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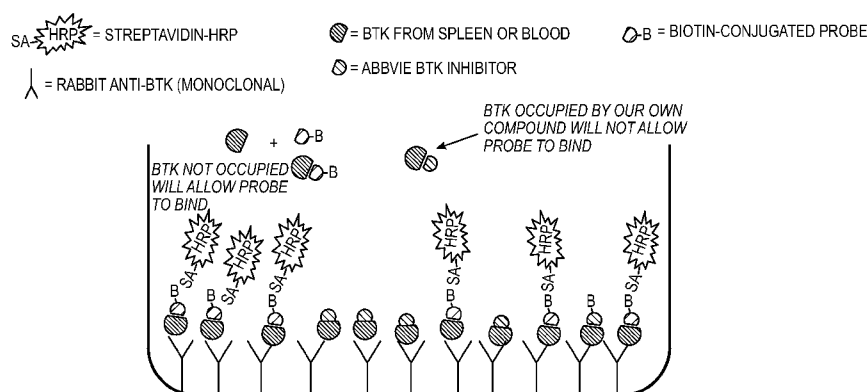
(54) **Title:** ENZYME OCCUPANCY ASSAY

FIG. 1A

(57) **Abstract:** The invention relates to methods and reagents for determining the extent of binding between a ligand and its binding partner. The methods have greatly improved sensitivity, and may be used to determine the percentage of a kinase (e.g., BTK) that is occupied / bound by an irreversible inhibitor of the kinase in a biological sample.

ENZYME OCCUPANCY ASSAY

REFERENCE TO RELATED APPLICATION

This application claims the benefit of the filing date under 35 USC 119(e) to U.S. Provisional Application No. 62/024780, filed on July 15, 2014, the entire content of which is incorporated herein by reference.

BACKGROUND OF THE INVENTION

The protein kinases represent a large family of proteins that play a central role in the regulation of a wide variety of cellular processes and maintenance of cellular function. A partial, non-limiting, list of these kinases include: non-receptor tyrosine kinases such as the Tec family (BTK, ITK, Tec, ETK/BMX & RLK/TXK), Janus kinase family (Jak1, Jak2, Jak3 and Tyk2); the fusion kinases, such as BCR-Abl, focal adhesion kinase (FAK), Fes, Lck and Syk; receptor tyrosine kinases such as epidermal growth factor receptor (EGFR), the platelet-derived growth factor receptor kinase (PDGF-R), the receptor kinase for stem cell factor, c-kit, the hepatocyte growth factor receptor, c-Met, and the fibroblast growth factor receptor, FGFR3; and serine/threonine kinases such as b-RAF, mitogen-activated protein kinases (*e.g.*, MKK6) and SAPK2 β . Aberrant kinase activity has been observed in many disease states including benign and malignant proliferative disorders as well as diseases resulting from inappropriate activation of the immune and nervous systems.

Bruton's tyrosine kinase (BTK) is a non-receptor tyrosine kinase with a key role in immunoreceptor signaling (BCR, Fc ϵ R, Fc γ R, DAP12, Dectin-1, GPVI, *etc.*) in a host of hematopoietic cells including B cells, platelets, mast cells, basophils, eosinophils, macrophages and neutrophils as well as osteoclasts involved in bone destruction (for reviews, see Brunner *et al.*, 2005 *Histol. Histopathol.*, 20:945-955; Mohamed *et al.*, 2009 *Immunol. Rev.*, 228(1):58-73). Mutations in BTK are known to lead to X-linked agammaglobulinemia (XLA) in humans and X-linked immunodeficiency (Xid) in mice, which are characterized by limited B-cell production & reduced antibody titers (Lindvall *et al.*, 2005 *Immunol. Rev.*, 203:200-215). The combined action of BTK in multiple cell types makes it an attractive target for autoimmune disease. BTK is related by sequence homology to other Tec family kinases (ITK, Tec, ETK/BMX & RLK/TXK).

In B-lymphocytes, BTK is required for B-cell development and for Ca^{2+} mobilization following B-cell receptor (BCR) engagement (Khan *et al.*, 1995 *Immunity* 3:283-299; Genevieve and Callard, 1997 *Clin. Exp. Immun.*, 110(3):386-391) where it is believed to be downstream of Src family kinases (such as Lyn), Syk & PI3K. BTK has been shown to be important for both thymus-dependent and thymus-independent type 2 responses to antigens (Khan *et al.*, 1995 *Immunity* 3:283-299). In mast cells, studies using BTK mouse knock-outs (Hata *et al.*, 1998 *J. Exp. Med.*, 187:1235-1247; Schmidt *et al.*, 2009 *Eur. J. Immun.*, 39:3228-3238) indicate a role for BTK in Fc ϵ RI induced signaling, histamine release and production of cytokines such as TNF, IL-2, & IL-4. In platelets, BTK is important for signaling through the glycoprotein VI (GPVI) receptor that responds to collagen and has been shown to promote platelet aggregation and contribute to cytokine production from fibroblast-like synoviocytes (Hsu *et al.*, 2013 *Immun. Letters*, 150:97-104). In monocytes and macrophages, the action of BTK is invoked in Fc γ RI induced signaling and may also have role in Toll-Like Receptor-induced cytokine responses including TLR2, TLR4, TLR8 & TLR9 (Horwood *et al.*, 2003 *J. Exp. Med.*, 197:1603-1611; Horwood *et al.*, 2006 *J. Immunol.*, 176:3635-3641; Perez de Diego *et al.*, 2006 *Allerg. Clin. Imm.*, 117:1462-1469; Doyle *et al.*, 2007 *J. Biol. Chem.*, 282:36953-36960; Hasan *et al.*, 2007 *Immunology*, 123:239-249; Sochorava *et al.*, 2007 *Blood*, 109:2553-2556; Lee *et al.*, 2008, *J. Biol. Chem.*, 283:11189-11198).

Therefore, inhibition of BTK is expected to intervene at several critical junctions of the inflammatory reactions resulting in an effective suppression of autoimmune response. As such diseases involving B-cell receptor activation, antibody-Fc receptor interactions & GPVI receptor signaling may be modulated by treatment with BTK inhibitors. BTK inhibition is likely to act on both the initiation of autoimmune disease by blocking BCR signaling and the effector phase by abrogation of FcR signaling on macrophages, neutrophils, basophils, and mast cells. Furthermore, blocking BTK would provide additional benefit via inhibition of osteoclast maturation and therefore attenuate the bone erosions & overall joint destruction associated with rheumatoid arthritis. Inhibiting BTK may be useful in treating a host of inflammatory and allergic diseases - for example (but not limited to), rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), multiple sclerosis (MS) and type I hypersensitivity reactions such as allergic rhinitis, allergic conjunctivitis, atopic dermatitis, allergic asthma and systemic anaphylaxis. For a review on targeting BTK as a treatment for inflammatory disorders and autoimmunity as well as leukemia and lymphomas, see Uckun

and Qazi, 2010 *Expert Opin. Ther. Pat.*, 20:1457-1470. Because BTK is highly expressed in cancers of the hematopoietic system & BTK-dependent signaling is believed to be dysregulated there, BTK inhibitors are expected to be useful treatments for B-cell lymphoma/leukemia & other oncologic disease - for example (but not limited to) acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL), non-Hodgkin's lymphoma (NHL), small lymphocytic lymphoma (SLL), and acute myeloid leukemia (for review, see Buggy & Elias 2012 *Int. Rev. Immunol.* 31:119). Taken together, BTK inhibitors provide a strong method to treat a host of inflammatory diseases and immunological disorders as well as hematologic cancers.

Recent study suggests that attrition rates in clinical development are highest in Phase 2 clinical trials, where lack of pharmacodynamic (PD) activity and/or efficacy leads to failure rates of ~ 1/3 of drug candidates (Kola and Landis, *Nat. Rev. Drug Discov.* 3:711-715, 2004). Thus, the development of novel strategies enabling earlier insights into PD activity is an intense area of research. Evans *et al.* (*J. Pharmacol. Exp. Ther.* 346:219-228, 2013) recently reported an assay to measure on-target activity of CC-292, a potent and selective inhibitor of BTK that has advanced into human clinical trial, which assay reportedly allows assessment of *in vivo* BTK occupancy from biological samples, thus facilitating both preclinical research and clinical development by enabling a quantitative understanding of the relationship among dose, exposure, target engagement, functional consequence, and efficacy. The assay reportedly provides quantitative measurements for CC-292 / BTK engagement by a direct ELISA assay that quantitates the amount of drug-free BTK protein. Using this assay, it was found that, *in vitro*, the concentrations of CC-292 required for inhibition of BTK activity and BTK occupancy were virtually equivalent ($EC_{50s} = 8$ versus 6 nM, respectively), demonstrating a near stoichiometric relationship. In freshly isolated primary human B lymphocytes, there was a close correlation between the concentration of CC-292 required to inhibit BTK signaling and B cell proliferation, and to achieve BTK occupancy, suggesting a quantitative relationship among inhibition of BTK kinase activity, target occupancy, and functional assays *in vitro*. This direct correlation supports the use of BTK occupancy as a surrogate marker for inhibition of BTK activity.

It is noteworthy that this relationship between the inhibition achieved and the extent of CC-292-target engagement measured was also maintained *in vivo*. Specifically, CC-292 demonstrated therapeutic efficacy in a mouse CIA (Collagen-Induced Arthritis) model, with 85 and 95% inhibition of disease observed at doses of 10 mg/kg per day and 30 mg/kg per

day, respectively. Remarkably, in addition to a full phenotypic response, once daily, oral dosing of 10 mg/kg CC-292 resulted in 84% BTK inhibition verified by BTK occupancy analysis assayed 2 hours after dose administration.

This finding is consistent with a previous study by Honigberg *et al.* (*PNAS* 107(29):13075-13080, 2010), in which occupancy of the BTK active site by another BTK inhibitor PCI-32765 was monitored *in vitro* and *in vivo* using a fluorescent affinity probe for BTK. It was found that active site occupancy of BTK was tightly correlated with the blockade of BCR signaling and *in vivo* efficacy of PCI-32765, as measured by the mouse CIA model and the MRL-Fas(lpr) lupus model.

However, one prominent issue with the reported BTK occupancy assays is their limited sensitivity, which restricts preclinical occupancy measurements to biological samples with high BTK levels, such as samples derived from spleen lysates or B-cells isolated from large volumes of blood. It is believed that about 12-15 mL of human blood is required for one assay. This, in addition to being a potential burden on the patient if multiple measurements are desired (especially for chronically ill patients who may require frequent testing), also prevents the use of the assays in small experimental animal models (*e.g.*, in mice or rats) where acquisition of such a large sample volume is difficult if not impossible.

A schematic representation of the reported BTK occupancy assay is shown in Figure 1B, which illustrates a probable reason for its low sensitivity of detection. Specifically, since the assay does not directly measure inhibitor-bound BTK, but measures instead “free BTK” not already occupied by the BTK inhibitor, it is crucial that substantially all free BTK molecules in the sample are bound by the probe (*e.g.*, the biotin-conjugated probe in Figure 1B) in the assay, such that any remaining free BTK not bound by the probe (and not bound by the inhibitor) won’t be falsely attributed to inhibitor-bound BTK. This may require the use of excess probe in the assay to ensure all free BTK molecules are bound by the probe. However, if the excess probe is not properly removed after the initial BTK-probe binding step, the probe will also compete with the probe-bound BTK for binding to the streptavidin-coated assay well, thus falsely reducing the amount of detectable probe-bound BTK, and resulting in a higher calculated percentage of inhibitor-occupied BTK.

Thus one way to avoid this inherent problem is to identify a concentration of the probe, such that it is sufficiently high to bind substantially all free BTK in the sample (which may not be known prior to carrying out the assay), and sufficiently low such that any

remaining probe, after binding to free BTK, will not significantly reduce the binding between the capture agent (*e.g.*, coated streptavidin) and the probe-BTK complex.

Alternatively, after the initial BTK-probe binding step, any excess probe should be removed without significantly disturbing probe-free BTK binding, such as through cumbersome dialysis or ultrafiltration.

Thus there remains a need to provide a BTK occupancy assay with improved assay sensitivity, in order to enable establishment of the optimal dose, dose frequency, and degree of BTK inhibition required for full disease modification in animal models.

SUMMARY OF THE INVENTION

One aspect of the invention provides a method for measuring binding between a ligand and a binding partner of the ligand in a sample, the method comprising: (1) contacting the sample with an affinity reagent that specifically binds the binding partner, in the presence of a ligand probe, and allowing binding to occur between the affinity reagent and the binding partner, wherein a first percentage of the binding partner in the sample is bound by the ligand, and a second percentage of the binding partner in the sample is bound by the ligand probe; (2) contacting binding partner bound by the affinity reagent with a first detection probe, wherein one of said first detection probe and said ligand probe comprises a multivalent agent, and allowing binding to occur between said first detection probe and said ligand probe through the multivalent agent; (3) optionally, contacting the multivalent agent with a second detection probe comprising a second detectable label and a second moiety that binds to the multivalent agent, and allowing binding to occur between the multivalent agent and the second moiety on the second detection probe; (4) detecting the presence or measuring the amount of the first detection probe or the second detectable label, thereby measuring binding between the ligand and the binding partner.

In certain embodiments, the first detection probe comprises the multivalent agent.

In certain embodiments, the ligand probe comprises the multivalent agent.

In certain embodiments, the binding partner is an enzyme.

In certain embodiments, the binding partner is a protein kinase (such as BTK, BLK, EGFR1, HER2/ERBB2, HER3/Erb-B3, ERBB4, JAK3, TEC, BMX, ITK, LKB1, and TXK).

In certain embodiments, the ligand probe comprises a first moiety covalently linked to the ligand, wherein the first moiety binds to the multivalent agent.

In certain embodiments, the first detection probe comprises a first moiety that binds to the multivalent agent, and a first detectable label.

In certain embodiments, the first moiety is the same (or different) from the second moiety in the second detection probe.

In certain embodiments, the ligand comprises a substrate or a substrate analog of the enzyme or the kinase, or an inhibitor of the enzyme or the kinase.

In certain embodiments, the inhibitor binds in the ATP-binding site of the kinase.

In certain embodiments, the inhibitor is covalently linked to the kinase via Michael reaction.

In certain embodiments, the inhibitor is covalently linked to the thiol group of a cysteine residue corresponding to Cys481 of the human BTK, isoform 1.

In certain embodiments, the inhibitor inhibits a phosphorylated or active conformation of BTK.

In certain embodiments, the affinity reagent is an antibody, an antigen-binding portion of an antibody, a nanobody, or a DVD-Ig.

In certain embodiments, binding between the antibody and the binding partner does not substantially affect binding between the binding partner and the ligand or the ligand probe (and vice versa).

In certain embodiments, the percentage of the binding partner bound by the ligand is 100%, or is less than 100%, 90%, 80%, 70%, 60%, 50%, 40%, 30%, 20%, 10%, 5%, 3%, 2%, 1% or less.

In certain embodiments, the first detection probe further comprises a first detectable label.

In certain embodiments, the first detectable label is the same (or different from) the second detectable label of the second detection probe.

In certain embodiments, the multivalent agent is streptavidin (SA) or avidin.

In certain embodiments, the multivalent agent is an antibody (such as a bi-specific Ab, or an Ab having multiple Ag-binding fragments - IgA, IgM *etc.*).

In certain embodiments, the first detectable label and/or the second detectable label is a dye, a photocrosslinker, a cytotoxic compound, a drug, an affinity label, a photo affinity label, a reactive compound, an antibody or antibody fragment, a biomaterial, a nanoparticle, a

spin label, a fluorophore, a metal-containing moiety, a radioactive moiety, a novel functional group, a group that covalently or noncovalently interacts with other molecules, a photocaged moiety, an actinic radiation excitable moiety, a ligand, a photoisomerizable moiety, biotin, a biotin analogue, a moiety incorporating a heavy atom, a chemically cleavable group, a photocleavable group, a redox-active agent, an isotopically labeled moiety, a biophysical probe, a phosphorescent group, a chemiluminescent group, an electrochemiluminescent group (*e.g.*, an MSD SULFO-TAG type electrochemiluminescent group), an enzyme, an electron dense group, a magnetic group, an intercalating group, a chromophore, an energy transfer agent, a biologically active agent, or a combination thereof.

In certain embodiments, the first detectable label and/or the second detectable label comprises a fluorophore, such as a Bodipy fluorophore.

In certain embodiments, the first detectable label and/or the second detectable label comprises an enzyme, such as HRP, peroxidase (POD), glucose oxidase (GOD), invertase (INV), β -D-galactosidase (BGase), glucose-6-phosphate dehydrogenase (G6PDH), or alkaline phosphatase (ALP).

In certain embodiments, the first detectable label and/or the second detectable label comprises an HRP, and wherein the method further comprises contacting the HRP with a biotinylated tyramide to produce an activated tyramide.

In certain embodiments, the method further comprises detecting / measuring / quantitating the activated tyramide by adding a streptavidin-labeled fluorophore, or a combination of a streptavidin-labeled peroxidase (*e.g.*, HRP) and a chromogenic reagent, wherein the chromogenic reagent is preferably 3,3',5,5'-tetramethylbenzidine (TMB).

In certain embodiments, the sample is derived from a small volume of body fluid (such as blood sample, knee lavage from an arthritic knee).

In certain embodiments, the sample is a lysate of a tissue (*e.g.*, biopsy, animal tissue, clinical sample, such as spleen homogenates), a lysate of isolated cells (*e.g.*, B cells or PBMCs isolated / purified / enriched from whole blood sample), or a lysate of a cell culture.

In certain embodiments, the sample is thawed from storage at -20°C, -80°C, or in liquid nitrogen. The storage time can be at least 30 min, 1 hr, 2 hrs, 6 hrs, 12 hrs, 1 day, 1 week, 1 month, 1 yr, 10 yrs *etc.*

In certain embodiments, the affinity reagent is immobilized on a solid support (such as a multi-well ELISA plate, or a resin bead for a column).

In certain embodiments, the method further comprises blocking the immobilized affinity reagent with a blocking agent (*e.g.*, BSA) prior to step (1).

In certain embodiments, the method further comprises removing binding partner unbound by the affinity reagent after step (1).

In certain embodiments, the method further comprises removing ligand probe unbound by the binding partner after step (1).

In certain embodiments, the method further comprises removing the first detection probe after step (2) and before step (3).

In certain embodiments, the method further comprises removing the second detection probe after step (3) and before step (4).

In certain embodiments, the method further comprises carrying out steps (1), (2), and (4), and optionally step (3), using a control sample comprising a pre-determined amount of the binding partner having a pre-determined portion thereof bound by the ligand.

In certain embodiments, the method comprises carrying out steps (1), (2), and (4), and optionally step (3), multiple times, either in parallel or sequentially, using two or more control samples having the same pre-determined amount of the binding partner but different pre-determined proportions bound by the ligand, in order to construct a standard curve.

In certain embodiments, step (4) is carried out using chemiluminescent (including ECL), electrochemiluminescent (*e.g.*, MSD SULFO-TAG based electrochemiluminescent), surface plasmon resonance (SPR)-based biosensor (*e.g.*, BIAcore), flow cytometry, ELISA, or Western blot.

Another aspect of the invention provides a method for assessing or predicting efficacy for a potential BTK inhibitor in a mammal, the method comprising: using the subject assay method, measuring the binding between the ligand and the binding partner in a sample derived from the mammal, wherein the ligand is the potential BTK inhibitor, the binding partner is BTK from the mammal, and the mammal has BTK inhibitor-naïve baseline measurements or previously been administered the potential BTK inhibitor, wherein a higher level / extent of binding between the potential BTK inhibitor and BTK is predictive of a higher level of efficacy.

Another aspect of the invention provides a method for assessing the pharmacodynamics (PD) of a BTK inhibitor in a mammal, the method comprising: using the subject assay method, measuring the binding between the ligand and the binding partner,

from a series of samples each derived from a different time point following administering the mammal with the ligand, wherein the ligand is the BTK inhibitor, and the binding partner is BTK from the mammal.

Another aspect of the invention provides a method for identifying a desired dose of a BTK inhibitor for achieving a pre-determined level of binding between the BTK inhibitor and BTK in a mammal, the method comprising: using the subject assay method, measuring the binding between the ligand and the binding partner in a sample derived from the mammal, wherein the ligand is the BTK inhibitor, the binding partner is BTK from the mammal, and the mammal has previously been administered a candidate dose of the BTK inhibitor, wherein a level of measured binding lower than the pre-determined level is indicative that a dose higher than the candidate dose is required to achieve the desired dose, and wherein a level of measured binding higher than the pre-determined level is indicative that a dose lower than the candidate dose is required to achieve the desired dose.

In certain embodiments, the method further comprises repeating the method using a higher or lower candidate dose.

Another aspect of the invention provides a ligand probe comprising a first moiety or a multivalent agent covalently linked to a ligand, wherein the first moiety binds to the multivalent agent, and wherein the ligand probe is represented by the following structure: X - L - M, wherein X is the ligand, M is the first moiety or the multivalent agent, and L is a linker moiety that covalently joins the ligand to the first moiety or the multivalent agent.

It is contemplated that all embodiments described herein, including those only described in the examples or drawings, and those only described under one aspect of the invention, can apply to any aspects of the invention, and can be combined with any other embodiments of the invention, except for those explicitly disclaimed.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A is a schematic drawing (not to scale) of an exemplary embodiment of a BTK occupancy assay. The assay avoids the inherent conflict of the Evans assay (shown in Figure 1B) of requiring more probe in the probe-free BTK binding step, and requiring less probe in the subsequent binding to affinity / capture reagent. As a result of the improved design, more BTK (inhibitor-bound or probe-bound) can be captured for stronger signal. The amount of capture BTK can also be adjusted based on the amount of the affinity reagent. Furthermore, no probe pre-incubation step may be required for probe-free BTK binding since

both the sample and the probe can be added to the assay container simultaneously if the probe has reasonably fast on-rate (*e.g.*, ≤ 2 hrs). Finally, excess probe, if present, no longer affects the assay since it can be easily washed away from binding.

Figure 1B is a schematic drawing (not to scale) of the BTK occupancy assay as described in Evans *et al.* (*J. Pharmacol. Exp. Ther.* 346:219-228, 2013).

Figure 2 shows a head-to-head comparison of two standard curves generated using recombinant human BTK (rhBTK) in both the “original” occupancy assay of Evans *et al.* (*supra*) and the present improved occupancy assay (“alternative” assay). The graphs at left and middle highlight the linear portion of both the “original” and the improved occupancy assay standard curves. The graph at right shows the entire data set for both standard curves. Note that the “original” occupancy assay allows for detection of ~100 ng of rhBTK at 50% detection, while the improved occupancy assay allows for detection of ~5 ng of rhBTK at 50% detection.

Figure 3 shows percent BTK occupancy in multiple biological compartments, as determined by the subject assay. Stars indicate statistical significance.

DETAILED DESCRIPTION OF THE INVENTION

1. Overview

The invention described herein provides a BTK occupancy assay with improved design and sensitivity, thus providing a marked acceleration of clinical PD evaluation, which is typically not available until Phase 2 of a clinical trial. The rapid identification of doses providing BTK target engagement provides an advantage in the design of subsequent human clinical trials and supports Phase 2 dose selection to incorporate, among other things, safety, tolerability, and on-target activity. The assay provides an appropriate assessment for the relationship between complete or partial BTK inhibition and therapeutic efficacy. Direct quantification of BTK engagement in these trials also reduces uncertainty about the dose required for target inhibition and enables selection of the optimal pharmacological dose and dosing schedule. By providing this information to clinicians early in clinical development, sub-therapeutic drug administration in initial patient cohorts may be avoided, and time efficiencies in clinical testing are achieved to more rapidly impact patient health.

On the other hand, the assay is just as useful to avoid unnecessarily high dosing in the patient, especially in immunology trials in which the intention may be dosing to above 100%

occupancy. The subject occupancy assay will allow one to stop dosing at some reasonable small margin above 100%, rather than blindly dosing much higher than what is necessary.

The assay also allows refining the number of cohorts required for dose finding, thus quickly providing recommended Phase 2 doses and permitting rapid advancement into Phase 2 testing.

Thus in an exemplary but non-limiting embodiment, the present invention provides a BTK occupancy assay as illustrated in Figure 1A, and further described in detail in Example 1. Specifically, Figure 1A shows an assay container (*e.g.*, a well of a 96-well plate) having a well bottom surface coated by a rabbit monoclonal antibody specific for BTK. After the well is washed to remove excess antibody, and blocked with a blocking agent to reduce or eliminate non-specific binding, a sample (such as a blood sample derived from a patient) containing BTK, which may be partially inhibited by an inhibitor, is mixed with a biotin-conjugated probe designed to bind to free BTK, but not to BTK inhibited / occupied by the BTK inhibitor. All BTK molecules in the sample, however, regardless of whether they are bound by the inhibitor or by the probe, are proportionally pulled down or bound to the rabbit anti-BTK antibody immobilized on the well. After removing any unbound BTK, BTK inhibitor, and the probe, HRP-conjugated streptavidin (SA-HRP) is added such that the biotin moiety on the probe will be bound by the multivalent streptavidin. Next, after removing any unbound SA-HRP, biotin-conjugated HRP is added such that they will occupy any unbound sites on the multivalent SA. The end result is that each pulled down BTK molecule bound by the biotin-conjugated probe will be labeled by one or more HRP via the biotin-SA interaction, while each pulled down BTK molecule initially bound by the inhibitor will not be labeled by any HRP.

Alternatively, in another exemplary but non-limiting embodiment, the biotin-conjugated probe is replaced by a streptavidin-conjugated probe designed to bind free BTK. After allowing the immobilized antibody to bind BTK, and allowing the SA-conjugated ligand probe to bind free BTK that is not already bound by the BTK inhibitor, any unbound BTK, BTK inhibitor, and the SA-conjugated ligand probe are removed by washing, and biotin-conjugated HRP (biotin-HRP) is added as a detection probe such that multiple biotin-HRP are bound by each SA-conjugated ligand probe now bound to BTK. The end result is that each pulled down BTK molecule bound by the SA-conjugated ligand probe will be labeled by one or more HRP via the biotin-SA interaction, while each pulled down BTK molecule initially bound by the inhibitor will not be labeled by any HRP.

In both cases, the presence and the amount of the HRP moiety can be easily detected / quantitated using chemiluminescence (such as ECL), and the signal strength is inversely proportional to the amount of BTK initially occupied by the inhibitor, which are not labeled by the detection label HRP. In other words, the methods of the invention directly measure binding between the binding partner (such as BTK) and a probe derived from a ligand (such as a BTK inhibitor), but not the binding between the binding partner and the ligand, the latter of which can be calculated based on the former, based on, for example, the total amount of BTK in the sample.

In either embodiments, the specificity of the signal to a BTK occupancy is determined at least at two levels, *e.g.*, by the selectivity of the tagged, covalent ligand probe for BTK over other kinases, and by the specificity of the detection antibody for BTK.

Although the invention described herein is suitable for BTK occupancy assay, the invention is not so limited, in that it is a sensitive assay that can be applied to any binding measurement between two binding partners, such as any enzymes (*e.g.*, kinases) and their inhibitors or substrates / substrate analogs. In certain embodiments, the two binding partners become covalently linked after the binding occurs.

Thus one aspect of the invention provides a method for measuring binding between a ligand (*e.g.*, a BTK inhibitor) and a binding partner of the ligand (*e.g.*, a BTK) in a sample, the method comprising: (1) contacting the sample with an affinity reagent (*e.g.*, an anti-BTK Ab) that specifically binds the binding partner (*e.g.*, the BTK), in the presence of a ligand probe (*e.g.*, a biotin- or SA-conjugated BTK inhibitor), and allowing binding to occur between the affinity reagent (*e.g.*, the anti-BTK Ab) and the binding partner (*e.g.*, the BTK), wherein a first percentage of the binding partner (*e.g.*, the BTK) in the sample is bound by the ligand (*e.g.*, the BTK inhibitor), and a second percentage of the binding partner (*e.g.*, the BTK) in the sample is bound by the ligand probe (*e.g.*, the biotin- or SA-conjugated BTK inhibitor); (2) contacting binding partner (*e.g.*, the BTK) bound by the affinity reagent (*e.g.*, the anti-BTK Ab) with a first detection probe (*e.g.*, an SA-HRP or a biotin-HRP), wherein one of said first detection probe (*e.g.*, an SA-HRP or a biotin-HRP) and said ligand probe (*e.g.*, the biotin- or SA-conjugated BTK inhibitor) comprises a multivalent agent (*e.g.*, SA), and allowing binding to occur between said first detection probe and said ligand probe through the multivalent agent (*e.g.*, SA); (3) optionally, contacting the multivalent agent (*e.g.*, SA) with a second detection probe (*e.g.*, biotin-HRP) comprising a second detectable label (*e.g.*, HRP) and a second moiety (*e.g.*, biotin) that binds to the multivalent agent (*e.g.*, SA),

and allowing binding to occur between the multivalent agent (*e.g.*, SA) and the second moiety (*e.g.*, biotin) on the second detection probe (*e.g.*, the biotin-HRP); and, (4) detecting the presence or measuring the amount of the first detection probe or the second detectable label (*e.g.*, HRP), thereby measuring binding between the ligand (*e.g.*, the BTK inhibitor) and the binding partner (*e.g.*, the BTK).

According to this aspect of the invention, the method applies to, but is not limited to BTK or a kinase, and any of its many covalent / irreversible inhibitors (see below). Rather, the method has general applicability to measure the extent of binding between any two or more binding partners where it is desirable to determine the extent or degree one binding partner engaged or bound by another binding partner at a given time.

For example, in certain embodiments, one binding partner may be a polypeptide, such as an enzyme or a kinase, and another binding partner may be a substrate of the enzyme or kinase, or a substrate analog that can bind to the enzyme or kinase, but otherwise cannot be turned into a product by the enzyme or kinase. In a specific embodiment, the binding partner is a kinase (*e.g.*, BTK, BLK, EGFR1, HER2/ERBB2, HER3/Erb-B3, ERBB4, JAK3, TEC, BMX, ITK, LKB1, and TXK), and the ligand that binds to the binding partner is an ATP analog which resembles ATP (a substrate of a kinase), and may be able to bind in the ATP binding site of the kinase.

In another embodiment, the binding partner may be a protein and the ligand may block a biologically or functionally active binding site of the protein. For example, in certain embodiments, the binding partner may be a cytokine or a cytokine receptor, and the ligand may be a small molecule that binds to the cytokine or cytokine receptor, and blocks binding between the cytokine and the receptor. In certain embodiments, the binding partner may be a monomer of a multimeric cytokine (*e.g.*, a monomer of a trimeric TNF α), or a cytokine receptor subunit (such as the common gamma chain, common beta chain, or common alpha chain of the various interleukine receptors) that is activated by dimerization or multimerization with one other subunit, and the ligand inhibits dimerization / multimerization of the cytokine monomers or the receptor subunits.

Other suitable but non-limiting binding partners may include: antibody and its antigen, polynucleotide and a complementary polynucleotide, metal-containing ligand and metal chelator, receptor and its ligand (including steroid such as glucocorticoid hormone and its receptor such as GR), vitamin and vitamin-binding protein, enzyme and enzyme cofactor (*e.g.*, co-enzyme), transcription factor and target DNA sequence, aptamer and a ligand that

binds the aptamer, *etc.* For each binding partner - ligand pair, the ligand may be derived to become the ligand probe such that it preserves the ability to bind to its binding partner, but may also comprise an additional moiety such as biotin, for binding the first detection probe.

In certain embodiments, the first detection probe comprises the multivalent agent (*e.g.*, SA). In this case, the ligand probe may comprise a first moiety (*e.g.*, biotin) covalently linked to the ligand (*e.g.* the BTK inhibitor), wherein the first moiety (*e.g.* biotin) binds to the multivalent agent (*e.g.* SA). The first moiety facilitates the binding of the ligand probe to the multivalent agent. Thus the first moiety may be the same (or different) from the second moiety in the second detection probe.

In certain other embodiments, the ligand probe comprises the multivalent agent (*e.g.*, SA). In this case, the first detection probe may comprise a first moiety (*e.g.*, biotin) that binds to the multivalent agent (*e.g.*, SA), and a first detectable label (*e.g.*, HRP).

In certain embodiments, the first moiety may be an epitope recognized by an antibody or antigen-binding sequence thereof, and the multivalent agent comprises the antibody, antigen-binding sequence thereof, or an antibody mimetic (such as DVD-Ig).

In another embodiment, the first moiety is biotin, and the multivalent agent is a streptavidin or avidin or analogs thereof having more than one biotin binding sites.

Streptavidin homo-tetramers have an extraordinarily high affinity for biotin (also known as vitamin B7 or vitamin H), with a dissociation constant (K_d) on the order of about 10^{-14} mol/L. The binding of biotin to streptavidin is one of the strongest non-covalent interactions known in nature, and the resulting streptavidin-biotin complex is resistance to organic solvents, denaturants (*e.g.* guanidinium chloride), detergents (*e.g.* SDS, Triton), proteolytic enzymes, and extremes of temperature and pH. Streptavidin is a tetramer and each subunit binds biotin with equal affinity. Streptavidin mutants unable to bind biotin have been produced, and thus Streptavidin tetramers comprising on average 1, 2, 3, or 4 functional streptavidin monomers can be produced for use in the methods of the invention.

Alternatively, avidin homo-tetramers have a similar or even higher affinity for biotin, with a dissociation constant (K_d) on the order of about 10^{-15} mol/L. Avidin only has about 30% sequence identity to streptavidin, but nearly identical secondary, tertiary and quaternary structures. Unlike streptavidin, however, avidin is glycosylated, positively charged, and has pseudo-catalytic activity. A commercially available avidin derivative, deglycosylated avidin (*e.g.*, Sigma-Aldrich (Extravidin), Thermo Scientific (NeutrAvidin), Invitrogen (NeutrAvidin), and Belovo (NeutraLite)) is more comparable to the size, pI and nonspecific

binding of streptavidin, and thus can also be used in the methods of the invention. Another available avidin derivative has gained reversible binding characteristics through nitration or iodination of the binding site tyrosine. This modified avidin exhibits strong biotin binding characteristics at pH 4 and releases biotin at a pH of 10 or higher (Morag *et al.*, *The Biochemical Journal* 316(1): 193-199, 1996), and can also be used in the methods of the invention.

In certain embodiments, the affinity reagent may be an antibody, or an antigen-binding portion thereof, or an antibody like affinity reagent, such as an antibody mimetic.

Antibodies that can be used as affinity reagents include immunoglobulin molecules comprised of four polypeptide chains, two heavy (H) chains and two light (L) chains, interconnected by disulfide bonds. Each heavy chain is comprised of a heavy chain variable region (abbreviated herein as HCVR or VH) and a heavy chain constant region. Each light chain is comprised of a light chain variable region (abbreviated herein as LCVR or VL) and a light chain constant region. In certain embodiments, the antibody is IgG, such as IgG1 or IgG2. In certain embodiments, the antibody includes bi-specific antibody, antibody having multiple Ag-binding fragments, or multivalent antibodies, such as all isotypes of IgG, IgA, IgD, IgM, and IgE.

Antigen-binding portion of an antibody (or simply “antibody portion”) includes one or more fragments of an antibody that retain the ability to specifically bind to an antigen (*e.g.*, the binding partner). Examples of binding fragments include: (i) an Fab fragment, a monovalent fragment consisting of the VL, VH, CL and CH1 domains; (ii) an F(ab')₂ fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; (iii) an Fd fragment consisting of the VH and CH1 domains; (iv) an Fv fragment consisting of the VL and VH domains of a single arm of an antibody, (v) a dAb fragment (Ward *et al.*, *Nature* 341:544-546, 1989), which consists of a VH domain, or a single-domain antibody (sdAb, also called Nanobody by the developer Ablynx), an antibody fragment consisting of a single monomeric variable antibody domain; and (vi) an isolated complementarity determining region (CDR). Furthermore, although the two domains of the Fv fragment, VL and VH, are coded for by separate genes, they can be joined, using recombinant methods, by a synthetic linker that enables them to be made as a single protein chain in which the VL and VH regions pair to form monovalent molecules (known as single chain Fv (scFv); see *e.g.*, Bird *et al. Science* 242:423-426, 1988: and Huston *et al.*, *Proc. Natl. Acad. Sci. USA* 85:5879-5883, 1988). Such single chain antibodies are also intended to

be encompassed within the scope of antigen-binding portion of an antibody. Other forms of single chain antibodies, such as diabodies are also encompassed. Diabodies are bivalent, bispecific antibodies in which 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).

Further antibody-like or engineered antibodies that may be used in the subject method (either as affinity reagent or the multivalent agent) include IgG with engineered Fc regions, bi- and multi-specific antibodies, dual-variable domain Ig (DVD-Ig) I which each DVD-Ig Fab binds two targets, and can be engineered from any two mAb with distinct or identical binding specificity (see, for example, Wu *et al.*, *Generation and Characterization of a Dual Variable Domain Immunoglobulin (DVD-Ig™) Molecule*, in *Antibody Engineering*, Volume 2 (Springer Protocols), Roland E. Kontermann (Editor) & Stefan Dübel (Editor), Springer; 2nd ed., March 26, 2010).

Antibody mimetics are organic compounds that function like antibodies (*e.g.*, specifically bind antigens), but are not structurally related to antibodies. They are usually artificial peptides or proteins with a molar mass of about 3 to 20 kDa. Nucleic acids and small molecules are sometimes considered antibody mimetics as well, but not artificial antibodies, antibody fragments and fusion proteins composed from these. Some types of antibody mimetics have an antibody-like beta-sheet structure. Common advantages of antibody mimetics over antibodies may include better solubility, tissue penetration, stability towards heat and enzymes, and comparatively low production costs.

Antibody mimetics have been developed as therapeutic and diagnostic agents, and may include: affibody molecules, such as those comprising the Z domain of Protein A; affilins, such as those comprising Gamma-B crystallin or ubiquitin; affitins, such as those comprising Sac7d (from *Sulfolobus acidocaldarius*); anticalins, such as those comprising lipocalins; avimers, such as those comprising a domain of various membrane receptors; DARPins, such as those comprising an ankyrin repeat motif; Fynomers, such as those comprising the SH3 domain of Fyn; Kunitz domain peptides, such as those comprising the Kunitz domains of various protease inhibitors; monobodies (or adnectins), such as those comprising the 10th type III domain of fibronectin, and Centyrins.

An antibody or antigen-binding portion thereof may be part of a larger immunoadhesion molecule formed by covalent or noncovalent association of the antibody or antibody portion with one or more other proteins or peptides. Examples of such immunoadhesion molecules include use of the streptavidin core region to make a tetrameric scFv molecule (Kipriyanov *et al.*, *Human Antibodies and Hybridomas* 6:93-101, 1995) and use of a cysteine residue, a marker peptide and a C-terminal polyhistidine tag to make bivalent and biotinylated scFv molecules (Kipriyanov *et al.*, *Mol. Immunol.* 31:1047-1058, 1994). Antibody portions, such as Fab and F(ab')₂ fragments, can be prepared from whole antibodies using conventional techniques, such as papain or pepsin digestion, respectively, of whole antibodies. Moreover, antibodies, antibody portions and immunoadhesion molecules can be obtained using standard recombinant DNA techniques. Such immunoadhesion molecules can be used as the affinity reagent as well as the multivalent agent.

In another embodiment, the affinity reagent is not an antibody, but may comprise a moiety or domain that specifically binds to the binding partner (*e.g.*, enzyme or kinase). For example, in certain embodiments, the binding partner or enzyme or kinase has a His6 tag, and the affinity reagent comprises nickel or cobalt (*e.g.*, Ni-NTA beads). Similarly, in certain embodiments, the binding partner or enzyme or kinase has a GST tag, and the affinity reagent comprises immobilized glutathione (GSH). In certain embodiments, the binding partner has a Maltose-Binding Protein (MBP) tag, and the affinity reagent comprises immobilized amylose that binds MBP. In certain embodiments, the binding partner comprises a moiety that is recognized by a nucleotide aptamer specific for the moiety, and the affinity reagent comprises the aptamer.

In certain embodiments, binding between the affinity reagent / antibody and the binding partner does not substantially affect binding between the binding partner and the ligand or the ligand probe. In certain embodiments, binding between the binding partner and the ligand or the ligand probe does not substantially affect binding between the affinity reagent / antibody and the binding partner. For example, the ligand or ligand probe may bind to the ATP binding pocket of a kinase, while the affinity reagent or antibody may bind to an epitope on the N-lobe or C-lobe of the kinase. The affinity reagent or antibody may be designed or prepared based on the binding site on the binding partner for the ligand or ligand probe.

In certain embodiments, the method of the invention can be used in samples wherein 100%, or less than 100%, 90%, 80%, 70%, 60%, 50%, 40%, 30%, 20%, 10%, 5%, 3%, 2%, 1% or less of the binding partner is bound by the ligand.

In certain embodiments, a standard curve may be constructed using the method of the invention, with a series of samples each comprising the same known amount of a binding partner (*e.g.*, a BTK) but increasing known amounts of a ligand (*e.g.*, a BTK inhibitor), so that the extent of binding between the binding partner and the ligand can be measured over the range of the ligand. Thus, in certain embodiments, the method may comprise carrying out steps (1), (2), and (4), optionally also step (3), using a control sample comprising a pre-determined amount of the binding partner having a pre-determined portion thereof bound by the ligand. In certain embodiments, the method may comprise carrying out steps (1), (2), and (4), and optionally also step (3), multiple times, either in parallel or sequentially, using two or more control samples having the same pre-determined amount of the binding partner but different pre-determined proportions bound by the ligand, in order to construct a standard curve.

In certain embodiments, the method further comprises determining an activity of the binding partner. For example, the binding partner may be a protein kinase (*e.g.*, BTK) or enzyme, and the measured kinase / enzyme occupancy by its inhibitor can be correlated with the remaining kinase / enzyme activity in the presence of the same amount of the inhibitor.

In certain embodiments, the first detection probe comprises a multivalent agent, such as an SA, avidin, or a multivalent antibody, and binds to the ligand probe through the multivalent agent while providing additional binding sites, such as vacant biotin binding sites on SA, for the second detection probe. In certain embodiments, the first detection probe optionally comprises a first detectable label, which may be the same or different from the second detectable label of the second detection probe.

In certain related embodiments, the ligand probe comprises a multivalent agent, such as an SA, avidin, or a multivalent antibody, and binds to the first detection probe through the multivalent agent. In these embodiments, the first detection probe also comprises a first detectable label, such as HRP, and it is not necessary to have the optional step (3) or the second detection probe.

In certain embodiments, the first detectable label and/or the second detectable label may be a dye, a photocrosslinker, a cytotoxic compound, a drug, an affinity label, a photo affinity label, a reactive compound, an antibody or antibody fragment, a biomaterial, a

nanoparticle, a spin label, a fluorophore, a metal-containing moiety, a radioactive moiety, a novel functional group, a group that covalently or noncovalently interacts with other molecules, a photocaged moiety, an actinic radiation excitable moiety, a ligand, a photoisomerizable moiety, biotin, a biotin analogue, a moiety incorporating a heavy atom, a chemically cleavable group, a photocleavable group, a redox-active agent, an isotopically labeled moiety, a biophysical probe, a phosphorescent group, a chemiluminescent group, an electrochemiluminescent group (*e.g.*, an MSD SULFO-TAG type electrochemiluminescent group), an enzyme, an electron dense group, a magnetic group, an intercalating group, a chromophore, an energy transfer agent, a biologically active agent, or a combination thereof.

For example, in certain embodiments, the first detectable label and/or the second detectable label may comprise a fluorophore, such as a Bodipy fluorophore. In other embodiments, the first detectable label and/or the second detectable label comprises an enzyme, such as HRP. The type of detectable labels that may be used in the subject invention are described in further detail below.

In certain embodiments, the first detectable label and/or the second detectable label comprises an HRP, and the method further comprises contacting the HRP with a biotinylated tyramide to produce an activated tyramide.

In certain embodiments, the method further comprises detecting / measuring / quantitating the activated tyramide by adding a streptavidin-labeled fluorophore, or a combination of a streptavidin-labeled peroxidase (*e.g.*, HRP) and a chromogenic reagent, wherein the chromogenic reagent is preferably 3,3',5,5'-tetramethylbenzidine (TMB).

This embodiment further enhances the sensitivity of the subject assay when HRP is used as the detection label. The enhancement is based on the catalyzed reporter deposition (CARD) technique using derivatized tyramide, or Tyramide Signal Amplification (TSA), in which hydrogen peroxide and HRP convert labeled substrate (*e.g.*, biotinylated tyramide) into a short-lived but extremely reactive intermediate. The activated tyramide substrate molecule then rapidly reacts with and covalently binds to electron rich regions of any adjacent proteins. Binding of the biotinylated activated tyramide molecules occurs only immediately adjacent to the sites at which the activating HRP enzyme is bound. Thus the signal is only amplified locally. However, multiple deposition of the biotinylated activated tyramide occurs in a very short time (generally within 3-10 minutes), which effectively amplifies the original (one) HRP detectable label into multiple HRP labels, if more SA-conjugated HRPs are added to turn each biotinylated tyramide into an HRP label. Subsequent detection of the label yields

an effectively large amplification of the original signal. The PerkinElmer ELAST® (ELISA Amplification System) is commercially available for carrying out this amplification.

In certain embodiments, the sensitivity of the subject method is vastly improved compared to traditional assays in which only one (on average) detectable label is associated with each binding partner (*e.g.*, kinase) pulled down by ligand probe. For example, the sensitivity or detection limit of the subject method can be defined or measured as the amount of binding partner (*e.g.*, kinase, such as BTK) that is required to achieve half maximum signal from the detectable label. For example, as in Example 1 and shown in Figure 2, in each 96-well plate well, the present method can detect about 4-4.5 units of absorbance at 450 nm using HRP-mediated chemiluminescence. The data also shows that about 5 ng of free BTK not bound by any irreversible inhibitor is needed in the assay well to achieve half the maximum signal of about 2.2 absorbance units. In contrast, the traditional method requires about 100 ng of free BTK to achieve its half max. absorbance of about 0.7 unit. Thus the sensitivity of the subject method is at least about 20-fold higher than the traditional method.

With the increased sensitivity of the subject method, a wider range of samples may be used in the subject invention, including samples of smaller amount or limited availability. For example, the sample may be derived from a body fluid, such as a blood sample, or knee lavage from an arthritic knee. The sample may also be derived from a biopsy with relatively small quantity of tissue samples.

In certain embodiments, the sample is derived from cells (such as peripheral blood leukocytes) obtained from no more than 200 μ L, 100 μ L, 50 μ L, 30 μ L, 20 μ L, or 10 μ L of whole blood. In certain embodiments, the sample is derived from cells (*e.g.*, isolated leukocytes) obtained from a single knee lavage in a rat inflicted with a rat model of collagen-induced arthritis (CIA), or equivalent volume of knee lavage from a human patient.

In certain embodiments, the sample is derived from a body secretion, such as lacrimal / salivary gland secretion in a Sjogren's syndrome patient. In certain embodiments, the sample is derived from a urine sample, such as a urine sample from a Lupus Nephritis patient. In certain embodiments, the sample is derived from a fecal sample, such as a fecal sample from an IBD (*e.g.*, Crohn's disease or ulcerative colitis) patient. Preferably, the sample contains equivalent amount of total binding partner (*e.g.*, BTK) as compared to that in no more than 200 μ L, 100 μ L, 50 μ L, 30 μ L, 20 μ L, or 10 μ L of whole blood, or that in a single knee lavage in a rat inflicted with a rat model of collagen-induced arthritis (CIA).

The sample may be treated before being used in the method of the invention. In certain embodiments, the sample is a lysate of a tissue, *e.g.*, biopsy, animal tissue, clinical sample, such as spleen homogenates; a lysate of isolated cells, *e.g.*, B cells or PBMCs isolated / purified / enriched from whole blood sample; or a lysate of a cell culture.

In certain embodiments, the sample is freshly obtained from a patient. In other embodiments, the sample can be frozen for a period of time before thawed and used in the methods of the invention. For example, the sample may be thawed from storage at -20°C, -80°C, or in liquid nitrogen. The length of storage can be at least 30 min, 1 hr, 2 hrs, 6 hrs, 12 hrs, 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 1 week, 2 weeks, 3 weeks, 1 month, 2 months, 3 months, 6 months, 9 months, 1 yr, 2 yrs, 3 yrs, 5 yrs, 10 yrs, or longer.

In certain embodiments, the affinity reagent may be immobilized on a solid support or solid surface, such as a multi-well ELISA plate, an electrochemiluminescence plate (*e.g.*, a Meso Scale Discovery or MSD plate for electrochemiluminescence), a gold coated glass surface with optional derivatization suitable for use in a BIAcore surface plasmon resonance (SPR) (*e.g.*, surface for BIAcore Sensor Chip CM5, Sensor Chip SA, Sensor Chip NTA, Sensor Chip HPA, or Pioneer Chip), or a resin bead for a column.

The solid support or surface may have a wide variety of forms, including membranes, slides, plates, micromachined chips, microparticles, beads, and the like. Solid support / surfaces may comprise a wide variety of materials including, but not limited to, glass, plastic, silicon, alkanethiolate derivatized gold, cellulose, low cross linked and high cross linked polystyrene, silica gel, polyamide, and the like, and can have various shapes and features (*e.g.*, wells, indentations, channels, *etc.*). The support / surface can be hydrophilic or capable of being rendered hydrophilic, and may comprise inorganic powders such as silica, magnesium sulfate, and alumina; natural polymeric materials, particularly cellulosic materials and materials derived from cellulose, such as fiber containing papers, *e.g.*, filter paper, chromatographic paper, *etc.*; synthetic or modified naturally occurring polymers, such as nitrocellulose, cellulose acetate, poly(vinyl chloride), polyacrylamide, cross linked dextran, agarose, polyacrylate, polyethylene, polypropylene, poly(4-methylbutene), polystyrene, polymethacrylate, poly(ethylene terephthalate), nylon, poly(vinyl butyrate), *etc.*; either used by themselves or in conjunction with other materials; glass available as Bioglass, ceramics, metals, and the like. Natural or synthetic assemblies such as liposomes, phospholipid vesicles, and cells can also be employed. The support / surface can have any one of a number of shapes, such as strip, rod, particle, including bead, and the like.

In some embodiments, the solid support / surface comprises a bead or plurality of beads. The beads may be of any convenient size and fabricated from any number of known materials. Example of such materials include: inorganics, natural polymers, and synthetic polymers. Specific examples of these materials include: cellulose, cellulose derivatives, acrylic resins, glass, silica gels, polystyrene, gelatin, polyvinyl pyrrolidone, co-polymers of vinyl and acrylamide, polystyrene cross-linked with divinylbenzene or the like (as described, *e.g.*, in Merrifield, *Biochemistry* 1964, 3, 1385-1390), polyacrylamides, latex gels, polystyrene, dextran, rubber, silicon, plastics, nitrocellulose, natural sponges, silica gels, control pore glass, metals, cross-linked dextrans (*e.g.*, Sephadex) agarose gel (Sephacrose), and other solid phase supports known to those of skill in the art. The beads are generally about 2 to about 100 μm in diameter, or about 5 to about 80 μm in diameter, in some cases, about 10 to about 40 μm in diameter. In some embodiments the beads can be magnetic, paramagnetic, or otherwise responsive to a magnetic field. Having beads responsive to a magnetic field can be useful for isolation and purification of the beads, such as by the application of a magnetic field and isolation of the beads (*e.g.* by removal of the beads from solution, or removal of solution from the beads). Non-limiting examples of beads responsive to a magnetic field include Dynabeads, manufactured by Life Technologies (Carlsbad, Calif.). Other methods to separate beads can also be used. For example, the capture beads may be labeled with a fluorescent moiety which would make the bead complex fluorescent. The target capture bead complex may be separated, for example, by flow cytometry or fluorescence cell sorter. Beads may also be separated by centrifugation. Isolation of affinity reagent captured binding partner by attachment to beads may further comprise the step of washing the beads, such as in a suitable wash buffer. Generally, purification of 10-fold, 50-fold, 100-fold, 500-fold, 1000-fold, 10000-fold, 100000-fold, or more may be achieved.

In certain embodiments, the method is carried out on a high throughput platform, such as one capable of simultaneously analyzing 4, 8, 16, 32, 64, 96, 192, 384, 1536, or more samples. For example, the assay format may be MSD assay using electrochemiluminescent detection.

In certain embodiments, each sample may be analyzed simultaneously for multiple (*e.g.*, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) analytes, each with a specific affinity capture reagent that may be immobilized on a pre-determined addressable location.

In certain embodiments, the methods of the invention further comprise blocking the (immobilized) affinity reagent with a blocking agent (*e.g.*, BSA) prior to step (1). The

blocking agent may reduce non-specific binding between the binding partner to the affinity reagent, or between the binding partner and the container (*e.g.*, well) in which the assay is carried out. In certain embodiments, the blocking agent is removed afterwards by a suitable buffer wash.

In certain embodiments, the method further comprises removing the binding partner unbound by the affinity reagent after step (1). For example, the unbound binding partner may be washed away by a suitable buffer whereas the bound binding partner remains bound by the (immobilized) affinity reagent.

In certain embodiments, the method further comprises removing ligand probe unbound by the binding partner after step (1). This step may be done concurrently with the removal of the unbound binding partner, or after the removal of the unbound binding partner.

In certain embodiments, the method further comprises removing the first detection probe after step (2) and before step (3). In certain embodiments, the method further comprises removing the second detection probe after step (3) and before step (4). By washing away the unbound first and second detection probes, assay background due to the excess unbound first and second detection probes will be reduced.

In certain embodiments, step (4) is carried out using chemiluminescence or ECL, flow cytometry, ELISA, or Western blot, or any art recognized method designed to detect the signal on the first and/or second detection probe.

A specific illustrative (and non-limiting) embodiment of the method of the invention is provided herein. The specific embodiment uses BTK as an example of the binding partner, an irreversible BTK inhibitor as the ligand, and a ligand-derived ligand probe to carry out the BTK occupancy assay. However, the invention generally applies to other ligand-binding partner pairs, especially other kinases and their inhibitors.

In particular, according to the method of the invention, a 96-well plate (such as a 96-well high-binding ELISA plate) is first coated with an affinity reagent, such as 100 μ L of rabbit anti-BTK monoclonal antibody (Cell Signaling Cat. #8547S) diluted 1:250 in bicarbonate buffer to each well, and incubated overnight at 4°C. The affinity reagent is then removed by washing the plate 3-5 times using 1 $\times$ PBS 0.05% Tween 20 Wash Buffer. The plate is then blocked with 300 μ L per well of 3% BSA in PBS for 1 hour at room temperature on an orbital shaker to reduce non-specific binding. After blocking, the blocking reagent is removed by washing the plate 3-5 times using 1 $\times$ PBS 0.05% Tween 20 Wash Buffer.

To initiate the assay, about 50 μ L of proprietary ligand probe (about 50 nM) is added to all wells. Then about 50 μ L of BTK-containing sample or standard is added to the wells, such that the total volume in each well is about 100 μ L, including ligand probe and sample (or standard). Incubate the ligand probe / sample on plate for 2 hours at room temperature on an orbital shaker to allow binding between the affinity reagent / Ab and BTK occur. Any free BTK not already occupied by the ligand / inhibitor will be bound by the ligand probe.

Next, the plate is washed 3-5 times using 1 $\times$ PBS 0.05% Tween 20 Wash Buffer to remove unbound reagents. Then about 100 μ L of the first detection probe - streptavidin-HRP diluted 1:2000 in dilution buffer - is added for 30 minutes at room temperature on an orbital shaker, with the plate covered in foil. Upon completion of the binding between the SA moiety on the first detection probe and the biotin moiety on the ligand probe, any unbound first detection probe is removed by washing the plate 3-5 times using 1 $\times$ PBS 0.05% Tween 20 Wash Buffer. Then about 100 μ L of the second detection probe - biotin-HRP diluted 1:50,000 - is added, and the mixture is incubated for 30 minutes at room temperature on an orbital shaker, covered in foil.

The second detection probe is then removed by washing the plate 3-5 times using 1 $\times$ PBS 0.05% Tween 20 Wash Buffer. Finally, about 100 μ L of TMB (3,3',5,5'-tetramethylbenzidine) reagent is added to each well, and the plate is covered with aluminum foil to allow ECL to proceed. The TMB is allowed to develop for about 15-20 minutes, then about 100 μ L of 1.5N sulfuric acid is added to each well to stop the reaction. Signals can then be read from the wells of the plate at 450 nm.

The free BTK in each sample can be calculated based on a rhBTK standard curve, and % occupancy is based on % reduction in free BTK comparing drug-treated sample to vehicle-treated sample. For example, in vehicle-treated (or sham / mock treated) sample in which no BTK is previously occupied by an inhibitor / substrate analog, the level of BTK as measured by the subject method can be used as a reference level for total free BTK. This level may be based on one sample, or the average of several similarly vehicle / sham / mock treated samples (*e.g.*, 1500 ng/mL of free BTK in vehicle-treated spleen lysate sample). In an inhibitor-treated sample, the method of the invention measures the level of BTK not already occupied by the inhibitor. Thus a similar experiment using the inhibitor-treated sample may yield a measured free BTK level of, for example, 400 ng/mL. For this particular sample, free BTK level is about 27% (400/1500) of the control, hence about 73% of the BTK in the

inhibitor-treated sample is pre-occupied by the inhibitor, yielding a 73% inhibitor occupancy on BTK.

Similar calculations can be effected for any ligand - binding partner.

Another aspect of the invention provides a method for assessing or predicting efficacy for a potential kinase (*e.g.*, BTK) inhibitor in a mammal, the method comprising: using the subject kinase occupancy assay method, measuring the binding between a ligand (*i.e.*, the potential kinase inhibitor) and its binding partner (*i.e.*, the kinase) in a sample derived from the mammal, wherein the mammal has kinase (*e.g.*, BTK) inhibitor-naïve baseline measurements or previously been administered the potential kinase (*e.g.*, BTK) inhibitor, wherein a higher level / extent of binding between the potential kinase (*e.g.*, BTK) inhibitor and the kinase (*e.g.*, BTK) is predictive of a higher level of efficacy.

In certain embodiments, the potential kinase inhibitor is a potential BTK inhibitor, and the kinase is BTK from the mammal.

In certain embodiments, the method further comprises determining the extent of kinase inhibition by the potential kinase inhibitor. The method may further comprising comparing the extent of kinase inhibition with the extent of kinase being occupied by the potential kinase inhibitor. For example, the concentration of the kinase inhibitor required to achieve 50% kinase activity may be compared to the concentration of the kinase inhibitor required to achieve 50% kinase occupancy (*e.g.*, 50% of the kinase is occupied by the potential kinase inhibitor).

Another aspect of the invention provides a method for assessing the pharmacodynamics (PD) of a kinase (*e.g.* BTK) inhibitor in a mammal, the method comprising: using the subject kinase occupancy assay method, measuring the binding between a ligand (*i.e.*, the potential kinase inhibitor) and its binding partner (*i.e.*, the kinase), from a series of samples each derived from a different time point following administering the mammal with the ligand. The method may further comprise constructing a PD curve to show the extent of kinase occupation by the potential kinase inhibitor over time.

In certain embodiments, the potential kinase inhibitor is a potential BTK inhibitor, and the kinase is BTK from the mammal.

In certain embodiments, the method may further comprise adjusting (increasing or decreasing) the dose of the kinase inhibitor before repeating the method to obtain a revised PD curve.

Another aspect of the invention provides a method for identifying a desired dose of a kinase (*e.g.*, BTK) inhibitor for achieving a pre-determined level of binding between the kinase (*e.g.*, BTK) inhibitor and kinase (*e.g.*, BTK) in a mammal, the method comprising: using the subject kinase occupancy assay method, measuring the binding between the ligand (*i.e.*, the potential kinase inhibitor) and the binding partner (*i.e.*, the kinase) in a sample derived from the mammal, wherein the mammal has previously been administered a candidate dose of the kinase (*e.g.*, BTK) inhibitor, wherein a level of measured binding lower than the pre-determined level is indicative that a dose higher than the candidate dose is required to achieve the desired dose, and wherein a level of measured binding higher than the pre-determined level is indicative that a dose lower than the candidate dose is required to achieve the desired dose.

In certain embodiments, the desired dose, or the pre-determined level of binding between the kinase (*e.g.*, BTK) inhibitor and kinase (*e.g.*, BTK), is based on or determined by a desired pharmacodynamic effect.

In certain embodiments, the potential kinase inhibitor is a potential BTK inhibitor, and the kinase is BTK from the mammal.

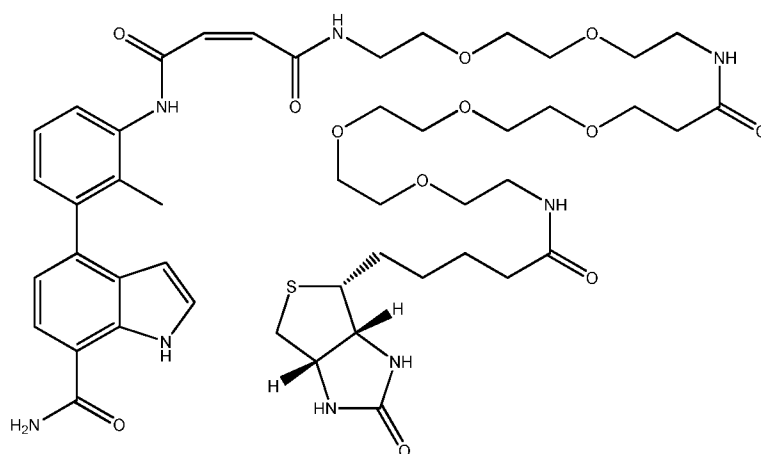
In certain embodiments, the method further comprises repeating the method using a higher or lower candidate dose.

Yet another aspect of the invention provides a ligand probe comprising a first moiety (*e.g.*, biotin) or a multivalent agent (*e.g.*, SA) covalently linked to a ligand (*e.g.*, BTK inhibitor), wherein the first moiety (*e.g.*, biotin) binds to the multivalent agent (*e.g.*, SA), and wherein the ligand probe is represented by the following structure: X - L - M, wherein X is the ligand, M is the first moiety or the multivalent agent, and L is a linker moiety that covalently joins the ligand to the first moiety or the multivalent agent.

In certain embodiments, the linker moiety is covalently linked to X through a -C(=O)-NH- bond. In certain embodiments, the linker moiety is covalently linked to M through a -NH-C(=O)- bond. In certain embodiments, the linker moiety comprises -(CH₂-CH₂-O)_n-, -(O-CH₂-CH₂)_m-, one or more -NHCO-, and/or one or more -CONH-, wherein m and n are each independently any one of the integers between 0-100. For example, n and m are each independently selected from 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10.

In certain embodiments, M is a biotin or a biotin analog, and the linker moiety is covalently linked to the terminal -C(=O)- group of biotin. An exemplary ligand moiety suitable for BTK occupancy assay, comprising a BTK inhibitor covalently linked to biotin

through a PEG repeat containing linker moiety, is represented below.



This ligand probe is derived from a specific BTK inhibitor. The specificity of BTK inhibition by the ligand probe shown above is demonstrated by the low IC_{50} value against BTK as compared to a panel of other kinases, as measured by the TR-FRET binding assay using a kinome panel (Invitrogen).

IC_{50} values (μM) of BTK ligand probe against a panel of kinases

Abl	>10	ACVR1	>10	Akt1	>10	ALK	>10
Aurora1	>10	Aurora2	>10	BRAF	>10	BTK	0.0166
CAMK1D	>10	CAMK2A	>10	CAMKK2	>10	CDK11	>10
CDK2	>10	CDK7 / Cyclin H / Mat1	>10	CDK8 / Cyclin C	>10	CDK9 / Cyclin K	>10
Ck1alpha1	>10	CLK2	>10	cMET	>10	CSF1R	>10
DDR1	>10	Dyrk1A	>10	Dyrk1B	>10	EGFR	>10
Erk2	>10	Zipk	>10	FAK	>10	FGFR1	>10
Flt1	>10	Fyn	>10	GRK5	>10	Gsk3a	>10
Gsk3b	>10	IGF1R	>10	IKKE	>10	InsR	>10
Wee1	>10	JAK2	>10	JAK3	1.21	JNK1	>10
JNK2	>10	Kdr	>10	Lck	>10	LTK	>10

MAP2K3	>10	MAP3K10	>10	MAP4K2	>10	MAP4K4	>10
MEK	>10	MEK1	>10	MST1	>10	Nek2	>10
p38 α	>10	PAK4KD	>10	PDGFRA V561D	>10	PDGFRB	>10
Pim1	>10	Pim2	>10	PKA	>10	PKCtheta	>10
PKCzeta	>10	PKG1A	>10	Plk3	>10	Prkn	>10
RET	>10	Rock1	>10	Rock2	>10	Rsk2	>10
SGK1	>10	Src	>10	STK16	>10	STK33	>10
Syk CatDom	>10	TAOK2	>10	TBK1	>10	TYRO3	>10
TNK2	>10	TrkA	>10	TrkB	>10	TrkC	>10

In certain embodiments, the linker or linker moiety is any one described in the section below entitled “Kinase occupancy assay.”

With the invention generally described above, certain aspects of the invention are described in further details in the separate sections below. Embodiments described in any parts of the specification, including those only described in the examples or those only specifically described under one but not the other aspects of the invention, are generally contemplated to be able to combine with any other embodiments described in any other parts of the specification.

2. *Kinase occupancy assay*

Protein kinases are enzymes that catalyze the transfer of the γ -phosphoryl group of ATP (ATP-Mg²⁺ complex) to the oxygen atom of the hydroxyl group of serine, threonine, or tyrosine residues in peptides and polypeptides (kinase substrates). Thus the kinase may be a Ser/Thr kinase, or a Tyr kinase, categorized according to their preferred substrate(s).

The methods of the invention can be used to determine the extent of a kinase that is bound / occupied by one of its substrates or substrate analogs or inhibitors (including irreversible inhibitors that may be covalently linked to the kinase) in a sample. For example, the methods of the invention (*e.g.*, kinase occupancy assay) may be used to measure the

extent of a kinase that is bound / occupied by any one of its inhibitors described below, using a probe that is substantially similar / identical in structure to the inhibitor in terms of binding to the ATP binding site on the kinase, but also contains a linker or extender that links another moiety, such as biotin, to the inhibitor (*e.g.*, biotin-conjugated inhibitor). Alternatively, the methods of the invention (*e.g.*, kinase occupancy assay) may also be used to measure the extent of a kinase that is bound / occupied by a proprietary inhibitor that does not have the same structure of any of the inhibitors described below, but nevertheless competitively binds to the ATP binding pocket of the kinase. In this embodiment, the same probe may be used to conduct the subject occupancy assay.

Thus for any of the kinase inhibitors described below, the inhibitor can be derived to become a corresponding probe comprising the inhibitor covalently linked to another moiety, such as biotin, through a linker or extender.

While there exist many different protein kinase sub-families within the broad grouping of protein kinases, they all share a common feature, and all act as ATP phosphotransferases. Thus, protein kinases share a very high degree of structural similarity in the region where the ATP is bound - the ATP binding pocket. The methods of the invention as applied to protein kinases - kinase occupancy assays - may be based on binding of a kinase inhibitor at the ATP binding pocket of the kinase.

Structural analysis of many protein kinases shows that the folding topology of the catalytic domain, responsible for the phosphotransfer activity, is very highly conserved. This domain is comprised of two lobes that are connected by a flexible hinge region. The amino-terminal lobe is comprised of a single alpha helix and five beta sheets, while the carboxy-terminal lobe is comprised of a four alpha helix bundle and a flexible loop called the activation loop. The ATP binding pocket (also referred to as the purine binding pocket) is formed at the interface between these two lobes. There are several highly conserved residues, including an invariant catalytic triad consisting of a single lysine and two aspartic acids. The lysine of this catalytic triad is responsible for properly positioning the γ -phosphate of ATP with the hydroxyl group of the residue in the substrate to which it is transferred (phosphoacceptor residue), while the first aspartic acid acts as a general base catalyst in the phosphotransfer reaction. Strikingly, these three crucial residues span the two lobes of the catalytic domain. Furthermore, the two aspartic acid residues within the catalytic triad are separated from each other by a second flexible region called the activation loop. To allow the phosphotransfer reaction, the structure of a substrate must conform to the geometric

constraints, surface electrostatics, and other features of the active site of the corresponding protein kinase. In turn, substrate binding can induce structural changes in a kinase that stimulate its catalytic activity. In particular, for enzyme - substrate interactions, residues within the activation loop and the catalytic loop need to be made available to make contacts with side chains in a substrate. Outside the conserved motifs crucial for catalytic activity (such as the ATP binding site), there are sequence differences in both loops that are critical for substrate recognition.

A list of representative protein kinases to which the method of the invention may be applied is provided in Table 1 of WO2003/081210 (incorporated herein by reference), which includes the type (Ser/Thr or Tyr), SwissProt, an NCBI Accession numbers, length, and literature references, and SEQ ID NOs for the amino acid sequence of each protein kinase. All references cited in Table 1 are also expressly incorporated herein by reference. An alignment of the sequences of the representative kinases is provided in Appendix A of WO2003/081210, which is also incorporated herein by reference.

Representative kinases to which the subject methods can be used to measure occupancy by an inhibitor, substrate or analog thereof may include, without limitation: AKT1; AKT2; AKT3; BLK; BTK; CDK1; CDK2; CDK3; CDK4; CDK5; CDK6; CDK7; CDK8; CDK9; CSK; EGFR1; ERBB2; ERBB4; ERK1; ERK2; ERK3; ERK4; ERK5; ERK6; FAK1; FGFR1; FGFR2; FGFR3; FGFR4; FYN; HCK; IKK-a; IKK-b; IKK-e; JAK1; JAK2; JAK3; JNK1; JNK2; JNK3; Lck; LYN; MAPK; NIK; PAK1; PAK2; PAK3; PAK4; PAK5; PDGFR-a; PDGFR-b; PIM1; A-Raf; B-Raf; C-Raf; SRC; SRC2; STK1; SYK; TEC; TGFR1; TGFR2; TIE1; TIE2; VEGFR1; VEGFR2; VEGFR3; YES; and ZAP-70.

In certain embodiments, representative kinases to which the subject methods can be used possess at least one naturally occurring cysteine at or near the ATP binding pocket of the kinase. Illustrative examples of such kinases include: BLK (C318); BTK (C481); EGFR1 (C797); HER2/ERBB2 (C805); ERBB4 (C803); JAK3 (C909); TEC (C449); BMX; ITK; LKB1; HER3/Erb-B3; and TXK. An additional about 200 kinases with at least one Cys near the ATP binding pocket are disclosed in Zhang *et al.* (*Nature Reviews Cancer* 9:28-39, 2009), incorporated herein by reference.

In certain embodiments, for certain kinases, one or more amino acids near the ATP binding pocket are mutated into a cysteine, before the mutant kinase is used in the method of the invention.

In certain embodiments, one or more other amino acids of the kinase not near the ATP binding pocket is a cysteine. Such Cys residue(s) may be used to immobilize the kinase to a solid support via, for example, reacting with a thiol reactive group on the solid support, such that it is not necessary to use an antibody specific for the kinase to pull down the kinase in the sample. Kinases in this group include: CDK5 (C53); ERK1 (C183); ERK2 (C166); ERK3 (C28); FGFR1 (C488); FGFR2 (C491); FGFR3 (C482); FGFR4 (C477); NIK (C533); PDGFR-a (C835); PDGFR-b (C843); SRC (C279); SRC2 (C273); STK1 (C828); TGFR2 (C396); VEGFR1 (C1039); VEGFR2 (C1045); VEGFR3 (C1054); YES (C287); ZAP-70 (C346).

In certain embodiments, one or more other amino acids not near the ATP binding pocket are mutated into a cysteine, before the mutant kinase is used in the method of the invention. Such mutation-generated Cys residue(s) may be used to immobilize the kinase to a solid support via, for example, reacting with a thiol reactive group on the solid support, such that it is not necessary to use an antibody specific for the kinase to pull down the kinase in the sample.

In these embodiments, the affinity reagent in step (1) of the method can be a thio reactive group that reacts with the binding partner (*e.g.*, kinase). Representative thiol reactive groups are well-known in the art, and may include (but are not limited to): pyridyldisulfides, nitropyridyldisulfides, maleimides, haloacetates and carboxylic acid chlorides.

For representative kinases, illustrative examples of their residues that can be mutated to Cys may include the following:

For the AKT1 kinase: L156C; K158C; T160C; F161C; K194C; E198C; M227C; E278C; T291C; K297C. For the AKT2 kinase: K158C; K160C; T162C; F163C; H196C; E200C; M229C; E279C; T292C; K298C. For the AKT3 kinase: L154C; K156C; T158C; F159C; H192C; E196C; M225C; E274C; T288C; K294C. For the BLK kinase: L246C; S248C; Q151C; F251C; A279C; E283C; T311C; A363C; A376C; R382C. For the BTK kinase: L408C; T410C; Q313C; F413C; E441C; E445C; T474C; R525C; S538C; R544C. For the CDK1 kinase: I10C; E12C; T14C; Y15C; S53C; E57C; F80C; Q432C; A145C; R151C. For the CDK2 kinase: I10C; E12C; T14C; Y15C; S53C; E57C; F80C; Q431C; A144C; R150C. For the CDK3 kinase: I10C; E12C; T14C; Y15C; S53C; E57C; F80C; Q431C; A144C; R150C. For the CDK4 kinase: I12C; V14C; A16C; Y17C; R55C; L59C; F93C; E153C; A157C; R163C. For the CDK5 kinase: I10C; E12C; T14C; Y15C; E57C; F80C; Q430C; A143C; R149C. For the CDK6 kinase: I19C; E21C; A23C; Y24C; A63C;

H67C; F98C; Q449C; A162C; R168C. For the CDK7 kinase: L18C; E20C; Q22C; F23C; R61C; L65C; F91C; N141C; A154C; K161C. For the CDK8 kinase: V27C; R29C; T31C; Y32C; R65C; L69C; F97C; A155C; A172C; H178C. For the CDK9 kinase: I25C; Q27C; T29C; F30C; R65C; I69C; F103C; A153C; A166C; R172C. For the CSK kinase: 1201 C; K203C; E205C; F206C; A232C; E236C; T266C; R318C; S331C; K337C. For the EGFR1 kinase: L718C; S720C; A722C; F723C; E758C; E762C; T790C; R841C; T854C; K860C. For the ERBB2 (also referred to as ErbB2) kinase: L726C; S728C; A730C; F731C; E766C; E770C; T798C; R849C; T862C; R868C. For the ERBB4 (also referred to as ErbB4) kinase: L724C; S726C; A728C; F729C; E764C; E768C; T796C; R847C; T860C; R864C. For the ERK1 kinase: I48C; E50C; A52C; Y53C; R84C; E88C; Q122C; S170C; R189C. For the ERK2 kinase: I31C; E33C; A35C; Y36C; R67C; E71C; Q105C; S153C; R172C. For the ERK3 kinase: L26C; G30C; N31C; H61C; E65C; Q108C; A156C; G170C; R176C. For the ERK4 kinase: L26C; F28C; V30C; N31C; H61C; E65C; Q105C; A153C; G167C; R173C. For the ERK5 kinase: I60C; N62C; A64C; Y65C; R97C; E101C; L136C; S185C; G198C; R204C. For the ERK6 kinase: V33C; S35C; A37C; Y38C; R70C; E74C; M109C; G157C; L170C; R176C. For the FAK1 kinase: I428C; E430C; Q333C; F433C; K467C; E471C; M499C; R550C; G563C; R569C. For the FGFR1 kinase: L484C; E486C; F489C; L528C; M532C; V561 C; R627C; A640C; R646C. For the FGFR2 kinase: L487C; E489C; F492C; L531C; M535C; V564C; R630C; A643C; R649C. For the FGFR3 kinase: L478C; E480C; F483C; L522C; M526C; V555C; R621C; A634C; R640C. For the FGFR4 kinase: L473C; E475C; F478C; L517C; M521C; V550C; R616C; A629C; R635C. For the FYN kinase: L276C; N278C; Q181C; F281C; S309C; E313C; T341C; A393C; A406C; R412C. For the HCK kinase: L268C; A270C; Q173C; F273C; A301C; E305C; T333C; A385C; A398C; R404C. For the IKK-a kinase: L21C; T23C; G25C; F26C; R57C; E61C; M95C; E148C; I164C; K170C. For the IKK-b kinase: L21C; T23Cb; G25C; F26C; R57C; E61C; M96C; E149C; I165C; K171C. For the IKK-e kinase: L15C; Q17C; A19C; T20C; V51C; E55C; M86C; G139C; T156C; R163C. For the JAK1 kinase: L870C; E872C; H874C; F875C; D909C; E913C; M944C; R995C; G1008C; K1014C. For the JAK2 kinase: L855C; L857C; N859C; F860C; D894C; E898C; M929C; R980C; G993C; K999C. For the JAK3 kinase: L828C; K830C; N832C; F833C; D867C; E871C; M902C; R953C; A966C; K972C. For the JNK1 kinase: I32C; S34C; A36C; Q37C; R69C; E73C; M108C; S155C; L168C; R174C. For the JNK2 kinase: I32C; S34C; A36C; Q37C; R69C; E73C; M108C; S155C; L168C; R174C. For the JNK3 kinase: I70C; S72C; A74C; Q75C; R107C; E111C; M146C; S193C; L206C; R212C. For the Lck kinase: L250C; A252C; Q155C; F255C; A283C; E287C; T315C;

A367C; A380C; R386C. For the LYN kinase: L252C; A254C; Q157C; F257C; A285C; E289C; T318C; A370C; A383C; D389C. For the MAPK kinase: V30C; S32C; A34C; Y35C; R67C; E71C; T106C; S154C; L167C; R173C. For the NIK kinase: L406C; R408C; S410C; F41 IC; F436C; E439C; M469C; D519C; V540C. For the PAK1 kinase: I276C; Q179C; A280C; S281C; N314C; V318C; M344C; D393C; T406C; A412C. For the PAK2 kinase: I255C; Q158C; A259C; S260C; N293C; V297C; M323C; D372C; T385C; A391C. For the PAK3 kinase: I274C; Q177C; A278C; S279C; N312C; V316C; M342C; D391C; T404C; A410C. For the PAK4 kinase: I327C; E329C; S331C; R332C; N365C; I369C; M395C; D444C; S457C; A463C. For the PAK5 kinase: I455C; E457C; S459C; T460C; N492C; I496C; M523C; D572C; D585C; A591C. For the PDGFR-a kinase: L599C; S601C; A603C; F604C; L641C; L645C; T674C; R822C; R841C. For the PDGFR-b kinase: L606C; S608C; A700C; F701C; L648C; L652C; T681C; R830C; R849C. For the PIM1 kinase: L44C; S46C; G48C; F49C; M87C; L91C; E121C; E171C; E171C; I185C; A192C. For the A-Raf kinase: I316C; T318C; S320C; F321C; A350C; E354C; T382C; N433C; G446C; T452C. For the B-Raf kinase: I462C; S464C; S466C; F467C; A496C; E500C; T528C; N579C; G592C; T598C. For the C-Raf kinase: I355C; S357C; S359C; F-360C; A389C; E393C; T421C; N472C; G485C; T491C. For the SRC kinase: L275C; Q178C; F280C; A308C; E402C; T340C; A392C; A405C; R41 IC. For the SRC2 kinase: L269C; T271C; F274C; A302C; E306C; T334C; A386C; A399C; R405C. For the STK1 kinase: L616C; S618C; A620C; F621C; L658C; L662C; F691C; R815C, R834C. For the SYK kinase: L377C; S379C; N381C; F382C; E416C; E420C; M448C; R498C; S511C; K518C. For the TEC kinase: L376C; S378C; L380C; F381C; D409C; E413C; T442C; R493C; S506C; R513C. For the TGFR1 kinase: I121C; K213C; R215C; F216C; F243C; E247C; S280C; K337C; A350C; V357C. For the TGFR2 kinase: V250C; K252C; R254C; F255C; K288C; D292C; T325C; S383C; L403C. For the TIE1 kinase: I845C; E847C; N849C; F850C; F884C; L888C; I917C; R983C; A996C1; R1002C. For the TIE2 kinase: I830C; E832C; N834C; F835C; F869C; L873C; I902C; R968C; A981C; R987C. For the VEGFR1 kinase: L834C; R836C; A838C; F839C; L876C; L880C; V910C; R1026C; R1045C. For the VEGFR2 kinase: L840C; R842C; A844C; F845C; L882C; L886C; V916C; R1032C; R1051C. For the VEGFR3 kinase: L851C; Y853C; A855C; F856C; L893C; L987C; V927C; R1041C; R1060C. For the YES kinase: L283C; Q286C; C287C; F288C; A316C; E320C; T348C; A400C; A413C; R419C. For the ZAP-70 kinase: L344C; N348C; F349C; E382C; E386C; M414C; R465C; S478C; and K485C.

For any of the kinase above, the methods of the invention may be applicable to any of their substrates or analogs thereof, or inhibitors thereof.

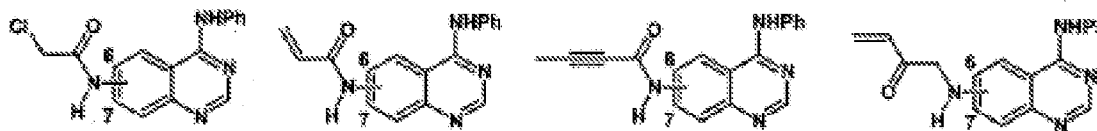
In certain embodiments, the methods of the invention are applicable to measure kinase occupancy by a kinase inhibitor. The inhibitor may be a reversible inhibitor, a covalent / irreversible inhibitor, a competitive inhibitor, or a non-competitive inhibitor.

In certain embodiments, the inhibitor is tethered, via a reversible or irreversible covalent bond, to the protein kinase. For example, the inhibitor may form an irreversible covalent bond through the nucleophile or electrophile, preferably nucleophile, on the protein kinase, thereby forming an irreversible protein kinase-inhibitor complex. The nucleophile on the kinase may be the sulfur of a thiol, usually a thiol group of a cysteine, which is reacted with a thiol reactive group capable of forming an irreversible (preferably under conditions that do not denature the kinase) covalent bond with the free thiol. Preferably, the thiol reactive group on the inhibitor is a group capable of undergoing SN₂-like attack by the thiol or forming a Michael-type adduct with the thiol to produce the irreversible reaction product.

For example, compounds of the form -RC=CR-Q, or -C≡C-Q, where Q is C(=O)H, C(=O)R (including quinines), COOR, C(=O)NH₂, C(=O)NHR, CN, NO₂, SOR, SO₂R, where each R is independently substituted or unsubstituted alkyl, aryl, hydrogen, halogen or another Q, can form Michael adducts with -SR (where R is H, glutathione or S-lower alkyl substituted with NH₂ or OH), OH and NH₂ on the target protein kinase. A Michael adduct can also be formed between a Cys thiol group of the kinase, and a Michael acceptor moiety on the inhibitor. Representative Michael acceptors include acrylamide, vinyl sulfonamide, and propargylamide.

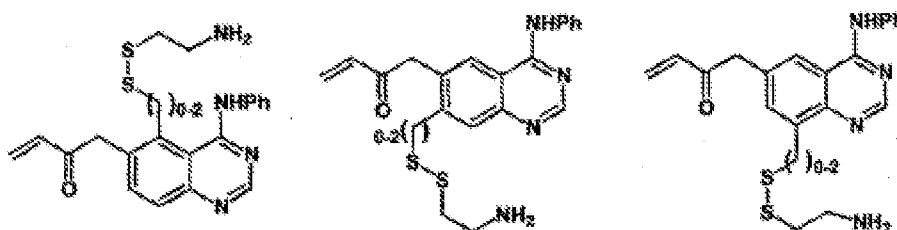
Quinazolines, broad specificity kinase inhibitors, are known to bind to the purine pocket of a myriad of protein kinases and are possibly the most commonly used of all the kinase inhibitor structural motifs. Furthermore, 6-arylamido quinazoline-based inhibitors are known to irreversibly label the EGFR1 kinase through reactivity with a cysteine residue (Cys773) located on the floor of the purine-binding pocket. In addition, 7-acrylamido quinazolines also irreversibly modify the EGFR1 kinase, indicating that despite the high affinity of these compounds for the purine pocket, they do have limited mobility within their binding site. Thus, similarly modified quinazolines can bind covalently but with loose specificity to the purine-binding pocket of any appropriately engineered protein kinase. For example, a set of eight 4-phenylamino quinazolines containing Michael acceptors in the 6 and 7 positions as shown below, can be used as broad spectrum inhibitors that bind the ATP

binding pocket of a kinase. These structures are shown below.



These compounds are designed to bind in the purine pocket of protein kinases, with little specificity for any particular kinase. Synthesis and purification of these compounds is relatively straightforward. Taken together, this panel accommodates possible slight variations in the position of the residue analogous to C773 of EGFR1 by varying the position, special orientation, and reactivity of the Michael acceptor.

These kinase inhibitors can be derived to become their corresponding probes by using a linker or extender. The exact extenders used partly depend on which of the 4-phenylamino quinazolines discussed above bind most efficiently to the kinase of interest. For example, as shown below, for any of the 4-phenylamino quinazolines, thiol groups can be appended to the 5, 6, 7, or 8 position of the best quinazolines, separated by a 1-, 2-, or 3-atom spacer.



The amino group at the end of the extender / linker can then be reacted with an amino reactive group (such as a carboxyl group) to attach biotin or other moieties. Such (biotin-linked) probes may be used in the subject kinase occupancy assay regardless of the identity of the kinase inhibitor, so long as the inhibitor and the probe do not simultaneously bind the ATP binding pocket of the kinase.

In certain embodiments, the linker / extender may comprise a bond, a substituted alkyl moiety, a substituted heterocycle moiety, a substituted amide moiety, a ketone moiety, a substituted carbamate moiety, an ester moiety, or any combination thereof.

In certain embodiments, the ligand probe of the invention may have the general structure of Ligand-X-linker-Y-1st moiety, wherein: X and Y are independently selected from the group consisting of: a bond, -O(C=O)-, -NR^a(C=O)-, -NR^a-, an N-containing heterocycle, -O-, -S-S-, -S-, -O-NR^a-, -O(C=O)O-, -O(C=O)NR^a-, -NR^a(C=O)NR^a-, -N=CR^a-, -S(C=O)-, -S(O)-, and -S(O)₂-; and R^a is hydrogen or alkyl.

The linker moiety may be selected from a bond, a polymer, a water soluble polymer, optionally substituted alkyl, optionally substituted heteroalkyl, optionally substituted heterocycloalkyl, optionally substituted cycloalkyl, optionally substituted heterocycloalkylalkyl, optionally substituted heterocycloalkylalkenyl, optionally substituted aryl, optionally substituted heteroaryl, and optionally substituted heterocycloalkylalkenylalkyl.

In some embodiments, the linker moiety is an optionally substituted heterocycle. In other embodiments, the heterocycle is selected from aziridine, oxirane, episulfide, azetidine, oxetane, pyrrolidine, tetrahydrofuran, tetrahydrothiophene, pyrrolidine, pyrazole, pyrrole, imidazole, triazole, tetrazole, oxazole, isoxazole, oxirane, thiazole, isothiazole, dithiolane, furan, thiophene, piperidine, tetrahydropyran, thiane, pyridine, pyran, thiapyrane, pyridazine, pyrimidine, pyrazine, piperazine, oxazine, thiazine, dithiane, and dioxane. In some embodiments, the heterocycle is piperazine. In further embodiments, the linker moiety is optionally substituted with halogen, CN, OH, NO₂, alkyl, S(O), and S(O)₂. In other embodiments, the water soluble polymer is a PEG group.

In other embodiments, the linker moiety provides sufficient spatial separation between the 1st moiety and the ligand. In further embodiments, the linker moiety is stable. In yet a further embodiment, the linker moiety does not substantially affect the binding of the 1st moiety (*e.g.*, biotin) to the 1st detection probe. In other embodiments the linker moiety provides chemical stability to the ligand probe. In further embodiments, the linker moiety provides sufficient solubility to the ligand probe.

In some embodiments, linkages such as water soluble polymers are coupled at one end to a ligand (*e.g.*, kinase inhibitor) and to a 1st moiety (*e.g.*, biotin) at the other end. In other embodiments, the water soluble polymers are coupled via a functional group or substituent of the ligand. In further embodiments, the water soluble polymers are coupled via a functional group or substituent of the 1st moiety. In other embodiments, covalent attachment of hydrophilic polymers to a ligand and the 1st moiety represents one approach to increasing water solubility (such as in a physiological environment), bioavailability, increasing serum half-life, increasing pharmacodynamic parameters, or extending the circulation time of the ligand probe, including proteins, peptides, and particularly hydrophobic molecules. In further embodiments, additional important features of such hydrophilic polymers include biocompatibility and lack of toxicity. In other embodiments, the polymer is pharmaceutically acceptable.

In some embodiments, examples of hydrophilic polymers include, but are not limited to: polyalkyl ethers and alkoxy-capped analogs thereof (*e.g.*, polyoxyethylene glycol, polyoxyethylene/propylene glycol, and methoxy or ethoxy-capped analogs thereof, polyoxyethylene glycol, the latter is also known as polyethylene glycol or PEG); polyvinylpyrrolidones; polyvinylalkyl ethers; polyoxazolines, polyalkyl oxazolines and polyhydroxyalkyl oxazolines; polyacrylamides, polyalkyl acrylamides, and polyhydroxyalkyl acrylamides (*e.g.*, polyhydroxypropylmethacrylamide and derivatives thereof); polyhydroxyalkyl acrylates; polysialic acids and analogs thereof; hydrophilic peptide sequences; polysaccharides and their derivatives, including dextran and dextran derivatives, *e.g.*, carboxymethyl dextran, dextran sulfates, aminodextran; cellulose and its derivatives, *e.g.*, carboxymethyl cellulose, hydroxyalkyl celluloses; chitin and its derivatives, *e.g.*, chitosan, succinyl chitosan, carboxymethylchitin, carboxymethylchitosan; hyaluronic acid and its derivatives; starches; alginates; chondroitin sulfate; albumin; pullulan and carboxymethyl pullulan; polyaminoacids and derivatives thereof, *e.g.*, polyglutamic acids, polylysines, polyaspartic acids, polyaspartamides; maleic anhydride copolymers such as: styrene maleic anhydride copolymer, divinylethyl ether maleic anhydride copolymer; polyvinyl alcohols; copolymers thereof; terpolymers thereof; mixtures thereof; and derivatives of the foregoing. In other embodiments, the water soluble polymer is any structural form including but not limited to linear, forked or branched. In some embodiments, polymer backbones that are water-soluble, with from 2 to about 300 termini, are particularly useful. In further embodiments, multifunctional polymer derivatives include, but are not limited to, linear polymers having two termini, each terminus being bonded to a functional group which is the same or different. In some embodiments, the water polymer comprises a poly(ethylene glycol) moiety. In further embodiments, the molecular weight of the polymer is of a wide range, including but not limited to, between about 100 Da and about 100,000 Da or more. In yet further embodiments, the molecular weight of the polymer is between about 100 Da and about 100,000 Da, including but not limited to, about 100,000 Da, about 95,000 Da, about 90,000 Da, about 85,000 Da, about 80,000 Da, about 75,000 Da, about 70,000 Da, about 65,000 Da, about 60,000 Da, about 55,000 Da, about 50,000 Da, about 45,000 Da, about 40,000 Da, about 35,000 Da, 30,000 Da, about 25,000 Da, about 20,000 Da, about 15,000 Da, about 10,000 Da, about 9,000 Da, about 8,000 Da, about 7,000 Da, about 6,000 Da, about 5,000 Da, about 4,000 Da, about 3,000 Da, about 2,000 Da, about 1,000 Da, about 900 Da, about 800 Da, about 700 Da, about 600 Da, about 500 Da, about 400 Da, about 300 Da, about 200 Da, and about 100 Da. In some embodiments, the molecular weight of the polymer is

between about 100 Da and 50,000 Da. In some embodiments, the molecular weight of the polymer is between about 100 Da and 40,000 Da. In some embodiments, the molecular weight of the polymer is between about 1,000 Da and 40,000 Da. In some embodiments, the molecular weight of the polymer is between about 5,000 Da and 40,000 Da. In some embodiments, the molecular weight of the polymer is between about 10,000 Da and 40,000 Da. In some embodiments, the poly(ethylene glycol) molecule is a branched polymer. In further embodiments, the molecular weight of the branched chain PEG is between about 1,000 Da and about 100,000 Da, including but not limited to, about 100,000 Da, about 95,000 Da, about 90,000 Da, about 85,000 Da, about 80,000 Da, about 75,000 Da, about 70,000 Da, about 65,000 Da, about 60,000 Da, about 55,000 Da, about 50,000 Da, about 45,000 Da, about 40,000 Da, about 35,000 Da, about 30,000 Da, about 25,000 Da, about 20,000 Da, about 15,000 Da, about 10,000 Da, about 9,000 Da, about 8,000 Da, about 7,000 Da, about 6,000 Da, about 5,000 Da, about 4,000 Da, about 3,000 Da, about 2,000 Da, and about 1,000 Da. In some embodiments, the molecular weight of the branched chain PEG is between about 1,000 Da and about 50,000 Da. In some embodiments, the molecular weight of the branched chain PEG is between about 1,000 Da and about 40,000 Da. In some embodiments, the molecular weight of the branched chain PEG is between about 5,000 Da and about 40,000 Da. In some embodiments, the molecular weight of the branched chain PEG is between about 5,000 Da and about 20,000 Da. The foregoing list for substantially water soluble backbones is by no means exhaustive and is merely illustrative, and in some embodiments, the polymeric materials having the qualities described above suitable for use in methods and compositions described herein.

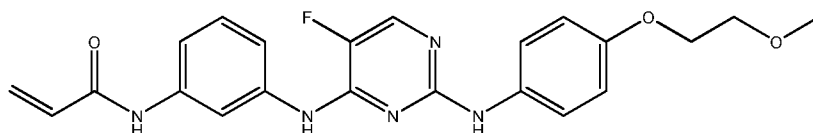
In further embodiments, the number of water soluble polymers linked to the ligand and the 1st moiety described herein is adjusted to provide an altered (including but not limited to, increased or decreased) pharmacologic, pharmacokinetic or pharmacodynamic characteristic such as *in vivo* half-life. In some embodiments, the half-life of the ligand probe is increased at least about 10, about 20, about 30, about 40, about 50, about 60, about 70, about 80, about 90 percent, about two fold, about five-fold, about 10-fold, about 50-fold, or at least about 100-fold over a ligand probe without a water soluble linker.

In another embodiment, X is selected from the group consisting of: a bond, -O(C=O)-, -NR^a(C=O)-, -NR^a-, an N-containing heterocycle, -O-, -S-, -S-S-, -O-NR^a-, -O(C=O)O-, -O(C=O)NR^a-, -NR^a(C=O)NR^a-, -N=CR^a-, -S(C=O)-, -S(O)-, and -S(O)₂-. In one embodiment, X is -NR^a(C=O)-. In another embodiment, X is a bond. In another

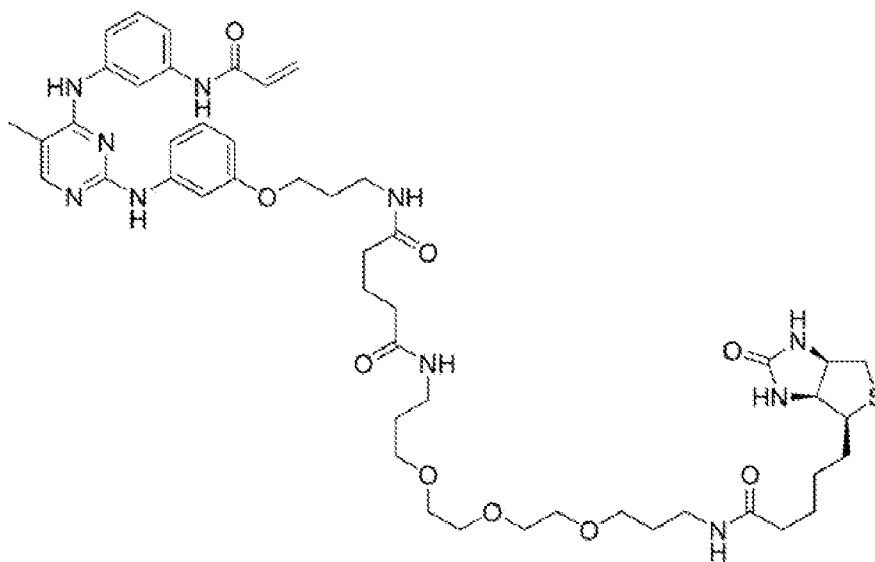
embodiment, X is $-O(C=O)-$. In a further embodiment, Y is selected from the group consisting of: a bond, $-O(C=O)-$, $-NR^a(C=O)-$, $-NR^a-$, an N-containing heterocycle, $-O-$, $-S-$, $-S-S-$, $-O-NR^a-$, $-O(C=O)O-$, $-O(C=O)NR^a-$, $-NR^a(C=O)NR^a-$, $-N=CR^a-$, $-S(C=O)-$, $-S(O)-$, and $-S(O)_2-$. In yet a further embodiment, Y is a bond. In one embodiment, Y is $-NR^a(C=O)-$. In yet another embodiment, R^a is hydrogen. In yet a further embodiment, R^a is alkyl.

In certain embodiments, the kinase occupancy assay of the invention is for a BTK of human or non-human origin, and an irreversible BTK inhibitor that forms a covalent bond with a Cys residue corresponding to Cys481 of the human BTK, isoform 1, via a Michael acceptor on the inhibitor, wherein the Michael acceptor is acrylamide, vinyl sulfonamide, or propargylamide.

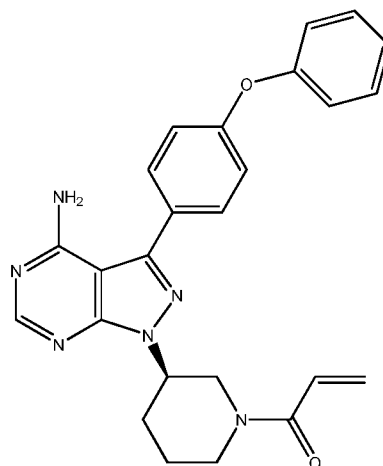
In certain embodiments, the BTK inhibitor is CC-292 (N-(3-(5-fluoro-2-(4-(2-methoxyethoxy)phenylamino)pyrimidin-4-ylamino)phenyl)acrylamide).



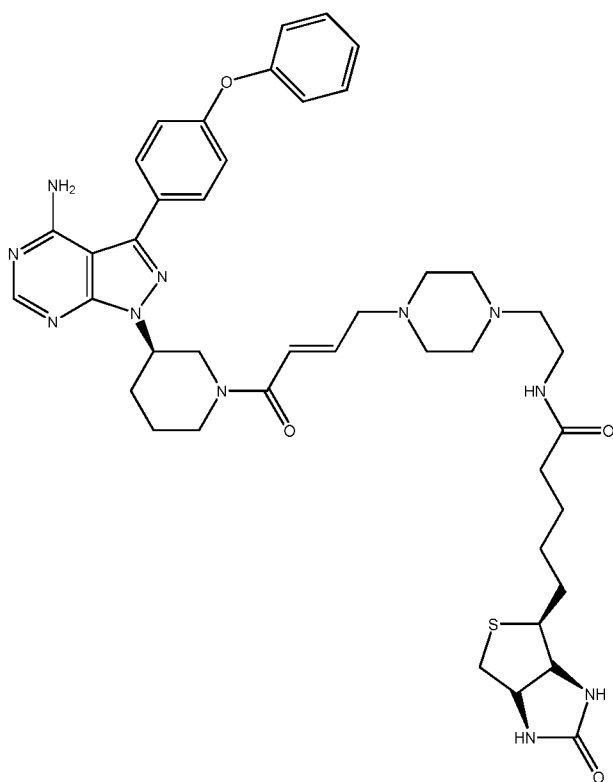
In certain embodiments, the probe is a biotin-linked CC-292 of the following structure:



In certain embodiments, the BTK inhibitor is (R)-1-(3-(4-amino-3-(4-phenoxyphenyl)-1H-pyrazolo[3,4-d]pyrimidin-1-yl)piperidin-1-yl)prop-2-en-1-one (or ibrutinib).



The corresponding probe may be a biotin-linked ibrutinib of the following structure:



In certain embodiments, the inhibitor and/or the ligand probe binds the active form of the kinase (*e.g.*, BTK). In other embodiments, the inhibitor and/or the ligand probe binds the inactive form of the kinase (*e.g.*, BTK). In certain embodiments, the inhibitor and/or the ligand probe preferentially (*e.g.*, having a binding affinity 50%, 100%, 2-fold, 3-, 5-, 10-, 20-, 50-, or 100-times or higher) binds the active form of the kinase (*e.g.*, BTK). In certain embodiments, the inhibitor and/or the ligand probe preferentially (*e.g.*, having a binding affinity 50%, 100%, 2-fold, 3-, 5-, 10-, 20-, 50-, or 100-times or higher) binds the inactive form of the kinase (*e.g.*, BTK). The active form of the BTK has Tyr phosphorylation at a

residue corresponding to Tyr551 of human BTK isoform 1, and may further comprise Tyr phosphorylation at a residue corresponding to Tyr223 of human BTK isoform 1.

In certain embodiments, the inhibitor and/or the ligand probe is cell permeable.

3. *BTK*

There are three known human BTK isoforms, one of which, NP_000052, relates to the 659-a.a. BTK isoform 1. Residue 481 of the human BTK isoform 1 is the Cys481 residue that can be irreversibly and covalently linked to a BTK inhibitor binding to the ATP binding pocket of the kinase, through Michael reaction. Isoform 2 (NP_001274274) has 483 a.a., and contains an alternate exon in the 5' UTR and lacks several exons in the 3' CDS (coding sequence) compared to isoform 1, resulting in a shorter protein compared to isoform 1. Isoform 3 (NP_001274273) has 693 a.a., and has a longer N-terminus than isoform 1. U.S. Pat. No. 6,326,469 discloses in its FIGs. 10A and 10B the 659-a.a. human BTK (also known as hATK) isoform 1 protein as SEQ ID NO: 8 (incorporated herein by reference).

Additional mammalian BTK sequences are known in the art, and can be retrieved from public database such as GenBank by, for example, performing a BLASTp search using the human BTK isoform 1 sequence as a query sequence.

For example, using the human BTK isoform 1 protein sequence as the query, the following BTK sequences from other species are identified: XP_003954082.1 from *Pan troglodytes* (chimpanzee); XP_004441045.1 from *Ceratotherium simum simum* (southern white rhinoceros); XP_001493268.1 from *Equus caballus* (horse); XP_004905120.1 from *Heterocephalus glaber* (naked mole-rat); XP_008051143.1 from *Tarsius syrichta* (Philippine tarsier); XP_005407008.1 from *Chinchilla lanigera* (long-tailed chinchilla); XP_003135290.2 from *Sus scrofa* (pig); XP_005072547.1 from *Mesocricetus auratus* (golden hamster); XP_549139.2 from *Canis lupus familiaris* (dog); XP_004283555.1 from *Orcinus orca* (killer whale); NP_038510.2 from *Mus musculus* (house mouse); NP_001029761.1 from *Bos taurus* (cattle); XP_004000760.1 from *Felis catus* (domestic cat); and NP_001007799.1 from *Rattus norvegicus* (Norway rat), all with > 98% sequence identity to the human BTK isoform 1.

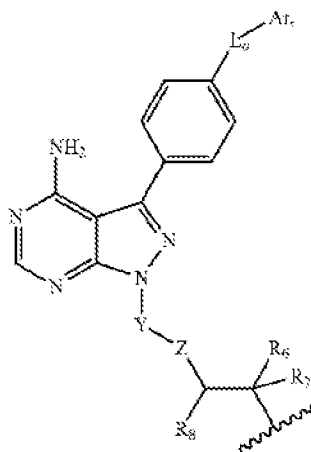
Thus the method of the invention can be applied to any and all BTK isoforms from any and all species, although the Cys481 residue in different BTK isoforms or BTK from different species will have a different residue number. Sequence alignments using any of art

recognized software, such as searching the nr database using the human BTK isoform 1 sequence as the query sequence, will readily reveal the Cys residue corresponding to Cys481 of the human BTK isoform 1. Specifically, the methods and reagents of the invention can be used for human and other non-human mammals, including but not limited to non-human primates, livestock (mammalian) animals, experimental / laboratory animals (*e.g.*, rats, mice, hamsters, or other rodents), mammalian pets (*e.g.*, cats or dogs), or marine mammals, such as those named above.

4. *BTK inhibitors*

The methods of the invention may be used for BTK occupancy assay for any of many known BTK inhibitors, including the various irreversible BTK inhibitors that form a covalent bond with a Cys residue corresponding to Cys481 of the human BTK, isoform 1.

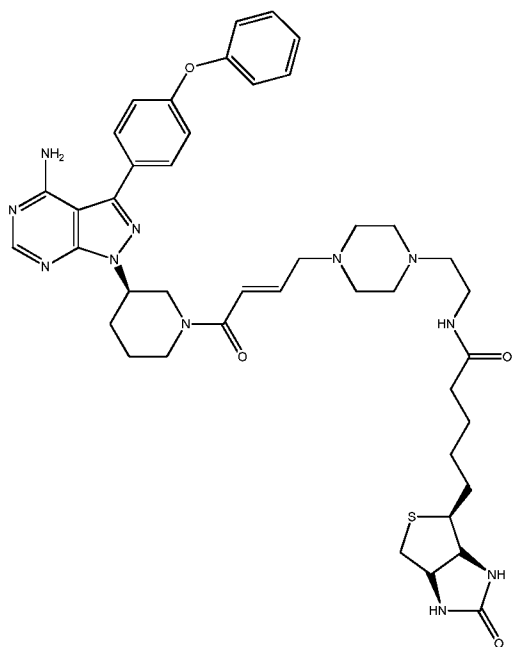
One exemplary BTK inhibitor is ibrutinib (*supra*), and its related compounds as described in U.S. Pat. Nos. 8,088,781 and 8,501,751 (incorporated herein by reference). Exemplary BTK inhibitors disclosed therein include a compound of the following structure:



wherein:

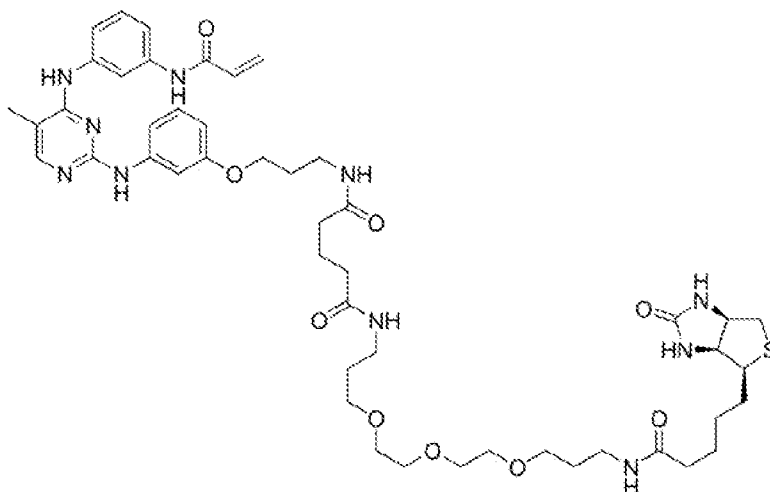
L_a is O or S; Ar is an unsubstituted phenyl; Y is a 4-, 5-, 6-, or 7-membered cycloalkyl ring, or Y is azetidiny, pyrrolidinyl, piperidinyl, or azepanyl; Z is C(O), OC(O), NHC(O), S(O)_x, or NHS(=O)_x, where x is 2; R₈ is H; R₇ is H, unsubstituted C₁-C₄ alkyl, C₁-C₆alkoxyalkyl, C₁-C₈alkylaminoalkyl, or C₁-C₄alkyl(phenyl); or R₇ and R₈ taken together form a bond; R₆ is H, unsubstituted C₁-C₆alkoxyalkyl, C₁-C₈alkylaminoalkyl, or C₁-C₄alkyl(phenyl); the wavy line indicates the point of attachment between the BTK inhibitor and the BTK tyrosine kinase.

Those BTK inhibitors can be derived to become ligand probes of the invention by, for example, linking to biotin through a linker. For example, for ibrutinib, the corresponding ligand probe may be a biotin-linked ibrutinib of the following structure:

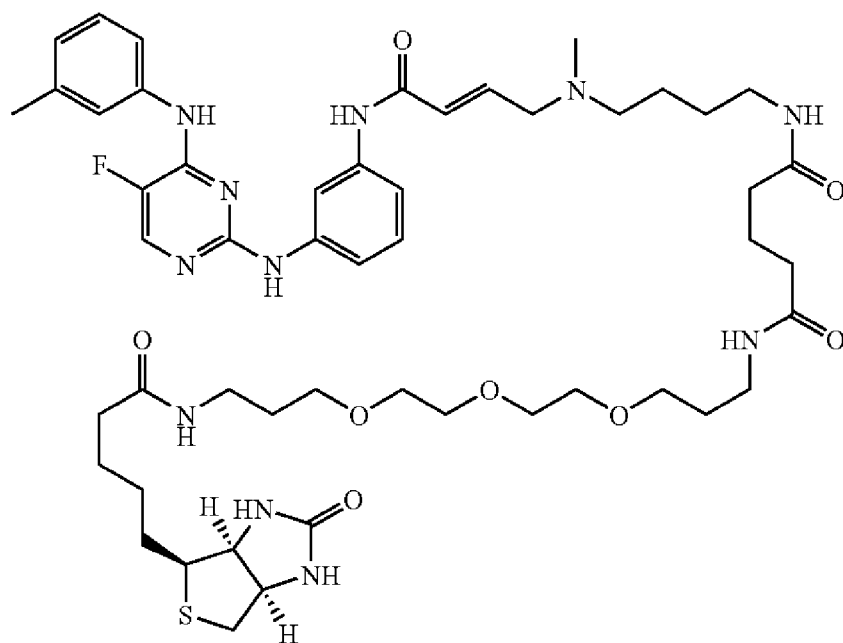
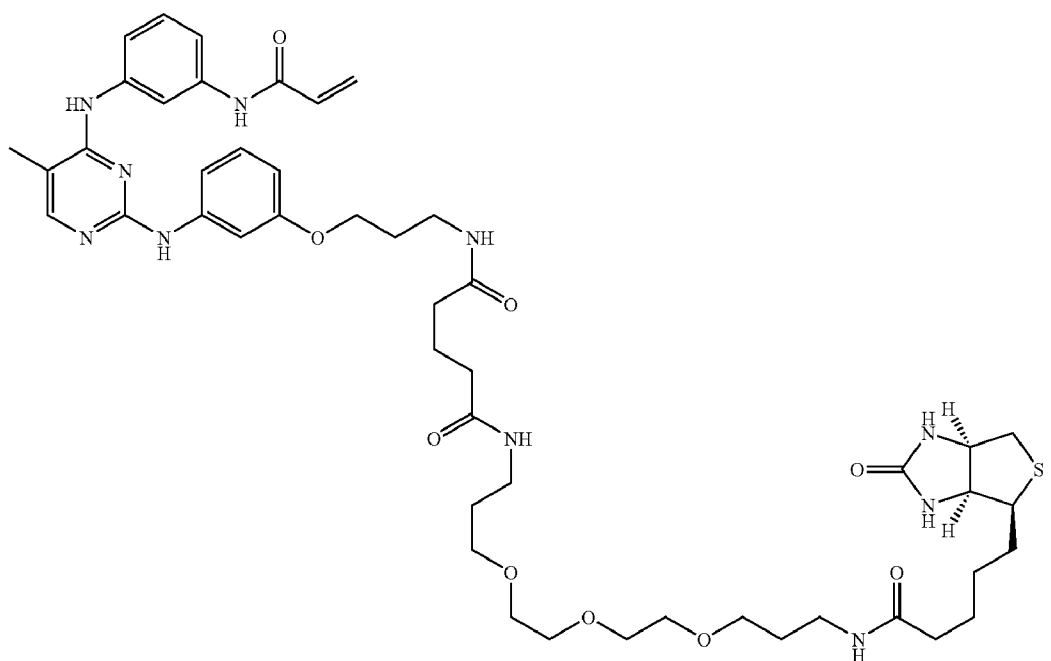


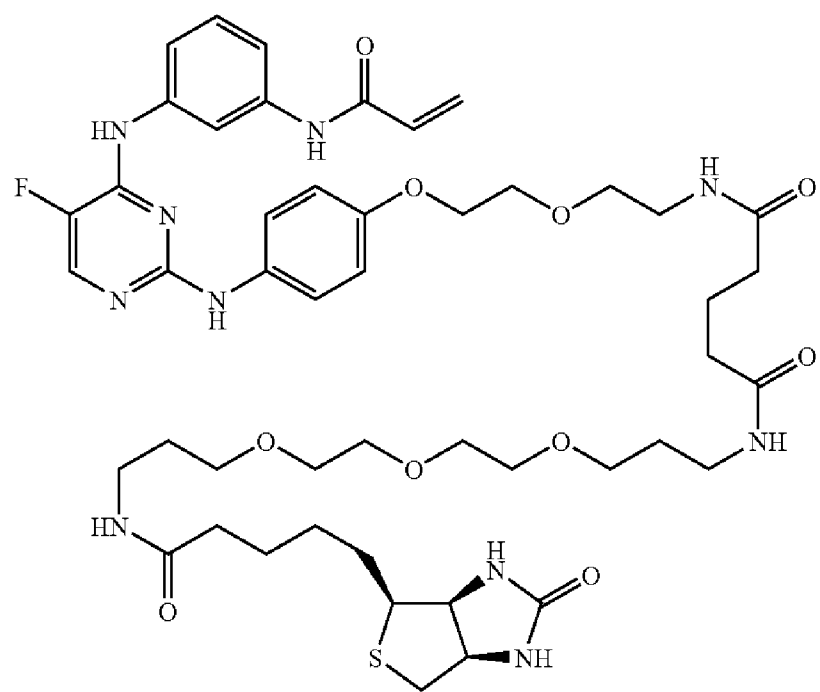
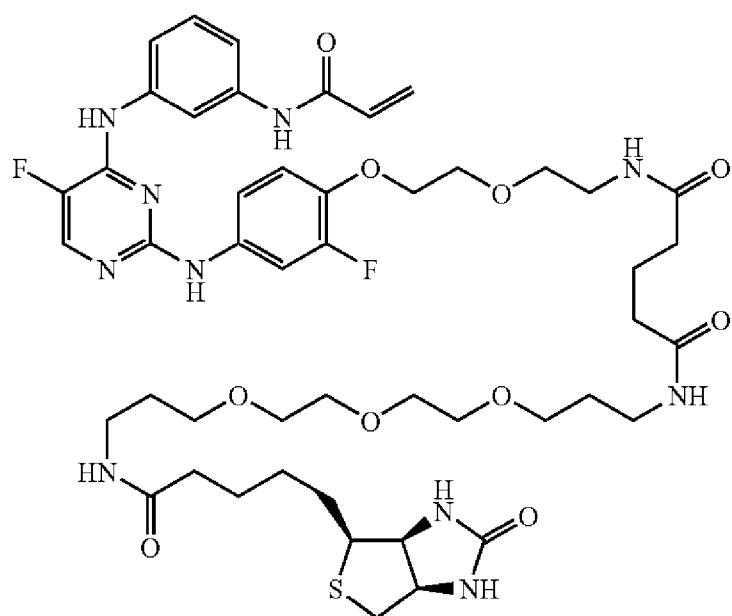
The same linkage reaction for ibrutinib and biotin as disclosed in Honigberg *et al.* (*PNAS* 107(29):13075-13080, 2010), and/or WO 2008/054827, both incorporate by reference, can also be used to produce biotin-conjugated BTK inhibitors for use in the methods of the invention.

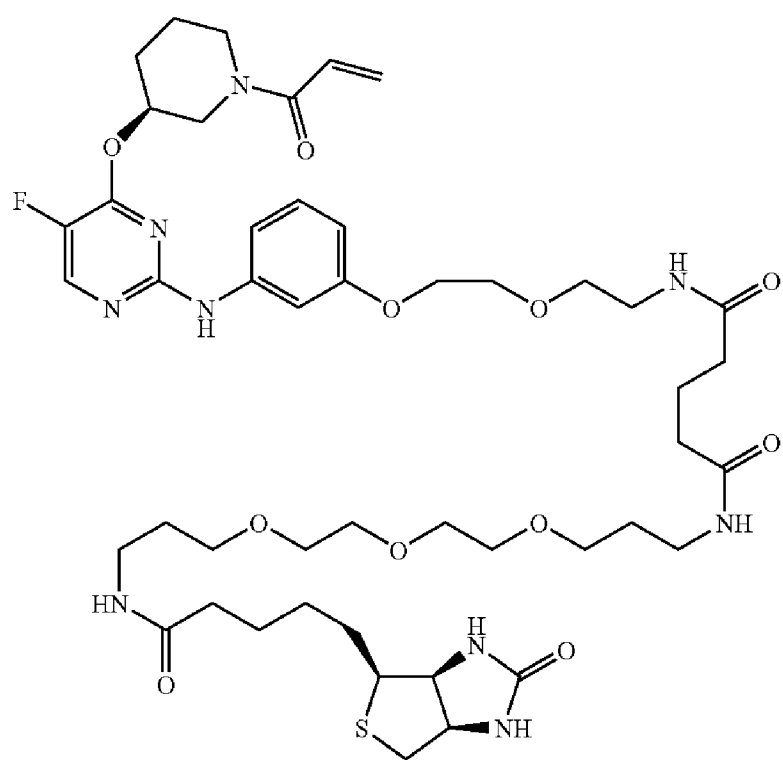
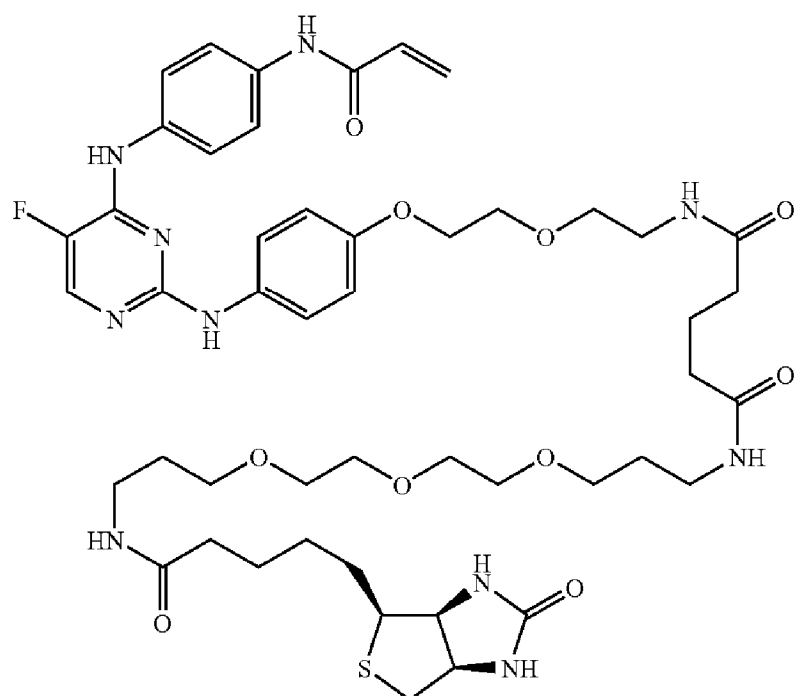
Another exemplary BTK inhibitor is CC-292 (*supra*) and its related compounds as disclosed in US 2010-0249092 A1 (incorporated herein by reference). A biotin-conjugated ligand probe of CC-292 is show below:

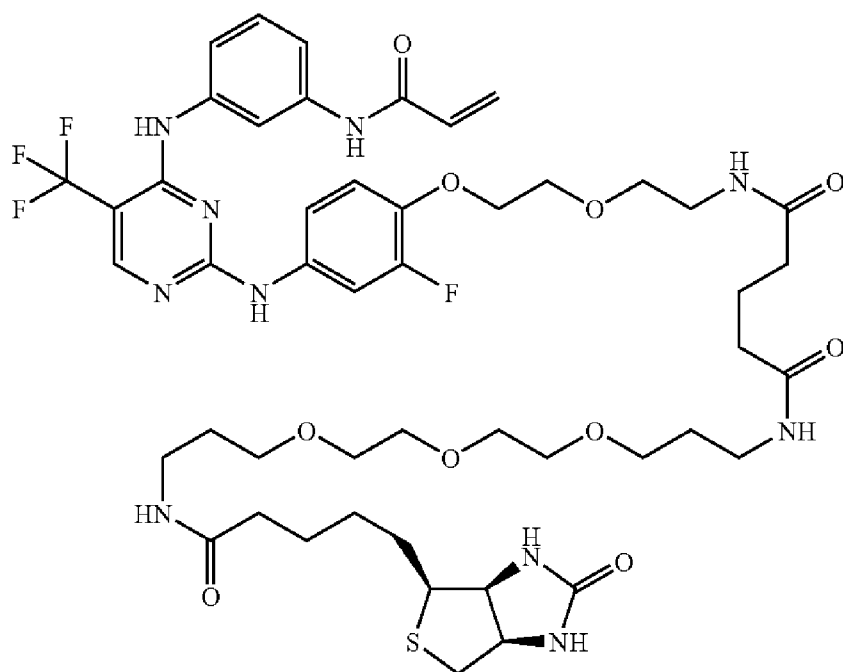


Additional similar ligand probe includes:

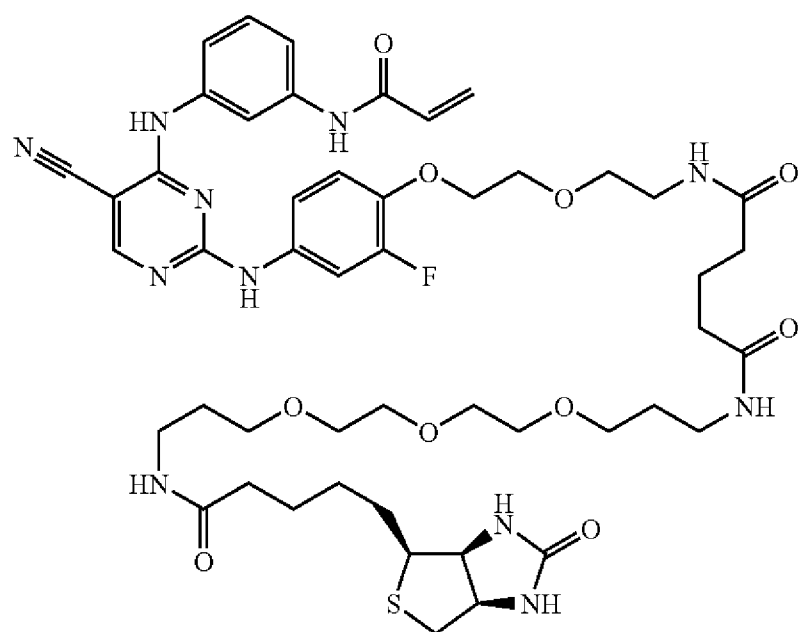




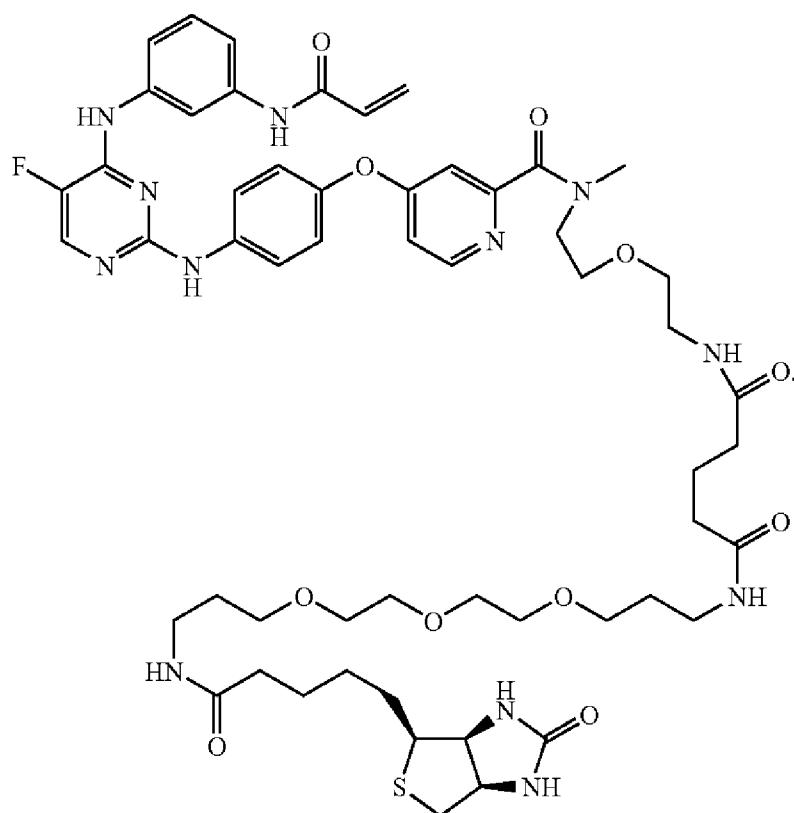




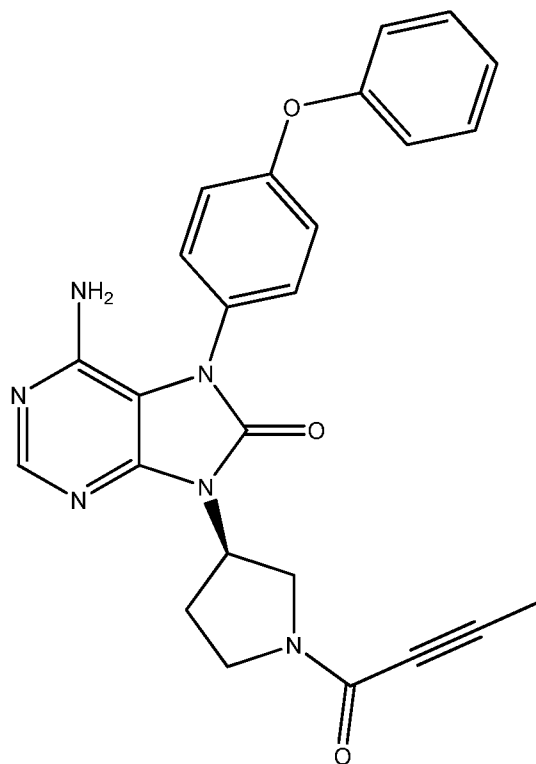
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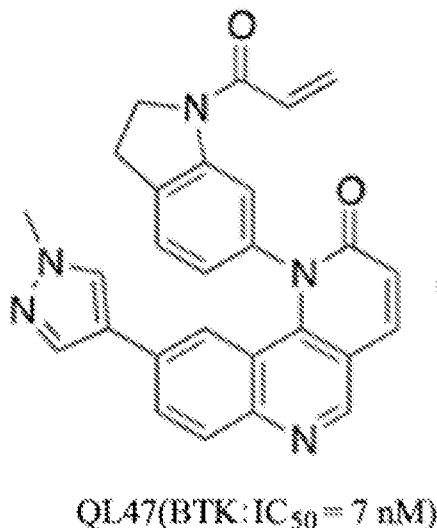
, and



Yet another exemplary BTK inhibitor shown below and its related compounds are disclosed in is U.S. Pat. No. 8,557,803 (incorporated herein by reference).

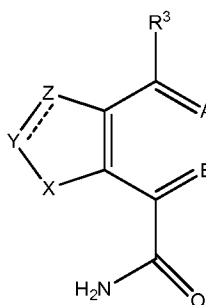


A further BTK inhibitor as described in Wu *et al.* (*Am. Chem. Soc.*, 2014) is reproduced below:



Biotin-conjugated version of these ligands / BTK inhibitors can also be made using any art-recognized methods.

Another BTK inhibitor is described in U.S. Provisional Application No. 61/839,729, filed on June 26, 2013, U.S. Provisional Application No. 61/897,577, filed on October 30, 2013, and U.S.S.N. 14/315,504, filed on June 26, 2014 (all incorporated herein by reference), in which the BTK inhibitor is a compound of Formula (I):



Formula (I)

or a pharmaceutically acceptable salt, pro-drug, biologically active metabolite, isomer, or stereoisomer thereof, wherein:

X is NR² or S;

Y is N or CR¹, and Z is N or CR¹; or, Y is CR¹R² and Z is CR¹R²;

A is N or CR⁴;

E is N or CR⁵;

R¹ is independently H, deuterium, CN, halogen, CF₃, -NR^cR^c, -N(R^a)C(O)R^b, optionally substituted (C₁-C₆)alkyl, optionally substituted (C₂-C₆)alkenyl, optionally

substituted aryl, optionally substituted (C₃-C₆)cycloalkyl, optionally substituted (C₃-C₆)cycloalkenyl, optionally substituted heteroaryl, or optionally substituted saturated or partially saturated heterocyclyl;

R² is independently H, deuterium, or optionally substituted (C₁-C₃)alkyl;

R³ is halogen, -N(R^a)₂, optionally substituted aryl, optionally substituted (C₃-C₇)cycloalkyl, optionally substituted saturated or partially saturated heterocyclyl, or optionally substituted heteroaryl; or

R³ is -R³⁰¹-L-R³⁰² wherein

R³⁰¹ is a bond, -O-, -OCH₂-, -NR^d-, or optionally substituted (C₁-C₃)alkylene, and

L is optionally substituted phenyl, optionally substituted (C₃-C₆)cycloalkyl, optionally substituted heteroaryl or a saturated or partially saturated heterocyclyl containing one or more heteroatoms, at least one of which is nitrogen; or

L is -L¹-L² wherein L¹ is attached to R³⁰¹ and

L¹ is optionally substituted phenyl, optionally substituted heteroaryl or optionally substituted saturated or partially saturated carbocycle or a saturated or partially saturated heterocyclyl; and

L² is a bond, CH₂, NR^d, CH₂N(H), S(O)₂N(H), or -O-;

R³⁰² is CN, -CH₂CN, optionally substituted -C(=O)R^{302a}, -(CH₂)_n- optionally substituted saturated or partly saturated heterocyclyl or optionally substituted -S(O)₂(C₂)alkenyl;

wherein R^{302a} is optionally substituted (C₁-C₄)alkyl, optionally substituted (C₂-C₄)alkenyl, (C₂-C₄)alkynyl, -C(O)-(C₁-C₄)alkyl, optionally substituted saturated or partially unsaturated (C₃-C₆)cycloalkyl, optionally substituted aryl, optionally substituted heteroaryl, -N(H)- optionally substituted heteroaryl or -(CH₂)_n-optionally substituted unsaturated or partly saturated heterocyclyl;

R⁴ is H, deuterium, CN, optionally substituted (C₁-C₃)alkyl, optionally substituted (C₃-C₆) cycloalkyl or optionally substituted saturated or partially saturated heterocyclyl, or optionally substituted heteroaryl;

wherein the optionally substituted saturated or partially saturated heterocyclyl; and optionally substituted heteroaryl contain at least one nitrogen atom; or

R^3 and R^4 , together with the carbon atoms to which they are attached, form an optionally substituted, saturated, unsaturated or partially unsaturated 5 or 6 membered carbocyclic ring or an optionally substituted, saturated, or partially unsaturated 5 or 6 membered heterocyclic ring containing one or more heteroatoms selected from N, S and O;

R^5 is H, deuterium, halogen, or optionally substituted (C₁-C₃)alkyl;

R^a is independently selected from H, -C(O)-optionally substituted (C₂-C₆)alkenyl, optionally substituted (C₁-C₆)alkyl, -(CH₂)_n-optionally substituted (C₃-C₆)cycloalkyl, -(CH₂)_n-optionally substituted heterocyclyl, or -(CH₂)_n-optionally substituted heteroaryl;

R^b is H, optionally substituted (C₁-C₆)alkyl, optionally substituted (C₂-C₆)alkenyl, optionally substituted (C₂-C₆)alkynyl, -CH₂-O-optionally substituted aryl, or -CH₂-O-optionally substituted heteroaryl;

R^c is independently H, optionally substituted (C₁-C₆)alkyl, optionally substituted (C₃-C₆)cycloalkyl, optionally substituted saturated or partially saturated heterocyclyl, optionally substituted aryl or optionally substituted heteroaryl;

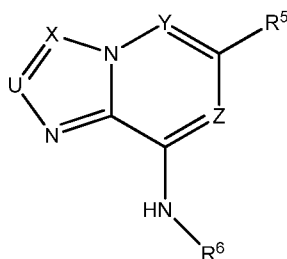
R^d is H, optionally substituted heterocyclyl, -(CH₂)_n-optionally substituted (C₃-C₆)cycloalkyl, -(CH₂)_n-optionally substituted heteroaryl or optionally substituted (C₁-C₃)alkyl;

R^f is optionally substituted (C₁-C₃)alkyl, optionally substituted (C₂-C₄)alkenyl or optionally substituted (C₂-C₄)alkynyl; and

n is independently 0 or 1.

The derived ligand probe comprising the 1st moiety (*e.g.*, biotin) may have the BTK inhibitor linked to the 1st moiety through a linker moiety, to a substituent group of R^3 , wherein R^3 is an optionally substituted aryl, such as phenyl.

Yet another BTK inhibitor is described in PCT/CN2014/075560 (incorporated herein by reference), filed on April 17, 2014, in which the BTK inhibitor is a compound of Formula (I):



Formula (1)

wherein

U is CR¹ or N;

X is CR² or N;

Y is CR³ or N;

Z is CR⁴ or N;

R¹ is independently H or deuterium;

R² is H, deuterium, optionally substituted (C₁-C₃)alkyl, or CF₃;

R³ is H, deuterium or optionally substituted (C₁-C₃)alkyl;

R⁴ is H or deuterium;

R⁵ is -R⁵⁰¹-L-R⁵⁰² wherein

R⁵⁰¹ is a bond, -O-, -OCH₂-, or optionally substituted (C₁-C₃)alkylene,

L is -C(=O)-, -CH₂N(H)C(=O)-, -N(H)C(=O)-, or -N(H)S(O)₂; or

L is a bond and R⁵⁰² is -CN; or

L is -L¹-L² wherein L¹ is attached to R⁵⁰¹ wherein

L¹ is optionally substituted phenyl, optionally substituted heteroaryl, optionally substituted saturated or partially saturated heterocyclyl, or optionally substituted saturated or partially saturated (C₃-C₆)cycloalkyl and L² is -CH₂N(R^a)-, -CH₂N(R^a)C(O)-, -N(R^a)C(O)-, -N(R^a)S(O)₂- or -N(R^a)-; or

L¹ is a saturated or partially saturated heterocyclyl containing one or more heteroatoms wherein at least one heteroatom is nitrogen and L² is a bond, C(O) or -S(O)₂;

R⁵⁰² is H, optionally substituted alkenyl, optionally substituted alkynyl, CN, or optionally substituted (C₃-C₆)cycloalkenyl;

R⁶ is optionally substituted (C₁-C₆)alkyl, optionally substituted (C₃-C₁₂)cycloalkyl, optionally substituted phenyl, optionally substituted heteroaryl, or optionally substituted heterocyclyl; and

R^a is independently H or optionally substituted (C₁-C₆)alkyl.

Optionally, the compound is not 2-(3-{8-[5-(morpholine-4-carbonyl)-pyridin-2-ylamino]-imidazo[1,2-*a*]pyridine-6-yl}-phenyl)-*N*-(5,5,5-trifluoro-4-hydroxy-4-methyl-pent-2-ynyl)-acetamide.

The derived ligand probe comprising the 1st moiety (*e.g.*, biotin) may have the BTK inhibitor linked to the 1st moiety through a linker, at a substituent group of R⁵.

5. Detectable label

Numerous detectable labels may be used in the first and second detection probe of the invention. The term “detectable label,” as used herein, refers to a label which is observable and/or quantifiable (in absolute, approximate or relative terms) using analytical techniques including, but not limited to, fluorescence, chemiluminescence, enhanced chemiluminescence (ECL), electron-spin resonance, ultraviolet/visible absorbance spectroscopy, mass spectrometry, nuclear magnetic resonance, magnetic resonance, and electrochemical methods.

In certain embodiments, the detectable label may include a label, a dye, an enzyme, a photocrosslinker, a cytotoxic compound, a drug, an affinity label, a photo affinity label, a reactive compound, an antibody or antibody fragment, a biomaterial, a nanoparticle, a spin label, a fluorophore, a metal-containing moiety, a radioactive moiety, a novel functional group, a group that covalently or noncovalently interacts with other molecules, a photocaged moiety, an actinic radiation excitable moiety, a ligand, a photoisomerizable moiety, biotin, a biotin analogue, a moiety incorporating a heavy atom, a chemically cleavable group, a photocleavable group, a redoxactive agent, an isotopically labeled moiety, a biophysical probe, a phosphorescent group, a chemiluminescent group, an electrochemiluminescent group (*e.g.*, an MSD SULFO-TAG type electrochemiluminescent group), an electron dense group, a magnetic group, an intercalating group, a chromophore, an energy transfer agent, a biologically active agent, a detectable label, or a combination thereof.

In certain embodiments, the detectable label is an electrochemiluminescent group, such as an MSD SULFO-TAG type electrochemiluminescent group. MESO SCALE

DISCOVERY (MSD) MULTI-ARRAY® technology (Meso Scale Diagnostics, LLC, Gaithersburg, MD) offers an excellent platform for the development of immunoassays for the measurement of biomarkers in life science, preclinical, and clinical samples. MSD electrochemiluminescence assays have ultra-low detection limits, provide up to five logs of linear dynamic range, use minimal sample, and handle difficult matrices easily. At the core of this technology are microplates that may take a variety of forms, including 96-well (each well having 4, 7, or 10 spots for simultaneous multiplexing analysis of different analytes in the same sample well) or 384-well (each well having 1 or 4 spots for multiplexing) commercial products that are customizable. The MSD plates have two different surface types: High Bind plates have a hydrophilic surface; and Standard plates have a hydrophobic surface. A combination of working electrode size and surface type determines the capacity of capture reagent that can be coated on the plate. Standard plates tend to offer higher sensitivity, while high-bind plates can facilitate the quantification of analytes at higher concentrations.

The bottom surface of each MSD MULTI-ARRAY® or MULTI-SPOT® microplate well has integrated working carbon electrode surface that is ideal for immobilizing most types of affinity reagents for biological assays, including antibodies, peptides (Wu *et al.*, *J. Gen. Virol.* 88:2719-2723, 2007), antigens (Mat *et al.*, *Nat. Biotechnol.* 28:1195-1202, 2010), carbohydrates / polysaccharides (Goldblatt, *Clin. Vaccine Immunol.* 18:1744-1751, 2011; Marchese, *Clin. Vaccine Immunol.* 16:387-396, 2009), lysates (Gillardon *et al.*, *J. Neurosci Methods*, 214(1):62-68, 2013; Chung *et al.*, *Proteomics Clin. Appl.* 2:1539-1547, 2008), cells (Lu *et al.*, *J. Immunol. Methods*, 314:74-79, 2006; Pang *et al.*, *J. Immunol Methods.* 362:176-179, 2010), membranes, and virus-like particles, according to the manufacturer. All references are incorporated by reference. These biological affinity reagents can be attached to the carbon electrode surface simply by passive adsorption, while retaining a high level of biological activity. The detection label for the MSD assays use electrochemiluminescent labels, such as the MSD SULFO-TAG type electrochemiluminescent group, for ultra-sensitive detection. These labels are non-radioactive, stable, and offer a choice of convenient coupling chemistries. For example, the SULFO-TAG Streptavidin label (Cat. No. R32AD-5) has a SULFO-TAG covalently linked to a multivalent streptavidin, and can bind multiple biotin labeled reagents. These electrochemiluminescent labels emit light when electrochemically stimulated. The detection process is initiated at electrodes located in the bottom of MSD's microplates. Only labels near the electrode are excited and detected,

enabling non-washed assays. MSD Read Buffers contain coreactants may also be used to further enhance the electrochemiluminescence signals. These coreactants are also stimulated when in proximity to the electrodes in the microplate. MSD's labels emit light at 620 nm, eliminating problems with color quenching. Few compounds interfere with the electrochemiluminescence process. Multiple excitation cycles of each label amplify the signal to enhance light levels and improve sensitivity. Background signals are minimal because the stimulation mechanism (electricity) is decoupled from the signal (light). The assay can be fully automated in a high throughput format, using plate readers designed for the MSD assay, such as SECTOR Imager 6000, SECTOR Imager 2400, or SECTOR PR reader from MSD.

In certain embodiments, the detectable label is a fluorophore, such as a Bodipy fluorophore (*e.g.*, a Bodipy FL fluorophore). In certain embodiments, the fluorophore is selected from the group consisting of: BODIPY 493/503, BODIPY FL, BODIPY R6G, BODIPY 530/550, BODIPY TMR, BODIPY 558/568, BODIPY 564/570, BODIPY 576/589, BODIPY 581/591, BODIPY TR, Fluorescein, 5(6)Carboxyfluorescein, 2,7-Dichlorofluorescein, N,N-Bis(2,4,6-trimethylphenyl)-3,4:9,10-perylenebis(dicarboximide), HPTS, Ethyl Eosin, DY-490XL MegaStokes, DY-485XL MegaStokes, Adirondack Green 520, ATTO 465, ATTO 488, ATTO 495, YOYO-1, 5-FAM, BCECF, BCECF, dichlorofluorescein, rhodamine 110, rhodamine 123, Rhodamine Green, YO-PRO-1, SYTOX Green, Sodium Green, SYBR Green I, Alexa Fluor 500, FITC, Fluo-3, Fluo-4, fluoro-emerald, YoYo-1 ssDNA, YoYo-1 dsDNA, YoYo-1, SYTO RNASelect, Diversa Green-FP, Dragon Green, EvaGreen, Surf Green EX, Spectrum Green, Oregon Green 488, NeuroTrace 500525, NBD-X, MitoTracker Green FM, LysoTracker Green DND-26, CBQCA, PA-GFP (post-activation), WEGFP (post-activation), FLASH-CCXXCC, Azami Green monomeric, Azami Green, EGFP (Campbell Tsien 2003), EGFP (patterson 2001), Fluorescein, Kaede Green, 7-Benzylamino-4-Nitrobenz-2-oxa-1,3-Diazole, Bexl, Doxorubicin, Lumio Green, and SuperGlo GFP.

In a further embodiment, the fluorophore is selected from the group consisting of: BODIPY 493/503, BODIPY FL, BODIPY R6G, BODIPY 530/550, BODIPY TMR, BODIPY 558/568, BODIPY 564/570, BODIPY 576/589, BODIPY 581/591, and BODIPY TR. In yet a further embodiment, the fluorophore is BODIPY FL. In certain embodiments, the fluorophore is not BODIPY 530. In some embodiments, the fluorophore has an excitation maxima of between about 500 and about 600 nm. In some other embodiments, the fluorophore has an excitation maxima of between about 500 and about 550 nm. In another

embodiment, the fluorophore has an excitation maxima of between about 550 and about 600 nm. In yet a further embodiment, the fluorophore has an excitation maxima of between about 525 and about 575 nm. In other embodiments, the fluorophore has an emission maxima of between about 510 and about 670 nm. In another embodiment, the fluorophore has an emission maxima of between about 510 and about 600 nm. In a further embodiment, the fluorophore has an emission maxima of between about 600 and about 670 nm. In another embodiment, the fluorophore has an emission maxima of between about 575 and about 625 nm.

In certain embodiments, the detectable label is an enzyme, such as an enzyme suitable for use in chemiluminescence or ECL. Such enzymes may include peroxidase (POD), glucose oxidase (GOD), invertase (INV), β -D-galactosidase (BGase), glucose-6-phosphate dehydrogenase (G6PDH), and alkaline phosphatase (ALP).

“Chemiluminescent group,” as used herein, refers to a group which emits light as a result of a chemical reaction without the addition of heat. By way of example, luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) reacts with oxidants like hydrogen peroxide (H_2O_2) in the presence of a base and a metal catalyst to produce an excited state product (3-aminophthalate, 3-APA).

Enhanced chemiluminescence (ECL) may be carried out using a horseradish peroxidase enzyme (HRP). HRP catalyzes the conversion of the enhanced chemiluminescent substrate into a sensitized reagent in the vicinity of the molecule of interest, which on further oxidation by hydrogen peroxide, produces a triplet (excited) carbonyl, which emits light when it decays to the singlet carbonyl. Enhanced chemiluminescence allows detection of minute quantities of a biomolecule. Proteins can be detected down to femtomole quantities, well below the detection limit for most assay systems.

The term “fluorophore,” as used herein, refers to a molecule which upon excitation emits photons and is thereby fluorescent.

The term “dye,” as used herein, refers to a soluble, coloring substance which contains a chromophore.

The term “chromophore,” as used herein, refers to a molecule which absorbs light of visible wavelengths, UV wavelengths, or IR wavelengths.

The term “electron dense group,” as used herein, refers to a group which scatters electrons when irradiated with an electron beam. Such groups include, but are not limited to,

ammonium molybdate, bismuth subnitrate cadmium iodide, carbohydrazide, ferric chloride hexahydrate, hexamethylene tetramine, indium trichloride anhydrous, lanthanum nitrate, lead acetate trihydrate, lead citrate trihydrate, lead nitrate, periodic acid, phosphomolybdic acid, phosphotungstic acid, potassium ferricyanide, potassium ferrocyanide, ruthenium red, silver nitrate, silver proteinate (Ag Assay: 8.0-8.5%) "Strong," silver tetraphenylporphyrin (S-TPPS), sodium chloroaurate, sodium tungstate, thallium nitrate, thiosemicarbazide (TSC), uranyl acetate, uranyl nitrate, and vanadyl sulfate.

The term "energy transfer agent," as used herein, refers to a molecule which either donates or accepts energy from another molecule. By way of example, fluorescence resonance energy transfer (FRET) is a dipole-dipole coupling process by which the excited-state energy of a fluorescence donor molecule is non-radiatively transferred to an unexcited acceptor molecule which then fluorescently emits the donated energy at a longer wavelength.

The term "moiety incorporating a heavy atom," as used herein, refers to a group which incorporates an ion of atom which is usually heavier than carbon. In some embodiments, such ions or atoms include, but are not limited to, silicon, tungsten, gold, lead, and uranium.

The term "photoaffinity label," as used herein, refers to a label with a group, which, upon exposure to light, forms a linkage with a molecule for which the label has an affinity. By way of example, in some embodiments, such a linkage is covalent or non-covalent.

The term "photocaged moiety," as used herein, refers to a group which, upon illumination at certain wavelengths, covalently or non-covalently binds other ions or molecules.

The term "photoisomerizable moiety," as used herein, refers to a group wherein upon illumination with light changes from one isomeric form to another.

The term "radioactive moiety," as used herein, refers to a group whose nuclei spontaneously give off nuclear radiation, such as alpha, beta, or gamma particles; wherein, alpha particles are helium nuclei, beta particles are electrons, and gamma particles are high energy photons.

The term "spin label," as used herein, refers to molecules which contain an atom or a group of atoms exhibiting an unpaired electron spin (*i.e.*, a stable paramagnetic group) that in some embodiments are detected by electron spin resonance spectroscopy and in other embodiments are attached to another molecule. Such spin-label molecules include, but are

not limited to, nitryl radicals and nitroxides, and in some embodiments are single spin-labels or double spin-labels.

To detect the labels, unless otherwise indicated, conventional methods of mass spectroscopy, NMR, HPLC, protein chemistry, biochemistry, recombinant DNA techniques and pharmacology are employed.

In certain embodiments, the detectable label may include isotope labeling, with isotopes of hydrogen, carbon, nitrogen, oxygen, fluorine and chlorine, such as ^2H , ^3H , ^{13}C , ^{14}C , ^{15}N , ^{18}O , ^{17}O , ^{35}S , ^{18}F , ^{36}Cl .

6. *Samples and sampling*

The methods of the invention involve analyzing one or more samples derived from an individual, such as a mammal (*e.g.*, a human or a non-human mammal). The sample may be obtained from tissues (*e.g.*, spleen), cells, or established cell lines thereof, including those of hematopoietic origin, such as a blood sample or B-cell lymphoma, or the sample may be derived from a target issue for which the status of kinase (*e.g.*, BTK) activity or inhibition is of interest, such as knee lavage from an arthritic knee.

Samples may be obtained once or multiple times from an individual. Multiple samples may be obtained from different locations in the individual (*e.g.*, blood samples, bone marrow samples and/or tissue samples), at different times from the individual (*e.g.*, a series of samples taken to monitor response to treatment or to monitor for return of a pathological condition), or any combination thereof.

These and other possible sampling combinations based on the sample type, location and time of sampling allows for the detection of the presence and the status of kinase (*e.g.*, BTK) inhibition, and by extension, status of diseases, or pre-pathological or pathological conditions.

When samples are obtained as a series, *e.g.*, a series of whole blood samples obtained after treatment or administration of a kinase (*e.g.*, BTK) inhibitor, the samples may be obtained at fixed intervals, at intervals determined by the status of the most recent sample or samples, or by other characteristics of the individual, or some combination thereof. For example, samples may be obtained at intervals of approximately 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 hours, at 1, 2, 3, or 4 weeks, at intervals of approximately 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or 11 months, at intervals of approximately 1, 2, 3, 4, 5, or more than 5 years, or some

combination thereof. It will be appreciated that an interval may not be exact, according to an individual's availability for sampling and the availability of sampling facilities. Thus approximate intervals corresponding to an intended interval scheme are encompassed by the invention.

As an example, an individual who has undergone treatment for a subject disease or condition may be sampled (*e.g.*, by blood draw) relatively frequently (*e.g.*, every hour, day, week, month or every three months) for the first six months to a year or two after treatment. Then if no abnormality is found, less frequently (*e.g.*, at times between six months and a year) thereafter. If, however, any abnormalities or other circumstances are found in any of the intervening times, or during the sampling, sampling intervals may be modified.

Generally, the most easily obtained samples are fluid samples. Fluid samples include normal and pathologic bodily fluids and aspirates of those fluids. Fluid samples also comprise rinses of organs and cavities (lavage and perfusions). Bodily fluids include whole blood, bone marrow aspirate, synovial fluid, cerebrospinal fluid, saliva, sweat, tears, semen, sputum, mucus, menstrual blood, breast milk, urine, lymphatic fluid, amniotic fluid, placental fluid and effusions such as cardiac effusion, joint effusion, pleural effusion, and peritoneal cavity effusion (ascites). Rinses can be obtained from numerous organs, body cavities, passage ways, ducts and glands. Sites that can be rinsed include lungs (bronchial lavage), stomach (gastric lavage), gastrointestinal track (gastrointestinal lavage), colon (colonic lavage), vagina, bladder (bladder irrigation), breast duct (ductal lavage), oral, nasal, sinus cavities, and peritoneal cavity (peritoneal cavity perfusion).

In some embodiments, the sample is a blood sample. In some embodiments, the sample or samples is whole blood sample. In some embodiments, the sample or samples is from a knee lavage. In some embodiments, the sample is a bone marrow sample. In some embodiments, the sample is a lymph node sample. In some embodiments, the sample is cerebrospinal fluid. In some embodiments, combinations of one or more of a blood, bone marrow, cerebrospinal fluid, and lymph node sample are used.

In certain embodiments, solid tissue samples may also be used, either alone or in conjunction with fluid samples. Solid samples may be derived from individuals by any method known in the art, including surgical specimens, biopsies, and tissue scrapings, including cheek scrapings. Surgical specimens include samples obtained during exploratory, cosmetic, reconstructive, or therapeutic surgery. Biopsy specimens can be obtained through numerous methods including bite, brush, cone, core, cytological, aspiration, endoscopic,

excisional, exploratory, fine needle aspiration, incisional, percutaneous, punch, stereotactic, and surface biopsy.

In one embodiment, a sample may be obtained from an apparently healthy individual, such as during a routine checkup, and analyzed so as to provide an assessment of the individual's status of a given kinase or an associated disease or condition.

In another embodiment, a sample may be taken to screen for a subject disease or condition of interest. Such screening may encompass testing for a single disease, a family of related diseases or a general screening for multiple, unrelated diseases. Screening can be performed weekly, biweekly, monthly, bi-monthly, every several months, annually, or in multi year intervals and may replace or complement existing screening modalities.

Certain fluid samples can be analyzed in their native state with or without the addition of a diluent or buffer. Alternatively, fluid samples may be further processed or derived to obtain enriched or purified cell populations prior to analysis. Numerous enrichment and purification methodologies for bodily fluids are known in the art. For example, a common method to separate cells from plasma in whole blood is through centrifugation using heparinized tubes. By incorporating a density gradient, further separation of the lymphocytes (such as B lymphocytes) from the red blood cells can be achieved. A variety of density gradient media are known in the art including sucrose, dextran, bovine serum albumin (BSA), FICOLL diatrizoate (Pharmacia), FICOLL metrizoate (Nycomed), PERCOLL (Pharmacia), metrizamide, and heavy salts such as cesium chloride. Alternatively, red blood cells can be removed through lysis with an agent such as ammonium chloride prior to centrifugation.

Whole blood can also be applied to filters that are engineered to contain pore sizes that select for the desired cell type or class. For example, rare pathogenic cells can be filtered out of diluted, whole blood following the lysis of red blood cells by using filters with pore sizes between 5 to 10 μm , as disclosed in U.S. patent application publication US 2002-0028431 A1 (incorporated by reference). Alternatively, whole blood can be separated into its constituent cells based on size, shape, deformability or surface receptors or surface antigens by the use of a micro fluidic device as disclosed in U.S. patent application publication US 2006-0134599 A1 (incorporated by reference).

Select cell populations can also be enriched for or isolated from whole blood through positive or negative selection based on the binding of antibodies or other entities that recognize cell surface or cytoplasmic constituents.

Solid tissue samples may require the disruption of the extracellular matrix or tissue stroma and the release of single cells for analysis. Various techniques are known in the art including enzymatic and mechanical degradation employed separately or in combination. An example of enzymatic dissociation using collagenase and protease can be found in Wolters *et al.*, “An analysis of the role of collagenase and protease in the enzymatic dissociation of the rat pancreas for islet isolation,” *Diabetologia*, 35:735-742, 1992. Examples of mechanical dissociation can be found in Singh, N P., “Technical Note: A rapid method for the preparation of single-cell suspensions from solid tissues,” *Cytometry*, 31:229-232 (1998). Alternately, single cells may be removed from solid tissue through microdissection including laser capture microdissection as disclosed in Emmert-Buck, M. R. *et al.*, “Laser Capture Microdissection,” *Science*, 274(8):998-1001, 1996. All incorporated herein by reference.

EXAMPLES

Example 1 Improved kinase (BTK) occupancy assay

The BTK kinase occupancy assay described herein has been designed to improve assay sensitivity over the BTK occupancy assay described in Evans (*supra*), and, due to its much improved sensitivity, permits measurements of BTK occupancy in samples of limited quantity, such as small volumes of blood PBMCs, without the lengthy B-cell isolation process. The improved assay also allows assessment of BTK occupancy in target organ tissue (ex. knee lavage).

The steps described herein is for illustrative purpose only, and can be readily adjusted or changed without departing from the spirit of the invention. Thus the conditions used herein should not be limiting.

1. Coat a 96-well high-binding ELISA plate with 100 μ L rabbit anti-BTK monoclonal antibody (Cell Signaling Cat. #8547S) diluted 1:250 in bicarbonate buffer to each well and incubate overnight at 4°C.
2. Wash plate 3-5 times using 1 $\times$ PBS 0.05% Tween 20 Wash Buffer. Block plate with 300 μ L per well of 3% BSA in PBS for 1 hour at room temperature on orbital shaker.
3. Wash plate 3-5 times using 1 $\times$ PBS 0.05% Tween 20 Wash Buffer. Add 50 μ L proprietary probe (50 nM) to all wells. Add 50 μ L of BTK-containing sample / standard to wells. Total volume in each well is 100 μ L, including ligand probe and sample (or

standard). Incubate probe / sample on plate for 2 hours at room temperature on orbital shaker.

4. Wash plate 3-5 times using 1× PBS 0.05% Tween 20 Wash Buffer. Add 100 µL streptavidin-HRP diluted 1:2000 in dilution buffer for 30 minutes at room temperature on orbital shaker, covered in foil.

5. Wash plate 3-5 times using 1× PBS 0.05% Tween 20 Wash Buffer. Add 100 µL biotin-HRP diluted 1:50,000 and incubate 30 minutes at room temperature on orbital shaker, covered in foil.

6. Wash plate 3-5 times using 1× PBS 0.05% Tween 20 Wash Buffer. Add 100 µL TMB reagent to each well and cover plate with aluminum foil. Develop TMB for 15-20 minutes then add 100 µL of 1.5N sulfuric acid to each well to stop the reaction. Read the plate at 450 nm.

7. Calculate free-BTK in each sample off of rhBTK standard curve, and then % occupancy based on % reduction in free-BTK comparing drug-treated sample to vehicle-treated sample.

As a comparison, the BTK occupancy assay as described in Evans *et al.* (*J. Pharmacol. Exp. Ther.* 346:219-228, 2013) was carried out under similar conditions as described below:

a. Pre-incubate 80 µL of sample containing free-BTK (ex. spleen lysate) with 80 µL proprietary probe (200 nM) in a 96-well plate (Thermo Cat. #266120) for 2 hours at room temperature at 450 rpm.

b. Add sample/probe mixture to streptavidin-coated plate (R&D Systems Cat. # CP004) and incubate for 1 hour at room temperature at 450 rpm.

c. Wash plate 3 times using 1× PBS 0.05% Tween 20 wash buffer. Add 100 µL of rabbit anti-BTK monoclonal antibody (Cell Signaling Cat. #8547S) diluted 1:1000 in buffer to each well and incubate 1 hour at room temperature at 450 rpm.

d. Wash plate 3 times using 1× PBS 0.05% Tween 20 wash buffer. Add 100 µL of goat anti-rabbit IgG conjugated to HRP (Invitrogen Cat. #G21234) diluted 1:5000 in buffer to each well and incubate 1 hour at room temperature at 450 rpm.

e. Wash plate 3 times using 1× PBS 0.05% Tween 20 wash buffer. Add 100 µL TMB reagent to each well and cover plate with aluminum foil. Develop TMB for 15-20

minutes then add 100 μ L 2N sulfuric acid to each well to stop the reaction. Read the plate at 450 nm.

f. Calculate free-BTK in each sample off of rhBTK standard curve, and then % occupancy based on % reduction in free-BTK comparing drug-treated sample to vehicle-treated sample.

The result showed that the subject BTK occupancy assay allowed a robust and sensitive method for assaying free-BTK in biological samples. Figure 2 shows a roughly 20-fold increase in sensitivity when comparing the alternative BTK occupancy assay to the assay described by Evans *et al.* (*J. Pharmacol. Exp. Ther.* 346:219-228, 2013), when standard curves were generated using recombinant human BTK in both the original / Evans assay, and the improved occupancy assays described herein.

Specifically, under the assay conditions, the Evans method was able to achieve detection of 50% of the max signal when there is about 100 ng of BTK in the sample. In contrast, the subject method was able to achieve detection of 50% of the max signal when there is about 5 ng of BTK in the sample - an increase of about 20-fold in terms of detection sensitivity.

The data presented herein demonstrates that the improved ELISA architecture described herein for BTK occupancy assay is not only possible, but also allows for increased sensitivity in free BTK measurements. This indicates the possibility of measuring BTK occupancy in smaller volumes of blood and/or in target organs such as arthritic knees, and has implications to improve both clinical and preclinical BTK occupancy measurements.

Example 2 Generation of a standard curve for the improved kinase occupancy assay

A 96-well polystyrene plate was coated with 100 μ L affinity reagent (rabbit anti-hBTK monoclonal antibody; Cell Signaling Cat. #8547S D3H5; diluted 1:250 in carbonate buffer). After an overnight incubation at 4°C, plate was washed with PBS-Tween 20, and coated with 300 μ L of 3% bovine serum albumin (BSA) in PBS to block wells. Dilution buffer consisting of 0.1% BSA in PBS was used to prepare all subsequent reagents. After a 1 hour incubation at room temperature on orbital shaker, plate was washed with PBS-Tween 20, and 50 μ L ligand probe (biotin-conjugated BTK inhibitor) was added at a concentration of 50 nM to all wells, and then 50 μ L binding partner of ligand (rhBTK; Life Technologies #PV3363) was also added to wells, in duplicate, at varying concentrations (see Figure 1A; two-fold dilutions starting at 1600 ng rhBTK per well and ending at 0.00000149 ng rhBTK

per well). Ligand probe and binding partner of ligand together in a total volume of 100 μ L per well were incubated for 2 hours at room temperature on an orbital shaker. Plate was then washed with PBS-Tween 20, and 100 μ L 1st detection probe was added (streptavidin-HRP; Jackson ImmunoResearch # 016-030-084; diluted 1:2000), covered in foil to protect from light, and incubated 30 minutes on an orbital shaker. Plate was then washed with PBS-Tween 20, and 100 μ L 2nd detection probe was added (biotin-HRP; Invitrogen #43-2040; diluted 1:50000), covered in foil to protect from light, and incubated 30 minutes on an orbital shaker. Plate was then washed with PBS-Tween 20, 100 μ L TMB substrate was added (Life Technologies #002023), and the plate was incubated without shaking in the dark for 20 minutes, then 100 μ L 1.5N sulfuric acid was added to each well to stop the reaction. Absorbance of each well was read with a plate reader at 450 nm (Molecular Devices Spectra Max 190, SoftMax Pro Software v5.2). Graphpad Prism5 software was used to plot the absorbance values.

To generate a standard curve for the occupancy assay of Evans (supra), a 96-well untreated microplate, 80 μ L ligand probe (biotin-conjugated BTK inhibitor; 200 nM) and 80 μ L binding partner of ligand (rhBTK; Life Technologies #PV3363) were mixed for a total volume of 160 μ L per well at varying rhBTK concentrations (two-fold dilutions starting at 1600 ng rhBTK per well and ending at 0.00000149 ng rhBTK per well), and incubated 2 hours at room temperature on an orbital shaker. After the mixing step, the entire 160 μ L mixture was transferred to a streptavidin-coated plate (R&D Systems # CP004), and incubated for 1 hour at room temperature on orbital shaker. Plate was then washed with PBS-Tween 20, and 100 μ L affinity reagent was added to wells (rabbit anti-hBTK monoclonal antibody; Cell Signaling #8547S D3H5; diluted 1:1000) and incubated for 1 hour at room temperature on orbital shaker. Plate was then washed with PBS-Tween 20, and 100 μ L of a secondary detection antibody was added (HRP-conjugated goat-anti-rabbit-IgG; Invitrogen #G21234; diluted 1:5000) and incubated 1 hour at room temperature on orbital shaker. Plate was then washed with PBS-Tween 20, 100 μ L TMB reagent was added, and the plate was incubated without shaking in the dark for 20 minutes, then 100 μ L 2N sulfuric acid was added to each well to stop the reaction. Absorbance of each well was read with a plate reader at 450 nm (Molecular Devices Spectra Max 250, SoftMax Pro Software v4.8). Graphpad Prism5 software was used to plot the absorbance values.

Both assays were run with a standard curve of rhBTK, starting with 1600 ng rhBTK and utilizing two-fold dilutions for a total of 32 points including diluent only for 0 ng rhBTK.

Comparing the dynamic range of the two assays, the subject assay exhibits a 3-fold larger assay dynamic range: $\Delta 3.76$ absorbance units in Alternative assay versus $\Delta 1.24$ absorbance units in the Evans assay. Comparing the sensitivities of the two assays, the subject assay exhibits a 20-fold increase in sensitivity when compared to the Evans assay: mid-point of subject assay curve ~ 5 ng rhBTK versus mid-point of the Evans assay curve ~ 100 ng. Altogether, the data here show the subject assay allows a robust and sensitive method for assaying free-BTK in biological samples, which can be used to assess % occupancy in samples containing ligand-bound BTK.

Example 3 Percent BTK occupancy in multiple biological compartments

Rats were dosed with Vehicle or a BTK inhibitor and euthanized 2 hours post-dosing. Spleens, B-cells, PBLs, and knee lavages were collected 2 hours post-dosing for occupancy measurements. Spleens and circulating B-cells were collected from dosed naïve rats, while PBLs and knee lavages were collected from dosed arthritic rats (collagen-induced arthritis). Samples were run using the subject method, using a standard curve generated from pooled naïve spleen B-cell homogenate. This data suggests that, with the enhanced assay sensitivity, target engagement (*i.e.*, BTK occupancy) can be detected in very small amounts of samples from the various relevant biological compartments, such as an arthritic knee of an experimental rat.

Collecting of the various biological samples is described in detail below.

Spleen: Rat spleens were flash-frozen at time of euthanasia and stored at -80°C prior to homogenization. To homogenize: on wet ice, protease inhibitor solution was added to frozen spleens in glass homogenizer vials, and held on wet ice to thaw slightly. Glass rod A was used to roughly crush spleen, followed with rod B to completely homogenize. Homogenized samples were poured into a 15 mL conical vial, then spun down at 4°C for 20 minutes at 2000 rpm. Supernatant was removed and stored in polypropylene 96-well plates at -80°C . 1 μL spleen lysate was used per well of occupancy assay.

Circulating B-cells: Rat whole blood was collected in heparinized tubes, mixed with 1X Pharm Lyse™ (BD 555899) and incubated at 37°C in the dark for 15 minutes. Cells were spun down for 5 minutes at 1000 rpm at room temperature, supernatant removed, and cells re-suspended in 1X Pharm Lyse. After spinning down for 5 minutes at 1000 rpm at room temperature, supernatant was removed and cells were re-suspended in BD Stain Buffer (554656). Cells were then strained (70 μm - BD 352350), counted using a Cellometer and

spun down at 1000 rpm for 5 minutes at 4°C, then re-suspended in 80 µL of cold Miltenyi Buffer (MACS BSA Stock Solution [130-091-376] diluted in autoMACS™ Rinsing Solution [130-091-222]). CD45R MicroBeads (Miltenyi 130-090-495) were added and cells mixed then incubated for 30 minutes on ice, washed in buffer, then spun down at 1000 rpm for 5 minutes at 4°C. Supernatant was then completely removed and cells re-suspended in buffer, MS columns placed in MACS Separators and rinsed with buffer, and cell suspensions applied to columns. Columns were then washed with buffer, removed, and placed in 5 mL FACS collection tube. Labeled cells were eluted by applying 1 mL of buffer to column and applying positive pressure with plunger. Unlabeled flow-through cells and labeled cell suspensions were transferred to 1.5 mL Eppendorf tubes and spun for 5 minutes at 5,000 g at 4°C, supernatant removed, and labeled cell pellets re-suspended in 45 µL of cell lysis buffer (R&D Systems sample diluent [DYC002] supplemented with 1X protease inhibitors [Calbiochem 539134]). Flow-through cell pellets were then lysed in 450 µL of cell lysis buffer for 20 minutes, then stored at -80°C and then cleared by spinning at 12,0000 g for 10 minutes at 4°C prior to analysis in occupancy ELISA. 5 µL PBL lysate was used per well of occupancy assay.

Knee lavage: Two hours post dosing BTK inhibitor, left and right knees were lavaged to collect cell infiltrate, red blood cells lysed with Gey's solution, and cell pellet resuspended in 500 µL FACS buffer. Lavages from 5 rats were pooled into one sample, for a total of 3 samples for each occupancy measurement. After counting total cell number, cells were spun down and lysed in 200 µL cell lysis buffer supplemented with protease inhibitors. Lysate was stored at -80°C and then cleared by spinning at 12,0000 g for 10 minutes at 4°C prior to analysis in occupancy ELISA, and 6.25 µL of knee lavage pool was used per well of occupancy assay.

Peripheral blood leukocytes (PBL): Two hours post dosing BTK inhibitor, rat whole blood was collected in heparinized tubes and pooled 4 mL per 50 mL tube, mixed with 1X Pharm Lyse™ (BD 555899) and incubated at 37°C in the dark for 15 minutes. Peripheral blood leukocytes were spun down for 5 minutes at 1000 rpm at RT (room temperature). Supernatant was removed and PBLs re-suspended in 1X Pharm Lyse. PBLs were spun down for 5 minutes at 1000 rpm at room temperature. Supernatant was removed and cells re-suspended with BD Stain Buffer (554656), then resuspended in FACS buffer and counted via hemacytometer. Supernatant was removed and cell pellets re-suspended in 100 µL of cell lysis buffer, then lysates incubated on ice for 20 minutes then stored at -80°C and then clear

by spinning at 12,0000 g for 10 minutes at 4°C prior to analysis in occupancy ELISA, and 1.5 µL PBL lysate was used per well of occupancy assay.

WE CLAIM:

1. A method for measuring binding between a ligand and a binding partner of the ligand in a sample, the method comprising:
 - (1) contacting the sample with an affinity reagent that specifically binds the binding partner, in the presence of a ligand probe, and allowing binding to occur between the affinity reagent and the binding partner, wherein a first percentage of the binding partner in the sample is bound by the ligand, and a second percentage of the binding partner in the sample is bound by the ligand probe;
 - (2) contacting binding partner bound by the affinity reagent with a first detection probe, wherein one of said first detection probe and said ligand probe comprises a multivalent agent, and allowing binding to occur between said first detection probe and said ligand probe through the multivalent agent;
 - (3) optionally, contacting the multivalent agent with a second detection probe comprising a second detectable label and a second moiety that binds to the multivalent agent, and allowing binding to occur between the multivalent agent and the second moiety on the second detection probe;
 - (4) detecting the presence or measuring the amount of the first detection probe or the second detectable label, thereby measuring binding between the ligand and the binding partner.
2. The method of claim 1, wherein said first detection probe comprises the multivalent agent.
3. The method of claim 1, wherein said ligand probe comprises the multivalent agent.
4. The method of claim 1, wherein the binding partner is an enzyme.
5. The method of claim 1, wherein the binding partner is a protein kinase (*e.g.*, BTK, BLK, EGFR1, HER2/ERBB2, HER3/Erb-B3, ERBB4, JAK3, TEC, BMX, ITK, LKB1, and TXK).
6. The method of claim 2, wherein the ligand probe comprises a first moiety covalently linked to the ligand, wherein the first moiety binds to the multivalent agent.
7. The method of claim 3, wherein the first detection probe comprises a first moiety that binds to the multivalent agent, and a first detectable label.

8. The method of claim 6, wherein the first moiety is the same from the second moiety in the second detection probe.
9. The method of claim 6 or 7, wherein the ligand comprises a substrate or a substrate analog of the enzyme or the kinase, or an inhibitor of the enzyme or the kinase.
10. The method of claim 9, wherein the inhibitor binds in the ATP-binding site of the kinase.
11. The method of claim 10, wherein the inhibitor is covalently linked to the kinase via Michael reaction.
12. The method of claim 11, and the inhibitor is covalently linked to the thiol group of a cysteine residue corresponding to Cys481 of the human BTK, isoform 1.
13. The method of claim 9, wherein the inhibitor inhibits a phosphorylated or active conformation of BTK.
14. The method of claim 1, wherein the affinity reagent is an antibody, an antigen-binding portion of an antibody, a nanobody, or a DVD-Ig.
15. The method of claim 14, wherein binding between the antibody and the binding partner does not substantially affect binding between the binding partner and the ligand or the ligand probe.
16. The method of claim 1, wherein the percentage of the binding partner bound by the ligand is 100%, or is less than 100%, 90%, 80%, 70%, 60%, 50%, 40%, 30%, 20%, 10%, 5%, 3%, 2%, 1% or less.
17. The method of claim 1, wherein the first detection probe further comprises a first detectable label.
18. The method of claim 17, wherein the first detectable label is the same as the second detectable label of the second detection probe.
19. The method of claim 1, wherein the multivalent agent is streptavidin (SA) or avidin.
20. The method of claim 1, wherein the multivalent agent is an antibody (*e.g.*, a bi-specific Ab, or an Ab having multiple Ag-binding fragments - IgA, IgM *etc.*).
21. The method of claim 1, wherein the first detectable label and/or the second detectable label is a dye, a photocrosslinker, a cytotoxic compound, a drug, an affinity label, a photo affinity label, a reactive compound, an antibody or antibody fragment, a

- biomaterial, a nanoparticle, a spin label, a fluorophore, a metal-containing moiety, a radioactive moiety, a novel functional group, a group that covalently or noncovalently interacts with other molecules, a photocaged moiety, an actinic radiation excitable moiety, a ligand, a photoisomerizable moiety, biotin, a biotin analogue, a moiety incorporating a heavy atom, a chemically cleavable group, a photocleavable group, a redox-active agent, an isotopically labeled moiety, a biophysical probe, a phosphorescent group, a chemiluminescent group, an electrochemiluminescent group (*e.g.*, an MSD SULFO-TAG type electrochemiluminescent group), an enzyme, an electron dense group, a magnetic group, an intercalating group, a chromophore, an energy transfer agent, a biologically active agent, or a combination thereof.
22. The method of claim 1, wherein the first detectable label and/or the second detectable label comprises a fluorophore, such as a Bodipy fluorophore.
 23. The method of claim 1, wherein the first detectable label and/or the second detectable label comprises an enzyme, such as HRP, peroxidase (POD), glucose oxidase (GOD), invertase (INV), β -D-galactosidase (BGase), glucose-6-phosphate dehydrogenase (G6PDH), or alkaline phosphatase (ALP).
 24. The method of claim 23, wherein the first detectable label and/or the second detectable label comprises an HRP, and wherein the method further comprises contacting the HRP with a biotinylated tyramide to produce an activated tyramide.
 25. The method of claim 24, further comprising detecting / measuring / quantitating the activated tyramide by adding a streptavidin-labeled fluorophore, or a combination of a streptavidin-labeled peroxidase (*e.g.*, HRP) and a chromogenic reagent, wherein the chromogenic reagent is preferably 3,3',5,5'-tetramethylbenzidine (TMB).
 26. The method of claim 1, wherein the sample is derived from a small volume of body fluid (*e.g.*, blood sample, knee lavage from an arthritic knee).
 27. The method of claim 1, wherein the sample is a lysate of a tissue (*e.g.*, biopsy, animal tissue, clinical sample, such as spleen homogenates), a lysate of isolated cells (*e.g.*, B cells or PBMCs isolated / purified / enriched from whole blood sample), or a lysate of a cell culture.
 28. The method of claim 1, wherein the sample is thawed from storage at -20°C, -80°C, or in liquid nitrogen.

29. The method of claim 1, wherein the affinity reagent is immobilized on a solid support (*e.g.*, a multi-well ELISA plate, or a resin bead for a column).
30. The method of claim 29, further comprising blocking the immobilized affinity reagent with a blocking agent (*e.g.*, BSA) prior to step (1).
31. The method of claim 29, further comprising removing binding partner unbound by the affinity reagent after step (1).
32. The method of claim 29, further comprising removing ligand probe unbound by the binding partner after step (1).
33. The method of claim 29, further comprising removing the first detection probe after step (2) and before step (3).
34. The method of claim 29, further comprising removing the second detection probe after step (3) and before step (4).
35. The method of claim 1, further comprising carrying out steps (1), (2), and (4), and optionally step (3), using a control sample comprising a pre-determined amount of the binding partner having a pre-determined portion thereof bound by the ligand.
36. The method of claim 35, comprising carrying out steps (1), (2), and (4), and optionally step (3), multiple times, either in parallel or sequentially, using two or more control samples having the same pre-determined amount of the binding partner but different pre-determined proportions bound by the ligand, in order to construct a standard curve.
37. The method of claim 1, wherein step (4) is carried out using chemiluminescent (including ECL), electrochemiluminescent (*e.g.*, MSD SULFO-TAG based electrochemiluminescent), surface plasmon resonance (SPR)-based biosensor (*e.g.*, BIAcore), flow cytometry, ELISA, or Western blot.
38. A method for assessing or predicting efficacy for a potential BTK inhibitor in a mammal, the method comprising: using the method of claim 1, measuring the binding between the ligand and the binding partner in a sample derived from the mammal, wherein the ligand is the potential BTK inhibitor, the binding partner is BTK from the mammal, and the mammal has BTK inhibitor-naïve baseline measurements or previously been administered the potential BTK inhibitor, wherein a higher level /

extent of binding between the potential BTK inhibitor and BTK is predictive of a higher level of efficacy.

39. A method for assessing the pharmacodynamics (PD) of a BTK inhibitor in a mammal, the method comprising: using the method of claim 1, measuring the binding between the ligand and the binding partner, from a series of samples each derived from a different time point following administering the mammal with the ligand, wherein the ligand is the BTK inhibitor, and the binding partner is BTK from the mammal.
40. A method for identifying a desired dose of a BTK inhibitor for achieving a pre-determined level of binding between the BTK inhibitor and BTK in a mammal, the method comprising: using the method of claim 1, measuring the binding between the ligand and the binding partner in a sample derived from the mammal, wherein the ligand is the BTK inhibitor, the binding partner is BTK from the mammal, and the mammal has previously been administered a candidate dose of the BTK inhibitor, wherein a level of measured binding lower than the pre-determined level is indicative that a dose higher than the candidate dose is required to achieve the desired dose, and wherein a level of measured binding higher than the pre-determined level is indicative that a dose lower than the candidate dose is required to achieve the desired dose.
41. The method of claim 40, further comprising repeating the method using a higher or lower candidate dose.
42. A ligand probe comprising a first moiety or a multivalent agent covalently linked to a ligand, wherein the first moiety binds to the multivalent agent, and wherein the ligand probe is represented by the following structure: X - L - M, wherein X is the ligand, M is the first moiety or the multivalent agent, and L is a linker moiety that covalently joins the ligand to the first moiety or the multivalent agent.

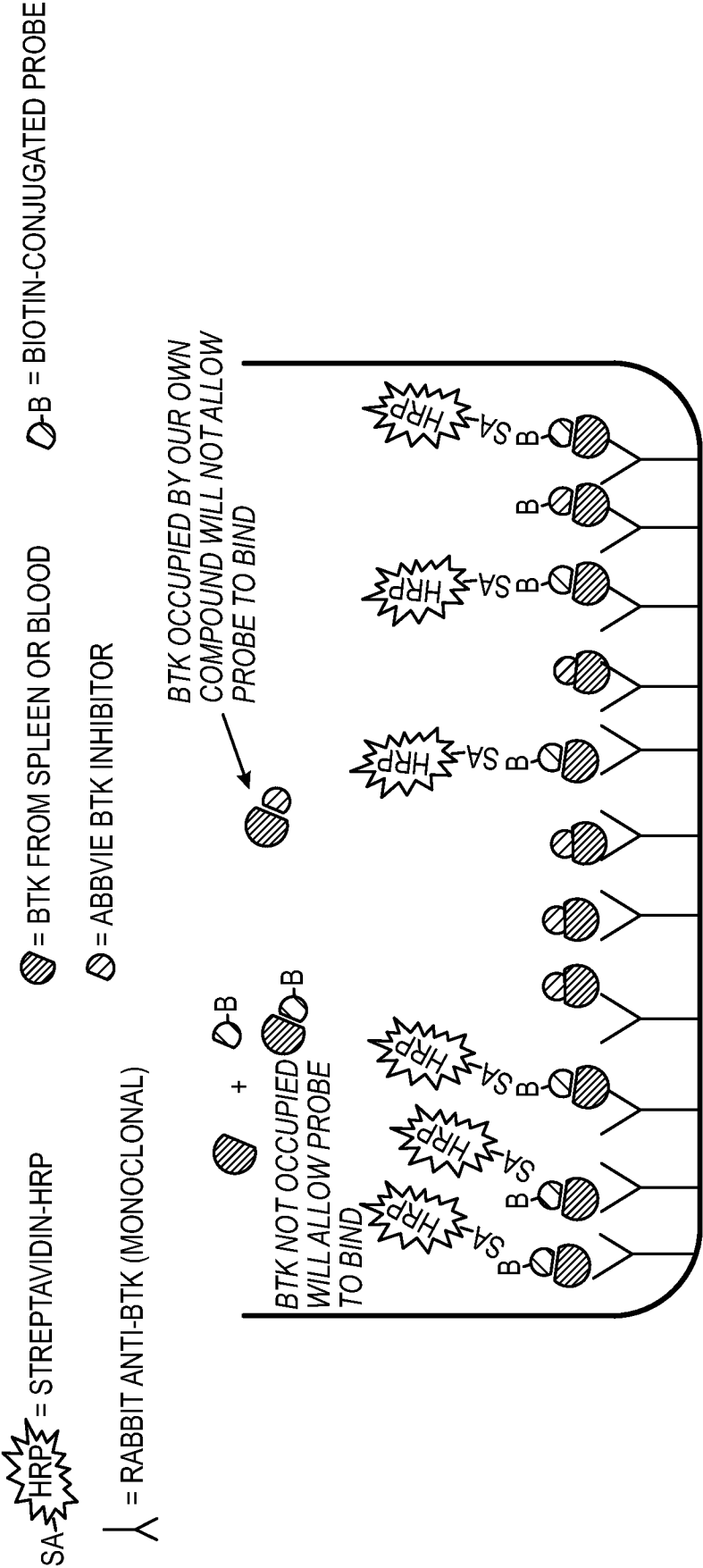


FIG. 1A

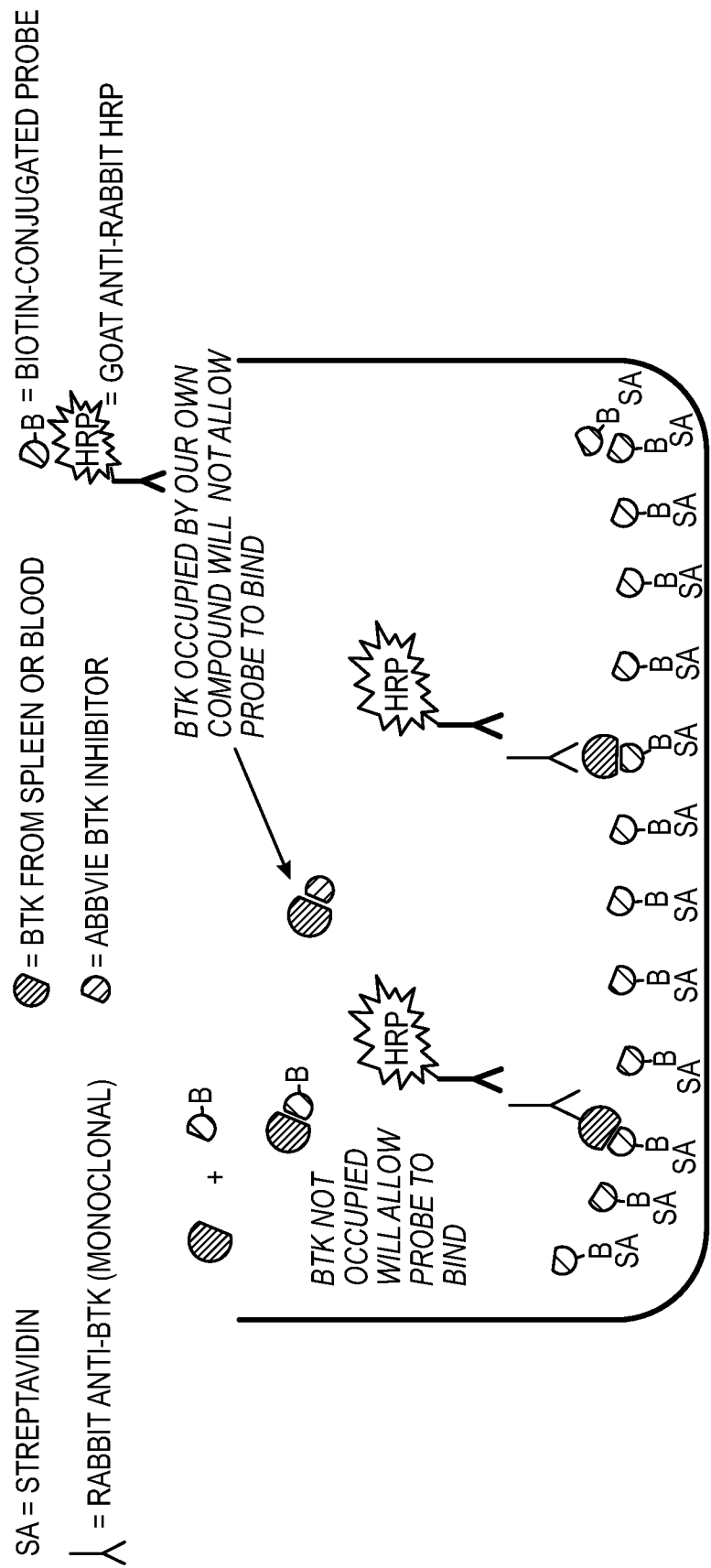
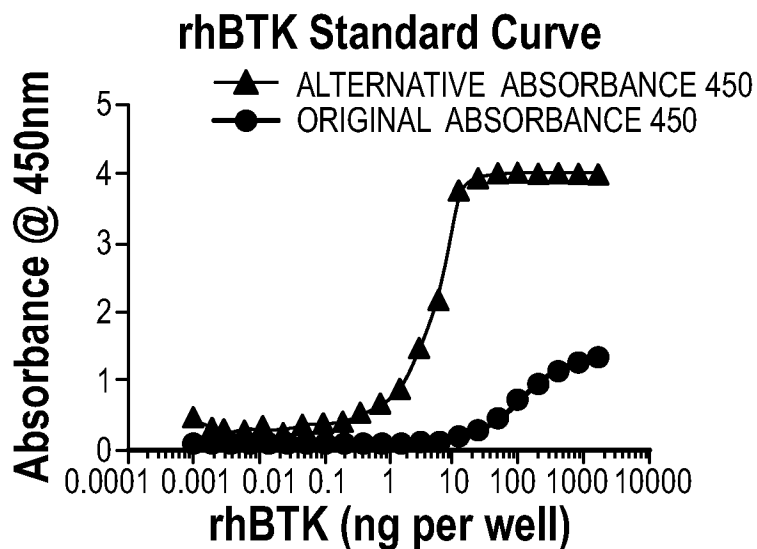
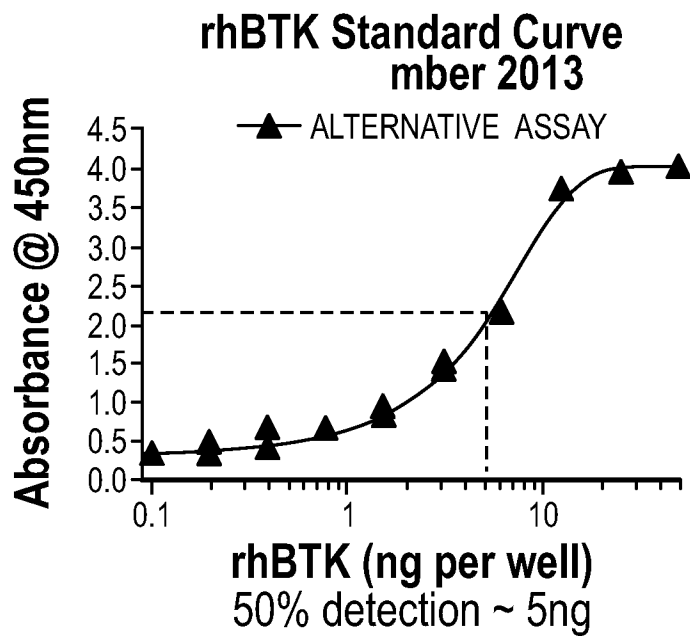
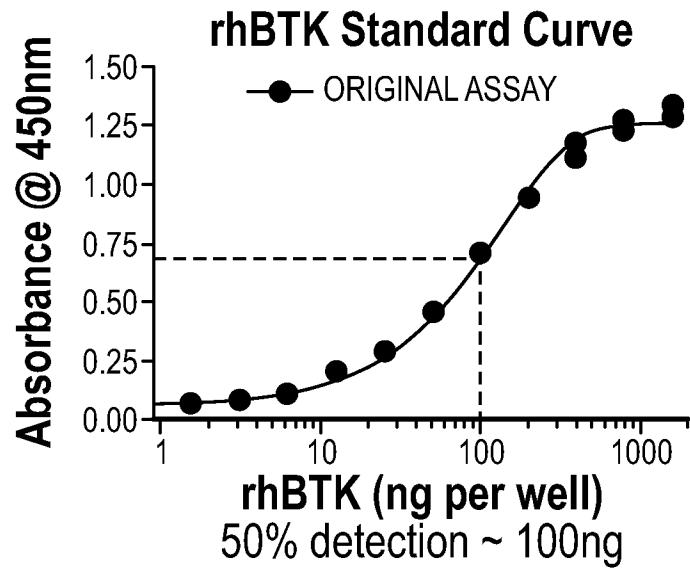


FIG. 1B

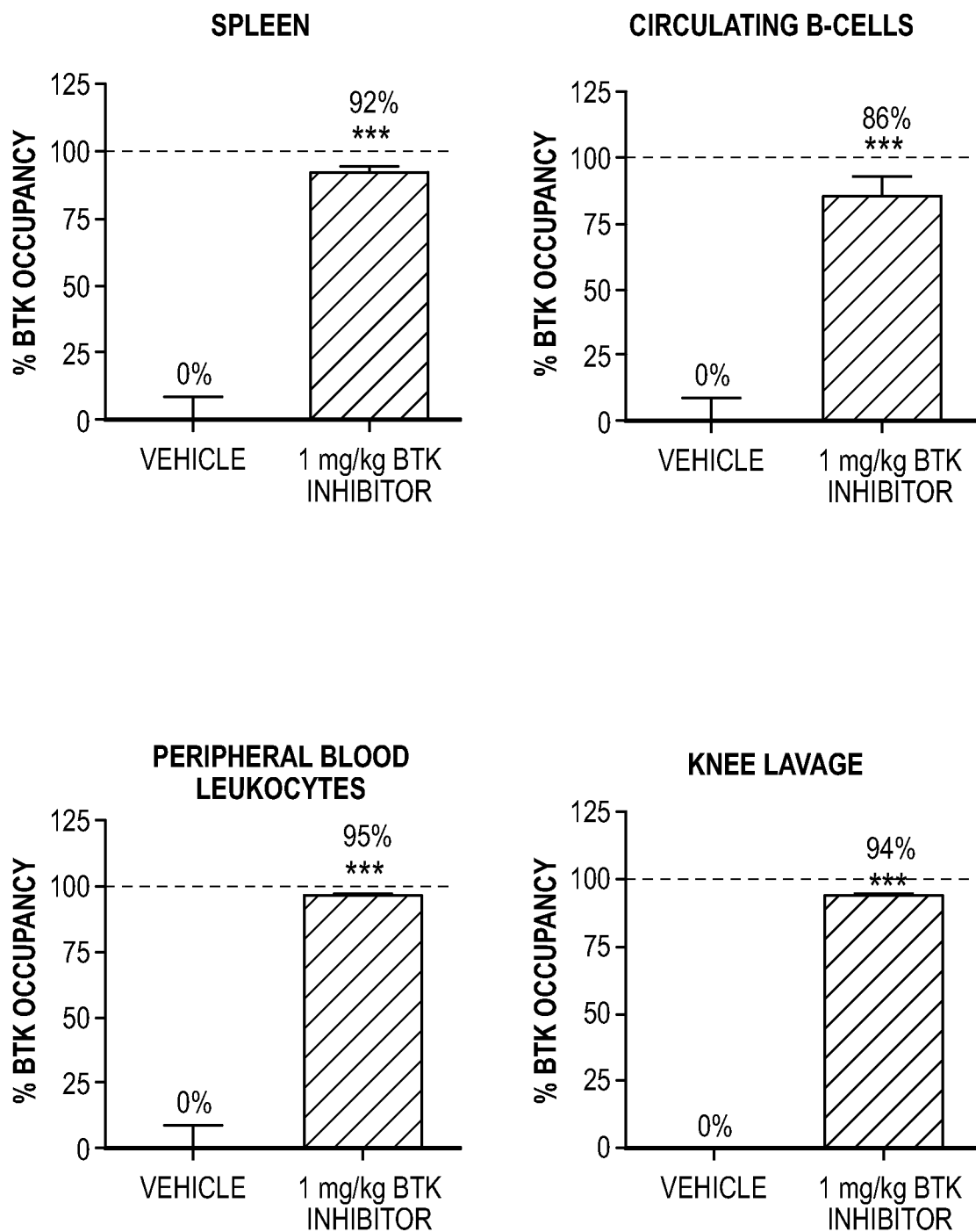
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FIG. 2



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FIG. 3



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 15/40269

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - G01N 33/53 (2015.01)

CPC - G01N 33/54386; C07K 16/18; G01N 33/535

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC(8): G01N 33/53 (2015.01)

CPC G01N 33/54386; C07K 16/18; G01N 33/535

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC 435/7.9, 287.1, 7.1 (Keyword limited, terms below)Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PatBase, Google Patents, Google Scholar (NPL); Keywords: measuring/assay occupancy/binding ligand/inhibitor/substrate binding partner/enzyme/protein kinase/BTK affinity reagent/antibody probe/first detection probe multivalent agent/streptavidin/avidin

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	WO 2014/059368 A1 (PHARMACYCLICS, INC.) 17 April 2014 (17.04.2014) para [0004], [0023], [0024], [0026], [0052], [0235], [0251], [0253], Figure 5, Figure 11, Figure 13	1-10, 13-19, 21-23, 26-37 ----- 11-12, 20, 24-25
Y	WO 2013/059738 A2 (PHARMACYCLICS, INC.) 25 April 2013 (25.04.2013) para [0238]	11-12
Y	US 2014/0134265 A1 (Buggy et al.) 15 May 2014 (15.05.2014) para [0008], [0091], [0218]	20
Y	US 2013/0108618 A1 (Zhu) 02 May 2013 (02.05.2013) para [0110], [0145]	24-25

☐ Further documents are listed in the continuation of Box C.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

04 November 2015 (04.11.2015)

Date of mailing of the international search report

07 DEC 2015

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents

P.O. Box 1450, Alexandria, Virginia 22313-1450

Facsimile No. 571-273-8300

Authorized officer:

Lee W. Young

PCT Helpdesk: 571-272-4300

PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 15/40269

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

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

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

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

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

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

This International Searching Authority found multiple inventions in this international application, as follows:
This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I: Claims 1-37, directed to a method for measuring binding between a ligand and a binding partner of the ligand in a sample

Group II: Claims 1 (in part), 38-39, directed to a method for assessing or predicting efficacy for a potential BTK inhibitor in amammal

Group III: Claims 1 (in part), 40-41, directed to a method for identifying a desired dose of a BTK inhibitor for achieving a predetermined level of binding between the BTK inhibitor and BTK in a mammal

--please see extra sheet--

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-37

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

Continued from Box No. III

Group IV: Claim 42, directed to a ligand probe comprising a first moiety or a multivalent agent covalently linked to a ligand

The inventions listed as Group I-IV do not relate to a single special technical feature under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Special Technical Feature:

Group I requires a method for measuring binding between a ligand and a binding partner of the ligand in a sample comprising contacting the sample with an affinity reagent that specifically binds the binding partner, in the presence of a ligand probe, and allowing binding to occur between the affinity reagent and the binding partner, wherein a first percentage of the binding partner in the sample is bound by the ligand, and a second percentage of the binding partner in the sample is bound by the ligand probe, not required by Groups II-IV

Group II requires a method for assessing or predicting efficacy for a potential BTK inhibitor in a mammal, not required by Groups I and III-IV

Group III requires a method for identifying a desired dose of a BTK inhibitor for achieving a predetermined level of binding between the BTK inhibitor and BTK in a mammal, not required by Groups I-II and IV

Group IV requires a ligand probe comprising a first moiety or a multivalent agent covalently linked to a ligand, wherein the first moiety binds to the multivalent agent, and wherein the ligand probe is represented by the following structure: X - L -M, not required by Groups I-III

Common Technical Features:

Group I and IV share the technical feature of a ligand probe. Groups I-III share the technical feature of A method for measuring binding between a ligand and a binding partner of the ligand in a sample, the method comprising: (1) contacting the sample with an affinity reagent that specifically binds the binding partner, in the presence of a ligand probe, and allowing binding to occur between the affinity reagent and the binding partner, wherein a first percentage of the binding partner in the sample is bound by the ligand, and a second percentage of the binding partner in the sample is bound by the ligand probe; (2) contacting binding partner bound by the affinity reagent with a first detection probe, wherein one of said first detection probe and said ligand probe comprises a multivalent agent, and allowing binding to occur between said first detection probe and said ligand probe through the multivalent agent; (3) optionally, contacting the multivalent agent with a second detection probe comprising a second detectable label and a second moiety that binds to the multivalent agent, and allowing binding to occur between the multivalent agent and the second moiety on the second detection probe; (4) detecting the presence or measuring the amount of the first detection probe or the second detectable label, thereby measuring binding between the ligand and the binding partner. However, these shared technical features do not represent a contribution over prior art, because the shared technical feature is being anticipated by WO 2014/059368 A1 to Chang et al. (hereinafter 'Chang'). Chang teaches a method for measuring binding between a ligand and a binding partner of the ligand in a sample (Figure 11, para [0253]), the method comprising: (1) contacting the sample with an affinity reagent that specifically binds the binding partner ('BTK kinases captured by the anti-BTK antibody,' para [0253]), in the presence of a ligand probe (inhibitor or drug, para [0235]), and allowing binding to occur between the affinity reagent and the binding partner, wherein a first percentage of the binding partner in the sample is bound by the ligand, and a second percentage of the binding partner in the sample is bound by the ligand probe ('The BTK kinases captured by the anti-BTK antibody include drug-occupied BTK kinases and unoccupied BTK kinases,' para [0253]); (2) contacting binding partner bound by the affinity reagent with a first detection probe ('The captured BTK kinases are contacted with a probe,'), wherein one of said first detection probe and said ligand probe comprises a multivalent agent (Streptavidin, para [0253]) (para [0253], Figure 13, para [0052]), and allowing binding to occur between said first detection probe and said ligand probe through the multivalent agent; (3) optionally, contacting the multivalent agent with a second detection probe comprising a second detectable label and a second moiety that binds to the multivalent agent, and allowing binding to occur between the multivalent agent and the second moiety on the second detection probe (para [0253], Figure 13, para [0052]); (4) detecting the presence or measuring the amount of the first detection probe or the second detectable label, thereby measuring binding between the ligand and the binding partner (para [0253]).

As the shared technical features were known in the art at the time of the invention, they cannot be considered common technical features that would otherwise unify the groups. Therefore, Groups I-IV lack unity under PCT Rule 13.