	***** Start Timed Steps *****
108	Rinse Slide With Reaction Buffer

An exemplary assay for the detection of a monoclonal antibody specific for the EGFR external domain (*e.g.*, clone 3C6) on a BenchMark™ series automated tissue stainer is described in Table 12.

5

Table 12: Exemplary method for detecting RTK extracellular domain

Step #	Anti-EGFR External Domain Antibody Staining (Automated Assay)
1	***** Select EZ Prep *****
2	***** Start Timed Steps *****
3	***** Mixers Off *****
4	Warm Slide to 75°C, and Incubate for 4 Minutes
5	Apply EZPrep Volume Adjust
6	Rinse Slide
7	Apply EZPrep Volume Adjust
8	Rinse Slide
9	Apply EZPrep Volume Adjust
10	Apply Coverslip
11	Warm Slide to 76°C, and Incubate for 4 Minutes
12	Rinse Slide
13	Apply Depar Volume Adjust

Step #	Anti-EGFR External Domain Antibody Staining (Automated Assay)
14	Apply Coverslip
15	Disable Slide Heater
16	***** Mixers On *****
17	Disable Slide Heater
18	***** Select SSC Wash *****
19	Rinse Slide With Reaction Buffer
20	Adjust Slide Volume With Reaction Buffer
21	Apply Coverslip
22	Rinse Slide With Reaction Buffer
23	Adjust Slide Volume With Reaction Buffer
24	Apply Coverslip
25	***** Procedure Synchronization *****
26	Warm Slide to 37°C, and Incubate for 4 Minutes
27	Rinse Slide With Reaction Buffer
28	Adjust Slide Volume With Reaction Buffer
29	Apply One Drop of I-VIEW INHIBITOR, Apply Coverslip, and Incubate for 4 Minutes
30	Rinse Slide With Reaction Buffer
31	Adjust Slide Volume With Reaction Buffer
32	Apply One Drop of [PROTEASE 1] (Enzyme), Apply Coverslip, and Incubate for [8 Minutes]
33	Rinse Slide With Reaction Buffer
34	Adjust Slide Volume With Reaction Buffer
35	Apply Coverslip
36	Warm Slide to 37°C, and Incubate for 4 Minutes
37	Rinse Slide With Reaction Buffer
38	Adjust Slide Volume With Reaction Buffer
39	Apply Coverslip
40	Apply One Drop of Antibody (e.g., clone 3C6), and Incubate for [0 Hr 32 Min]
41	Rinse Slide With Reaction Buffer
42	Adjust Slide Volume With Reaction Buffer
43	Apply Coverslip
44	Warm Slide to 37°C, and Incubate for 4 Minutes
45	Rinse Slide With Reaction Buffer
46	Adjust Slide Volume With Reaction Buffer
47	Apply One Drop of I-VIEW BIOTIN Ig, Apply Coverslip, and Incubate for 8 Minutes
48	Rinse Slide With Reaction Buffer
49	Adjust Slide Volume With Reaction Buffer
50	Apply One Drop of I-VIEW SA-HRP, Apply Coverslip, and Incubate for 8 Minutes
51	Rinse Slide With Reaction Buffer
52	Adjust Slide Volume With Reaction Buffer
53	Apply Coverslip
54	Rinse Slide With Reaction Buffer
55	Adjust Slide Volume With Reaction Buffer
56	Apply One Drop of I-VIEW DAB and One Drop of I-VIEW H ₂ O ₂ , Apply Coverslip, Incubate for 8 Minutes
57	Rinse Slide With Reaction Buffer

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Step #	Anti-EGFR External Domain Antibody Staining (Automated Assay)
58	Adjust Slide Volume With Reaction Buffer
59	Apply One Drop of I-VIEW COPPER, Apply Coverslip, and Incubate for 4 Minutes
60	Rinse Slide With Reaction Buffer
61	Adjust Slide Volume With Reaction Buffer
62	Apply One Drop of [HEMATOXYLIN II] (Counterstain), Apply Coverslip, and Incubate for [4 Minutes]
63	Rinse Slide With Reaction Buffer
64	Adjust Slide Volume With Reaction Buffer
65	Apply Coverslip
66	Rinse Slide With Reaction Buffer
67	Adjust Slide Volume With Reaction Buffer
68	Apply One Drop of [BLUING REAGENT] (Post Counterstain), Apply Coverslip, and Incubate for [4 Minutes]
69	Rinse Slide With Reaction Buffer
70	Apply Coverslip
71	Disable Slide Heater
72	***** Select Optional Wash *****
73	***** Select SSC Wash *****
74	***** Start Timed Steps *****
75	Rinse Slide With Reaction Buffer

Example 3

ANTIBODY SPECIFIC FOR EGFR REGULATORY DOMAIN EPITOPE UNEXPECTEDLY DOES NOT SUBSTANTIALLY BIND TO SOME EGFR-POSITIVE TISSUES

This Example demonstrates that RD-binding molecules, such as clone 5B7, exhibited differential binding to EGFR-positive tissues (as detected by an antibody specific for the EGFR external domain). As described in more detail below, but without being limited to a single theory, this differential binding is believed to be due to the differential expression of EGFR regulatory proteins (*e.g.*, SOCS proteins like SOCS1 or SOCS3) in EGFR-positive tissues. Such regulatory proteins, when directly associated with the EGFR regulatory domain, mask the epitopes of RD-binding molecules.

A. Normal Human Tissues

The staining by IHC of antibodies specific for the EGFR regulatory domain (*i.e.*, clone 5B7) and external domain (*i.e.*, clone 3C6) in FFPE 30 normal human tissues were compared. Tissue arrays were obtained from USBiomax (Igamsville,

MD; Cat. No. FDA801). Automated staining protocols as described in Example 2 were used to stain the tissue arrays on a BenchMark™ automated tissues stainer.

As shown in FIG. 5, extracellular-domain-specific clone 3C6 positively stained skin, testis, tonsil, liver and placenta tissues, which is consistent with known EGFR expression patterns in normal tissue. FIG. 5 also shows that regulatory-domain-specific clone 5B7 reacted positively with normal skin, testis, and tonsil. However, surprisingly, this antibody did not react with normal liver (see, e.g., FIG. 5B) and had variable reactivity with placental EGFR depending on the stage of development of the placenta (*Virchows Archiv A Pathol Anat.*, 420:385-393, 1992).

B. Human NSCLC Tumors

Non-small cell lung cancer (NSCLC) cells are known to express EGFR in approximately 75% of tumors. Regulatory-domain-specific clone 5B7 and external-domain-specific clone 3C6 were used to stain a cohort of NSCLC cases from three commercially available tissue micro arrays (Array LC801 and Array LC819 (Biomax; Ijamsville, MD) and Array IMH-305 (Imgenex (San Diego, CA)).

As shown in Table 13, Subpart A, clone 3C6 detected EGFR in 83% of the lung cases (as would be expected based on literature estimates of EGFR staining in NSCLC) while clone 5B7 stained positively 65% of lung tumors. This corresponds to an 18.5% discordance between clone 3C6 and clone 5B7 with the latter exhibiting no staining in 38 cases that were positive for clone 3C6 staining.

Table 13. Summary of NSCLC Immunohistochemistry Study

Subpart A			
5B7	3C6		
	Positive	Negative	Total
Positive	132	1	133
Negative	38	34	72
Total	170	35	205
Subpart B			
Sensitivity	78%		
Specificity	97%		
Overall	81%		
Kappa	67%		

As summarized in Table 13, Subpart B, the two antibodies each stained positively in 78% of cases (*i.e.*, Sensitivity (132/[132+38])); the two antibodies each stained negatively in 97% of cases (*i.e.*, Specificity (34/[1+34])). The overall agreement was 81% ([132+43]/205). The Kappa statistic, which is another measure of agreement, can be interpreted as follows: <0 =No agreement, 0.0-0.19=Poor agreement, 0.20-0.39=Fair agreement, 0.40-0.59=Moderate agreement, 0.60-0.79=Substantial agreement and 0.80-1.00=Almost perfect agreement (Landis and Koch, Biometrics, 33:159-174, 1977). The Kappa score for 3C6 versus 5B7 was 67% which falls into the substantial agreement category.

Particular examples demonstrating differences in the binding of EGFR regulatory-domain-specific clone 5B7 and EGFR external-domain-specific clone 3C6 to squamous cell carcinomas of the lung are shown in FIG. 6. Case 1 (left panel) showed equivalent staining of cells by the two antibodies, which indicates (among other things) that there is not a difference in the general sensitivity of clone 5B7 as compared to clone 3C6 when the epitope for each is accessible. Case 2 (right panel) showed distinctly different staining between the two antibodies with clone 5B7 being negative and clone 3C6 being 3+ positive (0-3+ scale).

The differential binding of clone 5B7 (specific for the EGFR intracellular regulatory domain) as compared to clone 3C6 (specific for the EGFR extracellular domain) in normal and neoplastic tissues as shown in this Example strongly supports the belief that the clone 5B7 epitope was accessible only in some tissues.

Example 4

EPITOPE MAPPING OF MONOCLONAL ANTIBODY CLONE 5B7

The epitope for the EGFR regulatory-domain-specific monoclonal antibody, clone 5B7, was mapped by peptide inhibition studies. Because the L2G Peptide was used as the immunogen and was used to screen for positive clones, it was known that the 5B7 epitope must be within that 19-amino acid sequence (see SEQ ID NO: 2). The L2G Peptide, a peptide containing the 13 C-terminal amino acid residues of the L2G Peptide, and a peptide containing the six N-terminal amino acid residues of the L2G Peptide plus three additional N-terminal residues (*i.e.*, QIS) corresponding to the respective positions in the human EGFR sequence. The amino

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acid sequences of the subject peptides are shown in FIG. 7A. The peptides were synthesized by Genemed Synthesis, Inc. (South San Francisco, CA).

A known EGFR-positive lung squamous cell carcinoma was chosen for the peptide inhibition study. The 5B7 antibody was pre-incubated with each peptide for
5 1 hour at room temperature before application to the tissue. A 1000-fold molar excess of peptide compared to antibody was used.

As shown in FIG. 7, Peptide 1 resulted in partial inhibition of 5B7 binding, which indicated that part of the epitope was contained within Peptide 1. Peptide 2 resulted in complete inhibition of 5B7 binding, which indicated that the primary
10 epitope was contained in Peptide 2. Logically, the three amino acids shared by Peptides 1 and 2 (*i.e.*, QQD) must contain at least part of the full epitope. Peptide 3 also completely inhibited 5B7 binding; thus, the tyrosine did not significantly contribute to the 5B7 epitope. An average epitope is on the order of 7-12 contiguous amino acids; thus, the boxed residues in FIG. 7 represent a likely full-length 5B7
15 epitope with some possibility for an additional 1-5 residues at the C-terminal end.

Example 5

SOCS3 KNOCKOUT UNMASKS CLONE 5B7 EPITOPE

The L2G Peptide of the EGFR sequence is within the binding region for
20 SOCS3 (Xia *et al.*, *J. Biol. Chem.*, 277(34):30716-23, 2002). Thus, it was postulated that SOCS3 may be masking the 5B7 epitope in some tissues. To test this hypothesis, livers from a hepatic-specific, SOCS3-knockout mouse were obtained from the laboratory that developed the model (Ogata *et al.*, *Gastroenterology*, 131(1):179-93, 2006). Sections of formalin-fixed, paraffin-embedded livers from
25 wild-type and SOCS3 mice were stained with clone 5B7 as described in Example 2.

As shown in the left two panels of FIG. 8, 5B7 failed to stain normal liver, which expresses SOCS3. In comparison, as shown in the right two panels of FIG. 8, 5B7 positively stained the membranes of cells in livers lacking SOCS3. These results indicate that the loss or absence of SOCS3 allows for the binding of
30 clone 5B7 to the regulatory domain of EGFR.

SOCS3 is only one example of a regulatory molecule that directly interacts with EGFR. The results demonstrated herein are widely applicable to other

interface-specific binding molecules that have epitopes in the interface between two components of a molecular complex, such as between EGFR and its many regulatory proteins.

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Example 6

**EGFR RD-BINDING MOLECULES, SUCH AS CLONE 5B7, PREDICT THE
RESPONSE OF NSCLC CANCER PATIENTS TO
EGFR-INHIBITOR THERAPY**

This Example demonstrates that a disclosed RD-binding molecule (*e.g.*,
10 clone 5B7) predicts the response of NSCLC cancer patients to EGFR-inhibitor
therapy (IRESSA™).

Tissue arrays containing biopsy samples from at least 100 NSCLC cancer
patients are obtained. Each patient is treated with IRESSA™ (EGFR-inhibitor)
therapy with a dosage of 250 mg/day given orally. Each patient has post-therapy
15 follow-up for up to 5 years. Each biopsy sample is fixed in 10% NBF and paraffin
embedded. Five (5) micron sections of each biopsy sample are cut and arrayed on
positively charged glass slides. The slides are stained with an RD-binding molecule
(*e.g.*, clone 5B7) and an ED-binding molecule (*e.g.*, clone 3C6) according to the
protocols in Example 2. The resulting stained array slides are scored by light
20 microscopy by a pathologist according to the following criteria:

Report Result	Staining Intensity Score	Microscope Observation
<u>Positive:</u> Any IHC staining of tumor cell membranes above background level whether it is complete or incomplete circumferential staining in more than 0% tumor cells	3+	<u>Strong reactivity:</u> Dark brown to black staining is usually, but not always, in a complete membrane pattern, producing a thick outline of the cell. Cytoplasmic reactivity may be absent or may be moderately intense when membrane staining is very intense. Submembranous cytoplasmic accentuation may be present.
	2.5	<u>Intense reactivity:</u> Shades of brown staining of medium darkness (intensity). Membranous reactivity is usually but not always complete, producing a circular outline of the neoplastic cell. Incomplete membrane reactivity of moderate intensity is also considered 2+. The cytoplasmic reactivity is of weaker intensity than the membrane reactivity.
	2+	<u>Moderate reactivity:</u> Shades of brown staining of intermediate darkness (intensity). Membranous reactivity is usually but not always complete, producing a circular outline of the neoplastic cell. Incomplete membrane reactivity of moderate intensity is also considered 2+. The cytoplasmic reactivity is of weaker intensity than the membrane reactivity.

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Report Result	Staining Intensity Score	Microscope Observation
	1.5	<u>Slight reactivity</u> : Staining of intermediate intensity that is membranous. Cytoplasmic reactivity that is uniform and involves all the cytoplasm may be present, but should not be evaluated for positivity.
	1+	<u>Weak reactivity</u> : Faint or light brown reactivity that is membranous. Cytoplasmic reactivity that is uniform and involves all the cytoplasm may be present, but should not be evaluated for positivity.
<u>Negative</u> : Absence of membrane staining above background in all tumor cells. Presence of cytoplasmic in the absence of membrane staining.	0.5	<u>Trace reactivity</u> : Trace brown reactivity where membranous and cytoplasmic localization is indeterminate.
	0	<u>No reactivity</u>

The score for each case is recorded in a database comparing the score for each binding molecule (*e.g.*, 5B7 or 3C6). The result of each case is assigned to 1 of the 4 categories described in Table 2. Approximately 65% of cases are expected to fall into category 1, 19% in category 2, <1% in category 3 and 15% in category 4. Patient outcome is directly related to the scoring category as indicated in Table 2 for an ID-based therapy. Patients in categories 1 and 3 will have an objective response to IRESSA™ therapy and patients in categories 2 and 4 will not significantly respond to IRESSA™ therapy.

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Example 7

EGFR RD-BINDING MOLECULES, SUCH AS CLONE 5B7, PREDICT THE RESPONSE OF NSCLC CANCER PATIENTS TO EGFR-INHIBITOR THERAPY

This Example demonstrates that a disclosed RD-binding molecule (*e.g.*, clone 5B7) predicts the response of NSCLC cancer patients to EGFR-inhibitor therapy (TARCEVA™).

Tissue arrays containing biopsy samples from at least 100 NSCLC cancer patients are obtained. Each patient is treated with TARCEVA™ (EGFR inhibitor) therapy with a dosage of 150 mg/day given orally. Each patient has post-therapy follow-up for up to 5 years. Each biopsy sample is fixed in 10% NBF and paraffin embedded. Five (5) micron sections of each biopsy sample are cut and arrayed on

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positively charged glass slides. The slides are stained with an RD-binding molecule (*e.g.*, clone 5B7) and an ED-binding molecule (*e.g.*, clone 3C6) according to the protocols in Example 2. The resulting stained array slides are scored by light microscopy by a pathologist according to the following criteria:

Report Result	Staining Intensity Score	Microscope Observation
<u>Positive:</u> Any IHC staining of tumor cell membranes above background level whether it is complete or incomplete circumferential staining in more than 0% tumor cells	3+	<u>Strong reactivity:</u> Dark brown to black staining is usually, but not always, in a complete membrane pattern, producing a thick outline of the cell. Cytoplasmic reactivity may be absent or may be moderately intense when membrane staining is very intense. Submembranous cytoplasmic accentuation may be present.
	2.5	<u>Intense reactivity:</u> Shades of brown staining of medium darkness (intensity). Membranous reactivity is usually but not always complete, producing a circular outline of the neoplastic cell. Incomplete membrane reactivity of moderate intensity is also considered 2+. The cytoplasmic reactivity is of weaker intensity than the membrane reactivity.
	2+	<u>Moderate reactivity:</u> Shades of brown staining of intermediate darkness (intensity). Membranous reactivity is usually but not always complete, producing a circular outline of the neoplastic cell. Incomplete membrane reactivity of moderate intensity is also considered 2+. The cytoplasmic reactivity is of weaker intensity than the membrane reactivity.
	1.5	<u>Slight reactivity:</u> Staining of intermediate intensity that is membranous. Cytoplasmic reactivity that is uniform and involves all the cytoplasm may be present, but should not be evaluated for positivity.
	1+	<u>Weak reactivity:</u> Faint or light brown reactivity that is membranous. Cytoplasmic reactivity that is uniform and involves all the cytoplasm may be present, but should not be evaluated for positivity.
<u>Negative:</u> Absence of membrane staining above background in all tumor cells. Presence of cytoplasmic in the absence of membrane staining.	0.5	<u>Trace reactivity:</u> Trace brown reactivity where membranous and cytoplasmic localization is indeterminate.
	0	<u>No reactivity</u>

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The score for each case is recorded in a database comparing the score for each binding molecule (*e.g.*, 5B7 or 3C6). The result of each case is assigned to 1 of the 4 categories described in Table 2. Approximately 65% of cases are expected to fall into category 1, 19% in category 2, <1% in category 3 and 15% in category 4.

10 Patient outcome is directly related to the scoring category as indicated in Table 2 for an ID-based therapy. Patients in categories 1 and 3 will have an objective response

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to TARCEVA™ therapy and Patients in categories 2 and 4 will not significantly respond to TARCEVA™ therapy.

Example 8

5 EGFR RD-BINDING MOLECULES, SUCH AS CLONE 5B7, PREDICT THE RESPONSE OF COLORECTAL CANCER PATIENTS TO EGFR-INHIBITOR THERAPY

This Example demonstrates that a disclosed RD-binding molecule (*e.g.*, clone 5B7) predicts the response of colorectal cancer patients to EGFR-inhibitor therapy (ERBITUX™).

Tissue arrays containing biopsy samples from at least 100 colorectal cancer patients are obtained. Each patient is treated with ERBITUX™ (EGFR inhibitor) therapy with a dosage of 400 mg/m² given i.v.. Each patient has post-therapy follow-up for up to 5 years. Each biopsy sample is fixed in 10% NBF and paraffin embedded. Five (5) micron sections of each biopsy sample are cut and arrayed on positively charged glass slides. The slides are stained with an RD-binding molecule (*e.g.*, clone 5B7) and an ED-binding molecule (*e.g.*, clone 3C6) according to the protocols in Example 2. The resulting stained array slides are scored by light microscopy by a pathologist according to the following criteria:

Report Result	Staining Intensity Score	Microscope Observation
<u>Positive:</u> Any IHC staining of tumor cell membranes above background level whether it is complete or incomplete circumferential staining in more than 0% tumor cells	3+	<u>Strong reactivity:</u> Dark brown to black staining is usually, but not always, in a complete membrane pattern, producing a thick outline of the cell. Cytoplasmic reactivity may be absent or may be moderately intense when membrane staining is very intense. Submembranous cytoplasmic accentuation may be present.
	2.5	<u>Intense reactivity:</u> Shades of brown staining of medium darkness (intensity). Membranous reactivity is usually but not always complete, producing a circular outline of the neoplastic cell. Incomplete membrane reactivity of moderate intensity is also considered 2+. The cytoplasmic reactivity is of weaker intensity than the membrane reactivity.
	2+	<u>Moderate reactivity:</u> Shades of brown staining of intermediate darkness (intensity). Membranous reactivity is usually but not always complete, producing a circular outline of the neoplastic cell. Incomplete membrane reactivity of moderate intensity is also considered 2+. The cytoplasmic reactivity is of weaker intensity than the membrane reactivity.

Report Result	Staining Intensity Score	Microscope Observation
	1.5	<u>Slight reactivity</u> : Staining of intermediate intensity that is membranous. Cytoplasmic reactivity that is uniform and involves all the cytoplasm may be present, but should not be evaluated for positivity.
	1+	<u>Weak reactivity</u> : Faint or light brown reactivity that is membranous. Cytoplasmic reactivity that is uniform and involves all the cytoplasm may be present, but should not be evaluated for positivity.
<u>Negative</u> : Absence of membrane staining above background in all tumor cells. Presence of cytoplasmic in the absence of membrane staining.	0.5	<u>Trace reactivity</u> : Trace brown reactivity where membranous and cytoplasmic localization is indeterminate.
	0	<u>No reactivity</u>

The score for each case is recorded in a database comparing the score for each binding molecule (*e.g.*, 5B7 or 3C6). The result of each case is assigned to 1 of the 4 categories described in Table 2. Approximately 65% of cases are expected to fall into category 1, 19% in category 2, <1% in category 3 and 15% in category 4. Patient outcome is directly related to the scoring category as indicated in Table 2 for an ED-based therapy. Patients in category 1 will have an objective response to ERBITUX™ therapy, and patients in categories 2, 3 and 4 will not significantly respond to ERBITUX™ therapy.

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Example 9

EGFR RD-BINDING MOLECULES, SUCH AS CLONE 5B7, PREDICT THE RESPONSE OF COLORECTAL CANCER PATIENTS TO EGFR-INHIBITOR THERAPY

This Example demonstrates that a disclosed RD-binding molecule (*e.g.*, clone 5B7) predicts the response of colorectal cancer patients to EGFR-inhibitor therapy (VECTIBIX™).

Tissue arrays containing biopsy samples from at least 100 colorectal cancer patients are obtained. Each patient is treated with VECTIBIX™ (EGFR inhibitor) therapy with a dosage of 6 mg/kg given i.v.. Each patient has post-therapy follow-up for up to 5 years. Each biopsy sample is fixed in 10% NBF and paraffin embedded. Five (5) micron sections of each biopsy sample are cut and arrayed on

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positively charged glass slides. The slides are stained with an RD-binding molecule (*e.g.*, clone 5B7) and an ED-binding molecule (*e.g.*, clone 3C6) according to the protocols in Example 2. The resulting stained array slides are scored by light microscopy by a pathologist according to the following criteria:

Report Result	Staining Intensity Score	Microscope Observation
<u>Positive:</u> Any IHC staining of tumor cell membranes above background level whether it is complete or incomplete circumferential staining in more than 0% tumor cells	3+	<u>Strong reactivity:</u> Dark brown to black staining is usually, but not always, in a complete membrane pattern, producing a thick outline of the cell. Cytoplasmic reactivity may be absent or may be moderately intense when membrane staining is very intense. Submembranous cytoplasmic accentuation may be present.
	2.5	<u>Intense reactivity:</u> Shades of brown staining of medium darkness (intensity). Membranous reactivity is usually but not always complete, producing a circular outline of the neoplastic cell. Incomplete membrane reactivity of moderate intensity is also considered 2+. The cytoplasmic reactivity is of weaker intensity than the membrane reactivity.
	2+	<u>Moderate reactivity:</u> Shades of brown staining of intermediate darkness (intensity). Membranous reactivity is usually but not always complete, producing a circular outline of the neoplastic cell. Incomplete membrane reactivity of moderate intensity is also considered 2+. The cytoplasmic reactivity is of weaker intensity than the membrane reactivity.
	1.5	<u>Slight reactivity:</u> Staining of intermediate intensity that is membranous. Cytoplasmic reactivity that is uniform and involves all the cytoplasm may be present, but should not be evaluated for positivity.
	1+	<u>Weak reactivity:</u> Faint or light brown reactivity that is membranous. Cytoplasmic reactivity that is uniform and involves all the cytoplasm may be present, but should not be evaluated for positivity.
<u>Negative:</u> Absence of membrane staining above background in all tumor cells. Presence of cytoplasmic in the absence of membrane staining.	0.5	<u>Trace reactivity:</u> Trace brown reactivity where membranous and cytoplasmic localization is indeterminate.
	0	<u>No reactivity</u>

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The score for each case is recorded in a database comparing the score for each binding molecule (*e.g.*, 5B7 or 3C6). The result of each case is assigned to 1 of the 4 categories described in Table 2. Approximately 65% of cases are expected to fall into category 1, 19% in category 2, <1% in category 3 and 15% in category 4.

- 10 Patient outcome is directly related to the scoring category as indicated in Table 2 for an ED-based therapy. Patients in category 1 will have an objective response to VECTIBIX™ therapy and, patients in categories 2, 3 and 4 will not significantly respond to VECTIBIX™ therapy.

Example 10

**EGFR RD-BINDING MOLECULES, SUCH AS CLONE 5B7, PREDICT THE
RESPONSE OF BREAST CANCER PATIENTS TO
EGFR-INHIBITOR THERAPY**

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This Example demonstrates that a disclosed RD-binding molecule (*e.g.*, clone 5B7) predicts the response of breast cancer patients to EGFR-inhibitor therapy or, more particularly, HER1 (EGFR)/HER2-inhibitor therapy (such as, lapatinib (TYKERB™)).

10

Tissue arrays containing biopsy samples from at least 100 breast cancer patients are obtained. Each patient is treated with lapatinib (TYKERB™) (HER1 (EGFR)/HER2-inhibitor) with a dosage of 1250-1500 mg/day given orally. Each patient has post-therapy follow-up for at least 20 months. Each biopsy sample is fixed in a standard fixative and paraffin embedded. Sections of each biopsy

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sample (*e.g.*, 5 µm thick) are cut and arrayed on positively charged glass slides. The slides are stained with an RD-binding molecule (*e.g.*, clone 5B7) and an ED-binding molecule (*e.g.*, clone 3C6) according to the protocols in Example 2. The resulting stained array slides are scored by light microscopy by a pathologist according to the following criteria:

Report Result	Staining Intensity Score	Microscope Observation
<u>Positive:</u> Any IHC staining of tumor cell membranes above background level whether it is complete or incomplete circumferential staining in more than 0% tumor cells	3+	<u>Strong reactivity:</u> Dark brown to black staining is usually, but not always, in a complete membrane pattern, producing a thick outline of the cell. Cytoplasmic reactivity may be absent or may be moderately intense when membrane staining is very intense. Submembranous cytoplasmic accentuation may be present.
	2.5	<u>Intense reactivity:</u> Shades of brown staining of medium darkness (intensity). Membranous reactivity is usually but not always complete, producing a circular outline of the neoplastic cell. Incomplete membrane reactivity of moderate intensity is also considered 2+. The cytoplasmic reactivity is of weaker intensity than the membrane reactivity.
	2+	<u>Moderate reactivity:</u> Shades of brown staining of intermediate darkness (intensity). Membranous reactivity is usually but not always complete, producing a circular outline of the neoplastic cell. Incomplete membrane reactivity of moderate intensity is also considered 2+. The cytoplasmic reactivity is of weaker intensity than the membrane reactivity.
	1.5	<u>Slight reactivity:</u> Staining of intermediate intensity that is membranous. Cytoplasmic reactivity that is uniform and involves all the cytoplasm may be present, but should not be evaluated for positivity.

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Report Result	Staining Intensity Score	Microscope Observation
	1+	<u>Weak reactivity</u> : Faint or light brown reactivity that is membranous. Cytoplasmic reactivity that is uniform and involves all the cytoplasm may be present, but should not be evaluated for positivity.
<u>Negative</u> : Absence of membrane staining above background in all tumor cells. Presence of cytoplasmic in the absence of membrane staining.	0.5	<u>Trace reactivity</u> : Trace brown reactivity where membranous and cytoplasmic localization is indeterminate.
	0	<u>No reactivity</u>

The score for each case is recorded in a database comparing the score for each binding molecule (*e.g.*, 5B7 or 3C6). The result of each case is assigned to 1 of the 4 categories described in Table 2. Approximately 20% of cases are expected to fall into category 1, 16% in category 2, 3% in category 3 and 61% in category 4. Patient outcome is directly related to the scoring category as indicated in Table 2 for an ID-based therapy. Patients in categories 1 and 3 will have an objective response to lapatinib (TYKERB™) therapy and Patients in categories 2 and 4 will not significantly respond to lapatinib (TYKERB™) therapy.

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Example 11

EGFR RD-BINDING MOLECULES, SUCH AS CLONE 5B7, PREDICT THE RESPONSE OF HEPATOCELLULAR CARCINOMA CANCER PATIENTS TO EGFR-INHIBITOR THERAPY

This Example demonstrates that a disclosed RD-binding molecule (*e.g.*, clone 5B7) predicts the response of hepatocellular carcinoma (“HCC”) (such as, resectable HCC) cancer patients to EGFR-inhibitor therapy (IRESSA™).

Tissue arrays containing biopsy samples from at least 100 HCC cancer patients are obtained (see, for example, samples collected in JS 0414, “A Pilot Study of Adjuvant Therapy of Gefitinib (Iressa, ZD1839) in Patients with Resectable Hepatocellular Carcinoma”, ClinicalTrials.gov Identifier No. NCT00228501). Each patient is treated with IRESSA™ (EGFR-inhibitor) therapy with a dosage of 200-500 mg/day given orally. Each patient has post-therapy follow-up for at least 12 months. Each biopsy sample is fixed in a standard fixative (*e.g.*, 10% NBF) and paraffin embedded. Sections of each biopsy sample (*e.g.*, 5µm thick) are cut and

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arrayed on positively charged glass slides. The slides are stained with an RD-binding molecule (*e.g.*, clone 5B7) and an ED-binding molecule (*e.g.*, clone 3C6) according to the protocols in Example 2. The resulting stained array slides are scored by light microscopy by a pathologist according to the following criteria:

Report Result	Staining Intensity Score	Microscope Observation
<u>Positive:</u> Any IHC staining of tumor cell membranes above background level whether it is complete or incomplete circumferential staining in more than 0% tumor cells	3+	<u>Strong reactivity:</u> Dark brown to black staining is usually, but not always, in a complete membrane pattern, producing a thick outline of the cell. Cytoplasmic reactivity may be absent or may be moderately intense when membrane staining is very intense. Submembranous cytoplasmic accentuation may be present.
	2.5	<u>Intense reactivity:</u> Shades of brown staining of medium darkness (intensity). Membranous reactivity is usually but not always complete, producing a circular outline of the neoplastic cell. Incomplete membrane reactivity of moderate intensity is also considered 2+. The cytoplasmic reactivity is of weaker intensity than the membrane reactivity.
	2+	<u>Moderate reactivity:</u> Shades of brown staining of intermediate darkness (intensity). Membranous reactivity is usually but not always complete, producing a circular outline of the neoplastic cell. Incomplete membrane reactivity of moderate intensity is also considered 2+. The cytoplasmic reactivity is of weaker intensity than the membrane reactivity.
	1.5	<u>Slight reactivity:</u> Staining of intermediate intensity that is membranous. Cytoplasmic reactivity that is uniform and involves all the cytoplasm may be present, but should not be evaluated for positivity.
	1+	<u>Weak reactivity:</u> Faint or light brown reactivity that is membranous. Cytoplasmic reactivity that is uniform and involves all the cytoplasm may be present, but should not be evaluated for positivity.
<u>Negative:</u> Absence of membrane staining above background in all tumor cells. Presence of cytoplasmic in the absence of membrane staining.	0.5	<u>Trace reactivity:</u> Trace brown reactivity where membranous and cytoplasmic localization is indeterminate.
	0	<u>No reactivity</u>

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The score for each case is recorded in a database comparing the score for each binding molecule (*e.g.*, 5B7 or 3C6). The result of each case is assigned to 1 of the 4 categories described in Table 2. Approximately 60% of cases are expected to fall into category 1, 13% in category 2, 5% in category 3 and 22% in category 4.

- 10 Patient outcome is directly related to the scoring category as indicated in Table 2 for an ID-based therapy. Patients in categories 1 and 3 will have an objective response to IRESSA™ therapy and patients in categories 2 and 4 will not significantly respond to IRESSA™ therapy.

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Example 12**CLONE 5B7 STATUS IS A CLEAR INDICATOR OF LUNG CANCER
PROGNOSIS**

This Example demonstrates that clone 5B7 predicts the prognosis of lung
5 cancer patients.

A tissue array containing lung biopsy samples from 109 Stage I or II NSCLC
patients was obtained (a subset of the larger cohort described in Olaussen *et al.*, *New
Engl. J. Med.*, 355(10):983-991, 2006). None of the patients from whom the
biopsies were obtained had been treated with an EGFR-based therapy
10 (*e.g.*, ERBITUX™, VECTIBIX™, IRESSA™, or TARCEVA™). Patient survival
post-diagnosis was monitored on a continuing basis. Each biopsy sample was
paraffin embedded, cancerous areas in the biopsy were identified, a core of the
cancerous area removed, and placed in a donor array paraffin block. Three to five
micron sections of the donor array block were cut and mounted on glass slides.
15 Slides containing serial sections of the donor array block were stained with
clone 5B7 or clone 3C6 according to the protocols in Example 2. The resulting
stained slides are scored by light microscopy by a pathologist according to the
following criteria:

Report Result	Staining Intensity Score	Microscope Observation
<u>Positive:</u> Any IHC staining of tumor cell membranes above background level whether it is complete or incomplete circumferential staining in more than 0% tumor cells	3+	<u>Strong reactivity:</u> Dark brown to black staining is usually, but not always, in a complete membrane pattern, producing a thick outline of the cell. Cytoplasmic reactivity may be absent or may be moderately intense when membrane staining is very intense. Submembranous cytoplasmic accentuation may be present.
	2.5	<u>Intense reactivity:</u> Shades of brown staining of medium darkness (intensity). Membranous reactivity is usually but not always complete, producing a circular outline of the neoplastic cell. Incomplete membrane reactivity of moderate intensity is also considered 2+. The cytoplasmic reactivity is of weaker intensity than the membrane reactivity.
	2+	<u>Moderate reactivity:</u> Shades of brown staining of intermediate darkness (intensity). Membranous reactivity is usually but not always complete, producing a circular outline of the neoplastic cell. Incomplete membrane reactivity of moderate intensity is also considered 2+. The cytoplasmic reactivity is of weaker intensity than the membrane reactivity.
	1.5	<u>Slight reactivity:</u> Staining of intermediate intensity that is membranous. Cytoplasmic reactivity that is uniform and involves all the cytoplasm may be present, but should not be evaluated for positivity.

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Report Result	Staining Intensity Score	Microscope Observation
	1+	<u>Weak reactivity</u> : Faint or light brown reactivity that is membranous. Cytoplasmic reactivity that is uniform and involves all the cytoplasm may be present, but should not be evaluated for positivity.
<u>Negative</u> : Absence of membrane staining above background in all tumor cells. Presence of cytoplasmic in the absence of membrane staining.	0.5	<u>Trace reactivity</u> : Trace brown reactivity where membranous and cytoplasmic localization is indeterminate.
	0	<u>No reactivity</u>

The score for each biopsy sample and the associated follow-up is shown in Table 14.

Table 14: Biopsy scores

Sample Number	3C6 Score	5B7 Score	Months at Recurrence	Months at Death
P003	2	2	None	None
P005	2.5	0.5	35	36
P006	0	2	6	36
P007	0	2	None	None
P008	0	1	5	25
P010	3	0.5	None	None
P011	2.5	0.5	42	57
P012	2.5	2	None	None
P014	2	2.5	70	None
P015	0	1	None	None
P016	1	1	None	None
P017	2.5	2	17	33
P018	2.5	2	None	None
P019	3	2	None	None
P020	0.5	1	None	None
P022	2	1.5	None	None
P023	0	2	17	None
P024	0	0.5	None	None
P025	0	2.5	None	None
P026	0	2.5	16	94
P029	0.5	1	47	24
P030	0	2	None	None
P031	2	1.5	36	70
P033	3	2	None	None
P037	0.5	4.5	None	96
P039	0	1	None	None
P041	0.5	1.5	None	None
P042	1	0	None	None
P044	1	1	None	None
P047	0	2	8	25
P050	1.5	0	None	None
P051	2.5	0.5	None	None
P057	1	0	90	None
P058	0	0.5	None	None
P061	2	1	None	None
P063	1.5	3	None	None
P064	1.5	2.5	65	None
P065	0	1.5	None	None
P066	0.5	0.5	None	None
P068	3	1	None	None
P070	1	1	5	7

Sample Number	3C6 Score	5B7 Score	Months at Recurrence	Months at Death
P163	2	1	None	None
P165	1	1	None	None
P167	0.5	1.5	None	None
P168	3	3	None	None
P169	2.5	2.5	None	None
P170	2	2.5	None	None
P171	1	1	None	None
P172	0	0.5	19	28
P173	0	1	None	None
P174	2	2	None	None
P175	0.5	2	None	None
P176	0.5	0.5	None	None
P177	0.5	2	None	None
P178	2	2	None	None
P179	3	2.5	None	None
P180	1.5	1	None	None
P181	2.5	2.5	None	None
P182	3	3	None	None
P184	3	3	16	44
P187	2	2.5	None	None
P188	1	1	None	None
P189	0	1	17	None
P190	3	3	17	44
P194	1.5	3	None	None
P197	0	1	None	None
P200	1	0.5	None	None
P202	3	3	8	20
P203	1.5	2	9	15
P207	1	2.5	None	None
P209	2.5	2.5	None	None
P210	2	1	None	None
P211	2	3	4	5
P212	1	1.5	None	None
P213	3	3	29	None
P214	2	2.5	None	None
P216	3	3	15	21
P217	3	3	19	None
P219	2	2	None	None
P220	2	2	None	None
P221	0	0.5	None	None
P222	0	1	6	12

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Sample Number	3C6 Score	5B7 Score	Months at Recurrence	Months at Death
P071	0.5	3	15	18
P072	3	2	22	25
P073	1	0.5	68	None
P074	2.5	0	None	None
P076	2	0.5	None	None
P078	3	1.5	None	None
P080	0.5	3	None	None
P161	0	0	None	None
P162	2	2	None	None

Sample Number	3C6 Score	5B7 Score	Months at Recurrence	Months at Death
P223	0	0.5	None	None
P224	3	2.5	24	53
P225	0	1	None	None
P226	1.5	2	5	9
P227	0.5	1.5	None	None
P228	1	2.5	None	None
P230	1.5	1.5	None	None
P231	1	2	None	None
P232	0	0.5	None	None

As shown in FIG. 9, clone 3C6 staining (whether negative or positive) has no correlation to NSCLC patient overall survivability while clone 5B7 clearly delineates two populations. In particular, positive 5B7 staining (score=1 or greater) identified NSCLC patients (n=80) having poor survivability, and negative 5B7 staining (score <1) identified NSCLC patients (n=20) with greater survivability. For example, as shown in FIG. 9B, approximately 82% of patients whose biopsy sample stained negative for clone 5B7 were still surviving at 8.3 years post-diagnosis. In comparison, approximately 65% of patients whose biopsy sample stained positive for clone 5B7 were surviving at the same time point.

As shown in FIG. 10, clone 3C6 staining (whether negative or positive) has no correlation to NSCLC patient disease-free survival (DFS) while clone 5B7 clearly delineates two populations. In particular, positive 5B7 staining (score=1 or greater) identified NSCLC patients (n=80) having poor DFS, and negative 5B7 staining (score <1) identified NSCLC patients (n=20) with greater DFS. For example, as shown in FIG. 10B, approximately 75% of patients whose biopsy sample stained negative for clone 5B7 were still surviving at 6 years post-diagnosis. In comparison, approximately 62% of patients whose biopsy sample stained positive for clone 5B7 were surviving at the same time point. The 5B7-positive and 5B7-negative curves converge around 90 months post-diagnosis most likely due to a statistical artifact caused by a decrease in the number of 5B7-negative samples at that (and later) time points. It is expected that 5B7-negative NSCLC patients will continue to have a better prognosis at 90 months and beyond when an even larger patient cohort is examined.

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This Example demonstrates that EGFR RD-binding molecules, such as clone 5B7, predict the prognosis (*e.g.*, overall survival and/or disease-free survival) of NSCLC patients (*e.g.*, early stage NSCLC patients) independent of treatment.

5

Example 13

**EGFR RD-BINDING MOLECULES, SUCH AS CLONE 5B7, ARE
INDICATORS OF COLORECTAL CANCER PROGNOSIS**

This Example demonstrates that a disclosed RD-binding molecule (*e.g.*, clone 5B7) predicts the prognosis of colorectal cancer patients.

10 Tissue arrays containing biopsy samples from at least 100 colorectal cancer patients are obtained. Each patient preferably will not have been treated with an EGFR-based therapy (*e.g.*, ERBITUX™, VECTIBIX™, IRESSA™, or TARCEVA™). Each patient is followed for up to 5 years post-diagnosis. Each biopsy sample is fixed in 10% NBF and paraffin embedded. Five (5) micron
15 sections of each biopsy sample are cut and arrayed on positively charged glass slides. The slides are stained with an RD-binding molecule (*e.g.*, clone 5B7) and an ED-binding molecule (*e.g.*, clone 3C6) according to the protocols in Example 2. The resulting stained array slides are scored by light microscopy by a pathologist according to the following criteria:

Report Result	Staining Intensity Score	Microscope Observation
<u>Positive:</u> Any IHC staining of tumor cell membranes above background level whether it is complete or incomplete circumferential staining in more than 0% tumor cells	3+	<u>Strong reactivity:</u> Dark brown to black staining is usually, but not always, in a complete membrane pattern, producing a thick outline of the cell. Cytoplasmic reactivity may be absent or may be moderately intense when membrane staining is very intense. Submembranous cytoplasmic accentuation may be present.
	2.5	<u>Intense reactivity:</u> Shades of brown staining of medium darkness (intensity). Membranous reactivity is usually but not always complete, producing a circular outline of the neoplastic cell. Incomplete membrane reactivity of moderate intensity is also considered 2+. The cytoplasmic reactivity is of weaker intensity than the membrane reactivity.
	2+	<u>Moderate reactivity:</u> Shades of brown staining of intermediate darkness (intensity). Membranous reactivity is usually but not always complete, producing a circular outline of the neoplastic cell. Incomplete membrane reactivity of moderate intensity is also considered 2+. The cytoplasmic reactivity is of weaker intensity than the membrane reactivity.

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	1.5	<u>Slight reactivity</u> : Staining of intermediate intensity that is membranous. Cytoplasmic reactivity that is uniform and involves all the cytoplasm may be present, but should not be evaluated for positivity.
	1+	<u>Weak reactivity</u> : Faint or light brown reactivity that is membranous. Cytoplasmic reactivity that is uniform and involves all the cytoplasm may be present, but should not be evaluated for positivity.
Negative: Absence of membrane staining above background in all tumor cells. Presence of cytoplasmic in the absence of membrane staining.	0.5	<u>Trace reactivity</u> : Trace brown reactivity where membranous and cytoplasmic localization is indeterminate.
	0	<u>No reactivity</u>

The score for each case is recorded in a database comparing the score for each binding molecule (*e.g.*, 5B7 and 3C6). The results of each case will fall into one of the 4 categories described in Table 2. Approximately 65% of cases are expected to fall into category 1, 19% in category 2, <1% in category 3 and 15% in category 4. Patient outcome will be directly related to the scoring category as indicated in FIG. 3 and Table 2. Patients in category 1 and 3 will have a poor prognosis, and patients in categories 2 and 4 will have a better prognosis.

10

Example 14

EGFR RD-BINDING MOLECULES, SUCH AS CLONE 5B7, ARE INDICATORS OF HEAD AND NECK CANCER PROGNOSIS

This Example demonstrates that a disclosed RD-binding molecule (*e.g.*, clone 5B7) predicts the prognosis of head and neck cancer patients.

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Tissue arrays containing biopsy samples from at least 100 head and neck cancer patients are obtained. Each patient preferably will not have been treated with an EGFR-based therapy (*e.g.*, ERBITUX™, VECTIBIX™, IRESSA™, or TARCEVA™). Each patient is followed for up to 5 years post-diagnosis. Each biopsy sample is fixed in 10% NBF and paraffin embedded. Five (5) micron sections of each biopsy sample are cut and arrayed on positively charged glass slides. The slides are stained with an RD-binding molecule (*e.g.*, clone 5B7) and an ED-binding molecule (*e.g.*, clone 3C6) according to the protocols in Example 2. The resulting stained array slides are scored by light microscopy by a pathologist according to the following criteria:

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Report Result	Staining Intensity Score	Microscope Observation
Positive: Any IHC staining of tumor cell membranes above background level whether it is complete or incomplete circumferential staining in more than 0% tumor cells	3+	<u>Strong reactivity</u> : Dark brown to black staining is usually, but not always, in a complete membrane pattern, producing a thick outline of the cell. Cytoplasmic reactivity may be absent or may be moderately intense when membrane staining is very intense. Submembranous cytoplasmic accentuation may be present.
	2.5	<u>Intense reactivity</u> : Shades of brown staining of medium darkness (intensity). Membranous reactivity is usually but not always complete, producing a circular outline of the neoplastic cell. Incomplete membrane reactivity of moderate intensity is also considered 2+. The cytoplasmic reactivity is of weaker intensity than the membrane reactivity.
	2+	<u>Moderate reactivity</u> : Shades of brown staining of intermediate darkness (intensity). Membranous reactivity is usually but not always complete, producing a circular outline of the neoplastic cell. Incomplete membrane reactivity of moderate intensity is also considered 2+. The cytoplasmic reactivity is of weaker intensity than the membrane reactivity.
	1.5	<u>Slight reactivity</u> : Staining of intermediate intensity that is membranous. Cytoplasmic reactivity that is uniform and involves all the cytoplasm may be present, but should not be evaluated for positivity.
	1+	<u>Weak reactivity</u> : Faint or light brown reactivity that is membranous. Cytoplasmic reactivity that is uniform and involves all the cytoplasm may be present, but should not be evaluated for positivity.
Negative: Absence of membrane staining above background in all tumor cells. Presence of cytoplasmic in the absence of membrane staining.	0.5	<u>Trace reactivity</u> : Trace brown reactivity where membranous and cytoplasmic localization is indeterminate.
	0	<u>No reactivity</u>

The score for each case is recorded in a database comparing the score for each binding molecule (*e.g.*, 5B7 and 3C6). The results of each case will fall into one of the 4 categories described in Table 2. Approximately 65% of cases are expected to fall into category 1, 19% in category 2, <1% in category 3 and 15% in category 4. Patient outcome will be directly related to the scoring category as indicated in FIG. 3 and Table 2. Patients in category 1 and 3 will have a poor prognosis, and patients in categories 2 and 4 will have a better prognosis.

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Example 15

EGFR RD-BINDING MOLECULES, SUCH AS CLONE 5B7, ARE INDICATORS OF GASTRIC CANCER PROGNOSIS

This Example demonstrates that a disclosed RD-binding molecule (*e.g.*, clone 5B7) predicts the prognosis of gastric cancer patients.

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Tissue arrays containing biopsy samples from at least 100 gastric cancer patients are obtained. Each patient preferably will not have been treated with an EGFR-based therapy (*e.g.*, ERBITUX™, VECTIBIX™, IRESSA™, or TARCEVA™). Each patient is followed for up to 5 years post-diagnosis. Each

5 biopsy sample is fixed in 10% NBF and paraffin embedded. Five (5) micron sections of each biopsy sample are cut and arrayed on positively charged glass slides. The slides are stained with an RD-binding molecule (*e.g.*, clone 5B7) and an ED-binding molecule (*e.g.*, clone 3C6) according to the protocols in Example 2. The resulting stained array slides are scored by light microscopy by a pathologist

10 according to the following criteria:

Report Result	Staining Intensity Score	Microscope Observation
<u>Positive:</u> Any IHC staining of tumor cell membranes above background level whether it is complete or incomplete circumferential staining in more than 0% tumor cells	3+	<u>Strong reactivity:</u> Dark brown to black staining is usually, but not always, in a complete membrane pattern, producing a thick outline of the cell. Cytoplasmic reactivity may be absent or may be moderately intense when membrane staining is very intense. Submembranous cytoplasmic accentuation may be present.
	2.5	<u>Intense reactivity:</u> Shades of brown staining of medium darkness (intensity). Membranous reactivity is usually but not always complete, producing a circular outline of the neoplastic cell. Incomplete membrane reactivity of moderate intensity is also considered 2+. The cytoplasmic reactivity is of weaker intensity than the membrane reactivity.
	2+	<u>Moderate reactivity:</u> Shades of brown staining of intermediate darkness (intensity). Membranous reactivity is usually but not always complete, producing a circular outline of the neoplastic cell. Incomplete membrane reactivity of moderate intensity is also considered 2+. The cytoplasmic reactivity is of weaker intensity than the membrane reactivity.
	1.5	<u>Slight reactivity:</u> Staining of intermediate intensity that is membranous. Cytoplasmic reactivity that is uniform and involves all the cytoplasm may be present, but should not be evaluated for positivity.
	1+	<u>Weak reactivity:</u> Faint or light brown reactivity that is membranous. Cytoplasmic reactivity that is uniform and involves all the cytoplasm may be present, but should not be evaluated for positivity.
<u>Negative:</u> Absence of membrane staining above background in all tumor cells. Presence of cytoplasmic in the absence of membrane staining.	0.5	<u>Trace reactivity:</u> Trace brown reactivity where membranous and cytoplasmic localization is indeterminate.
	0	<u>No reactivity</u>

The score for each case is recorded in a database comparing the score for each binding molecule (*e.g.*, 5B7 and 3C6). The results of each case will fall into

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one of the 4 categories described in Table 2. Approximately 65% of cases are expected to fall into category 1, 19% in category 2, <1% in category 3 and 15% in category 4. Patient outcome will be directly related to the scoring category as indicated in FIG. 3 and Table 2. Patients in category 1 and 3 will have a poor prognosis, and patients in categories 2 and 4 will have a better prognosis.

Example 16

EGFR RD-BINDING MOLECULES, SUCH AS CLONE 5B7, ARE INDICATORS OF GLIOBLASTOMA CANCER PROGNOSIS

This Example demonstrates that a disclosed RD-binding molecule (*e.g.*, clone 5B7) predicts the prognosis of glioblastoma cancer patients.

Tissue arrays containing biopsy samples from at least 100 glioblastoma cancer patients are obtained. Each patient preferably will not have been treated with an EGFR-based therapy (*e.g.*, ERBITUX™, VECTIBIX™, IRESSA™, or TARCEVA™). Each patient is followed for up to 5 years post-diagnosis. Each biopsy sample is fixed in 10% NBF and paraffin embedded. Five (5) micron sections of each biopsy sample are cut and arrayed on positively charged glass slides. The slides are stained with an RD-binding molecule (*e.g.*, clone 5B7) and an ED-binding molecule (*e.g.*, clone 3C6) according to the protocols in Example 2. The resulting stained array slides are scored by light microscopy by a pathologist according to the following criteria:

Report Result	Staining Intensity Score	Microscope Observation
Positive: Any IHC staining of tumor cell membranes above background level whether it is complete or incomplete circumferential staining in more than 0% tumor cells	3+	<u>Strong reactivity</u> : Dark brown to black staining is usually, but not always, in a complete membrane pattern, producing a thick outline of the cell. Cytoplasmic reactivity may be absent or may be moderately intense when membrane staining is very intense. Submembranous cytoplasmic accentuation may be present.
	2.5	<u>Intense reactivity</u> : Shades of brown staining of medium darkness (intensity). Membranous reactivity is usually but not always complete, producing a circular outline of the neoplastic cell. Incomplete membrane reactivity of moderate intensity is also considered 2+. The cytoplasmic reactivity is of weaker intensity than the membrane reactivity.

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Report Result	Staining Intensity Score	Microscope Observation
	2+	<u>Moderate reactivity</u> : Shades of brown staining of intermediate darkness (intensity). Membranous reactivity is usually but not always complete, producing a circular outline of the neoplastic cell. Incomplete membrane reactivity of moderate intensity is also considered 2+. The cytoplasmic reactivity is of weaker intensity than the membrane reactivity.
	1.5	<u>Slight reactivity</u> : Staining of intermediate intensity that is membranous. Cytoplasmic reactivity that is uniform and involves all the cytoplasm may be present, but should not be evaluated for positivity.
	1+	<u>Weak reactivity</u> : Faint or light brown reactivity that is membranous. Cytoplasmic reactivity that is uniform and involves all the cytoplasm may be present, but should not be evaluated for positivity.
<u>Negative</u> : Absence of membrane staining above background in all tumor cells. Presence of cytoplasmic in the absence of membrane staining.	0.5	<u>Trace reactivity</u> : Trace brown reactivity where membranous and cytoplasmic localization is indeterminate.
	0	<u>No reactivity</u>

The score for each case is recorded in a database comparing the score for each binding molecule (*e.g.*, 5B7 and 3C6). The results of each case will fall into one of the 4 categories described in Table 2. Approximately 65% of cases are expected to fall into category 1, 19% in category 2, <1% in category 3 and 15% in category 4. Patient outcome will be directly related to the scoring category as indicated in FIG. 3 and Table 2. Patients in category 1 and 3 will have a poor prognosis, and patients in categories 2 and 4 will have a better prognosis.

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Example 17

EGFR RD-BINDING MOLECULES, SUCH AS CLONE 5B7, ARE INDICATORS OF HEPATOCELLULAR CARCINOMA PROGNOSIS

This Example demonstrates that a disclosed RD-binding molecule (*e.g.*, clone 5B7) predicts the prognosis of HCC (such as, resectable HCC) cancer patients

Tissue arrays containing biopsy samples from at least 100 HCC cancer patients are obtained (see, for example, control arm of samples collected in JS 0414, “A Pilot Study of Adjuvant Therapy of Gefitinib (Iressa, ZD1839) in Patients with Resectable Hepatocellular Carcinoma”, ClinicalTrials.gov Identifier No. NCT00228501). Each patient preferably will not have been treated with an

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EGFR-based therapy (*e.g.*, ERBITUX™, VECTIBIX™, IRESSA™, or TARCEVA™). Each patient is followed for up to 5 years post-diagnosis. Each biopsy sample is fixed in a standard fixative (*e.g.*, 10% NBF) and paraffin embedded. Sections of each biopsy sample (*e.g.*, 5 µm thick) are cut and arrayed on positively charged glass slides. The slides are stained with an RD-binding molecule (*e.g.*, clone 5B7) and an ED-binding molecule (*e.g.*, clone 3C6) according to the protocols in Example 2. The resulting stained array slides are scored by light microscopy by a pathologist according to the following criteria:

Report Result	Staining Intensity Score	Microscope Observation
<u>Positive:</u> Any IHC staining of tumor cell membranes above background level whether it is complete or incomplete circumferential staining in more than 0% tumor cells	3+	<u>Strong reactivity:</u> Dark brown to black staining is usually, but not always, in a complete membrane pattern, producing a thick outline of the cell. Cytoplasmic reactivity may be absent or may be moderately intense when membrane staining is very intense. Submembranous cytoplasmic accentuation may be present.
	2.5	<u>Intense reactivity:</u> Shades of brown staining of medium darkness (intensity). Membranous reactivity is usually but not always complete, producing a circular outline of the neoplastic cell. Incomplete membrane reactivity of moderate intensity is also considered 2+. The cytoplasmic reactivity is of weaker intensity than the membrane reactivity.
	2+	<u>Moderate reactivity:</u> Shades of brown staining of intermediate darkness (intensity). Membranous reactivity is usually but not always complete, producing a circular outline of the neoplastic cell. Incomplete membrane reactivity of moderate intensity is also considered 2+. The cytoplasmic reactivity is of weaker intensity than the membrane reactivity.
	1.5	<u>Slight reactivity:</u> Staining of intermediate intensity that is membranous. Cytoplasmic reactivity that is uniform and involves all the cytoplasm may be present, but should not be evaluated for positivity.
	1+	<u>Weak reactivity:</u> Faint or light brown reactivity that is membranous. Cytoplasmic reactivity that is uniform and involves all the cytoplasm may be present, but should not be evaluated for positivity.
<u>Negative:</u> Absence of membrane staining above background in all tumor cells. Presence of cytoplasmic in the absence of membrane staining.	0.5	<u>Trace reactivity:</u> Trace brown reactivity where membranous and cytoplasmic localization is indeterminate.
	0	<u>No reactivity</u>

The score for each case is recorded in a database comparing the score for each binding molecule (*e.g.*, 5B7 and 3C6). The results of each case will fall into one of the 4 categories described in Table 2. Approximately 60% of cases are expected to fall into category 1, 13% in category 2, 5% in category 3 and 22% in

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category 4. Patient outcome will be directly related to the scoring category as indicated in FIG. 3 and Table 2. Patients in category 1 and 3 will have a poor prognosis, and patients in categories 2 and 4 will have a better prognosis.

- 5 While this disclosure has been described with an emphasis upon particular embodiments, it will be obvious to those of ordinary skill in the art that variations of the particular embodiments may be used and it is intended that the disclosure may be practiced otherwise than as specifically described herein. Accordingly, this disclosure includes all modifications encompassed within the spirit and scope of the
- 10 disclosure as defined by the following claims:

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CLAIMS

1. A method of identifying a diagnostic reagent, comprising:
identifying a receptor tyrosine kinase (RTK), which is a component of a cellular pathway that confers a growth advantage on a cancer cell, wherein inhibition
5 of the RTK decreases the cancer cell growth advantage and wherein a function of the RTK protein is positively or negatively regulated by a regulatory protein when the RTK and the regulatory protein form a protein-protein interaction, thereby producing a protein-protein interface; and
producing a specific binding reagent that specifically binds to at least one
10 RTK amino acid of the protein-protein interface in the absence of the protein-protein interaction and fails to specifically bind the at least one RTK amino acid of the protein-protein interface in the presence of the protein-protein interaction, wherein the specific binding reagent is a diagnostic reagent.
- 15 2. The method of claim 1, further comprising identifying five or more amino acid residues that form the protein-protein interface, and wherein the specific binding reagent specifically binds to the five or more amino acid residues that form the protein-protein interface in the absence of the protein-protein interaction and fails to specifically bind the five or more amino acid residues that form the
20 protein-protein interface in the presence of the protein-protein interaction.
3. The method of claim 1, wherein the RTK protein comprises an RTK listed in Table 2.
- 25 4. The method of claim 1, wherein a function of the RTK protein is negatively regulated by a regulatory protein when the RTK and the regulatory protein form a protein-protein interaction, and wherein the negative regulation decreases the cancer cell growth advantage.
- 30 5. The method of claim 1, wherein a function of the RTK protein is positively regulated by a regulatory protein when the RTK and the regulatory

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protein form a protein-protein interaction, and wherein the positive regulation increases the cancer cell growth advantage.

6. The method of claim 1, wherein the specific binding reagent comprises an antibody or fragment thereof, or an aptamer.

7. The method of claim 1, wherein identifying the five or more amino acid residues comprises competitively binding peptides and the regulatory protein to the RTK, wherein the peptide comprises at least 5 amino acid residues of the protein-protein interface.

8. The method of claim 1, wherein the protein-protein interface comprises an RTK intracellular domain.

9. The method of claim 8, wherein the specific binding reagent specifically binds to at least 5 amino acids of the RTK intracellular domain that form the protein-protein interface.

10. The method of claim 1, wherein producing a specific binding reagent comprises:

identifying peptide sequences of less than about 20 amino acids that form the protein-protein interface and that have significant antigenicity; and

administering at least one of the peptide sequences to an animal under conditions that permit production of the specific binding reagent in the animal.

11. The method of claim 1, wherein the specific binding reagent does not specifically bind to an RTK extracellular domain, an RTK transmembrane domain, or the regulatory protein.

12. A diagnostic reagent identified using the method of claim 1.

13. A kit comprising:

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the diagnostic reagent of claim 12 in a container; and
a labeled secondary antibody.

14. A method of diagnosing a subject having cancer, comprising:
- 5 contacting a sample obtained from the subject with the diagnostic reagent of claim 12, wherein the diagnostic reagent specifically binds the one or more amino acid residues in the absence of the protein-protein interaction between the RTK protein and a negative regulatory protein; and
- detecting binding of the diagnostic reagent with a protein in the sample,
- 10 wherein the presence of detectable binding indicates that the RTK protein did not form a protein-protein interaction with the negative regulatory protein and the subject is diagnosed to have a cancer with worse prognosis and absence of detectable binding indicates that the RTK protein formed a protein-protein interaction with the negative regulatory protein and the subject is diagnosed to have
- 15 cancer with a good prognosis.

15. The method of claim 14, wherein the method is a method of prognosing a sensitivity of the cancer to an RTK inhibitor, wherein the presence of detectable binding indicates that the cancer will be sensitive to the RTK inhibitor and absence
- 20 of detectable binding indicates that the cancer will be resistant to the RTK inhibitor.

16. The method of claim 15, wherein the RTK inhibitor comprises a tyrosine kinase inhibitor (TKI).

- 25 17. The method of any of claims 14 to 16, further comprising:
- contacting the sample with an agent that permits detection of an RTK extracellular domain, wherein the presence of a detectable signal from the agent indicates that an RTK extracellular domain is present in the sample, and the absence of a detectable signal from the agent indicates that the RTK extracellular domain is
- 30 absent or mutated.

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18. The method of claim 17, wherein the method is a method of prognosing a sensitivity of the cancer to an RTK extracellular domain-based inhibitor,

wherein the presence of detectable binding by the diagnostic reagent that specifically binds the one or more amino acid residues in the absence of the protein-protein interaction between the RTK protein and a negative regulatory protein and
5 the presence of detectable binding by the agent that permits detection of an RTK extracellular domain indicates that the cancer will be sensitive to RTK inhibitors that target the RTK extracellular or intracellular domain;

wherein the absence of detectable binding by the diagnostic reagent that
10 specifically binds the one or more amino acid residues in the absence of the protein-protein interaction between the RTK protein and a negative regulatory protein and the presence of detectable binding by the agent that permits detection of an RTK extracellular domain indicates that the cancer will be resistant to RTK inhibitors that target the RTK extracellular or intracellular domain;

wherein the presence of detectable binding by the diagnostic reagent that specifically binds the one or more amino acid residues in the absence of the protein-protein interaction between the RTK protein and a negative regulatory protein and
15 the absence of detectable binding by the agent that permits detection of an RTK extracellular domain indicates that the cancer will be resistant to RTK extracellular domain-based inhibitors but sensitive to RTK inhibitors that target the RTK
20 intracellular domain; and

wherein the absence of detectable binding by the diagnostic reagent that specifically binds the one or more amino acid residues in the absence of the protein-protein interaction between the RTK protein and a negative regulatory protein and
25 the absence of detectable binding by the agent that permits detection of an RTK extracellular domain indicates that the cancer will not be responsive to RTK inhibitors that target the RTK extracellular or intracellular domain.

19. The method of claim 18, wherein the RTK extracellular domain-based
30 inhibitor comprises an antibody specific for the RTK extracellular domain.

20. A method of diagnosing a subject, comprising:

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contacting a sample obtained from the subject with the diagnostic reagent of claim 12, wherein the diagnostic reagent specifically binds the one or more amino acid residues in the absence of the protein-protein interaction between the RTK protein and a positive regulatory protein; and

- 5 detecting binding of the diagnostic reagent with a protein in the sample, wherein the presence of detectable binding indicates that the RTK protein did not form a protein-protein interaction with the positive regulatory protein and the subject is diagnosed to have a cancer with good prognosis and absence of detectable binding indicates that the RTK protein formed a protein-protein interaction with the positive
10 regulatory protein and the subject is diagnosed to have cancer with a poor prognosis.

21. The method of any of claims 14 to 20, wherein the sample comprises a formalin-fixed, paraffin-embedded tissue sample.

15

FIG. 1

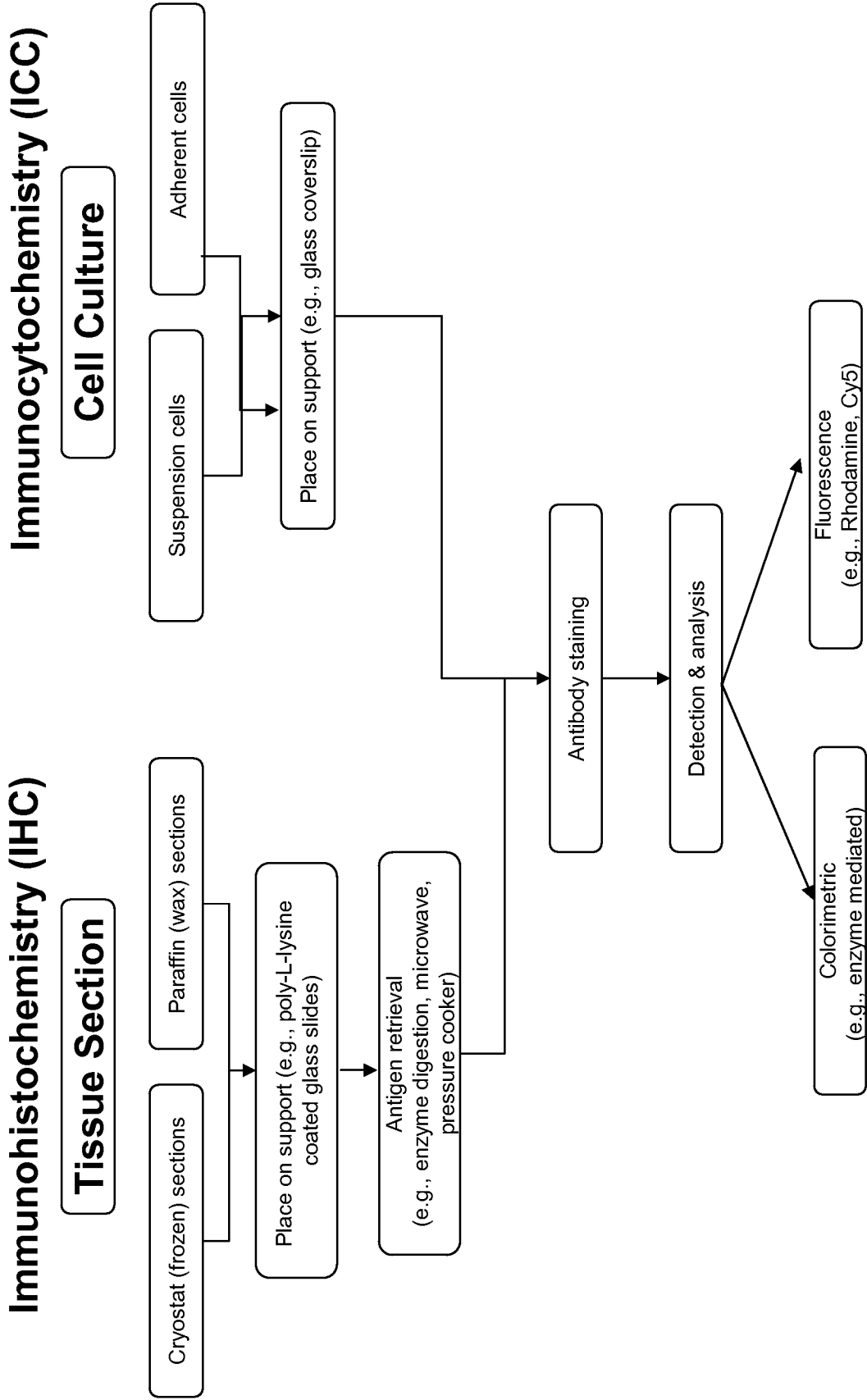


FIG. 2

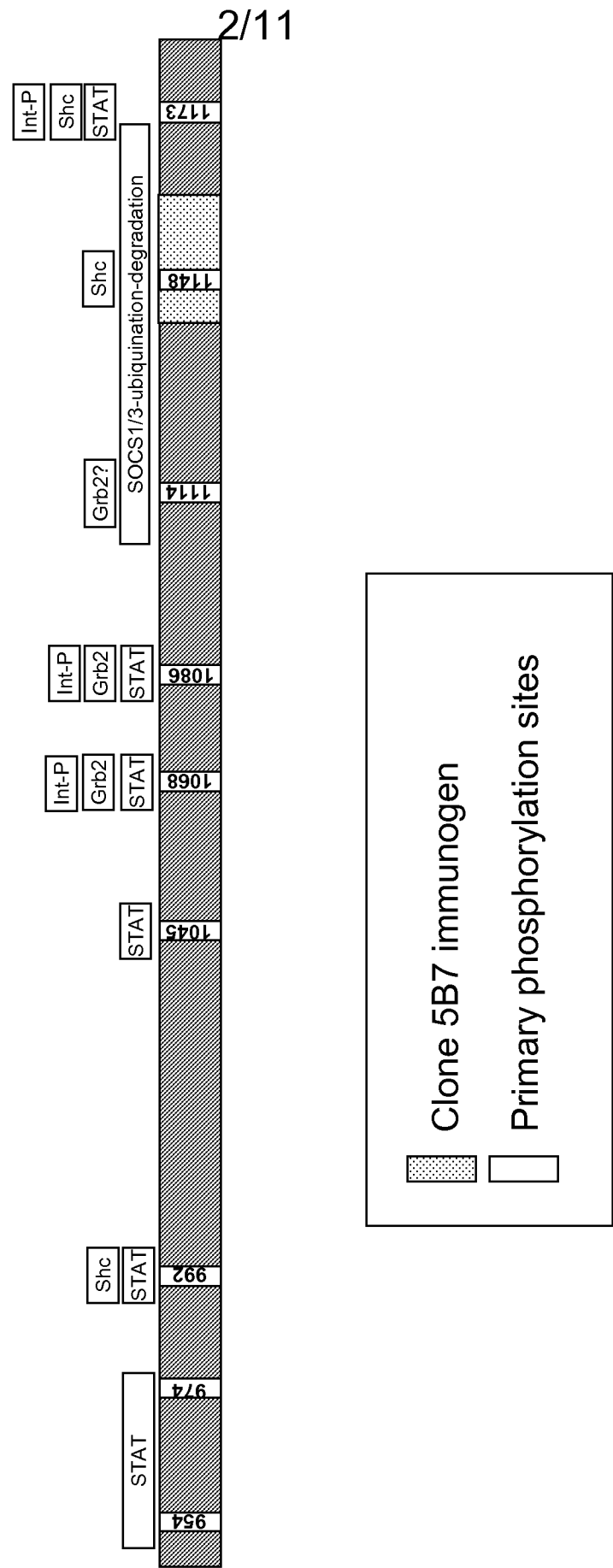


FIG. 3A

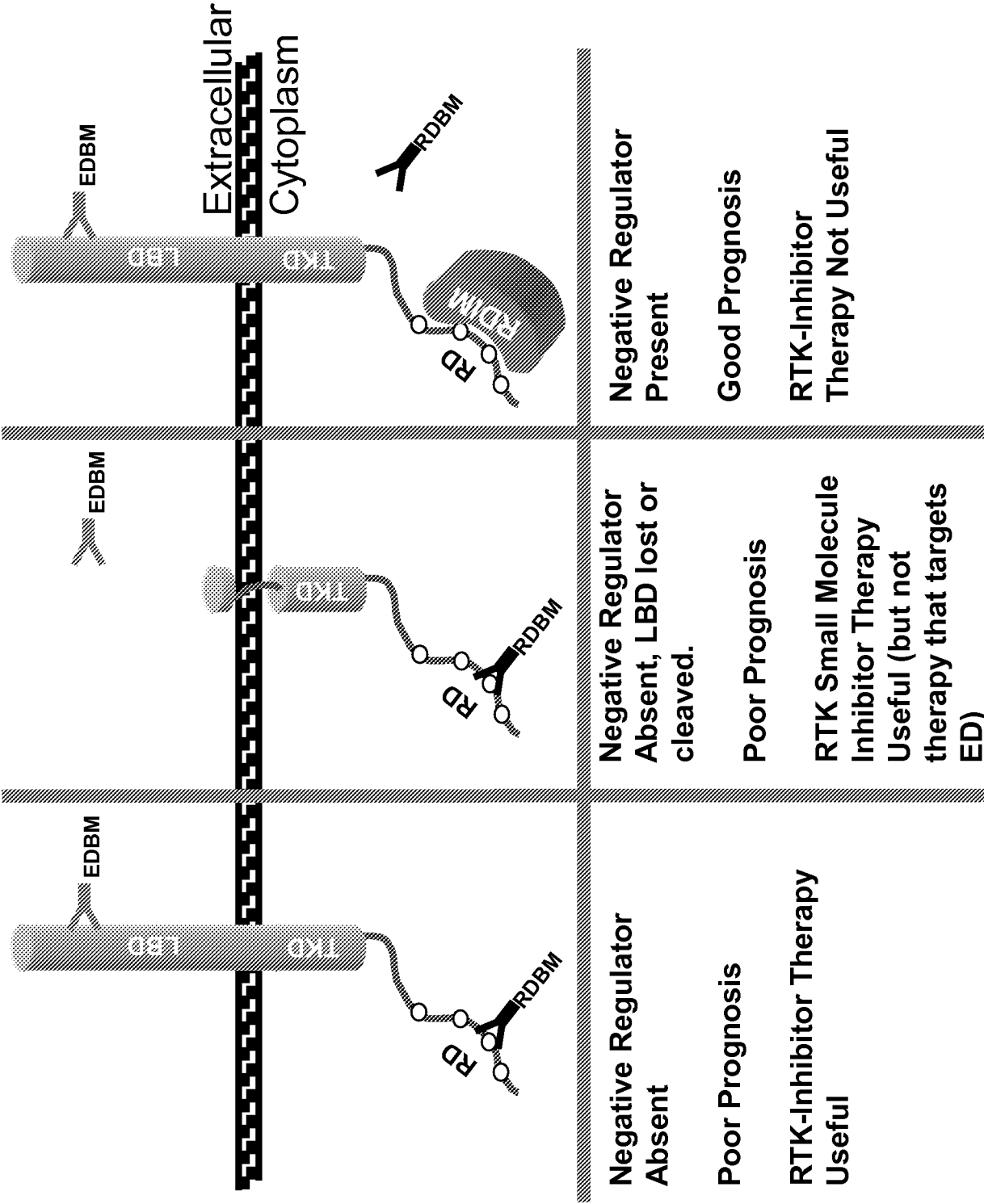


FIG. 3B

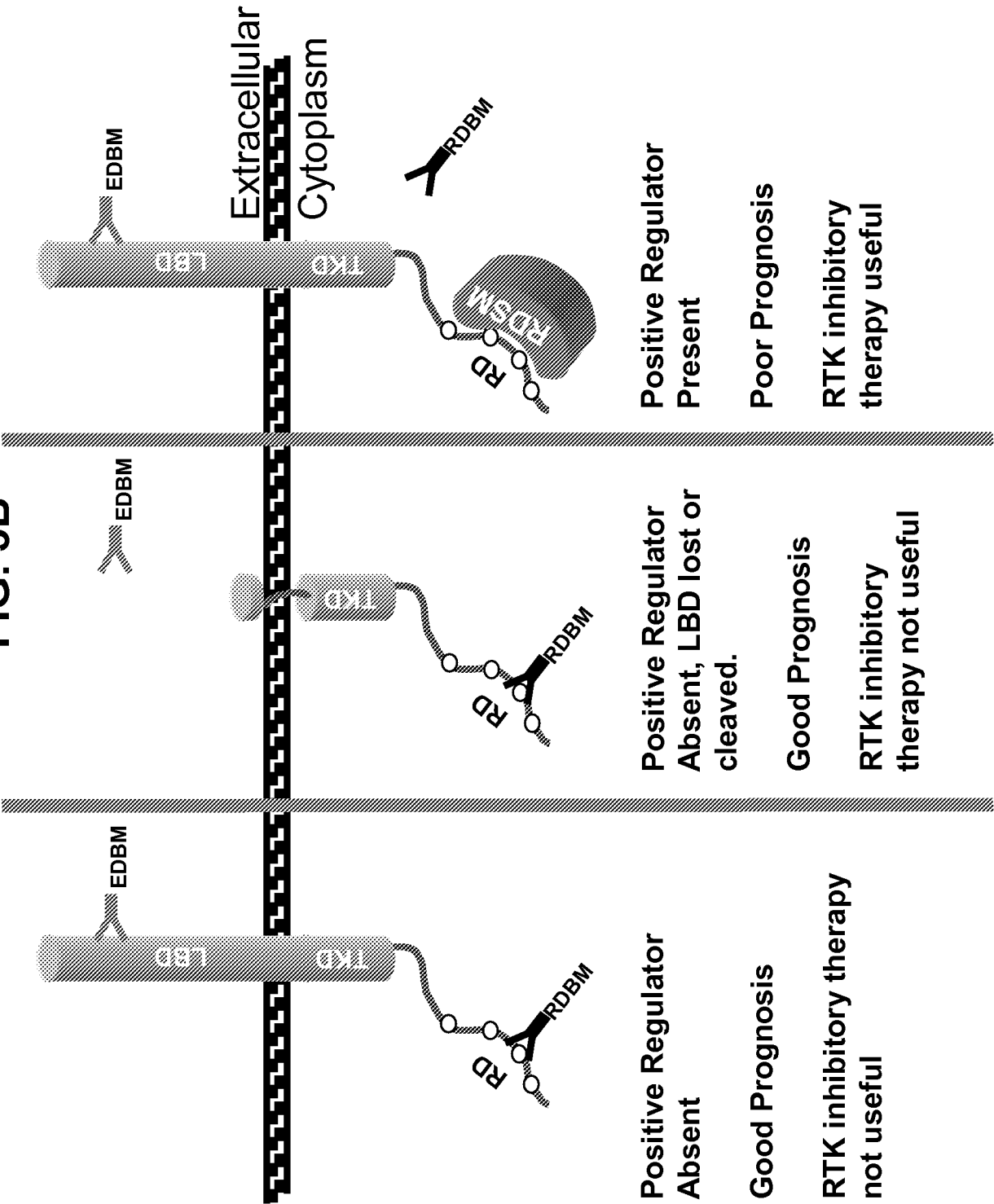


FIG. 4

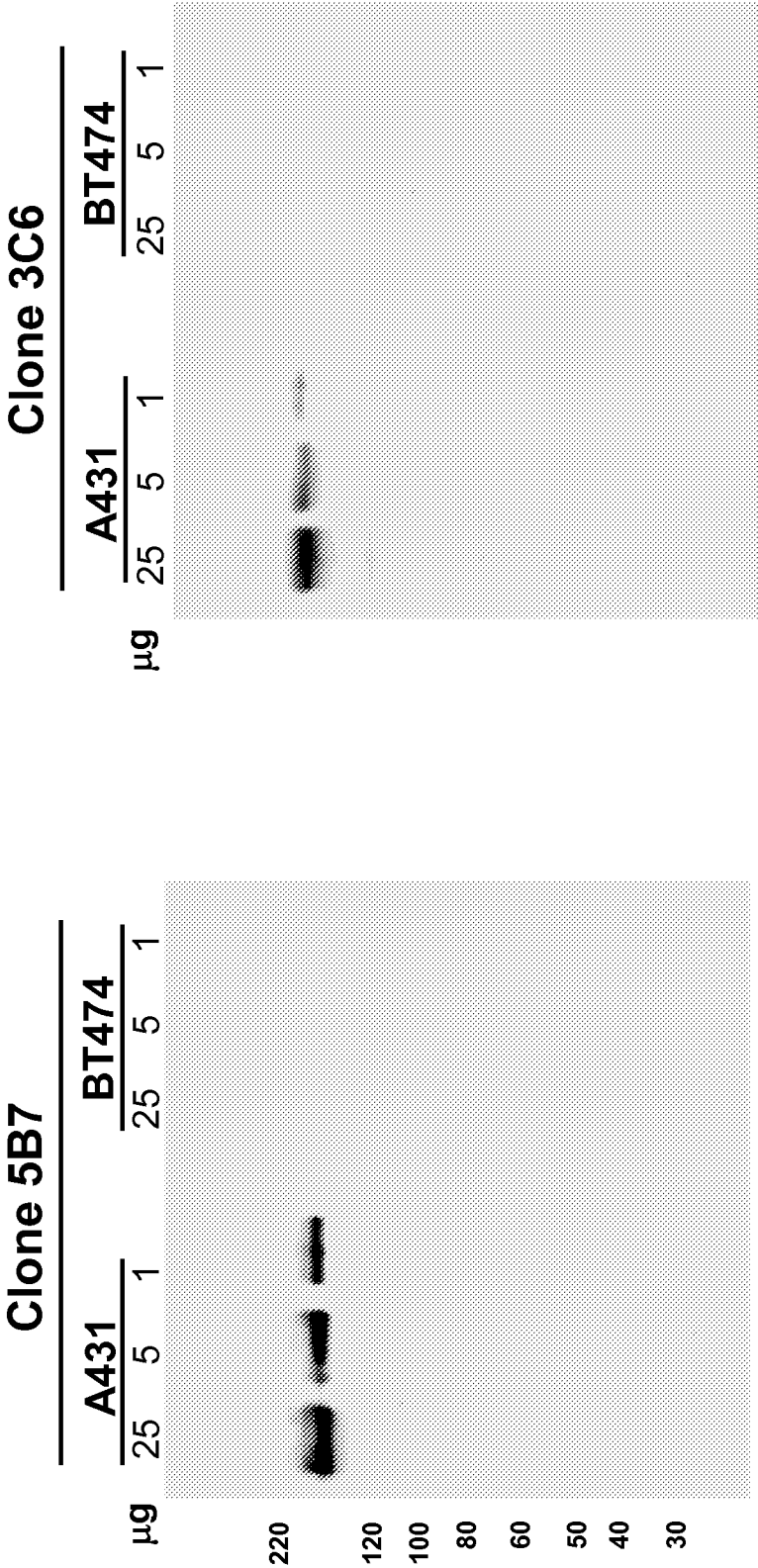


FIG. 5

A

Tissue	3C6	5B7
Skin	Pos	Pos
Testis	Pos	Pos
Tonsil	Pos	Pos
Liver	Pos	Neg
Placenta	Pos	Neg

B

Normal Liver

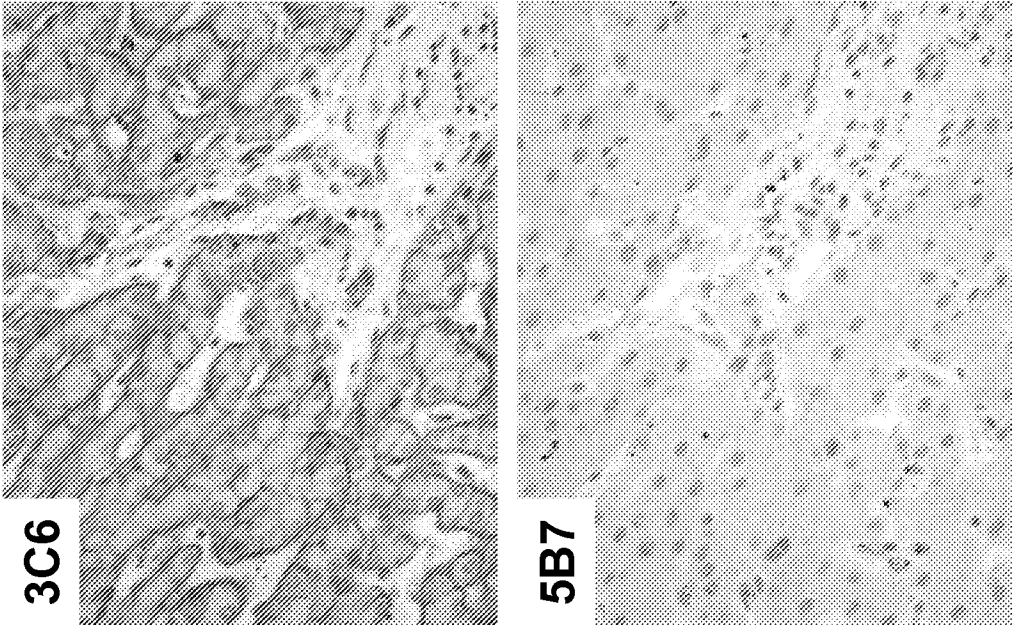


FIG. 6

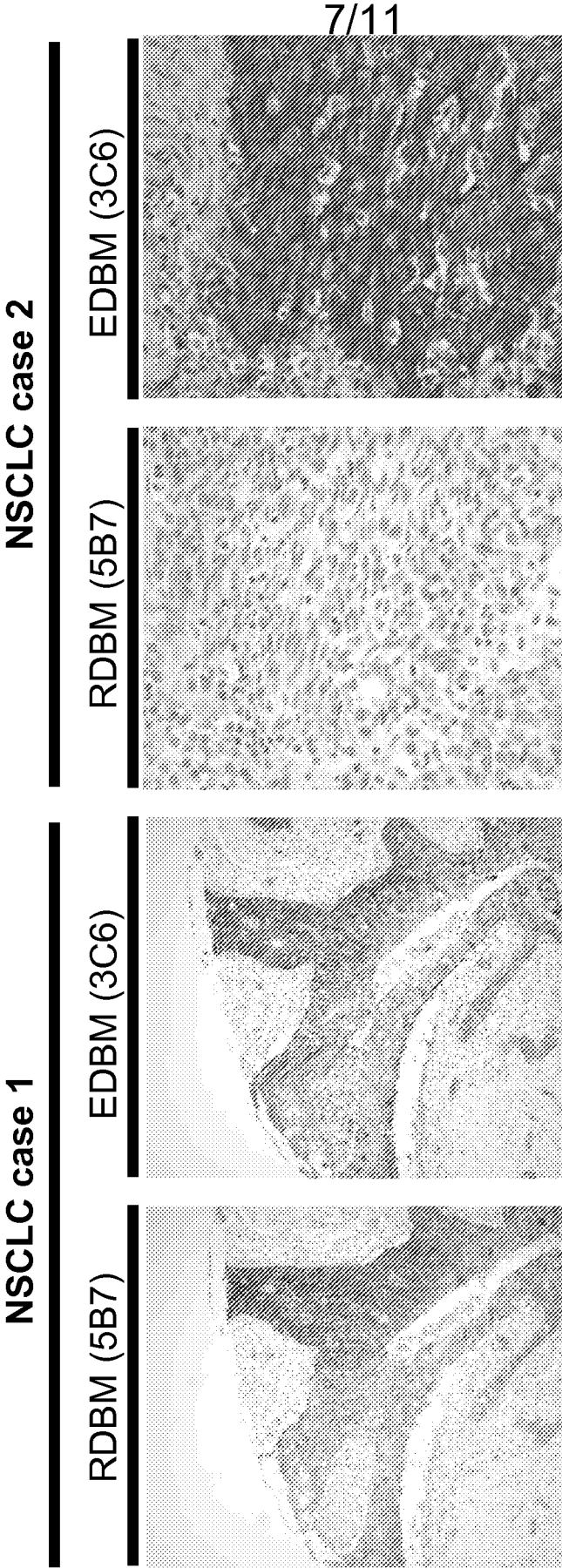


FIG. 7

A

Peptide number	Sequence	Level of inhibition
Peptide 1	QISLDNPDYQQD	Partial
Peptide 2	QQDFFPKEAKPNG	Complete
Peptide 3	LDNPDYQQDFFPKEAKPNG	Complete

B

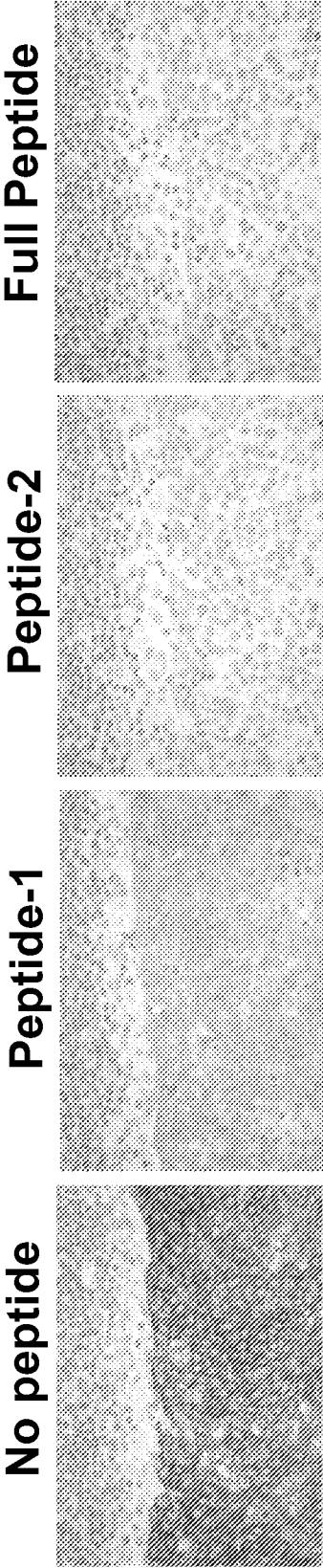
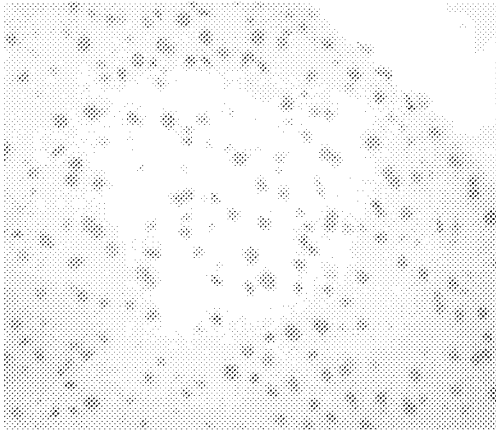


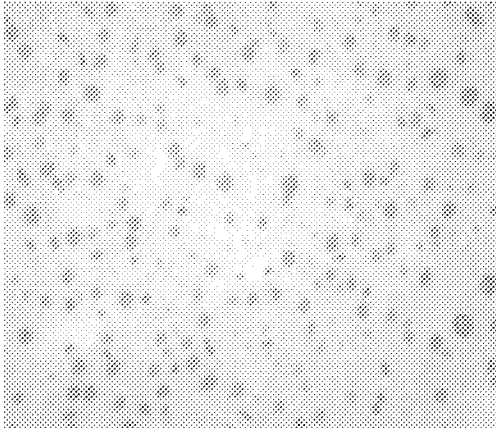
FIG. 8

Normal mouse livers (2) stained with
RDBM (5B7)

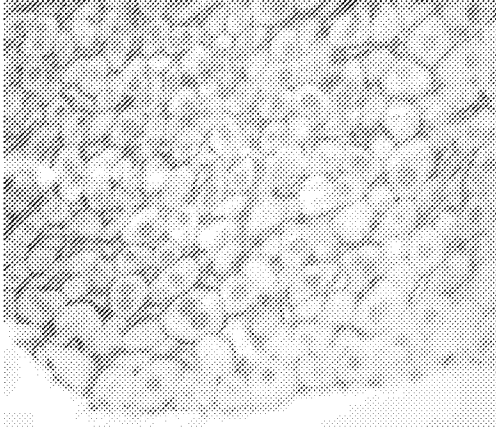
SOCS-3 knockout mouse livers (2)
stained with RDBM (5B7)



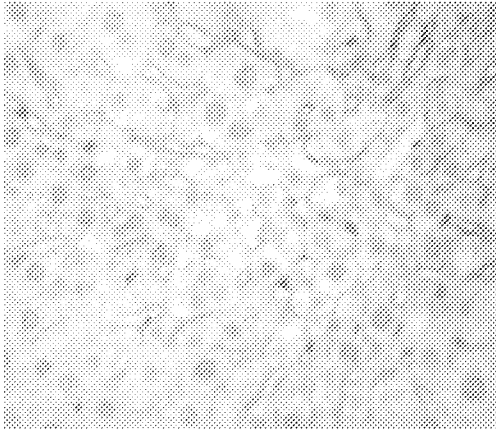
Negative membrane



Negative membrane



Positive membrane



Positive membrane

FIG. 9B

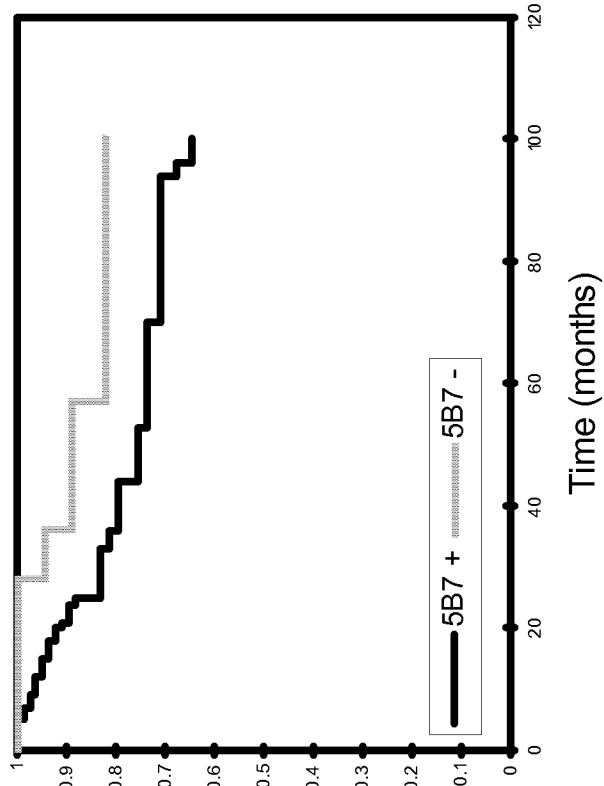


FIG. 9A

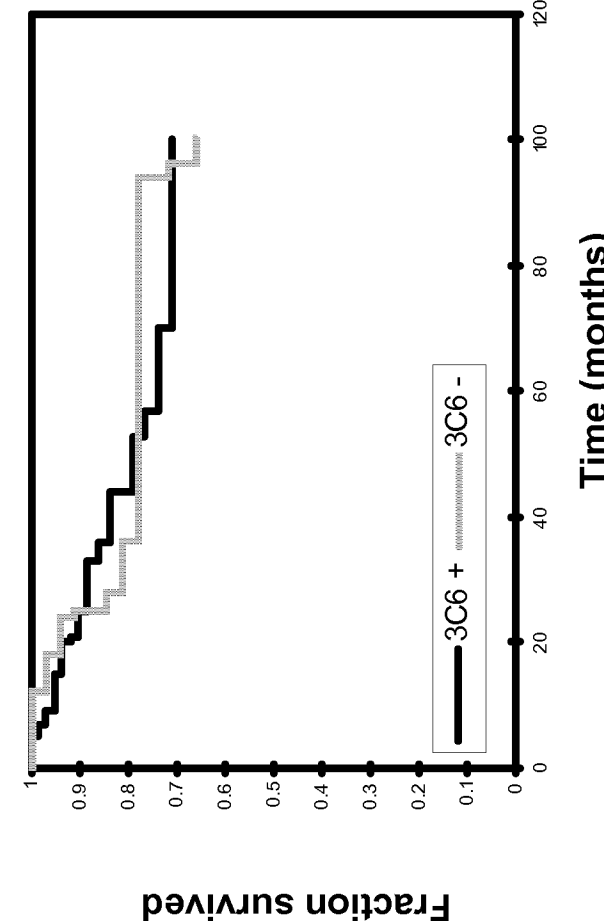


FIG. 10B

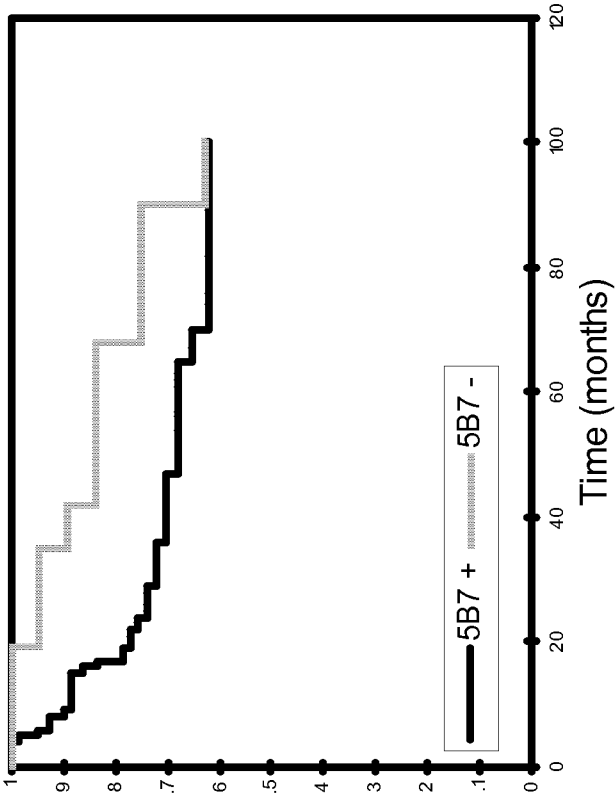


FIG. 10A

