(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
17 April 2014 (17.04.2014)

(10) International Publication Number
WO 2014/059368 Al

(51) International Patent Classification:
C07D 403/12 (2006.01) *G01N 33/68* (2006.01)
A61K 31/506 (2006.01) *G01N 33/532* (2006.01)

(21) International Application Number: PCT/US20 13/064688

(22) International Filing Date: 11 October 2013 (11.10.2013)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/712,675 11 October 2012 (11.10.2012) US

(71) Applicant: PHARMACYCLICS, INC. [US/US]; 995 East Arques Avenue, Sunnyvale, CA 94085 (US).

(72) Inventors: CHANG, Betty, Y.; 10375 Lindsay Avenue, Cupertino, CA 95014 (US). CHANG, Stella; 2195 Summerton Drive, San Jose, CA 95122 (US).

(74) Agent: BEST-MARTIN, Jennifer, L.; Wilson Sonsini Goodrich & Rosati, 650 Page Mill Road, Palo Alto, CA 94304 (US).

(81) Designated States (*unless otherwise indicated, for every kind of national protection available*): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

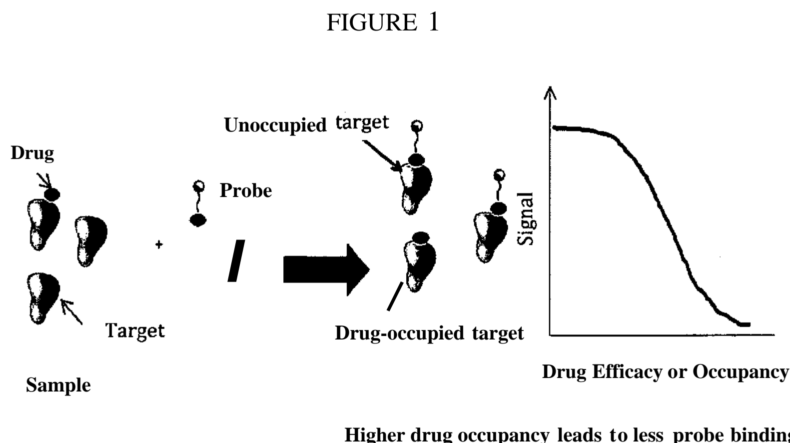
(84) Designated States (*unless otherwise indicated, for every kind of regional protection available*): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

(54) Title: COMPANION DIAGNOSTICS FOR TEC FAMILY KINASE INHIBITOR THERAPY

FIGURE 1



Higher drug occupancy leads to less probe binding

(57) Abstract: The invention provides methods, assays and systems for determining the efficacy of a TEC family kinase inhibitor on a target kinase. The methods, assays and systems relate to determining the occupancy of a target kinase by a TEC family kinase inhibitor (e.g., BTK inhibitors). Such quantitative measurements are used to inform therapeutic treatment and the over-all health care management of a subject. For example, diagnostic kits for diagnosing, prognosing, and monitoring a disease or indication benefitting from treatment with a TEC family kinase inhibitor are provided. In another example, diagnostic kits for identifying responders to TEC family kinase inhibitor therapy, determining therapeutic regimens, and detecting resistance to TEC family kinase inhibitor also are provided.

WO 2014/059368 A1

COMPANION DIAGNOSTICS FOR TEC FAMILY KINASE INHIBITOR THERAPY

RELATED APPLICATION

[0001] The present application claims the benefit of priority from U.S. Provisional Patent Application No. 61/712,675, filed October 11, 2012, which is herein incorporated byreference in its entirety.

FIELD OF THE INVENTION

[0002] Described herein are companion diagnostic methods and kits for use in combination with a therapy comprising administration of a TEC family kinase inhibitor.

BACKGROUND OF THE INVENTION

[0003] The TEC kinase family is a subfamily of non-receptor protein-tyrosine kinases (PTKs). The TEC kinase family is composed of five members, TEC, BTK (Bruton's Tyrosine Kinase), ITK (interleukin-2-inducible T-cell kinase)/EMT/TSK, BMX and TXK/RLK. A characteristic feature of this family is the presence of a pleckstrin homology (PH) domain, which is known to bind phosphoinositides. The TEC family kinases participate in phosphotyrosine-mediated and phospholipid-mediated signaling systems. Many TEC family proteins are abundantly expressed in hematopoietic tissues, and play important roles in the growth and differentiation processes of blood cells. Mutations in the BTK gene cause X chromosome-linked agammaglobulinemia (XLA) in humans and X chromosome-linked immunodeficiency (Xid) in mice, indicating that BTK activity is indispensable for B-cell ontogeny. ITK is functionally important for the development and effector function of Th2 and Thl7 cells. In addition, TEC family kinases have been shown to be involved in the intracellular signaling mechanisms of cytokine receptors, lymphocyte surface antigens, heterotrimeric G-protein-coupled receptors and integrin molecules. Inhibitors of TEC kinases have been developed for the treatment of a variety of diseases associated with activation of TEC family kinases, including cancer, autoimmune disorders, and inflammatory diseases.

SUMMARY OF THE INVENTION

[0004] Described herein are companion diagnostic methods and kits for use in combination with a therapy comprising administration of a TEC family kinase inhibitor. In some embodiments, the companion diagnostic methods provided involve protein occupancy assays for one or more inhibitors of the TEC kinase family. Accordingly, described herein are protein occupancy assays for kinase inhibitors of the TEC kinase family. Further described herein are protein occupancy assays for irreversible kinase inhibitors of the TEC kinase family. Further described herein are protein occupancy assays for reversible kinase inhibitors of the TEC kinase family. In some embodiments, the TEC kinase family inhibitor is an inhibitor of one or more kinases of the TEC kinase family. In some embodiments, the TEC kinase family inhibitor is an

inhibitor of BTK, ITK, BMX, TXK, TEC, or any combination thereof. In some embodiments, the TEC kinase family inhibitor is an inhibitor of one or more kinases of the TEC kinase family and also is an inhibitor of one or more structurally homologous kinases. In some embodiments, the TEC kinase family inhibitor is an inhibitor of one or more kinases of the TEC kinase family and also is an inhibitor of one or more structurally homologous tyrosine kinases (e.g., a kinase that has a structurally homologous active site). In some embodiments, the TEC kinase family inhibitor is an inhibitor of one or more kinases of the TEC kinase family and also is an inhibitor of a kinase of the EGFR family. In some embodiments, the TEC kinase family inhibitor is an inhibitor of one or more kinases of the TEC kinase family and also is an inhibitor of HERl (EGFR, ErbBl), HER2/c-neu (ErbB2), HER3 (ErbB3) and HER4 (ErbB4), or JAK3. In some embodiments, the TEC kinase family inhibitor is an inhibitor of one or more kinases of the TEC kinase family and also is an inhibitor of a SRC family tyrosine kinase. In some embodiments, the TEC kinase family inhibitor is an inhibitor of one or more kinases of the TEC kinase family and also is an inhibitor of B lymphoid kinase (BLK). Further described herein are exemplary reagents and probes for use in the protein occupancy assays provided.

[0005] Described herein in certain embodiments is protein occupancy assay that is an ELISA probe assay. In some embodiments, the ELISA probe assay is plate based electrochemilummescent assay to determine the relative amount of a TEC family kinase that has not been bound by a TEC family kinase inhibitor. In some embodiments, TEC family kinase inhibitor is an irreversible TEC family kinase inhibitor. For example, in some embodiments, the TEC family kinase inhibitor binds to the active site of the TEC family kinase and forms a disulfide bond with a cysteine residue. In some embodiments, the assays involve binding a probe to TEC family kinases that have not been bound by the TEC family kinase inhibitor. In some embodiments, the probe comprises a TEC family kinase inhibitor attached to a detectable label (e.g., biotin) via a linker (e.g., a long chain linker). In some embodiments, the TEC family kinase inhibitor is a BTK inhibitor. In some embodiments, the TEC family kinase inhibitor is an irreversible BTK inhibitor. In some embodiments, the TEC family kinase inhibitor is ibrutinib. In some embodiments, the probe is Compound 1-5 described herein, which consists of ibrutinib linked to biotin via a long chain linker. Labeling of samples with the probe allows for the detection of BTK not occupied by the TEC family kinase inhibitor. In some embodiments, the probe conjugated with the TEC family kinase (i.e. probe-bound kinase) is captured by a streptavidin coated plate. In some embodiments, excess un-conjugated probe competes with probe-bound kinase for binding to streptavidin.

[0006] Also described herein are methods for determining the efficacy of inhibitors of the TEC kinase family. Further described herein are methods for using the protein occupancy assays

in the diagnosis, prognosis, and determination and modification of therapeutic regimens in the treatment of diseases associated with activation of one or more members of the TEC kinase family, including diseases wherein inhibition of one or more members of the TEC kinase family provides therapeutic benefit to a patient having the disease. In some embodiments, the patent is diagnosed as having a disease or disorder associated with aberrant activation of a TEC family kinase, such as, for example, cancer, an autoimmune disorder, and/or an inflammatory disease.

[0007] In one aspect, provided herein is a protein occupancy assay comprising a plate-based system. In some embodiments, the plate-based protein occupancy assay comprises an electrochemiluminescent assay.

[0008] Described herein are methods for determining the amount of TEC family kinases in a sample that have not been bound by a TEC family kinase inhibitor (e.g., active sites that are not occupied by the inhibitor). In some embodiments, the methods comprise determining the amount of BTK in a sample that have not been bound by a TEC family kinase inhibitor. In some embodiments, the TEC family kinase inhibitor is a BTK inhibitor. In some embodiments, the BTK inhibitor is ibrutinib. In some embodiments, the BTK inhibitor is AVL-292. In some embodiments, the BTK inhibitor is ONO-WG-307. In some embodiments, the methods comprise determining the amount of a TEC family kinase in a sample that is not bound to ibrutinib. In some embodiments, the methods comprise determining the amount of BTK in a sample that is not bound to ibrutinib. In some embodiments, the methods comprise determining the amount of ITK in a sample that is not bound to ibrutinib. In some embodiments, the methods comprise determining the amount of BMX in a sample that is not bound to ibrutinib. In some embodiments, the methods comprise determining the amount of TEC in a sample that is not bound to ibrutinib. In some embodiments, the methods comprise determining the amount of TXK in a sample that is not bound to ibrutinib. In some embodiments, the methods comprise determining the amount of BLK in a sample that is not bound to ibrutinib.

[0009] In some embodiments, the methods comprise determining the number of BTK kinase active sites in a sample that have not been bound by a TEC family kinase inhibitor. In some embodiments, the methods comprise determining the number of ITK kinase active sites in a sample that have not been bound by a TEC family kinase inhibitor. In some embodiments, the methods comprise determining the number of BMX active sites in a sample that have not been bound by a TEC family kinase inhibitor. In some embodiments, the methods comprise determining the number of TXK active sites in a sample that have not been bound by a TEC family kinase inhibitor. In some embodiments, the methods comprise determining the number of TEC active sites in a sample that have not been bound by a TEC family kinase inhibitor. In some embodiments, the methods comprise determining the number of BLK active sites in a sample

that have not been bound by a TEC family kinase inhibitor. In some embodiments, the TEC family kinase inhibitor inhibits two or more members of the TEC kinase family. In some embodiments, the TEC family kinase inhibitor inhibits BTK, ITK, BMX, TXK, TEC or any combination thereof. In some embodiments, the TEC family kinase inhibitor inhibits an EGFR or SRC family tyrosine kinase.

[0010] In some embodiments, the methods comprise determining the number of BTK kinase active sites in a sample that have not been bound by ibrutinib. In some embodiments, the methods comprise determining the number of ITK kinase active sites in a sample that have not been bound by ibrutinib. In some embodiments, the methods comprise determining the number of BMX active sites in a sample that have not been bound by ibrutinib. In some embodiments, the methods comprise determining the number of TXK active sites in a sample that have not been bound by ibrutinib. In some embodiments, the methods comprise determining the number of TEC active sites in a sample that have not been bound by ibrutinib. In some embodiments, the methods comprise determining the number of BLK active sites in a sample that have not been bound by ibrutinib.

[0011] In some embodiments, the protein occupancy assay comprises contacting a sample with a probe, wherein the probe comprises a TEC family kinase inhibitor attached to a label via linker; and detecting a TEC family kinase bound to the probe (i.e. probe-bound kinase). In some embodiments, the probe is a derivative of ibrutinib, where ibrutinib is attached to a label via a linker. In some embodiments, the label is biotin or a derivative thereof. In some embodiments, the probe is selected from among probe of Formula (I), (II), or (III) as described herein. In some embodiments, the probe is selected from among probe compounds 1-1, 1-2, 1-3, 1-4, or 1-5 as described herein. In some embodiments, the probe is probe compound 1-5.

[0012] Disclosed herein in some embodiments are companion diagnostic kits for the detection of protein occupancy in a sample from a patient that has been administered a TEC family kinase inhibitor. In some embodiments, the kit comprise a probe that binds to the TEC family kinase not bound to the TEC family kinase inhibitor. In some embodiments, the probe comprises an inhibitor that binds to TEC family kinase (e.g., the probe is a derivative of a TEC family kinase inhibitor). In some embodiments, the inhibitor is attached to a label. In some embodiments, the probe further comprises a linker, wherein the linker is capable of attaching the label to the inhibitor. In some embodiments, the probe is a derivative of a TEC family kinase inhibitor. In some embodiments, the probe is a TEC family kinase inhibitor attached to a label via a linker. In some embodiments, the probe is a derivative of ibrutinib. In some embodiments, the probe consists of ibrutinib attached to a label via a linker. In some embodiments, the probe consists of ibrutinib attached to biotin via a linker. In some embodiments, the probe is a

compound of Formula (I), (II) or (III). In some embodiments, the probe is compound 1-1, 1-2, 1-3, 1-4 or 1-5. In some embodiments, the kit further comprises one or more solid supports.

[0013] Further disclosed herein are methods comprising: (a) contacting a sample comprising a TEC family kinase, or a homologous tyrosine kinase, with a probe; (b) detecting the amount of the probe-bound kinase; and (c) determining occupancy of the kinase based on the amount of the probe-bound kinase in the sample. In some embodiments, the method further comprises contacting the probe-bound kinase with a primary detection agent. In some embodiments, the method further comprises contacting the primary detection agent with a secondary detection agent.

[0014] Further disclosed herein are methods for determining a therapeutic regimen comprising: (a) contacting a sample comprising a TEC family kinase with a probe; (b) detecting the amount of a probe-bound kinase; (c) determining occupancy of a kinase based on the amount of the probe-bound kinase; and (d) determining a therapeutic regimen based on the occupancy of the kinase. In some embodiments, determining the therapeutic regimen comprises administering a TEC family kinase inhibitor. In some embodiments, determining the therapeutic regimen comprises modifying a therapeutic regimen with a TEC family kinase inhibitor. In some embodiments, modifying a therapeutic regimen comprises increasing, decreasing, initiating, or terminating a therapeutic regimen with a TEC family kinase inhibitor. In some embodiments, the therapeutic regimen with a TEC family kinase inhibitor is modified when the occupancy of the target increases. In some embodiments, the therapeutic regimen with a TEC family kinase inhibitor is modified when the occupancy of the target decreases. In some embodiments, the method further comprises contacting the probe-bound kinase with a primary detection agent. In some embodiments, the method further comprises contacting the primary detection agent with a secondary detection agent.

[0015] Further disclosed herein are methods for determining efficacy of a TEC family kinase inhibitor comprising: (a) contacting a sample comprising a TEC family kinase with a probe; (b) detecting the presence or absence of a probe-bound kinase; (c) determining occupancy of the kinase based on the amount of the probe-bound kinase; and (d) determining efficacy of the TEC family kinase inhibitor based on the occupancy of the kinase. In some embodiments, the TEC family kinase inhibitor is effective when the occupancy of the target is at least about 70%. In some embodiments, the TEC family kinase inhibitor is ineffective when the occupancy of the target is less than about 50%. In some embodiments, the method further comprises contacting the probe-bound kinase with a primary detection agent. In some embodiments, the method further comprises contacting the primary detection agent with a secondary detection agent.

[0016] Further disclosed herein are methods for identifying responders of a TEC family kinase inhibitor therapy comprising: (a) contacting a sample comprising a TEC family kinase with a probe, wherein the sample is from a subject having received at least one administration of the TEC family kinase inhibitor; (b) detecting the amount of the probe-bound kinase; (c) determining occupancy of the TEC family kinase based on the amount of the probe-bound kinase; and (d) identifying the subject as a TEC family kinase inhibitor responder or TEC family kinase inhibitor non-responder based on the occupancy of the kinase. In some embodiments, the subject is identified as a TEC family kinase inhibitor responder when the occupancy of the target is at least about 70%. In some embodiments, the subject is identified as a TEC family kinase inhibitor non-responder when the occupancy of the target is less than about 50%. In some embodiments, the method further comprises contacting the probe-bound kinase with a primary detection agent. In some embodiments, the method further comprises contacting the primary detection agent with a secondary detection agent.

[0017] Further disclosed herein are methods for determining TEC family kinase inhibitor resistance comprising: (a) contacting a sample comprising a TEC family kinase with a probe; (b) detecting the amount of the probe-bound kinase; (c) determining occupancy of the TEC family kinase based on the amount of the probe-bound kinase; and (d) determining TEC family kinase inhibitor resistance based on the occupancy of the TEC family kinase. In some embodiments, TEC family kinase inhibitor resistance is determined when the occupancy of the target is less than about 50%. In some embodiments, the method further comprises contacting the probe-bound kinase with a primary detection agent. In some embodiments, the method further comprises contacting the primary detection agent with a secondary detection agent.

[0018] Further disclosed herein are methods for validating a TEC family kinase inhibitor comprising: (a) contacting a sample comprising a TEC family kinase with a probe to form a probe-bound kinase; (b) detecting the amount of the probe-bound kinase; and (c) determining occupancy of the kinase by the TEC family kinase inhibitor based on the amount of the probe-bound target; and (d) validating the TEC family kinase inhibitor based on the occupancy of the TEC family kinase. In some embodiments, validating the TEC family kinase inhibitor comprises determining the efficacy of the TEC family kinase inhibitor on a TEC family kinase. In some embodiments, determining occupancy of the TEC family kinase by the TEC family kinase inhibitor comprises quantifying the amount of probe-bound kinases. In some embodiments, the drug is effective when the occupancy of the TEC family kinase is at least about 70%. In some embodiments, the method further comprises contacting the probe-bound kinase with a primary detection agent. In some embodiments, the method further comprises contacting the primary detection agent with a secondary detection agent.

[0019] Further disclosed herein are methods method for identifying TEC family kinase modulators that bind to TEC family kinases comprising: (a) contacting a sample comprising a TEC family kinase with a probe; (b) detecting the presence or absence of a probe-bound kinase; and (c) identifying TEC family kinase modulators based on the amount of the probe-bound kinase. In some embodiments, the method further comprises contacting the probe-bound kinase with a primary detection agent. In some embodiments, the method further comprises contacting the primary detection agent with a secondary detection agent. In some embodiments, the sample is a sample that pre-treated with the putative TEC family kinase modulator. In some embodiments, the sample is pre-treated *in vitro*. In some embodiments, the sample is a sample from a subject (e.g., a patient) that has been administered the putative TEC family kinase modulator.

[0020] Further disclosed herein are methods method for identifying TEC family kinase inhibitor comprising: (a) contacting a sample comprising a TEC family kinase with a probe; (b) detecting the presence or absence of a probe-bound kinase; and (c) identifying TEC family kinase inhibitor based on the amount of the probe-bound kinase. In some embodiments, the method further comprises contacting the probe-bound kinase with a primary detection agent. In some embodiments, the method further comprises contacting the primary detection agent with a secondary detection agent. In some embodiments, the sample is a sample that pre-treated with the putative TEC family kinase inhibitor. In some embodiments, the sample is pre-treated *in vitro*. In some embodiments, the sample is a sample from a subject (e.g., a patient) that has been administered the putative TEC family kinase inhibitor.

[0021] In some embodiments, the methods, kits or compositions disclosed herein comprise a TEC family kinase inhibitor. In some embodiments, the inhibitor is an irreversible TEC family kinase inhibitor. In some embodiments, the inhibitor covalently binds to a TEC family kinase. In some embodiments, the inhibitor binds to a cysteine residue of a TEC family kinase. In some embodiments, the inhibitor is a small molecule, polypeptide, antibody, or nucleic acid. In some embodiments, the TEC family kinase inhibitor is an inhibitor of a Bruton's tyrosine kinase (BTK). In some embodiments, the inhibitor of a Bruton's tyrosine kinase (BTK) is ibrutinib. In some embodiments, the inhibitor of a Bruton's tyrosine kinase (BTK) is AVL-292, AVL-291, AVL-101, CNX-774, ONO-WG-307. In some embodiments, the TEC family kinase inhibitor is an inhibitor of an ITK. In some embodiments, the TEC family kinase inhibitor is an inhibitor of a TEC kinase. In some embodiments, the TEC family kinase inhibitor is an inhibitor of a BMX kinase. In some embodiments, the TEC family kinase inhibitor is an inhibitor of a BLK. In some embodiments, the TEC family kinase inhibitor is an inhibitor of a kinase selected from HER 1, HER2, HER3, HER4 and JAK3.

[0022] In some embodiments, the methods, kits or compositions disclosed herein comprise a target kinase. In some embodiments, the target kinase is a TEC family kinase. In some embodiments, the kinase is a Bruton's tyrosine kinase (BTK). In some embodiments, the kinase is an ITK. In some embodiments, the kinase is a BLK. In some embodiments, the kinase is a TEC kinase. In some embodiments, the kinase is a TXK. In some embodiments, the kinase is a BMX kinase. In some embodiments, the kinase is ITK. In some embodiments, the kinase is HER1, HER2, HER3, HER4, or JAK3.

[0023] In some embodiments, the methods, kits or compositions disclosed herein comprise one or more solid supports. In some embodiments, the one or more solid supports is a plate. In some embodiments, the one or more solid supports is a bead or a plurality of beads. In some embodiments, the kit comprises two or more solid supports. In some embodiments, the two or more solid supports comprise (a) a plate; and (b) a bead or a plurality of beads. In some embodiments, the plate is a microplate. In some embodiments, the microplate is a streptavidin-coated microplate. In some embodiments, the microplate is a MSD microplate. In some embodiments, the bead is a streptavidin bead. In some embodiments, the bead is a magnetic bead. In some embodiments, the solid support is coated to form a coated solid support. In some embodiments, the coated solid support is coated with streptavidin. In some embodiments, the coated solid support is coated with an antibody. In some embodiments, the coated solid support is capable of capturing probe. In some embodiments, the coated solid support is capable of capturing the label. In some embodiments, the coated solid support is capable of capturing a target (e.g., a TEC family kinase).

[0024] In some embodiments, the methods disclosed herein further comprise contacting the probe-bound target with a primary detection agent. In some embodiments, the primary detection agent comprises an antibody, a bead, a dye, a label, a tag, a fluorophore, or any combination thereof. In some embodiments, the primary detection agent comprises an antibody. In some embodiments, the antibody is an anti-BTK antibody. In some embodiments, the primary detection agent is conjugated to a tag. In some embodiments, the primary detection agent is conjugated to an electrochemiluminescent tag. In some embodiments, the electrochemiluminescent tag comprises Tris(bipyridine)ruthenium(II) dichloride. In some embodiments, the electrochemiluminescent tag is Ruthenium (II) tris-bipyridine, N-hydroxysuccinimide. In some embodiments, the primary detection agent is conjugated to a SULFO tag. In some embodiments, the primary detection agent is a bead.

[0025] In some embodiments, the methods disclosed herein further comprise contacting the primary detection agent with a secondary detection agent. In some embodiments, the secondary detection agent comprises an antibody, a bead, a dye, a label, a tag, a fluorophore, or any

combination thereof. In some embodiments, the secondary detection agent comprises an antibody. In some embodiments, the antibody is an anti-IgG antibody. In some embodiments, the antibody is an anti-IgA antibody. In some embodiments, the secondary detection agent is conjugated to a tag. In some embodiments, the secondary detection agent is conjugated to an electrochemilummescent tag. In some embodiments, the electrochemilummescent tag comprises Tris(bipyridine)ruthenium(II) dichloride. In some embodiments, the electrochemilummescent tag is Ruthenium (II) tris-bipyridine, N-hydroxysuccinimide. In some embodiments, the secondary detection agent is conjugated to a SULFO tag. In some embodiments, the secondary detection agent is a bead.

[0026] In some embodiments, the methods, kits or compositions disclosed herein comprise a primary detection agent. In some embodiments, the methods, kits or compositions disclosed herein comprise a secondary detection agent. In some embodiments, the detection agent comprises an antibody, a bead, a dye, a label, a tag, a fluorophore, or any combination thereof. In some embodiments, the detection agent detection agent comprises an antibody. In some embodiments, the detection agent is conjugated to a tag. In some embodiments, the detection agent is conjugated to an electrochemilummescent tag. In some embodiments, the electrochemilummescent tag comprises Tris(bipyridine)ruthenium(II) dichloride. In some embodiments, the electrochemilummescent tag is Ruthenium (II) tris-bipyridine, N-hydroxysuccinimide. In some embodiments, the detection agent conjugated to a SULFO tag. In some embodiments, the detection agent is a bead. In some embodiments, the detection agent is conjugated to an enzyme. In some embodiments, the antibody is conjugated to horseradish peroxidase (HRP) or alkaline phosphatase (AP). In some embodiments, the antibody is an anti-BTK antibody. In some embodiments, the antibody targets a TEC family kinase. In some embodiments, the antibody is an anti-ITK antibody. In some embodiments, the antibody is an anti-TEC antibody. In some embodiments, the antibody is an anti-BMX antibody. In some embodiments, the antibody is an anti-BLK antibody. In some embodiments, the antibody is an anti-HERl antibody, anti-HER2 antibody, anti-HER3 antibody, or anti-HER4 antibody.

[0027] In some embodiments, the methods, kits or compositions disclosed herein comprise a label. In some embodiments, the label is biotin. In some embodiments, the label is a fluorophore.

[0028] In some embodiments, the methods disclosed herein further comprise capturing the target kinase. In some embodiments, the methods disclosed herein further comprise capturing the probe-bound target kinase. In some embodiments, the target is captured by an antibody. In some embodiments, the antibody is an anti-target antibody. In some embodiments, the probe-bound target is captured by a bead.

[0029] In some embodiments, the methods, kits or compositions disclosed herein comprise

an antibody. In some embodiments, the antibody is attached to a solid support. In some embodiments, the bead is attached to a solid support. In some embodiments, the solid support is a microplate. In some embodiments, the microplate is a MSD microplate. In some embodiments, the solid support is a bead.

[0030] In some embodiments, the methods, kits, and compositions disclosed herein comprise a bead. In some embodiments, the bead is a streptavidin bead. In some embodiments, the bead is a magnetic bead. In some embodiments, the bead is a coated bead. In some embodiments, the bead is a coated streptavidin bead. In some embodiments, the coated bead is coated with a tag. In some embodiments, the tag is an electrochemiluminescent tag. In some embodiments, the electrochemiluminescent tag comprises Tris(bipyridine)ruthenium(II) dichloride. In some embodiments, the electrochemiluminescent tag is Ruthenium (II) tris-bipyridine, N-hydroxysuccinimide. In some embodiments, the bead is a SULFO tag streptavidin bead. In some embodiments, the bead is a SULFO tag bead. In some embodiments, the bead interacts with the probe. In some embodiments, the probe comprises a label. In some embodiments, the bead interacts with the label. In some embodiments, the label comprises biotin. In some embodiments, the bead forms a conjugate with the probe-bound target. In some embodiments, the bead binds to the probe.

[0031] In some embodiments, the methods disclosed herein comprise detecting the presence or absence of the probe-bound target or a portion thereof. In some embodiments, detecting the presence or absence of the probe-bound target comprises detecting the probe-bound target or a portion thereof. In some embodiments, detecting the presence or absence of the probe-bound target comprises detecting the bead or a portion thereof. In some embodiments, detecting the presence or absence of the probe-bound target comprises detecting the coated bead. In some embodiments, detecting the presence or absence of the probe-bound target comprises detecting a electrochemiluminescent tag. In some embodiments, the electrochemiluminescent tag comprises Tris(bipyridine)ruthenium(II) dichloride. In some embodiments, the electrochemiluminescent tag is Ruthenium (II) tris-bipyridine, N-hydroxysuccinimide. In some embodiments, detecting the presence or absence of the probe-bound target comprises detecting a SULFO tag. In some embodiments, detecting the presence or absence of the probe-bound target comprises luminescence. In some embodiments, detecting the presence or absence of the probe-bound target comprises electrochemiluminescence. In some embodiments, the probe-bound target is an unoccupied target. In some embodiments, the probe-bound target is a drug-occupied target.

[0032] In some embodiments, the methods disclosed herein further comprise purification of the probe-bound target. In some embodiments, purification of the probe-bound target comprises magnetic separation of probe-bound targets from non-probe-bound targets. In some

embodiments, the sample is a pre-treated sample, wherein the pre-treated sample is contacted with a TEC family kinase inhibitor prior to contact with the probe. In some embodiments, the sample is a non-treated sample, wherein the sample is not contacted with a TEC family kinase inhibitor prior to contact with the label. In some embodiments, the sample is a sample from a patient that has been administered a TEC family kinase inhibitor. In some embodiments, the sample is a control sample from a patient that has not been administered a TEC family kinase inhibitor. In some embodiments, the sample is a whole blood sample, peripheral blood sample, lymph sample, tissue sample, tumor biopsy sample, or bone marrow sample. In some embodiments, the sample is a sample containing one or more cell types, or a lysate thereof, derived from a whole blood sample, peripheral blood sample, lymph sample, tissue sample, tumor biopsy sample, or bone marrow sample.

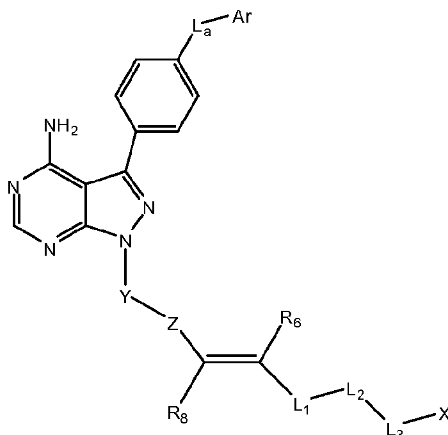
[0033] In some embodiments, the methods, kits, and compositions disclosed herein comprise a probe. In some embodiments, the probe comprises an inhibitor. In some embodiments, the inhibitor binds to a target. In some embodiments, the inhibitor is an irreversible inhibitor. In some embodiments, the inhibitor covalently binds to a target. In some embodiments, the inhibitor binds to a cysteine residue of a target. In some embodiments, the inhibitor is a small molecule, polypeptide, antibody, or nucleic acid. In some embodiments, the inhibitor is an inhibitor of a TEC family kinase. In some embodiments, the inhibitor is an inhibitor of a Bruton's tyrosine kinase (BTK). In some embodiments, the inhibitor binds to a cysteine residue of a Bruton's tyrosine kinase (BTK). In some embodiments, the inhibitor binds to a cysteine 481 of a Bruton's tyrosine kinase (BTK). In some embodiments, the inhibitor of a Bruton's tyrosine kinase (BTK) is ibrutinib. In some embodiments, the inhibitor of a Bruton's tyrosine kinase (BTK) is AVL-292, AVL-291 AVL-101, CNX-774, ONO-WG-307. In some embodiments, the agent is an inhibitor of an ITK. In some embodiments, the agent is an inhibitor of a TEC kinase. In some embodiments, the agent is an inhibitor of a BMX kinase. In some embodiments, the agent is an inhibitor of a BLK. In some embodiments, the agent is an inhibitor of a kinase selected from HER1, HER2, HER3, HER4 and JAK3.

[0034] In some embodiments, the methods, kits, and compositions disclosed herein comprise a target. In some embodiments, the target is a kinase. In some embodiments, the kinase is a Bruton's tyrosine kinase (BTK). In some embodiments, the kinase is ITK, BLK, TEC, TXK, or BMX. In some embodiments, the kinase is HER1, HER2, HER3, HER4, or JAK3. In some embodiments, the target is a protein.

[0035] In some embodiments, the methods, kits, and compositions disclosed herein comprise a sample. In some embodiments, the sample is from a subject suffering from a cancer. In some embodiments, the cancer is a sarcoma. In some embodiments, the cancer is a carcinoma. In some

embodiments, the cancer is a lymphoma. In some embodiments, the lymphoma is a Hodgkin's lymphoma. In some embodiments, the lymphoma is a non-Hodgkin's lymphoma (NHL). In some embodiments, the cancer is a leukemia. In some embodiments, the cancer is a chronic lymphocytic leukemia. In some embodiments, the cancer is a small lymphocytic leukemia. In some embodiments, the cancer is Waldenstrom's macroglobulinemia. In some embodiments, the cancer is a follicular lymphoma. In some embodiments, the cancer is a mantle cell lymphoma. In some embodiments, the cancer is a diffuse large B-cell lymphoma. In some embodiments, the cancer is a multiple myeloma. In some embodiments, the cancer is a solid tumor. In some embodiments, the sample is from a subject suffering from a an autoimmune or inflammatory disorder.

[0036] Described herein, in particular embodiments, are kits for determining drug target occupancy in a patient receiving a TEC family kinase inhibitor therapy, comprising a probe having the structure of Formula (II) comprising:



Formula (II);

wherein:

La is CH2, O, NH or S;

Ar is optionally substituted aryl or optionally substituted heteroaryl;

Y is optionally substituted alkyl, optionally substituted heteroalkyl, optionally substituted cycloalkyl, optionally substituted heterocycloalkyl, optionally substituted aryl, or optionally substituted heteroaryl;

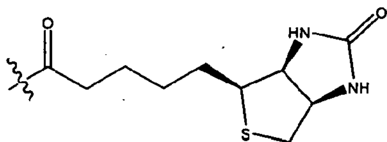
Z is C(O), OC(O), NHC(O), C(S), S(0)n, OS(0)n, NHS(0)n, where n is 1 or 2;

R6 and R8 are independently selected from H, optionally substituted alkyl, or optionally substituted heteroalkyl;

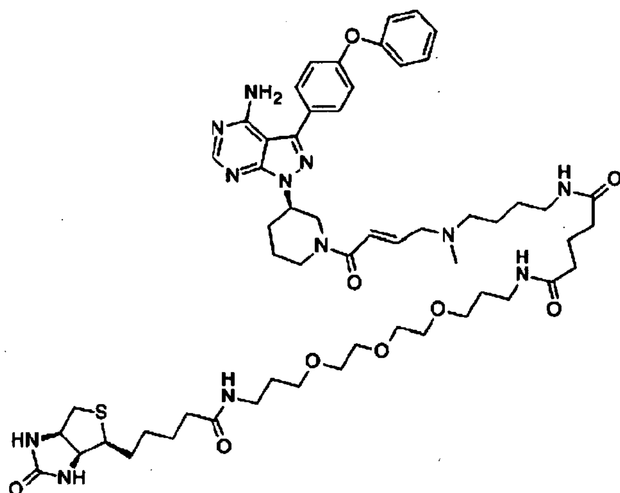
LI is optionally substituted alkyl or optionally substituted heteroalkyl;

L2 is a bond, optionally substituted heterocycloalkyl, or -N(H)C(0)(CH2)mC(0)N(H), where m is 2-6;

L3 is optionally substituted alkyl or optionally substituted heteroalkyl; and X is a detectable label, wherein the probe binds to a TEC family kinase. In some embodiments, X is:

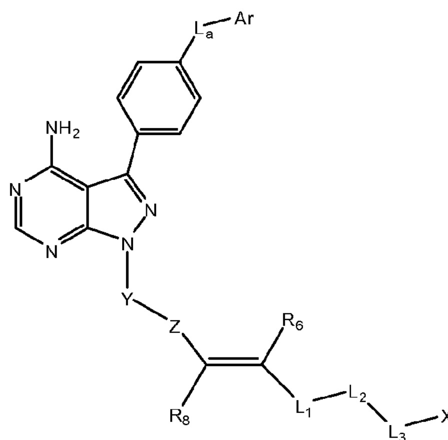


. In some embodiments, the probe has the structure of:



. In some embodiments, the TEC family kinase is Bruton's tyrosine kinase (BTK), ITK, TEC, BMX, or TXK. In some embodiments, the probe binds to BLK ,HER1, HER2, HER3, HER4 or JAK3. In some embodiments, the kits further comprise one or more solid supports. In some embodiments, the one or more solid supports are selected from among a plate, a microplate, a bead or a plurality of beads. In some embodiments, the solid support is coated with a capture agent to form a coated solid support, wherein the capture agent binds to the probe. In some embodiments, the capture agent is streptavidin or an antibody. In some embodiments, the kits further comprise a primary detection agent, and optionally, a secondary detection agent that binds to the primary detection agent. In some embodiments, the primary detection agent or secondary detection agent comprises an antibody, a bead, a dye, a label, a tag, a fluorophore, or any combination thereof. In some embodiments, the primary detection agent is an antibody that is an anti-BTK antibody, an anti-ITK antibody, an anti-TEC antibody, an anti-TXK antibody, an anti-BMX antibody, or an anti-BLK antibody. In some embodiments, the primary detection agent is an antibody that is an anti-HER1 antibody, an anti-HER2 antibody, an anti-HER3 antibody, or an anti-HER4 antibody. In some embodiments, the primary or secondary detection agent is conjugated to an electrochermluminescent tag. In some embodiments, chemiluminescent tag is a Ruthenium (II) tris-bipyridine, N-hydroxysucciriimide tag. In some embodiments, the TEC kinase inhibitor therapy is an irreversible TEC kinase inhibitor. In some embodiments, the TEC kinase inhibitor therapy is an irreversible BTK inhibitor. In some embodiments, the TEC kinase inhibitor therapy is ibrutinib.

[0037] Described herein, in certain embodiments are methods for determining drug target occupancy in a patient receiving a TEC family kinase inhibitor therapy, comprising: (a) contacting a sample comprising a TEC family kinase with a probe to form a probe-bound kinase, wherein the sample is obtained from the patient following administration of at least one dose of an irreversible TEC family kinase inhibitor; (b) detecting the amount of probe-bound kinase in the sample; and (c) determining target occupancy of the TEC family kinase based on the amount of probe-bound kinase detected in the sample, wherein the probe has the structure of Formula (II) comprising:



Formula (II);

wherein:

La is CH2, O, NH or S;

Ar is optionally substituted aryl or optionally substituted heteroaryl;

Y is optionally substituted alkyl, optionally substituted heteroalkyl, optionally substituted cycloalkyl, optionally substituted heterocycloalkyl, optionally substituted aryl, or optionally substituted heteroaryl;

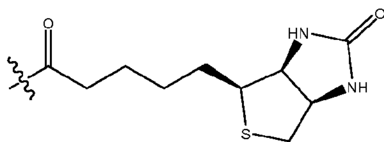
Z is C(O), OC(O), NHC(O), C(S), S(0)n, OS(0)n, NHS(0)n, where n is 1 or 2;

R6 and R8 are independently selected from H, optionally substituted alkyl, or optionally substituted heteroalkyl;

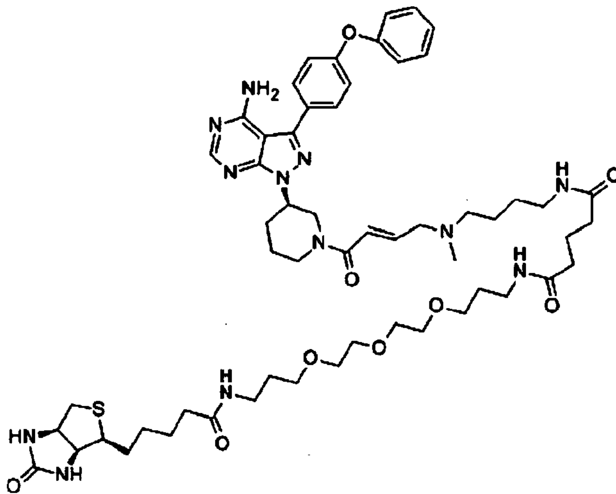
LI is optionally substituted alkyl or optionally substituted heteroalkyl;

L2 is a bond, optionally substituted heterocycloalkyl, or -N(H)C(0)(CH2)mC(0)N(H), where m is 2-6;

L3 is optionally substituted alkyl or optionally substituted heteroalkyl; and X is a detectable label, wherein the probe binds to a TEC family kinase. In some embodiments, X is:



. In some embodiments, the probe has the structure of:



. In some embodiments, determining target occupancy comprises i) determining the number of binding sites not bound to the TEC family kinase inhibitor based on the amount of probe-bound kinase detected in the sample and ii) comparing said number to the total amount of active TEC family kinases in the sample. In some embodiments, the control is the amount of probe-bound kinase that is present when the method is performed on an untreated sample. In some embodiments, the methods further comprise determining or modifying a therapeutic regimen based on the target occupancy of the TEC family kinase. Described herein are methods for monitoring drug target occupancy in a patient receiving a TEC family kinase inhibitor therapy, comprising performing the methods provided herein for determining protein occupancy of the kinase at two or more time points over the course of the therapy. In some embodiments, the methods further comprise modifying a therapeutic regimen if the target occupancy increases or decreases over the course of the therapy. In some embodiments, the methods further comprise: i) increasing the dosage or frequency of administration of the TEC family kinase inhibitor if the target occupancy is less than about 50%, ii) decreasing the dosage or frequency of administration of the TEC family kinase inhibitor if the target occupancy is above at least about 70%, iii) maintaining the same therapeutic regimen of the TEC family kinase inhibitor or iv) discontinuing the therapeutic regimen. In some embodiments, the dosage of the TEC family kinase inhibitor is increased if the target occupancy is less than about 50%. In some embodiments, the dosage of the TEC family kinase inhibitor is decreased if the target occupancy is above at least about 70%. In some embodiments, the dosage of the TEC family kinase inhibitor is maintained if the target occupancy is above at least about 70%. In some embodiments, the frequency of adrninistration of the TEC family kinase inhibitor is increased if the target occupancy is less than about 50%. In some embodiments, the frequency of administration of the TEC family kinase inhibitor is decreased if the target occupancy is above at least about 70%. In some embodiments, the frequency of administration of the TEC family kinase inhibitor is maintained if the target occupancy is above at least about 70%. In

some embodiments, the methods further comprise determining the efficacy of the TEC family kinase inhibitor therapy based on the target occupancy. In some embodiments, the TEC family kinase inhibitor is effective when the occupancy of the TEC family kinase is at least about 70%. In some embodiments, the TEC family kinase inhibitor is ineffective when the occupancy of the TEC family kinase is less than about 50%. In some embodiments, the TEC family kinase inhibitor an inhibitor of a Bruton's tyrosine kinase (BTK). In some embodiments, the TEC family kinase inhibitor is ibrutinib, AVL-292, AVL-291, AVL-101, CNX-774, or ONO-WG-307. In some embodiments, the TEC family kinase inhibitor is ibrutinib. In some embodiments, the at least one dosage of ibrutinib is about 10 mg to about 2000 mg, such as, for example, 140 mg, 420 mg, 560 mg or 840 mg. In some embodiments, where protein occupancy is monitored over the course of a therapy, the patient is receiving a daily dosage of ibrutinib is about 10 mg per day to about 2000 mg per day, such as, for example, a daily dosage of about 140 mg per day, 420 mg per day, 560 mg per day or 840 mg per day. In a particular embodiment, the patient is receiving a maintenance dosage of ibrutinib of about 420 mg per day. In some embodiments, the methods further comprise capturing probe-bound kinase with a capture agent. In some embodiments, the capture target is streptavidin or an antibody. In some embodiments, the capture target is attached to a solid support. In some embodiments, the solid support is a plate, a microplate, a bead or a plurality of beads. In some embodiments, the methods further comprise contacting the probe-bound kinase with a primary detection agent, and optionally, a secondary detection agent that binds to the primary detection agent. In some embodiments, the primary detection agent or secondary detection agent comprises an antibody, a bead, a dye, a label, a tag, a fluorophore, or any combination thereof. In some embodiments, the primary detection agent is an antibody binds to a TEC family kinase. In some embodiments, the primary detection agent is an antibody that is an anti-BTK antibody, an anti-ITK antibody, an anti-TXK antibody, anti-TEC antibody, anti-BMX antibody, or anti-BLK antibody. In some embodiments, the primary detection agent is an antibody that is an anti-HERl antibody, anti-HER2 antibody, anti-HER3 antibody, or anti-HER4 antibody. In some embodiments, the methods further comprise contacting the primary detection agent with a secondary detection agent. In some embodiments, the secondary detection agent comprises an antibody, a bead, a dye, a label, a tag, a fluorophore, or any combination thereof. In some embodiments, the primary or secondary detection agent is conjugated to a chemiluminescent tag. In some embodiments, the chemiluminescent tag is a Ruthenium (II) tris-bipyridine, N-hydroxysuccinimide tag. In some embodiments, the patient is suffering from a cancer, an autoimmune disease or an inflammatory disorder. In some embodiments, the cancer is a sarcoma, carcinoma, myeloma, leukemia or a lymphoma. In some embodiments, the cancer is Hodgkin's lymphoma or a non-Hodgkin's lymphoma (NHL). In

some embodiments, the cancer is chronic lymphocytic leukemia (CLL), small lymphocytic lymphoma (SLL), mantle cell leukemia (MCL), follicular lymphoma (FL), diffuse large B-cell lymphoma (DLBCL), Waldenstrom macroglobulinemia or multiple myeloma (MM). In some embodiments, the sample is a blood sample, a lymph sample or tumor biopsy sample.

INCORPORATION BY REFERENCE

[0038] All publications and patent applications mentioned in this specification are herein incorporated by reference in their entireties to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference. The publications discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the inventors described herein are not entitled to antedate such disclosure by virtue of prior invention or for any other reason.

BRIEF DESCRIPTION OF THE DRAWINGS

[0039] The skilled artisan will understand that the drawings described below are for illustration purposes only. The drawings are not intended to limit the scope of the present teachings in any way.

[0040] **FIGURE 1** illustrates an overview of the Protein Occupancy Assay

[0041] **FIGURE 2** illustrates components of a Protein Occupancy Assay Kit

[0042] **FIGURE 3** illustrates an overview of a Protein occupancy assay method for the detection of drug-bound targets

[0043] **FIGURE 4** illustrates an overview of a Protein occupancy assay for the detection of unoccupied targets

[0044] **FIGURE 5** illustrates an overview of a Plate-based Protein occupancy assay for the detection of drug-bound targets

[0045] **FIGURE 6** illustrates an overview of a Plate-based Protein occupancy assay for the detection of probe-bound targets

[0046] **FIGURE 7** illustrates an overview of a Plate-based protein occupancy assay for the detection of drug-bound targets

[0047] **FIGURE 8** illustrates an overview of a Plate-based protein occupancy assay for the detection of unoccupied targets

[0048] **FIGURE 9** illustrates an overview of a Probe-coated plate-based protein occupancy for the detection of probe-bound targets

[0049] **FIGURE 10** illustrates an overview of a Probe-coated plate-based protein occupancy assay for the detection of unoccupied targets

[0050] **FIGURE 11** illustrates exemplary BTK occupancy assay formats. Figure 11A

presents an illustrative overview of the streptavidin detection method. Figure 1IB presents an illustrative overview of the streptavidin capture method.

[0051] **FIGURE 12** illustrates a Streptavidin detection BTK occupancy assay. Figure 12A presents an illustrative plate layout. Figure 12 B presents illustrative data showing the results for the streptavidin detection method

[0052] **FIGURE 13** presents illustrative results for a streptavidin detection BTK occupancy assay using two different BTK capture antibodies.

[0053] **FIGURE 14** illustrates a Streptavidin-capture BTK occupancy assay. Figure 14A presents an overview of the Streptavidin-capture method. Figure 14B presents an illustrative plate layout. Figure 14C presents illustrative data showing the results for the streptavidin-capture method

[0054] **FIGURE 15** presents illustrative results for a streptavidin-capture BTK occupancy assay using two different BTK detection antibodies.

[0055] **FIGURE 16** illustrates a comparison of streptavidin detection and streptavidin capture methods

[0056] **FIGURE 17** presents illustrative results for a probe optimization experiment for a streptavidin capture BTK occupancy assay.

[0057] **FIGURE 18** illustrates the results for a titration experiment for a streptavidin capture BTK occupancy assay.

[0058] **FIGURE 19** illustrates the results for a titration experiment for a streptavidin capture BTK occupancy assay.

[0059] **FIGURE 20** illustrates a SI2400 MSD SECTOR IMAGER plate.

[0060] **FIGURE 21** illustrates exemplary probe compounds 1-1, 1-2, 1-3, 1-4 and 1-5.

[0061] **FIGURE 22** presents illustrative results for exemplary probe compounds 1-1, 1-2, 1-3, 1-4 and 1-5 for a probe optimization experiment for a streptavidin capture BTK occupancy assay.

[0062] **FIGURE 23** illustrates raw signal data for various capture antibody/detection antibody pairs tested for quantifying total BLK. Top, MSD high bind plate. Bottom, MSD standard plate.

[0063] **FIGURE 24** illustrates signal to background ratios for the different capture antibody/detection antibody pairs tested for quantifying total BLK. Top, MSD high bind plate. Bottom, MSD standard plate.

[0064] **FIGURE 25** illustrates raw signal data for dose titration of the capture antibody/detection antibody pairs tested for quantifying total BLK.

[0065] **FIGURE 26** illustrates signal to background ratios for the dose titration of the capture antibody/detection antibody pairs tested for quantifying total BLK.

[0066] **FIGURE 27** illustrates a plot of the signal values for recombinant BLK protein using 1 µ¾m1 capture antibody and **0.5** µg/ml detection antibody.

[0067] **FIGURE 28** illustrates the results for a probe titration experiment for a streptavidin capture BLK occupancy assay (A) and ITK occupancy assay (B).

[0068] **FIGURE 29** illustrates the results for a drug titration experiment for a streptavidin capture ITK occupancy assay (A) and % ITK inhibition (B).

[0069] **FIGURE 30** illustrates the results for a drug titration experiment for a streptavidin capture ITK occupancy assay using PBMC lysates. Results are expressed as % ITK inhibition.

DETAILED DESCRIPTION OF THE INVENTION

[0070] Described herein are companion diagnostic methods and kits for use in combination with a therapy comprising administration of a TEC family kinase inhibitor. In some embodiments, the companion diagnostic methods provided involve protein occupancy assays for one or more inhibitors of the TEC kinase family. Accordingly, described herein are protein occupancy assays for kinase inhibitors of the TEC kinase family. Further described herein are protein occupancy assays for irreversible kinase inhibitors of the TEC kinase family. Further described herein are protein occupancy assays for reversible kinase inhibitors of the TEC kinase family. In some embodiments, the TEC kinase family inhibitor is an inhibitor of one or more kinases of the TEC kinase family. In some embodiments, the TEC kinase family inhibitor is an inhibitor of BTK, ITK, BMX, TXK, TEC, or any combination thereof. In some embodiments, the TEC kinase family inhibitor is an inhibitor of one or more kinases of the TEC kinase family and also is an inhibitor of one or more structurally homologous kinases. In some embodiments, the TEC kinase family inhibitor is an inhibitor of one or more kinases of the TEC kinase family and also is an inhibitor of one or more structurally homologous tyrosine kinases. In some embodiments, the TEC kinase family inhibitor is an inhibitor of one or more kinases of the TEC kinase family and also is an inhibitor of a kinase of the EGFR family. In some embodiments, the TEC kinase family inhibitor is an inhibitor of one or more kinases of the TEC kinase family and also is an inhibitor of HER1 (EGFR, ErbBl), HER2/c-neu (ErbB2), HER3 (ErbB3) and HER4 (ErbB4), or JAK3. In some embodiments, the TEC kinase family inhibitor is an inhibitor of one or more kinases of the TEC kinase family and also is an inhibitor of a SRC family tyrosine kinase. In some embodiments, the TEC kinase family inhibitor is an inhibitor of one or more kinases of the TEC kinase family and also is an inhibitor of B lymphoid kinase (BLK). Further described herein are exemplary reagents and probes for use in the protein occupancy assays provided.

[0071] Described herein in certain embodiments is protein occupancy assay that is an ELISA probe assay. In some embodiments, the ELISA probe assay is plate based

electrochemilummescent assay to determine the relative amount of a TEC family kinase that has not been bound by a TEC family kinase inhibitor. In some embodiments, TEC family kinase inhibitor is an irreversible TEC family kinase inhibitor. For example, in some embodiments, the TEC family kinase inhibitor binds to the active site of the TEC family kinase and forms a disulfide bond with a cysteine residue. In some embodiments, the assays involves binding an activity probe to free TEC family kinases that have not been bound by the TEC family kinase inhibitor. In some embodiments, the activity probe comprises a TEC family kinase inhibitor attached to a detectable label (e.g., biotin) via a linker (e.g., a long chain linker). In some embodiments, the TEC family kinase inhibitor a BTK inhibitor. In some embodiments, the TEC family kinase inhibitor is an irreversible BTK inhibitor. In some embodiments, the TEC family kinase inhibitor is ibrutinib. In some embodiments, the probe is Compound 1-5, which consists of ibrutinib linked to biotin via a long chain linker. Labeling of samples with the probe allows for the detection of BTK not occupied by drug. In some embodiments, the probe conjugated with the TEC family kinase is captured by a streptavidin coated plate. In some embodiments, excess un-conjugated probe competes with probe labeled BTK for binding to streptavidin.

[0072] Also described herein are methods for determining the efficacy of inhibitors of the TEC kinase family. Further described herein are methods for using the protein occupancy assays in the diagnosis, prognosis, and determination and modification of therapeutic regimens in the treatment of diseases associated with activation of one or more members of the TEC kinase family, including diseases wherein inhibition of one or more members of the TEC kinase family provides therapeutic benefit to a patient having the disease. In some embodiments, the patent is diagnosed as having a disease or disorder associated with aberrant activation of a TEC family kinase, such as, for example, cancer, an autoimmune disorder, and/or an inflammatory disease.

[0073] Further disclosed herein are diagnostic assays for diagnosing, prognosing, and monitoring a disease or condition benefitting from treatment with a TEC family kinase inhibitor. Also disclosed herein are diagnostic assays for identifying responders to TEC family kinase inhibitor therapy, determining therapeutic regimens, and detecting resistance to TEC family kinase inhibitor therapy.

Certain Terminology

[0074] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of skill in the art to which the claimed subject matter belongs. In the event that there is a plurality of definitions for terms herein, those in the section prevail. Where reference is made to a URL or other such identifier or address, it is understood that such identifiers can change and particular information on the internet can come and go, but equivalent information can be found by searching the internet. Reference thereto

evidences the availability and public dissemination of such information.

[0075] It is to be understood that the foregoing general description and the following detailed description are exemplary and explanatory only and are not restrictive of any subject matter claimed. In this application, the use of the singular includes the plural unless specifically stated otherwise. It must be noted that, as used in the specification and the appended claims, the singular forms "a," "an" and "the" include plural referents unless the context clearly dictates otherwise. Unless stated otherwise, the use of "or" means "and/or". Furthermore, use of the term "including" as well as other forms, such as "include", "includes," and "included," is not limiting.

[0076] The section headings used herein are for organizational purposes only and are not to be construed as limiting the subject matter described. All documents, or portions of documents, cited in the application including, but not limited to, patents, patent applications, articles, books, manuals, and treatises are hereby expressly incorporated by reference in their entirety for any purpose.

[0077] Definition of standard chemistry terms may be found in reference works, including Carey and Sundberg "ADVANCED ORGANIC CHEMISTRY 4TH ED." Vols. A (2000) and B (2001), Plenum Press, New York. Unless otherwise indicated, conventional methods of mass spectroscopy, NMR, HPLC, protein chemistry, biochemistry, recombinant DNA techniques and pharmacology, within the skill of the art are employed. Unless specific definitions are provided, the nomenclature employed in connection with, and the laboratory procedures and techniques of, analytical chemistry, synthetic organic chemistry, and medicinal and pharmaceutical chemistry described herein are those known in the art. Standard techniques can be used for chemical syntheses, chemical analyses, pharmaceutical preparation, formulation, and delivery, and treatment of patients. Standard techniques can be used for recombinant DNA, oligonucleotide synthesis, and tissue culture and transformation (e.g., electroporation, lipofection). Reactions and purification techniques can be performed e.g., using kits of manufacturer's specifications or as commonly accomplished in the art or as described herein. The foregoing techniques and procedures can be generally performed of conventional methods well known in the art and as described in various general and more specific references that are cited and discussed throughout the present specification.

[0078] It is to be understood that the methods and compositions described herein are not limited to the particular methodology, protocols, cell lines, constructs, and reagents described herein and as such may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the methods and compositions described herein, which will be limited only by the appended claims.

[0079] All publications and patents mentioned herein are incorporated herein by reference in their entirety for the purpose of describing and disclosing, for example, the constructs and methodologies that are described in the publications, which might be used in connection with the methods, compositions and compounds described herein. The publications discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the inventors described herein are not entitled to antedate such disclosure by virtue of prior invention or for any other reason.

[0080] "Alkyl" refers to a straight or branched hydrocarbon chain radical consisting solely of carbon and hydrogen atoms, containing no unsaturation, having from one to fifteen carbon atoms *(e.g.,* C1-C15 alkyl). In certain embodiments, an alkyl comprises one to thirteen carbon atoms *(e.g.,* C1-C13 alkyl). In certain embodiments, an alkyl comprises one to eight carbon atoms *(e.g.,* $Ci-C_8$ alkyl). In other embodiments, an alkyl comprises five to fifteen carbon atoms *(e.g.,* C_5-C15 alkyl). In other embodiments, an alkyl comprises five to eight carbon atoms *(e.g.,* C_5-C_8 alkyl). The alkyl is attached to the rest of the molecule by a single bond, for example, methyl (Me), ethyl (Et), n-propyl, 1-methylethyl (wo-propyl), n-butyl, n-pentyl, 1,1-dimethylethyl (z-butyl), 3-methylhexyl, 2-methylhexyl, and the like. Unless stated otherwise specifically in the specification, an alkyl group is optionally substituted by one or more of the following substituents: halo, cyano, nitro, oxo, thioxo, trimethylsilanyl, $-OR^a$, $-SR^a$, $-OC(0)-R^a$, $-N(R^a)_2$, $-C(0)R^a$, $-C(0)OR^a$, $-C(0)N(R^a)_2$, $-N(R^a)C(0)OR^a$, $-N(R^a)C(0)R^a$, $-N(R^a)S(0)_tR^a$ (where t is 1 or 2), $-S(0)_tOR^a$ (where t is 1 or 2) and $-S(0)tN(R^a)_2$ (where t is 1 or 2) where each R^a is independently hydrogen, alkyl, fluoroalkyl, carbocyclyl, carbocyclylalkyl, aryl, aralkyl, heterocyclyl, heterocyclylalkyl, heteroaryl or heteroarylalkyl.

[0081] The alkyl group could also be a "lower alkyl" having 1 to 6 carbon atoms.

[0082] As used herein, $Ci-C_x$ includes $Ci-C_2$, C1-C3 . . . $Ci-C_x$.

[0083] "Aryl" refers to a radical derived from an aromatic monocyclic or multicyclic hydrocarbon ring system by removing a hydrogen atom from a ring carbon atom. The aromatic monocyclic or multicyclic hydrocarbon ring system contains only hydrogen and carbon from six to eighteen carbon atoms, where at least one of the rings in the ring system is fully unsaturated, *i.e.,* it contains a cyclic, delocalized (4n+2) π-electron system in accordance with the Huckel theory. Aryl groups include, but are not limited to, groups such as phenyl, fluorenyl, and naphthyl. Unless stated otherwise specifically in the specification, the term "aryl" or the prefix "ar-" (such as in "aralkyl") is meant to include aryl radicals optionally substituted by one or more substituents independently selected from alkyl, alkenyl, alkynyl, halo, fluoroalkyl, cyano, nitro, optionally substituted aryl, optionally substituted aralkyl, optionally substituted aralkenyl,

optionally substituted aralkynyl, optionally substituted carbocyclyl, optionally substituted carbocyclylalkyl, optionally substituted heterocyclyl, optionally substituted heterocyclylalkyl, optionally substituted heteroaryl, optionally substituted heteroarylalkyl, $-R^b-OR^a$, $-R^b-OC(0)-R^a$, $-R^b-N(R^a)_2$, $-R^b-C(0)R^a$, $-R^b-C(0)OR^a$, $-R^b-C(0)N(R^a)_2$, $-R^b-0-R^c-C(0)N(R^a)_2$, $-R^b-N(R^a)C(0)OR^a$, $-R^b-N(R^a)C(0)R^a$, $-R^b-N(R^a)S(0)_tR^a$ (where t is 1 or 2), $-R^b-S(0)_tOR^a$ (where t is 1 or 2) and $-R^b-S(0)_tN(R^a)_2$ (where t is 1 or 2), where each R^a is independently hydrogen, alkyl, fluoroalkyl, cycloalkyl, cycloalkylalkyl, aryl (optionally substituted with one or more halo groups), aralkyl, heterocyclyl, heterocyclylalkyl, heteroaryl or heteroarylalkyl, each R^b is independently a direct bond or a straight or branched alkylene or alkenylene chain, and R^c is a straight or branched alkylene or alkenylene chain, and where each of the above substituents is unsubstituted unless otherwise indicated.

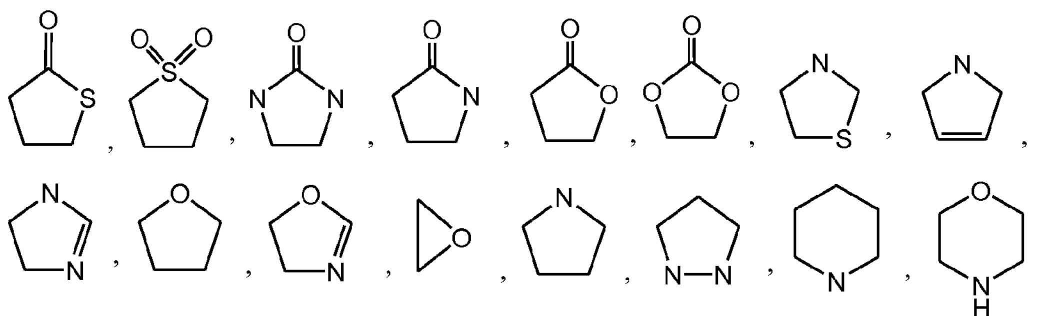
[0084] "Carbocyclyl" refers to a stable non-aromatic monocyclic or polycyclic hydrocarbon radical consisting solely of carbon and hydrogen atoms, which includes fused or bridged ring systems, having from three to fifteen carbon atoms. In certain embodiments, a carbocyclyl comprises three to ten carbon atoms. In other embodiments, a carbocyclyl comprises five to seven carbon atoms. The carbocyclyl is attached to the rest of the molecule by a single bond. Carbocyclyl is optionally saturated, *(i.e.,* containing single C-C bonds only) or unsaturated *(i.e.,* containing one or more double bonds or triple bonds.) A fully saturated carbocyclyl radical is also referred to as "cycloalkyl." Examples of monocyclic cycloalkyls include, *e.g.,* cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, cycloheptyl, and cyclooctyl. An unsaturated carbocyclyl is also referred to as "cycloalkenyl." Examples of monocyclic cycloalkenyls include, *e.g.,* cyclopentenyl, cyclohexenyl, cycloheptenyl, and cyclooctenyl. Polycyclic carbocyclyl radicals include, for example, adamantyl, norbornyl *(i.e.,* bicyclo[2.2.1]heptanyl), norbornenyl, decalinyl, 7,7-dimethyl-bicyclo[2.2.1]heptanyl, and the like. Unless otherwise stated specifically in the specification, the term "carbocyclyl" is meant to include carbocyclyl radicals that are optionally substituted by one or more substituents independently selected from alkyl, alkenyl, alkynyl, halo, fluoroalkyl, oxo, thioxo, cyano, nitro, optionally substituted aryl, optionally substituted aralkyl, optionally substituted aralkenyl, optionally substituted aralkynyl, optionally substituted carbocyclyl, optionally substituted carbocyclylalkyl, optionally substituted heterocyclyl, optionally substituted heterocyclylalkyl, optionally substituted heteroaryl, optionally substituted heteroarylalkyl, $-R^b-OR^a$, $-R^b-SR^a$, $-R^b-OC(0)-R^a$, $-R^b-N(R^a)_2$, $-R^b-C(0)R^a$, $-R^b-C(0)OR^a$, $-R^b-C(0)N(R^a)_2$, $-R^b-0-R^c-C(0)N(R^a)_2$, $-R^b-N(R^a)C(0)OR^a$, $-R^b-N(R^a)C(0)R^a$, $-R^b-N(R^a)S(0)_tR^a$ (where t is 1 or 2), $-R^b-S(0)_tOR^a$ (where t is 1 or 2) and $-R^b-S(0)_tN(R^a)_2$ (where t is 1 or 2), where each R^a is independently hydrogen, alkyl, fluoroalkyl, cycloalkyl, cycloalkylalkyl, aryl, aralkyl, heterocyclyl, heterocyclylalkyl, heteroaryl or heteroarylalkyl, each R^b is independently a

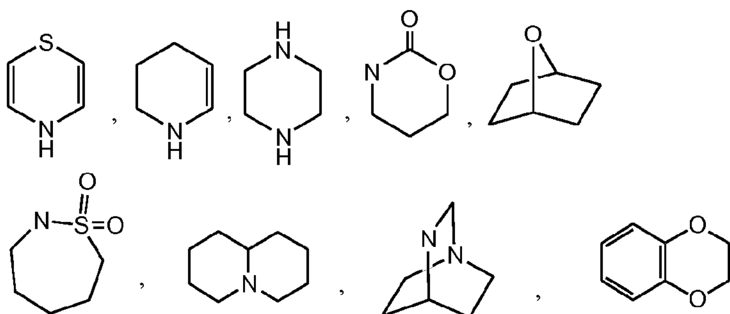
direct bond or a straight or branched alkylene or alkenylene chain, and R^c is a straight or branched alkylene or alkenylene chain, and where each of the above substituents is unsubstituted unless otherwise indicated.

[0085] "Halo" or "halogen" refers to bromo, chloro, fluoro or iodo substituents.

[0086] The terms "haloalkyl," "haloalkenyl," "haloalkynyl" and "haloalkoxy" include alkyl, alkenyl, alkynyl and alkoxy structures in which at least one hydrogen is replaced with a halogen atom. In certain embodiments in which two or more hydrogen atoms are replaced with halogen atoms, the halogen atoms are all the same as one another. In other embodiments in which two or more hydrogen atoms are replaced with halogen atoms, the halogen atoms are not all the same as one another.

[0087] As used herein, the term "non-aromatic heterocycle", "heterocycloalkyl" or "heteroalicyclic" refers to a non-aromatic ring wherein one or more atoms forming the ring is a heteroatom. A "non-aromatic heterocycle" or "heterocycloalkyl" group refers to a cycloalkyl group that includes at least one heteroatom selected from nitrogen, oxygen and sulfur. The radicals may be fused with an aryl or heteroaryl. Heterocycloalkyl rings can be formed by three, four, five, six, seven, eight, nine, or more than nine atoms. Heterocycloalkyl rings can be optionally substituted. In certain embodiments, non-aromatic heterocycles contain one or more carbonyl or thiocarbonyl groups such as, for example, oxo- and thio-containing groups. Examples of heterocycloalkyls include, but are not limited to, lactams, lactones, cyclic imides, cyclic thioimides, cyclic carbamates, tetrahydrothiopyran, 4H-pyran, tetrahydropyran, piperidine, 1,3-dioxin, 1,3-dioxane, 1,4-dioxin, 1,4-dioxane, piperazine, 1,3-oxathiane, 1,4-oxathiin, 1,4-oxathiane, tetrahydro-l,4-thiazine, 2H- 1,2-oxazine, maleimide, succinimide, barbituric acid, thiobarbituric acid, dioxopiperazine, hydantoin, dihydrouracil, morpholine, trioxane, hexahydro-1,3,5-triazine, tetrahydrothiophene, tetrahydrofuran, pyrroline, pyrrolidine, pyrrolidone, pyrrolidione, pyrazoline, pyrazolidine, imidazoline, imidazolidine, 1,3-dioxole, 1,3-dioxolane, 1,3-dithiole, 1,3-dithiolane, isoxazoline, isoxazolidine, oxazoline, oxazolidine, oxazolidinone, thiazoline, thiazolidine, and 1,3-oxathiolane. Illustrative examples of heterocycloalkyl groups, also referred to as non-aromatic heterocycles, include:





and the like. The term heteroalicyclic also includes all ring forms of the carbohydrates, including but not limited to the monosaccharides, the disaccharides and the oligosaccharides. Depending on the structure, a heterocycloalkyl group can be a monoradical or a diradical (i.e., a heterocycloalkylene group).

[0088] "Heteroaryl" refers to a radical derived from a 3- to 18-membered aromatic ring radical that comprises two to seventeen carbon atoms and from one to six heteroatoms selected from nitrogen, oxygen and sulfur. As used herein, the heteroaryl radical is a monocyclic, bicyclic, tricyclic or tetracyclic ring system, wherein at least one of the rings in the ring system is fully unsaturated, *i.e.,* it contains a cyclic, delocalized (4n+2) π-electron system in accordance with the Hiickel theory. Heteroaryl includes fused or bridged ring systems. The heteroatom(s) in the heteroaryl radical is optionally oxidized. One or more nitrogen atoms, if present, are optionally quatemized. The heteroaryl is attached to the rest of the molecule through any atom of the ring(s). Examples of heteroaryls include, but are not limited to, azepinyl, acridinyl, benzimidazolyl, benzindolyl, 1,3-benzodioxolyl, benzofuranyl, benzooxazolyl, benzo[d]thiazolyl, benzothiadiazolyl, benzo[Z>][l,4]dioxepinyl, benzo[b][l,4]oxazinyl, 1,4-benzodioxanyl, benzonaphthofuranyl, benzoxazolyl, benzodioxolyl, benzodioxinyl, benzopyranyl, benzopyranonyl, benzofuranyl, benzofuranonyl, benzothienyl (benzothiophenyl), benzothieno[3,2-d]pyrimidinyl, benzotriazolyl, benzo[4,6]imidazo[l,2-a]pyridinyl, carbazolyl, cinnolinyl, cyclopenta[d]pyrimidinyl, 6,7-dihydro-5H-cyclopenta[4,5]thieno[2,3-d]pyrimidinyl, 5,6-dihydrobenzo[h]quinazolinyl, 5,6-dihydrobenzo[h] cinnolinyl, 6,7-dihydro-5H-benzo[6,7]cyclohepta[l,2-c]pyridazinyl, dibenzofuranyl, dibenzothiophenyl, furanyl, furanonyl, furo[3,2-c]pyridinyl, 5,6,7,8,9, 10-hexahydrocycloocta[d]pyrimidinyl, 5,6,7,8,9, 10-hexahydrocycloocta[d]pyridazinyl, 5,6,7, 8,9,10-hexahydrocycloocta[d]pyridinyl,isothiazolyl, imidazolyl, indazolyl, indolyl, indazolyl, isoindolyl, indolinyl, isoindolinyl, isoquinolyl, indolizinyl, isoxazolyl, 5,8-methano-5,6,7,8-tetrahydroquinazolinyl, naphthyridinyl, 1,6-naphthyridinonyl, oxadiazolyl, 2-oxoazepinyl, oxazolyl, oxiranyl, 5,6,6a,7,8,9,10,10a-octahydrobenzo[h]quinazolinyl, 1-phenyl-1*H*-pyrrolyl, phenazinyl, phenothiazinyl, phenoxazinyl, phthalazinyl, pteridinyl, purinyl, pyrrolyl, pyrazolyl, pyrazolo[3,4-d]pyrimidinyl, pyridyl, pyrido[3,2-d]pyrimidinyl,

pyrido[3,4-d]pyrimidinyl, pyrazinyl, pyrimidinyl, pyridazinyl, pyrrolyl, quinazolinyl, quinoxalinyl, quinolinyl, isoquinolinyl, tetrahydroquinolinyl, 5,6,7,8-tetrahydroquinazolinyl, 5.6.7. 8-tetrahydrobenzo[4,5]thieno[2,3-d]pyrimidinyl, 6.7.8.9-tetrahydro-5H-cyclohepta[4,5]thieno[2,3-d]pyrimidinyl, 5,6,7, 8-tetrahydropyrido[4,5-c]pyridazinyl, thiazolyl, thiadiazolyl, triazolyl, tetrazolyl, triazinyl, thieno[2,3-d]pyrimidinyl, thieno[3,2-d]pyrimidinyl, thieno[2,3-c]pridinyl, and thiophenyl *(i.e.* thienyl). Unless stated otherwise specifically in the specification, the term "heteroaryl" is meant to include heteroaryl radicals as defined above which are optionally substituted by one or more substituents selected from alkyl, alkenyl, alkynyl, halo, fluoroalkyl, haloalkenyl, haloalkynyl, oxo, thioxo, cyano, nitro, optionally substituted aryl, optionally substituted aralkyl, optionally substituted aralkenyl, optionally substituted aralkynyl, optionally substituted carbocyclyl, optionally substituted carbocyclylalkyl, optionally substituted heterocyclyl, optionally substituted heterocyclylalkyl, optionally substituted heteroaryl, optionally substituted heteroarylalkyl, $-R^b-OR^a$, $-R^b-SR^a$, $-R^b-OC(0)-R^a$, $-R^b-N(R^a)_2$, $-R^b-C(0)R^a$, $-R^b-C(0)OR^a$, $-R^b-C(0)N(R^a)_2$, $-R^b-0-R^c-C(0)N(R^a)_2$, $-R^b-N(R^a)C(0)OR^a$, $-R^b-N(R^a)C(0)R^a$, $-R^b-N(R^a)S(0)_tR^a$ (where t is 1 or 2), $-R^b-S(0)_tOR^a$ (where t is 1 or 2) and $-R^b-S(0)_tN(R^a)_2$ (where t is 1 or 2), where each R^a is independently hydrogen, alkyl, fluoroalkyl, cycloalkyl, cycloalkylalkyl, aryl, aralkyl, heterocycloalkyl, heterocycloalkylalkyl, heteroaryl or heteroarylalkyl, each R^b is independently a direct bond or a straight or branched alkylene or alkenylene chain, and R^c is a straight or branched alkylene or alkenylene chain, and where each of the above substituents is unsubstituted unless otherwise indicated.

[0089] "Sulfanyl" refers to the -S- radical.

[0090] "Sulfmyl" refers to the -S(=0)- radical.

[0091] "Sulfonyl" refers to the $-S(=0)_2-$ radical.

[0092] "Amino" refers to the $-NH_2$ radical.

[0093] "Cyano" refers to the -CN radical.

[0094] "Nitro" refers to the $-N0_2$ radical.

[0095] "Oxa" refers to the -O- radical.

[0096] "Oxo" refers to the =0 radical.

[0097] An "alkoxy" group refers to a (alkyl)O- group, where alkyl is as defined herein.

[0098] An "aryloxy" group refers to an (aryl)O- group, where aryl is as defined herein.

[0099] As used herein, the terms "heteroalkyl" "heteroalkenyl" and "heteroalkynyl" include optionally substituted alkyl, alkenyl and alkynyl radicals in which one or more skeletal chain atoms is a heteroatom, *e.g.,* oxygen, nitrogen, sulfur, silicon, phosphorus or combinations thereof. The heteroatom(s) may be placed at any interior position of the heteroalkyl group or at

the position at which the heteroalkyl group is attached to the remainder of the molecule. Examples include, but are not limited to, $-CH_2-0-CH_3$, $-CH_2-CH_2-0-CH_3$, $-CH_2-NH-CH_3$, $-CH_2-CH_2-NH-CH_3$, $-CH_2-N(CH_3)-CH_3$, $-CH_2-CH_2-NH-CH_3$, $-CH_2-CH_2-N(CH_3)-CH_3$, $-CH_2-S-CH_2-CH_3$, $-CH_2-CH_2,-S(0)-CH_3$, $-CH_2-CH_2-S(0)_2-CH_3$, $-CH=CH-0-CH_3$, $-Si(CH_3)_3$, $-CH_2-CH=N-OCH_3$, and $-CH=CH-N(CH_3)-CH_3$. In addition, up to two heteroatoms may be consecutive, such as, by way of example, $-CH_2-NH-OCH_3$ and-$CH_2-0-Si(CH_3)_3$.

[00100] The term "heteroatom" refers to an atom other than carbon or hydrogen. Heteroatoms are typically independently selected from among oxygen, sulfur, nitrogen, silicon and phosphorus, but are not limited to these atoms. In embodiments in which two or more heteroatoms are present, the two or more heteroatoms can all be the same as one another, or some or all of the two or more heteroatoms can each be different from the others.

[00101] The term "bond" or "single bond" refers to a chemical bond between two atoms, or two moieties when the atoms joined by the bond are considered to be part of larger substructure.

[00102] The term "moiety" refers to a specific segment or functional group of a molecule. Chemical moieties are often recognized chemical entities embedded in or appended to a molecule.

[00103] "Carboxy" means a -C(0)OH radical.

[00104] As used herein, the substituent "R" appearing by itself and without a number designation refers to a substituent selected from among from alkyl, cycloalkyl, aryl, heteroaryl (bonded through a ring carbon) and non-aromatic heterocycle (bonded through a ring carbon).

[00105] An "amide" is a chemical moiety with the formula -C(0)NHR or -NHC(0)R, where R is selected from among alkyl, cycloalkyl, aryl, heteroaryl (bonded through a ring carbon) and heteroalicyclic (bonded through a ring carbon). An amide moiety may form a linkage between an amino acid or a peptide molecule and a compound described herein, thereby forming a prodrug. Any amine, or carboxyl side chain on the compounds described herein can be amidifted. The procedures and specific groups to make such amides are known to those of skill in the art and can readily be found in reference sources such as Greene and Wuts, Protective Groups in Organic Synthesis, 3rd Ed., John Wiley & Sons, New York, NY, 1999, which is incorporated herein by reference in its entirety.

[00106] The term "ester" refers to a chemical moiety with formula -COOR, where R is selected from among alkyl, cycloalkyl, aryl, heteroaryl (bonded through a ring carbon) and heteroalicyclic (bonded through a ring carbon). Any hydroxy, or carboxyl side chain on the compounds described herein can be esterified. The procedures and specific groups to make such esters are known to those of skill in the art and can readily be found in reference sources such as Greene and Wuts, Protective Groups in Organic Synthesis, 3rd Ed., John Wiley & Sons, New

York, NY, 1999, which is incorporated herein by reference in its entirety.

[00107] As used herein, the term "ring" refers to any covalently closed structure. Rings include, for example, carbocycles (e.g., aryls and cycloalkyls), heterocycles (e.g., heteroaryls and non-aromatic heterocycles), aromatics (e.g., aryls and heteroaryls), and non-aromatics (e.g., cycloalkyls and non-aromatic heterocycles). Rings can be optionally substituted. Rings can be monocyclic or polycyclic.

[00108] As used herein, the term "ring system" refers to one, or more than one ring.

[00109] The term "membered ring" can embrace any cyclic structure. The term "membered" is meant to denote the number of skeletal atoms that constitute the ring. Thus, for example, cyclohexyl, pyridine, pyran and thiopyran are 6-membered rings and cyclopentyl, pyrrole, furan, and thiophene are 5-membered rings.

[00110] The term "fused" refers to structures in which two or more rings share one or more bonds.

[00111] The term "optionally substituted" or "substituted" means that the referenced group may be substituted with one or more additional group(s) individually and independently selected from alkyl, cycloalkyl, aryl, heteroaryl, heteroalicyclic, hydroxy, alkoxy, aryloxy, alkylthio, arylthio, alkylsulfoxide, arylsulfoxide, alkylsulfone, arylsulfone, cyano, halo, acyl, nitro, haloalkyl, fluoroalkyl, amino, including mono- and di-substituted amino groups, and the protected derivatives thereof. By way of example an optional substituents may be L_SR_S, wherein each L_s is independently selected from a bond, -0-, -C(=0)-, -S-, -S(=0)-, -S(=0)2-, -NH-, -NHC(O)-, -C(0)NH-, $S(=0)_2NH$-, $-NHS(=0)_2$, -OC(0)NH-, -NHC(0)0-, -(substituted or unsubstituted C_1-C_6 alkyl), or -(substituted or unsubstituted C_2-C_6 alkenyl); and each R_s is independently selected from H, (substituted or unsubstituted Ci-C_4alkyl), (substituted or unsubstituted C_3-C_6cycloalkyl), heteroaryl, or heteroalkyl. The protecting groups that may form the protective derivatives of the above substituents are known to those of skill in the art and may be found in references such as Greene and Wuts, above.

[00112] The term "target" refers to a biological molecule wherein a protein modulator can interact with. Non-limiting examples of targets include proteins, such as cell cycle regulators, transcription factors, translation initiation factors, cyclins, receptors, cell signaling proteins, ligands, enzymes, and kinases.

[00113] The term "drug" as used herein refers to a protein modulator. Non-limiting examples of protein modulators include kinase inhibitors, kinase antagonists, and kinase agonists. For example, a drug can be a BTK inhibitor. In another example, a drug is a BMK antagonist.

[00114] The term "agent" refers to a compound that interacts with a target. In some instances, the agent is identical to the drug. In other instances, the agent is similar to the drug. In another

instance, the agent is different from the drug.

[00115] The term "probe" refers to a compound or molecule for the detection of a target. In some instances, the probe comprises an agent, a linker, a label, or any combination thereof. In some instances, the probe comprises an agent. In other instances, the probe comprises an agent and a linker. In another instance, the probe comprises an agent and a label. In another instance, the probe comprises a label. In some instances, the probe comprises a label and a linker. In some instances, the probe comprises an agent, a linker, and a label. In some instances, the agent is attached to the linker. In other instances, the label is attached to the linker. In some embodiments, the agent is attached to the label via the linker. Alternatively, the agent is attached to the label.

[00116] The term "unoccupied target" refers to a target wherein a drug is not bound to.

[00117] As used herein, the term "drug-occupied target" or "drug-bound target" refers to a target wherein one or more drugs are bound to. Binding comprises any type of bond, including, but not limited to, covalent, non-covalent, ionic, hydrogen, disulfide, or van der Waals. Binding can also include hydrophilic or hydrophobic interactions.

[00118] The term "probe-bound target," or "probe-bound kinase," refers to a target, or kinase, wherein one or more probes are bound to. Binding comprises any type of bond, including, but not limited to, covalent, non-covalent, ionic, hydrogen, disulfide, van der Waals. Binding can also include hydrophilic or hydrophobic interactions. In some instances, a "probe-bound target" comprises a drug-occupied target with a probe attached thereto. In other instances, a "probe-bound target" comprises an unoccupied target with a probe attached thereto.

[00119] A "treated sample" refers to a sample wherein one or more drugs have been administered to. As used herein, a treated sample from a patient means that the sample is from a patient that has been administered one or more drugs (e.g., a TEC family kinase inhibitor).

[00120] An "untreated sample" refers to a sample wherein a drug has not been administered to. As used herein, an untreated sample from a patient means that the sample is from a patient that has not been administered one or more drugs (e.g., a TEC family kinase inhibitor).

Protein Occupancy Assay

[00121] Disclosed herein are methods for determining the efficacy of a protein modulator (e.g., inhibitor drug) on a target (e.g., target protein kinase). In some embodiments, methods are provided for determining the efficacy of a TEC family kinase inhibitor on a target kinase (e.g., a TEC family kinase or homologous kinase). In some embodiments, the method comprises: (a) contacting a sample comprising a TEC family kinase with a probe to form a probe-bound target kinase; (b) detecting the amount of the probe-bound target kinase in the sample; and (c) determining the efficacy of the TEC family kinase inhibitor based on the amount of probe-bound target kinase. In some embodiments, the method further comprises contacting the sample with

the TEC family kinase inhibitor prior to step (a) (e.g., combining the sample with the probe). In some embodiments, detecting the amount of the probe-bound target kinase comprises administering a compound, reagent or buffer to detect the probe-bound kinase. In some embodiments, the compound, reagent or buffer comprises horseradish peroxidase (HRP), detection antibody buffer, read buffer, wash buffer. In some embodiments, detecting the presence or absence of the probe-bound target kinase comprises quantifying the amount of probe-bound target kinase. In some embodiments, the quantifying step comprises fluorescence, immunofluorescence, chemiluminescence, or electrochemiluminescence. In some embodiments, determining the efficacy of the TEC family kinase inhibitor comprises determining occupancy of the target kinase by the TEC family kinase inhibitor. In some embodiments, the amount of probe-bound target kinase inversely correlates with the efficacy of the TEC family kinase inhibitor. For example, as shown in Figures 8 and 10, if a drug-treated sample (e.g., a sample that is contacted with the drug prior to contact with the probe) is contacted with the probe, then as the amount of probe-bound target kinases (e.g., unoccupied target kinases) detected increases, the efficacy of the drug decreases. In another example, if a drug-treated sample is contacted with the probe, then as the amount of probe-bound target kinase (e.g., unoccupied target kinases) detected decreases, the efficacy of the drug increases. In some embodiments, the amount of probe-bound target kinases directly correlates with the efficacy of the drug. For example, as shown in Figure 9, if an untreated sample (e.g., a sample that is not contacted with the drug prior to contact with the probe) is contacted with the probe, then as the amount of probe-bound target kinase detected increases, the efficacy of the drug also increases. In another example, if an untreated sample (e.g., a sample that is not contacted with the drug prior to contact with the probe) is contacted with the probe, then as the amount of probe-bound target kinase detected decreases, the efficacy of the drug decreases. In some embodiments, a drug is determined to be effective when the drug binds at least about 50% of the target kinases. Alternatively, a drug is determined to be effective when the drug binds at least about 60% of the target kinases. In some embodiments, a drug is determined to be effective when the drug binds at least about 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, or 99% of the targets.

[00122] In some embodiments, the assay is performed on a sample obtained from a patient that has been administered a TEC family kinase. In some embodiments, the sample is obtained about 1 hour, 2 hours, 4 hours, 6 hours, 8 hours, 10 hours, 12 hours, 14 hours, 16 hours, 18 hours, 20 hours, 22 hours, 24 hours, 30 hours, 36 hours, 42 hours, 48 hours, 3 days, 4, days, 5 days, 6 days, 1 week, 2 weeks or longer after administration of the TEC family kinase inhibitor.

[00123] In some embodiments, the probe comprises an agent and a label. In some instances, the agent is fused to the label. In other instances, the agent is attached to the label. In another

instance, the agent is attached to the label by a linker. In some embodiments, the agent and the drug are essentially the same. In some embodiments, the probe comprises a label. In some embodiments, the probe comprises a label and a linker. In some embodiments, the agent and the drug are at least about 20% identical, at least about 30% identical, at least about 40% identical, at least about 50% identical, at least about 60% identical, at least about 70% identical, at least about 80% identical, at least about 90% identical, or at least about 95% identical. In other embodiments, the agent and the drug are different. In some embodiments, the agent and the drug are at least about 5% different, at least about 10% different, at least about 20% different, at least about 30% different, at least about 40% different, at least about 50% different, at least about 60%) different, at least about 70% different, at least about 80% different, at least about 90% different, or at least about 95% different.

Targets

[00124] As disclosed herein are methods, assays and systems for determining the efficacy of a the TEC family kinase inhibitor on a target kinase (e.g., TEC family kinase or a homologous tyrosine kinase). In some embodiments, the methods provided herein can be adapted to other target proteins, such as, but not limited to, cell cycle regulators, receptors, ligands, transcriptional regulators, transcription initiation factors, enzymes, cell signaling proteins, and other protein kinases. In particular embodiments, the target kinase is a tyrosine kinase. In particular embodiments, the target kinase is a ser/threonine kinase.

[00125] In some embodiments, the target kinase is a member of the TEC family of non-receptor tyrosine kinases. The TEC kinase family comprises TEC, BMX (Bone marrow kinase on the X chromosome; also named Etk), BTK (Bruton's tyrosine kinase), ITK (IL-2-inducible T cell kinase; also known as Emt), and Rlk (Resting lymphocyte kinase; also designated TXK). In some instances, the target kinase is BTK. In other instances, the target kinase is ITK. In other instances, the target kinase is TXK. In other instances, the target kinase is BMX. In other instances, the target kinase is TEC.

[00126] In some embodiments, the target kinase is a member of the epidermal growth factor receptor (EGFR). In some embodiments, the target kinase is HER1 (EGFR, ErbBl), HER2/c-neu (ErbB2), HER3 (ErbB3) and HER4 (ErbB4), or JAK3.

[00127] In some embodiments, the target kinase is a member of the SRC kinase family. In some embodiments, the target kinase is BLK.

[00128] Additional exemplary target kinases for use in the methods and compositions provided include, but are not limited to, Abl, activated Cdc42-associated kinase- 1(ACK1), Akt/PKB, Abl-related gene (Arg), apoptosis signal-regulating kinase (Ask-1), Aurora A, Aurora B, Aurora C, Axl, Calcium/calmodulin dependent kinase-Iδ (CaMKK), Calcium/calmodulin

dependent kinase Πβ (CaMKIip), CaMKIIy, CaMKIK, casein kinases (CK, CKlyl, CKly2, CKly3), Cyclin-dependent kinases (Cdk), Cyclin-dependent protein kinase-9/cyclin Tl (CDK9/cyclin Tl), Casein kinase-2ct2 (CK2a2), Chk, c-kit, cdc-like kinase-2 (CLK2), Cotl, C-terminal c-Src kinase (Csk), Death-associated protein kinase- 1 (DAPK1), doublecortin and CAM kinase-like-2 (DCAMKL2), Discoidin domain receptors 1 and 2 (DDR1 and DDR2), Eph receptors, Focal adhesion kinase (FAK), Fer, Fibroblast growth factor receptor (FGFR), Fgr, Fns-like tyrosine kinase (Fit), Fms-like tyrosine kinase-4 (Flt4), Fms/CSF-1 R, Fyn, G protein-coupled receptor kinases (GRKs), G protein-coupled receptor kinase-7 (GRK7), Glycogen synthase kinase (GSK), Hematopoietic cell kinase (Hck), Homeodomain-interacting protein kinase-1 (HIPK1), HIPK2, HIPK3, Insulin-like growth factors (IGF), 1KB kinase (IKK), insulin receptor, IL-1 receptor associated kinase (IRAK), stress-activated protein kinase 1 (SAPK), Kinase insert domain-containing receptor (KDR), c-Kit, Lck, LIM kinase (LIMK), Lymphocyte-oriented kinase (LOK), Lyn, MAPK/Erk, MAPK-activated protein kinases (MAPKAP K or MK), MAP kinase/Erk Kinase (MEK), Maternal embryonic leucine zipper kinase (MELK), Met, Mer, Misshapen/NIK-related kinase (MINK), Mitogen activated protein kinase kinase (MKK), Mixed lineage kinase-1 (MLK1), Myotonic dystrophy kinase-related Cdc42-binding kinasea (MRCKa), Mitogen-and-stress-activated protein kinase 1 (MSK1), Mammalian STE20-like kinase (MST), Mammalian STE20-like protein kinase-3 (MST3), target of rapamycin (mTOR, FRAP, RAFT), mTor/FKBP12, NIMA-related protein kinase-3 (NEK3), NEK9, P2 1-activated kinases (PAK), PAK3, PAR-1 kinase, Platelet-derived Growth Factor Receptors (PDGFR), PI (3,4,5) P3-dependent kinase 1 (PDK1), Phosphorylase kinase (PhK), Phosphatidylinositol (PI) 3-kinase, Polo-like kinase-1 (PLK1), PIM kinases, Protein kinase C, PKD2, cGMP-dependent protein kinase-la (PKGla), double-stranded RNA-activated protein kinase (PKR), P38-regulated/activated protein kinase (PRAK), Protein tyrosine kinase-5 (PTK5), Proline rich kinase (Pyk)2, Raf kinases (Raf-1, A-Raf, B-Raf), Ret, Receptor-interacting serine/threonine kinase 2 (RIPK2), Ron, Ros, Rse (Brt, BYK, Dtk, Etk3, Sky, Tif, or sea-related receptor tyrosine kinase), Ribosomal protein S6 kinase-4 (Rsk4), P70 S6 kinase, SAPK, Serum- and glucocorticoid-induced kinase (SGK), c-Src, Syk, TGF-β activated kinase (TAK1), thousand and one amino acid protein kinase-1 (TAOl), Tyrosine kinase with Ig- and EGF-homology domains-2 (Tie2/TEK), Tousled-like kinases (TLKs), Trk, Testis specific serine kinases (TSSK), Unc-51-like kinase-2 (ULK2), ULK3, Vaccinia-related kinase-2, Wee, Yes, ZAP-70, and Zipper interacting protein kinase (ZIPK).

Drugs

[00129] As disclosed herein are methods, assays, and systems for determining the efficacy of a drug on a target. Suitable drugs disclosed herein comprise protein modulators. Protein

modulators comprise protein inhibitors, protein antagonists, and protein agonists. In some embodiments, the drug is a protein inhibitor. Examples of protein inhibitors include, but are not limited to, protein kinase inhibitors.

[00130] In some embodiments, the drug is a protein kinase inhibitor. In some embodiments, the protein kinase inhibitor is a tyrosine kinase inhibitor. In some embodiments, the tyrosine kinase inhibitor is any one of dasatinib, imatinib, nilotinib, sunitinib, gefitinib, erlotinib.

[00131] In some embodiments, the tyrosine kinase inhibitor is a TEC family kinase inhibitor. In some embodiments, the tyrosine kinase inhibitor is a BTK inhibitor. In some embodiments, the BTK inhibitor is a reversible BTK inhibitor. In some embodiments, the reversible BTK inhibitor is LFM-A13 or terreic acid. In some embodiments, the BTK inhibitor is an irreversible BTK inhibitor. Examples of irreversible BTK inhibitors include ibrutinib, AVL-291, AVL-101, AVL-292, or ONO-WG-307. In some embodiments, the irreversible BTK inhibitor is ibrutinib. In some instances, the BTK inhibitor is RN486. In some embodiments, the drug is an ITK inhibitor. In some instances, the ITK inhibitor is CTA056. In some embodiments, the drug is a TEC kinase inhibitor. In some embodiments, the drug is a TXK inhibitor. In some embodiments, the drug is a BMX inhibitor. In some embodiments, the drug is a BLK inhibitor.

[00132] In some embodiments, the drug inhibits a kinase. In some embodiments, the drug inhibits a tyrosine kinase. In some embodiments, the drug inhibits a receptor tyrosine kinase. In some embodiments, the drug inhibits a non-receptor tyrosine kinase. In some embodiments, the drug inhibits a serine/threonine kinase.

[00133] In some instances, the kinase is a member of the AGC kinase family. In other instances, the kinase is a member of the CaM kinase family. In some embodiments, the kinase is a member of the TK kinase family. Alternatively, the kinase is a member of the CKI kinase family. In some embodiments, the kinase is a member of the CMGC kinase family. In some instances, the kinase is a member of the STE kinase family. In some embodiments, the kinase is a member of the STK kinase family. In some instances, the kinase is a member of the TKL kinase family.

Protein Occupancy Assay Kit

[00134] Disclosed herein are protein occupancy assay kits comprising a linker, a label, an agent, or any combination thereof. In one aspect is a protein occupancy assay kit comprising a linker and a label, wherein the linker is capable of attaching the label to an agent and the agent is a protein modulator. In another aspect is a protein occupancy assay kit comprising an agent, a linker, and a label, wherein the linker is capable of attaching to the agent and the label, thereby attaching the agent to the label. In some embodiments is a protein occupancy assay kit comprising a probe, wherein the probe comprises an agent attached to a label. In some

embodiments is a protein occupancy assay kit comprising a probe, wherein the probe comprises an agent attached to a linker.

[00135] In some embodiments is a protein occupancy assay kit comprising an agent and a solid support, wherein the agent is attached to the solid support. In another embodiment is a protein occupancy assay kit comprising a label and a solid support, wherein the label is attached to the solid support. In another embodiment is a protein occupancy assay kit comprising a probe and a solid support, wherein the probe comprises an agent, a linker, a label, or any combination thereof. In some embodiments is a protein occupancy assay kit comprising a target (e.g., protein) and a solid support, wherein the target is attached to the solid support.

[00136] In some aspects, any of the kits disclosed herein further comprise a label. In some aspects, any of the kits disclosed herein further comprise a linker. In some aspects, any of the kits disclosed herein further comprise an agent. In some aspects, any of the kits disclosed herein further comprise a plurality of linkers, wherein the linkers are capable of attaching to another linker, an agent, a label, or any combination thereof. In some aspects, any of the kits disclosed herein further comprise a probe. In some aspects the probe comprises an agent, a linker, a label, or any combination thereof. In some aspects, any of the kits disclosed herein further comprise a target (e.g., protein). Exemplary embodiments of agents, linkers, labels, probes, solid supports, and targets are disclosed herein. Further disclosed herein are exemplary methods for attaching probes or targets to solid supports.

Probe

[00137] In some embodiments, the methods, kits, and compositions disclosed herein comprise a probe. In some embodiments, the probe comprises an agent and a label. In some embodiments, the agent and label are attached. In other embodiments, the probe comprises an agent and a linker. In some embodiments, the agent and linker are attached. In another embodiment, the probe comprises an agent, a linker, and a label. In some embodiments, the agent, linker and/or label are attached to each other. In some embodiments, the probe comprises a label. In another embodiment, the probe comprises a label and a linker. In some embodiments, the label and the linker are attached. In some embodiments, attachment is by chemical methods, enzymatic methods, or crosslinking methods. In some embodiments, the probe is attached to a solid support. Exemplary embodiments of agents, linkers, labels, and solid supports are disclosed herein.

Agent

[00138] In some embodiments, the methods, kits, and compositions disclosed herein comprise an agent. Suitable agents comprise protein modulators (e.g., inhibitors, antagonists, and agonists). In some embodiments, the agent is a drug. Suitable drugs disclosed herein comprise protein modulators. Protein modulators comprise protein inhibitors, protein antagonists, and

protein agonists. In some embodiments, the drug is a protein inhibitor. Examples of protein inhibitors include, but are not limited to, protein kinase inhibitors.

[00139] In some embodiments, the drug is a protein kinase inhibitor. In some embodiments, the protein kinase inhibitor is a tyrosine kinase inhibitor. In some embodiments, the tyrosine kinase inhibitor is any one of dasatinib, imatinib, nilotinib, sunitinib, gefitinib, erlotinib.

[00140] In some embodiments, the tyrosine kinase inhibitor is a TEC family kinase inhibitor. In some embodiments, the tyrosine kinase inhibitor is a BTK inhibitor. In some embodiments, the BTK inhibitor is a reversible BTK inhibitor. In some embodiments, the reversible BTK inhibitor is LFM-A13 or terreic acid. In some embodiments, the BTK inhibitor is an irreversible BTK inhibitor. Examples of irreversible BTK inhibitors include ibrutinib, AVL-291, AVL-101, AVL-292, or ONO-WG-307. In some embodiments, the irreversible BTK inhibitor is ibrutinib. In some instances, the BTK inhibitor is RN486. In some embodiments, the drug is an ITK inhibitor. In some instances, the ITK inhibitor is CTA056. In some embodiments, the drug is a TEC kinase inhibitor. In some embodiments, the drug is a TXK inhibitor. In some embodiments, the drug is a BMX inhibitor. In some embodiments, the drug is a BLK inhibitor.

[00141] In some embodiments, the drug inhibits a kinase. In some embodiments, the drug inhibits a tyrosine kinase. In some embodiments, the drug inhibits a receptor tyrosine kinase. In some embodiments, the drug inhibits a non-receptor tyrosine kinase. In some embodiments, the drug inhibits a serine/threonine kinase.

[00142] In some instances, the kinase is a member of the AGC kinase family. In other instances, the kinase is a member of the CaM kinase family. In some embodiments, the kinase is a member of the TK kinase family. Alternatively, the kinase is a member of the CKI kinase family. In some embodiments, the kinase is a member of the CMGC kinase family. In some instances, the kinase is a member of the STE kinase family. In some embodiments, the kinase is a member of the STK kinase family. In some instances, the kinase is a member of the TKL kinase family.

Linker

[00143] In some embodiments, the methods, kits, and compositions disclosed herein comprise a linker. Suitable linkers comprise any chemical or biological compound capable of attaching to a label and/or agent disclosed herein. If the linker attaches to both the label and the agent, then a suitable linker would be capable of sufficiently separating the label and the agent. Suitable linkers would not significantly interfere with the ability of the agent to bind to a target (e.g., protein). Suitable linkers would not significantly interfere with the ability of the label to be detected. In some embodiments, the linker is rigid. In other embodiments, the linker is flexible. In another embodiment, the linker is semi rigid. In some embodiments, the linker is

proteolytically stable (e.g., resistant to proteolytic cleavage). In another embodiment, the linker is proteolytically unstable (e.g., sensitive to proteolytic cleavage). In some embodiments, the linker is helical. In some embodiments, the linker is non-helical. In some embodiments, the linker is coiled. In some embodiments, the linker is β-stranded. In some embodiments, the linker comprises a turn conformation. In some embodiments, the linker is a single chain. In some embodiments, the linker is a long chain. In some embodiments, the linker is a short chain. In some embodiments, the linker comprises at least about 5 residues, at least about 10 residues, at least about 15 residues, at least about 20 residues, at least about 25 residues, at least about 30 residues, or at least about 40 residues.

[00144] Examples of linkers include, but are not limited to, hydrazone, disulfide, thioether, and peptide linkers. In some embodiments, the linker is a peptide linker. In some embodiments, the peptide linker comprises a proline residue. In some embodiments, the peptide linker comprises an arginine, phenylalanine, threonine, glutamine, glutamate, or any combination thereof. In some embodiments, the linker is a heterobifunctional crosslinker. In some embodiments, the heterobifunctional crosslinker is Sulfo-SMCC.

Label

[00145] In some embodiments, the methods, kits, and compositions disclosed herein comprise a label. Examples of labels include, but are not limited to, chemical, biochemical, biological, colorimetric, enzymatic, fluorescent, luminescent labels, chemiluminescent labels, and electrochemiluminescent labels, which are well known in the art. In some embodiments, the label is selected from the group consisting of a dye, a photocrosslinker, a cytotoxic compound, a drug, an affinity label, a photoaffmity 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 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.

[00146] In some embodiments, the label is a chemical label. Examples of chemical labels can include, but are not limited to, biotin and radioisotopes (e.g., iodine, carbon, phosphate, hydrogen).

[00147] In some embodiments, the methods, kits, and compositions disclosed herein comprise a biological label. In some embodiments, biological labels comprise metabolic labels, including,

but not limited to, bioorthogonal azide-modifted amino acids, sugars, and other compounds.

[00148] In some embodiments, the methods, kits, and compositions disclosed herein comprise an enzymatic label. Enzymatic labels can include, but are not limited to horseradish peroxidase (HRP), alkaline phosphatase (AP), glucose oxidase, and β-galactosidase. In some embodiments, the enzymatic label is luciferase.

[00149] In some embodiments, the methods, kits, and compositions disclosed herein comprise a fluorescent label. In some embodiments, the fluorescent label is an organic dye (e.g., FITC), biological fluorophore (e.g., green fluorescent protein), or quantum dot. A non-limiting list of fluorescent labels includes fluorescein isothiocyante (FITC), DyLight Fluors, fluorescein, rhodamine (tetramethyl rhodamine isothiocyanate, TRITC), coumarin, Lucifer Yellow, and BODIPY. In some embodiments, the label is a fluorophore. Exemplary fluorophores include, but are not limited to, indocarbocyanine (C3), indodicarbocyanine (C5), Cy3, Cy3.5, Cy5, Cy5.5, Cy7, Texas Red, Pacific Blue, Oregon Green 488, Alexa Fluor®-355, Alexa Fluor 488, Alexa Fluor 532, Alexa Fluor 546, Alexa Fluor-555, Alexa Fluor 568, Alexa Fluor 594, Alexa Fluor 647, Alexa Fluor 660, Alexa Fluor 680, JOE, Lissamine, Rhodamine Green, BODIPY, fluorescein isothiocyanate (FITC), carboxy-fluorescein (FAM), phycoerythrin, rhodamine, dichlororhodamine (dRhodamine), carboxy tetramethylrhodamine (TAMRA), carboxy-X-rhodamine (ROX™), LIZ™, VIC™, NED™, PET™, SYBR, PicoGreen, RiboGreen, and the like. In some embodiments, the fluorescent label is a green fluorescent protein (GFP), red fluorescent protein (RFP), yellow fluorescent protein, phycobiliproteins (e.g., allophycocyanin, phycocyanin, phycoerythrin, and phycoerythrocyanin).

Solid support

[00150] In some embodiments, the methods, kits, and compositions disclosed herein comprise a solid support. A solid support comprises any solid platform to which a probe or antibody can be attached. In some embodiments, the solid support comprises a bead, plate, and array. In some embodiments, the solid support comprises a bead attached to a plate. For example, as shown in Figure 1IB, a streptavidin bead is attached to a plate. In some embodiments, the solid support comprises a plate. In another embodiment, the solid support comprises an antibody attached to a plate. For example, as shown in Figure 11A, an anti-BTK antibody is attached to a plate.

[00151] In some embodiments, the methods, kits, and compositions disclosed herein comprise a bead. Examples of beads include, but are not limited to, streptavidin beads, agarose beads, magnetic beads, Dynabeads®, MACS® microbeads, antibody conjugated beads (e.g., anti-immunoglobulin microbead), protein A conjugated beads, protein G conjugated beads, protein A/G conjugated beads, protein L conjugated beads, oligo-dT conjugated beads, silica beads, silica-like beads, anti-biotin microbead, anti-fluorochrome microbead, and BcMag™ Carboxy-

Terminated Magnetic Beads.

[00152] In some embodiments, the methods, kits, and compositions disclosed herein comprise a plate. Examples of plates include, but are not limited to, MSD multi-array plates, MSD Multi-Spot® plates, microplate, ProteOn microplate, AlphaPlate, DELFIA plate, IsoPlate, and LumaPlate.

Methods for attaching Agents, Linkers, and/or Labels

[00153] In some embodiments, the methods, kits, and compositions disclosed herein comprise an agent, linker, label, or any combination thereof. In some embodiments, the agent, linker, and/or label are attached. Methods for attaching agents, linkers, and/or labels include, but are not limited to, chemical labeling and enzymatic labeling.

[00154] In some embodiments, methods to attach labels to linkers and/or agents comprise chemical labeling techniques. In some embodiments, chemical labeling techniques comprise a chemically reactive group. Common reactive groups include, but are not limited to, amine-reactive isothiocyanate derivatives including FITC, amine-reactive succinimidyl esters such as NHS-fluorescein or NHS-rhodamine, and sulfhydryl-reactive maleimide-activated fluors such as fluorescein-5-maleimide. In some embodiments, reaction of any of these reactive dyes with another molecule results in a stable covalent bond formed between a fiuorophore and the linker and/or agent. In some embodiments, the reactive group is isothiocyanates. In some embodiments, a label is attached to an agent through the primary amines of lysine side chains. In some embodiments, chemical labeling comprises a NHS-ester chemistry method.

[00155] In some embodiments, methods to attach labels to linkers and/or agents comprise enzymatic labeling and affinity labeling. Enzymatic labeling can include, but is not limited to, an acyl carrier protein/phosphopantetheine transferase (ACP/PPTase), Q-tag/transglutaminase (TGase) (Lin, C.W. and Ting, A.Y. Transglutaminase-catalyzed site-specific conjugation of small-molecule probes to proteins *in vitro* and on the surface of living cells. *J. Am. Chem. Soc.* 2006, *128,* 4542-4543), biotin acceptor peptide/biotin ligase (AP/Bir A) (Chen, I., et al., Site-specific labeling of cell surface proteins with biophysical probes using biotin ligase. *Nat. Meth.* 2005, *2,* 99-104.), farnesylation motif/protein farnesyltransferase (PFTase) (Duckworth, B.P., et al, Selective labeling of proteins by using protein farnesyltransferase. *ChemBioChem* 2007, *8,* 98-105), aldehyde tag/formylglycine-generating enzyme (Carrico, I.S., et al., Introducing genetically encoded aldehydes into proteins. *Nat. Chem. Biol.* 2007, *3,* 321-322), human O^6-alkylguanine transferase (hAGT) (Keppler, A., et al., A general method for the covalent labeling of fusion proteins with small molecules *in vivo. Nat. Biotechnol.* 2003, *21,* 86-89; Keppler, A., et al, Labeling of fusion proteins with synthetic fluorophores in live cells. *Proc. Natl. Acad. Sci. USA* 2004, *101,* 9955-9959), and mutated prokaryotic dehalogenase (HaloTagTM) methods (Los,

G., et al., Halotag™ technology: cell imaging and protein analysis. *Cell Notes* 2006, *14,* 10-14). Affinity labeling can include, but is not limited to, noncovalent methods utilizing dihydrofolate reductase (DHFR) (Miller, L.W., et al., Methotrexate conjugates: a molecular *in vivo* protein tag. *Angew. Chem. Int. Ed. Engl.* 2004, *43,* 1672-1675; Miller, L.W., et al, In vivo protein labeling with trimethoprim conjugates: a flexible chemical tag. *Nat. Meth.* 2005, *2,* 255-257) and Phe36Val mutant of FK506-binding protein 12 (FKBP12(F36V)) (Marks, K.M., Braun, P.D., Nolan, G.P., A general approach for chemical labeling and rapid, spatially controlled protein inactivation. *Proc. Natl. Acad. Sci. USA* 2004, *101,* 9982-9987), as well as metal-chelation methods.

[00156] In some embodiments, crosslinking reagents are used to attach labels, linkers, and/or agents. In some embodiments, the crosslinking reagent is glutaraldehyde. In some embodiments, glutaraldehyde reacts with amine groups to create crosslinks by one of several routes. In some embodiments, under reducing conditions, the aldehydes on both ends of glutaraldehyde couple with amines to form secondary amine linkages.

[00157] In some embodiments, attachment of labels, linkers, and/or agents comprises periodate-activation followed by reductive amination. For example, preiodate-activation followed by reductive amination is used to conjugate of HRP and other glycoproteins to a linker and/or agent. In some instances, treatment of a glycosylated enzyme with periodate results in oxidation of sugar cis-diol groups (especially sialic acid, which is common in glycoprotein polysaccharides), resulting in formation of aldehyde groups. In some instances, these aldehyde groups react (in the presence of the mild reductant cyanoborohydride) with primary amines of an added antibody or other molecule.

[00158] In some embodiments, Sulfo-SMCC or other heterobifunctional crosslinkers are used to conjugate labels to linkers and/or agents. For example, Sulfo-SMCC is used to conjugate an enzyme to a drug. In some embodiments, the enzyme is activated and purified in one step and then conjugated to the drug in a second step. In some embodiments, the directionality of crosslinking is limited to one specific orientation (e.g., amines on the enzyme to sulfhydryl groups on the antibody).

[00159] In some embodiments, a linkage is formed between the linker and the label and/or agent. The term "linkage," as used herein to refer to bonds or a chemical moiety formed from a chemical reaction between the functional group of a linker and another molecule (e.g., label, agent). In some embodiments, such bonds include, but are not limited to, covalent linkages and non-covalent bonds, while such chemical moieties include, but are not limited to, esters, carbonates, imines, phosphate esters, hydrazones, acetals, orthoesters, peptide linkages, and oligonucleotide linkages. Hydrolytically stable linkages means that the linkages are substantially

stable in water and do not react with water at useful pH values, including but not limited to, under physiological conditions for an extended period of time, perhaps even indefinitely. Hydrolytically unstable or degradable linkages means that the linkages are degradable in water or in aqueous solutions, including for example, blood. In other embodiments, enzymatically unstable or degradable linkages means that the linkage is degraded by one or more enzymes. By way of example only, PEG and related polymers include degradable linkages in the polymer backbone or in the linker group between the polymer backbone and one or more of the terminal functional groups of the polymer molecule. Such degradable linkages include, but are not limited to, ester linkages formed by the reaction of PEG carboxylic acids or activated PEG carboxylic acids with alcohol groups on a biologically active agent, wherein such ester groups generally hydrolyze under physiological conditions to release the biologically active agent. Other hydrolytically degradable linkages include but are not limited to carbonate linkages; imine linkages resulted from reaction of an amine and an aldehyde; phosphate ester linkages formed by reacting an alcohol with a phosphate group; hydrazone linkages which are reaction product of a hydrazide and an aldehyde; acetal linkages that are the reaction product of an aldehyde and an alcohol; orthoester linkages that are the reaction product of a formate and an alcohol; peptide linkages formed by an amine group, including but not limited to, at an end of a polymer such as PEG, and a carboxyl group of a peptide; and oligonucleotide linkages formed by a phosphoramidite group, including but not limited to, at the end of a polymer, and a 5' hydroxyl group of an oligonucleotide.

Methodsfor attaching Probes or Targets (e.g., proteins) to Solid Supports

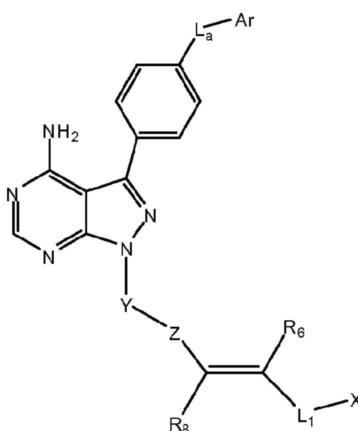
[00160] In some embodiments, methods for attaching probes or targets (e.g., proteins) to solid supports include chemical and/or enzymatic methods. In some embodiments, the chemical methods are disclosed herein. In some embodiments, the enzymatic methods disclosed herein. In some embodiments, methods for attaching probes or targets to a solid support comprises coating the solid support with a probe or target. Methods for coating a microplate with an antibody are well known in the art and can include diluting the antibody in a coating buffer and adding the diluted antibody to a well in the microplate. The unbound antibody can be removed by washing the plate with a wash buffer.

TEC Family Kinase Probe Compounds

[00161] The TEC family kinase probe compounds described herein are composed of a moiety comprising a TEC family kinase inhibitor, a linker moiety, and a detectable label. In some embodiments, the TEC family kinase inhibitor is an irreversible TEC family kinase inhibitor. In some embodiments, the TEC family kinase inhibitor is a Btk inhibitor. In some embodiments, the inhibitor of Btk is an irreversible inhibitor. In another embodiment, the irreversible inhibitor

of Btk binds to a non-catalytic residue in the ATP binding pocket of Btk. In further embodiments, the non-catalytic residue is a cysteine residue. In some embodiments, the Btk probe forms a covalent bond with at least one non-catalytic residue of Btk. In some embodiments, the TEC family kinase probe compound is a derivative of an irreversible Btk inhibitor. In some embodiments, the TEC family kinase probe compound is a derivative of ibrutinib. In some embodiments, the TEC family kinase probe compound is a derivative of ibrutinib. In some embodiments, the TEC family kinase probe compound consists of ibrutinib attached to a label via a linker. In some embodiments, the TEC family kinase probe compound is a derivative of AVL-292, AVL-291, AVL-101, CNX-774,or ONO-WG-307. In some embodiments, the TEC family kinase probe compound consists of AVL-292, AVL-291, AVL-101, CNX-774,or ONO-WG-307 attached to a label via a linker.

[00162] In one aspect is a TEC family kinase probe of Formula (I) comprising:



Formula (I);

wherein:

L_a is CH_2, O, NH or S;

Ar is optionally substituted aryl or optionally substituted heteroaryl;

Y is optionally substituted alkyl, optionally substituted heteroalkyl, optionally substituted cycloalkyl, optionally substituted heterocycloalkyl, optionally substituted aryl, or optionally substituted heteroaryl;

Z is C(O), OC(O), NHC(O), C(S), $S(0)_n$, $OS(0)_n$, $NHS(0)_n$, where n is 1 or 2;

R_6 and R_8 are independently selected from H, optionally substituted alkyl, or optionally substituted heteroalkyl;

Li is selected from the group consisting of a bond, optionally substituted alkyl, optionally substituted heteroalkyl, optionally substituted cycloalkyl, optionally substituted heterocycloalkyl, optionally substituted aryl, optionally substituted heteroaryl, an optionally substituted amide moiety, an optionally ketone moiety, an optionally

substituted carbamate moiety, and an optionally ester moiety, or any combination thereof; and

X is a detectable label.

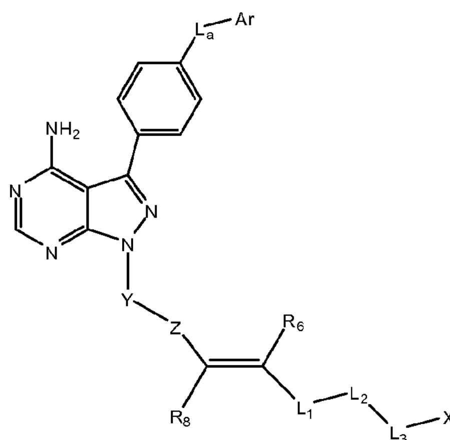
[00163] In some embodiments, L_a is CH_2, O, or NH. In other embodiments, L_a is O or NH. In some embodiments, L_a is O. In some embodiments, Ar is a substituted or unsubstituted aryl. In some embodiments, Ar is a 6-membered aryl. In some other embodiments, Ar is phenyl. In some embodiments, Z is **C(=0), OC(=0), NHC(=0), S(=0)** $_x$**, OS(=0)** $_2$**,** or **NHS(=0)** $_2$**.** In some embodiments, Z is **C(=0), NHC(=0),** or **S(=0)** $_2$**.** In some embodiments, Z is **C(=0).** In some embodiments, Z is **NHC(=0).** In some embodiments, Y is an optionally substituted group selected from among alkyl, heteroalkyl, cycloalkyl, and heterocycloalkyl. In some embodiments, Y is an optionally substituted group selected from among Ci-C_6alkyl, Ci-C_6heteroalkyl, 4-, 5-, 6- or 7-membered cycloalkyl, and 4-, 5-, 6- or 7-membered heterocycloalkyl. In some embodiments, Y is an optionally substituted group selected from among Ci-Cealkyl, Ci-Ceheteroalkyl, 5-, or 6-membered cycloalkyl, and 5-, or 6-membered heterocycloalkyl containing 1 or 2 N atoms. In some embodiments, Y is a 5-, or 6-membered cycloalkyl, or a 5-, or 6-membered heterocycloalkyl containing 1 or 2 N atoms. In some embodiments, Y is a pyrrolidine ring. In some embodiments, Y is a piperidine ring. In some embodiments, Re and R_8 are independently selected from among H, unsubstituted c 1-C4 alkyl, substituted Ci-C4alkyl, unsubstituted Ci-C_4heteroalkyl, and substituted Ci-C_4heteroalkyl. In some embodiments, R_6 and R_8 are each H.

[00164] In some embodiments, Li is selected from the group consisting of a bond, optionally substituted alkyl, optionally substituted heteroalkyl, optionally substituted cycloalkyl, optionally substituted heterocycloalkyl, optionally substituted aryl, optionally substituted heteroaryl, an optionally substituted amide moiety, an optionally ketone moiety, an optionally substituted carbamate moiety, and an optionally ester moiety. In some embodiments, Li is selected from any combination of at least two groups selected from optionally substituted alkyl, optionally substituted heteroalkyl, optionally substituted cycloalkyl, optionally substituted heterocycloalkyl, optionally substituted aryl, optionally substituted heteroaryl, an optionally substituted amide moiety, an optionally ketone moiety, an optionally substituted carbamate moiety, and an optionally ester moiety. In some embodiments, Li is selected from any combination of at least three groups selected from optionally substituted alkyl, optionally substituted heteroalkyl, optionally substituted cycloalkyl, optionally substituted heterocycloalkyl, optionally substituted aryl, optionally substituted heteroaryl, an optionally substituted amide moiety, an optionally ketone moiety, an optionally substituted carbamate moiety, and an optionally ester moiety. In some embodiments, L_1 is selected from any combination of at least four groups selected from

optionally substituted alkyl, optionally substituted heteroalkyl, optionally substituted cycloalkyl, optionally substituted heterocycloalkyl, optionally substituted aryl, optionally substituted heteroaryl, an optionally substituted amide moiety, an optionally ketone moiety, an optionally substituted carbamate moiety, and an optionally ester moiety.

[00165] In some embodiments, X is a detectable label selected from the group consisting of a dye, a photocrosslinker, a cytotoxic compound, a drug, an affinity label, a photoaffmity 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 electron dense group, a magnetic group, an intercalating group, a chromophore, an energy transfer agent, a biologically active agent, or a combination thereof. In some embodiments, X is a fluorophore. In some embodiments, X is biotin. In some embodiments, X is biotin analogue.

[00166] In another embodiment is a TEC family kinase probe of Formula (II) comprising:



Formula (II);

wherein:

L_a is CH_2, O, NH or S;

Ar is optionally substituted aryl or optionally substituted heteroaryl;

Y is optionally substituted alkyl, optionally substituted heteroalkyl, optionally substituted cycloalkyl, optionally substituted heterocycloalkyl, optionally substituted aryl, or optionally substituted heteroaryl;

Z is C(O), OC(O), NHC(O), C(S), $S(0)_n$, $OS(0)_n$, $NHS(0)_n$, where n is 1 or 2;

R_6 and R_8 are independently selected from H, optionally substituted alkyl, or

optionally substituted heteroalkyl;

Li is optionally substituted alkyl or optionally substituted heteroalkyl;

L_2 is a bond, optionally substituted heterocycloalkyl, or -N(H)C(0)(CH $_2)_m$C(0)N(H)-, where m is 2-6;

L_3 is optionally substituted alkyl or optionally substituted heteroalkyl; and

X is a detectable label.

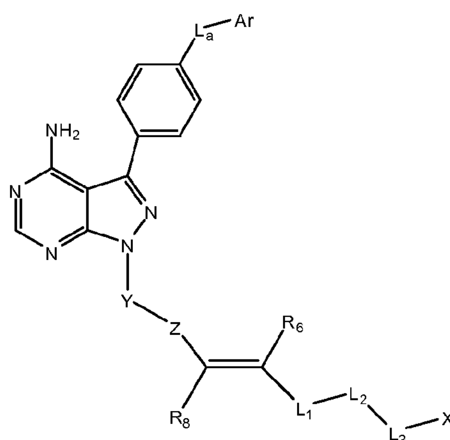
[00167] In some embodiments, L_a is CH_2, O, or NH. In other embodiments, L_a is O or NH. In some embodiments, L_a is O. In some embodiments, Ar is a substituted or unsubstituted aryl. In some embodiments, Ar is a 6-membered aryl. In some other embodiments, Ar is phenyl. In some embodiments, Z is C(=0), OC(=0), NHC(=0), S(=0) $_x$, OS(=0) $_2$, or NHS(=0) $_2$. In some embodiments, Z is C(=0), NHC(=0), or S(=0) $_2$. In some embodiments, Z is C(=0). In some embodiments, Z is NHC(=0). In some embodiments, Y is an optionally substituted group selected from among alkyl, heteroalkyl, cycloalkyl, and heterocycloalkyl. In some embodiments, Y is an optionally substituted group selected from among Ci-C_6alkyl, Ci-Ceheteroalkyl, 4-, 5-, 6- or 7-membered cycloalkyl, and 4-, 5-, 6- or 7-membered heterocycloalkyl. In some embodiments, Y is an optionally substituted group selected from among Ci-Cealkyl, Ci-Ceheteroalkyl, 5-, or 6-membered cycloalkyl, and 5-, or 6-membered heterocycloalkyl containing 1 or 2 N atoms. In some embodiments, Y is a 5-, or 6-membered cycloalkyl, or a 5-, or 6-membered heterocycloalkyl containing 1 or 2 N atoms. In some embodiments, Y is a pyrrolidine ring. In some embodiments, Y is a piperidine ring. In some embodiments, ¾ and R_8 are independently selected from among H, unsubstituted C l-C4 alkyl, substituted Ci-C4alkyl, unsubstituted Ci-C_4heteroalkyl, and substituted Ci-C_4heteroalkyl. In some embodiments, R.6 and R-8 are each H.

[00168] In some embodiments, L_1 is optionally substituted alkyl. In some embodiments, Li is optionally substituted heteroalkyl. In some embodiments, L_2 is a bond. In some embodiments, L_2 is optionally substituted heterocycloalkyl. In some embodiments, L_2 is optionally substituted piperazine. In some embodiments, L_2 is optionally substituted piperidine. In some embodiments, L_2 is -N(H)C(0)(CH $_2)_2$C(0)N(H)-. In some embodiments, L_2 is -N(H)C(0)(CH $_2)_3$C(0)N(H)-. In some embodiments, L_2 is -N(H)C(0)(CH $_2)_4$C(0)N(H)-. In some embodiments, L_2 is -N(H)C(0)(CH $_2)_5$C(0)N(H)-. In some embodiments, L_2 is -N(H)C(0)(CH $_2)_6$C(0)N(H)-. In some embodiments, L_3 is optionally substituted alkyl. In some embodiments, L_3 is optionally substituted heteroalkyl.

[00169] In some embodiments, X is a detectable label selected from the group consisting of a dye, a photocrosslinker, a cytotoxic compound, a drug, an affinity label, a photoafrmity 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 electron dense group, a magnetic group, an intercalating group, a chromophore, an energy transfer agent, a biologically active agent, or a combination thereof. In some embodiments, X is a fluorophore. In some embodiments, X is biotin. In some embodiments, X is biotin analogue.

[00170] In another embodiment is a TEC family kinase probe of Formula (III) comprising:



Formula (III);

wherein:

L_a is O;

Ar is optionally substituted phenyl;

Y is optionally substituted cycloalkyl or optionally substituted heterocycloalkyl;

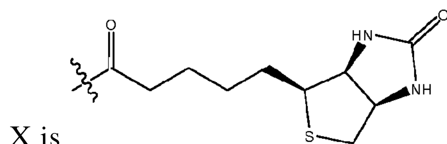
Z is C(O) orNHC(O);

R_6 and R_8 are independently selected from H, optionally substituted alkyl, or optionally substituted heteroalkyl;

Li is optionally substituted alkyl or optionally substituted heteroalkyl;

L_2 is a bond, optionally substituted heterocycloalkyl, or -N(H)C(0)(CH $_2)_m$C(0)N(H)-, where m is 2-6;

L_3 is optionally substituted alkyl or optionally substituted heteroalkyl; and

X is 

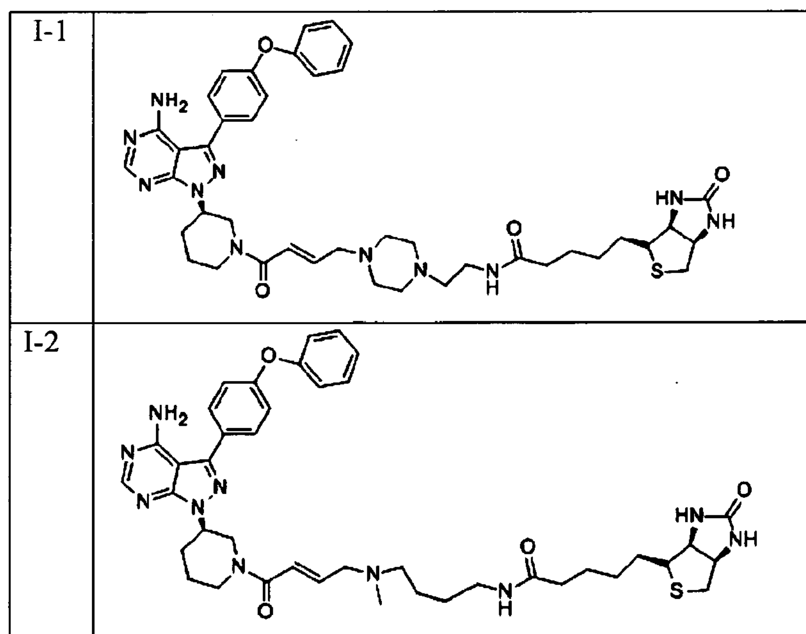
.

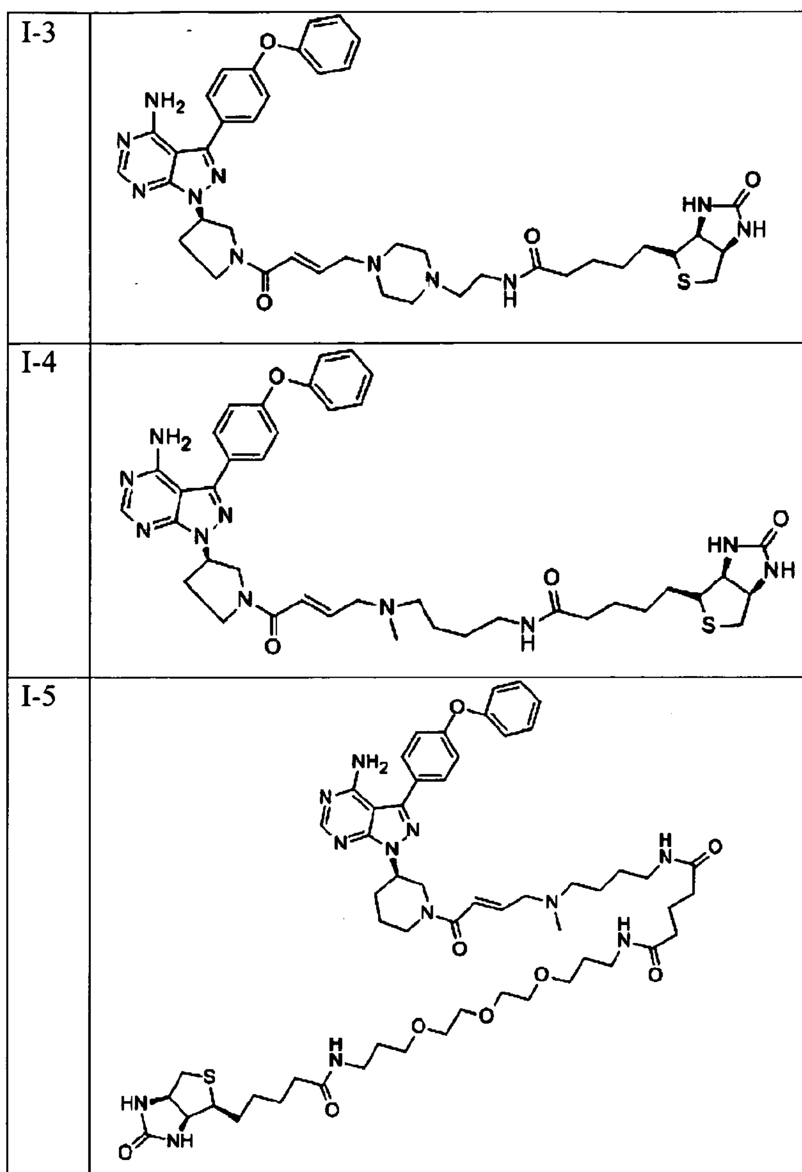
[00171] In some embodiments, Z is C(=0). In some embodiments, Z is NHC(=0). In some

embodiments, Y is an optionally substituted cycloalkyl. In some embodiments, Y is an optionally substituted heterocycloalkyl. In some embodiments, Y is a 5-, or 6-membered cycloalkyl, or a 5-, or 6-membered heterocycloalkyl containing 1 or 2 N atoms. In some embodiments, Y is a cyclohexyl ring. In some embodiments, Y is a pyrrolidine ring. In some embodiments, Y is a piperidine ring. In some embodiments, R_6 and R_8 are independently selected from among H, unsubstituted **C_1-C4** alkyl, substituted **Ci-C_4alkyl,** unsubstituted Cr **C_4heteroalkyl,** and substituted **Ci-C_4heteroalkyl.** In some embodiments, **R_6** and ¾ are each H.

[00172] In some embodiments, L_1 is optionally substituted alkyl. In some embodiments, L_1 is optionally substituted heteroalkyl. In some embodiments, L_2 is a bond. In some embodiments, L_2 is optionally substituted heterocycloalkyl. In some embodiments, L_2 is optionally substituted piperazine. In some embodiments, L_2 is optionally substituted piperidine. In some embodiments, L_2 is -N(H)C(0)(CH $_2$)$_2$C(0)N(H)-. In some embodiments, L_2 is -N(H)C(0)(CH $_2$)$_3$C(0)N(H)-. In some embodiments, L_2 is -N(H)C(0)(CH $_2$)$_4$C(0)N(H)-. In some embodiments, L_2 is -N(H)C(0)(CH $_2$)$_5$C(0)N(H)-. In some embodiments, L_2 is -N(H)C(0)(CH $_2$)$_6$C(0)N(H)-. In some embodiments, L_3 is optionally substituted alkyl. In some embodiments, L_3 is optionally substituted heteroalkyl.

[00173] In some embodiments, the probe comprises biotin attached to ibrutinib via a linker (i.e. a biotinylated ibrutinib). In some embodiments, the probe is selected from among:





Detection Methods

[00174] In some embodiments, the methods, assays, and systems disclosed herein comprise detection of the targets (e.g., the target kinases). In some instances, the targets are probe-bound targets. In some instances, the probe-bound targets are drug-occupied targets. In other instances, the probe-bound targets are unoccupied targets.

[00175] In some embodiments, detection of the targets comprises contacting the sample with an antibody. In some embodiments, the antibody is labeled antibody. In some embodiments, the antibody is labeled with an electrochermluminescent tag. In some embodiments, the electrochemiluminescent tag comprises Tris(bipyridine)ruthenium(II) dichloride. In some embodiments, the electrochemiluminescent tag is Ruthenium (II) tris-bipyridine, N-hychoxysuccinimide. In some embodiments, the labeled antibody is a SULFO TAG labeled

antibody. In some embodiments, the labeled antibody is a horseradish peroxidase labeled antibody. In some embodiments, the antibody is used as a primary antibody. In another embodiment, the antibody is used as a secondary antibody.

[00176] In some embodiments, detection of the targets comprises chemiluminescence, luminescence, fluorescence, immunofluorescence, calorimetry, or electrochemiluminescence methods. In some embodiments, detection of the targets comprises a fluorescence detection instrument. In some embodiments, the fluorescence detection instrument comprises an excitation light source. In some embodiments, the light source is a laser, photodiode, or lamps. In some embodiments, the lamp is a xenon arc or mercury. In some embodiments, the fluorescence detection instrument comprises a fluorophore. In some embodiments, the fluorescence detection instrument comprises a filter. In some embodiments, the filter isolates specific wavelengths to excite different fluorophores. In some embodiments, the fluorescence detection instrument comprises a detector that records the output. In some embodiments, the output is an electronic signal. In some embodiments, the fluorescence detection instrument is a fluorescent microscope. In some embodiments, the fluorescent microscope detects the localized fluors. In some embodiments, detection occurs in two and/or three dimensions. In some embodiments, the fluorescence detection instrument is a fluorescence scanner. In some embodiments, the fluorescence scanner is a microarray reader. In some embodiments, the microarray reader detects localized fluors in two dimensions. In some embodiments, the fluorescence detection instrument is a spectrofluorometer. In some embodiments, the fluorescence detection instrument is a microplate reader. In some embodiments, the fluorescence detection instrument records the average fluorescence. In some embodiments, the fluorescence detection instrument is a flow cytometer. In some embodiments, the flow cytometer analyzes the fluorescence of individual cells in a sample population.

[00177] In some embodiments, detection of the targets comprises the use of a microplate reader. In some instances, the microplate reader is an xMark™ microplate absorbance spectrophotometer, iMark microplate absorbance reader, EnSpire® Multimode plate reader, EnVision Multilabel plate reader, VICTOR X Multilabel plate reader. Fluoroskan Ascent FL Microplate Fluoremeter and Luminometer, Fluoroskan Ascent Microplate Fluoremeter, Luminoskan Ascent Microplate Luminometer, Multiskan EX Microplate Photometer, Muliskan FC Microplate Photometer, and Muliskan GO Microplate Photometer. In some instances, the microplate reader detects absorbance, fluorescence, luminescence, time-resolved fluorescence, and light scattering. In some embodiments, the microplate reader detects dynamic light scattering. Alternatively, the microplate reader detects static light scattering.

[00178] In some embodiments, detection of the targets comprises the use of a microplate

imager. In some instances, the microplate imager comprises ViewLux uHTS microplate imager and BioRad microplate imaging system.

[00179] In some embodiments, computer-based systems are employed in the detection methods for determining protein occupancy described herein. In some embodiments, the computer-based systems include a digital processing device which analyzes the data and signals obtained from an devices or instrument such as a multiwell plate assay. In some embodiments, provided herein is computer readable storage media encoded with a computer program including instructions executable by a digital processing device for performance of the detection methods for determining protein occupancy described herein.

[00180] In further embodiments, the digital processing device includes one or more hardware central processing units (CPU) that carry out the device's functions. In still further embodiments, the digital processing device further comprises an operating system configured to perform executable instructions. In some embodiments, the digital processing device is optionally connected a computer network. In further embodiments, the digital processing device is optionally connected to the Internet such that it accesses the World Wide Web. In still further embodiments, the digital processing device is optionally connected to a cloud computing infrastructure. In other embodiments, the digital processing device is optionally connected to an intranet. In other embodiments, the digital processing device is optionally connected to a data storage device.

[00181] In some embodiments, provided herein is an analytical system for determining protein occupancy of a target kinase, comprising: (a) a probe ELISA assay comprising patient samples comprising a target kinase and a probe as described herein; (b) an analytical instrument for detecting probe-bound target kinases for determining protein occupancy; (c) a digital processing device comprising an operating system configured to perform executable instructions and a memory; and (d) a computer program, provided to the digital processing device, including executable instructions that create a target occupancy application comprising: (i) a database of threshold levels for target occupancy; (ii) a software module configured to receive signal data from the analytical instrument; (iii) a software module configured to apply an algorithm to the signal data to identify the level of target kinase occupancy in the sample.

Applications

Drug Research and Validation

[00182] Any of the assays and systems disclosed herein can be useful in researching and validating drugs. Provided herein are methods for validating a drug comprising (a) contacting a sample comprising a target with a probe to form a probe-bound target; (b) detecting the presence or absence of the probe-bound target; and (c) determining occupancy of the target by a drug

based on the presence or absence of the probe-bound target, thereby validating the drug.

[00183] Further provided herein are methods for determining occupancy of a target comprising: a) combining a sample comprising a target with a probe; b) detecting the presence or absence of a probe-bound target; and c) determining occupancy of the target by a drug based on the presence or absence of the probe-bound target.

[00184] In some embodiments, the method further comprises capturing the target prior to step (a) contacting the sample with the probe. In some embodiments, the target is captured by an antibody. In some embodiments, the antibody is an anti-target antibody. In some embodiments, the antibody is attached to a solid support. In some embodiments, the solid support is a microplate. In some embodiments, the microplate is a MSD microplate.

[00185] In some embodiments, the method further comprises contacting the probe-bound target with a primary detection agent. In some embodiments, the primary detection agent comprises an antibody, a bead, a dye, or a fluorophore. In some embodiments, the primary detection agent comprises an antibody. In some embodiments, the antibody is an anti-BTK antibody. In some embodiments, the method further comprises contacting the detection agent with a secondary detection agent. In some embodiments, the secondary detection agent comprises an antibody, a bead, a dye, or a fluorophore. In some embodiments, the primary detection agent is labeled. In some embodiments, the secondary detection agent is labeled. In some embodiments, the label is an electrochemiluminescent tag. In some embodiments, the electrochemiluminescent tag comprises Tris(bipyridine)ruthenium(II) dicfiloride. In some embodiments, the electrochemiluminescent tag is Ruthenium (II) tris-bipyridine, N-hydroxysuccinimide. In some embodiments, the label is a SULFO TAG.

[00186] In some embodiments, detecting the presence or absence of the probe-bound target comprises contacting the sample with a solid support. In some embodiments, the solid support comprises a bead. In some embodiments, the bead is a streptavidin bead. In some embodiments, the bead is a magnetic bead. In some embodiments, the bead is a labeled bead. In some embodiments, the bead is a labeled streptavidin bead. In some embodiments, the bead is a labeled with an electrochemiluminescent tag. In some embodiments, the electrochemiluminescent tag comprises Tris(bipyridine)ruthenium(II) dicfiloride. In some embodiments, the electrochemiluminescent tag is Ruthenium (II) tris-bipyridine, N-hydroxysuccinimide. In some embodiments, the bead is a SULFO TAG bead. In some embodiments, the bead is a SULFO TAG streptavidin bead.

[00187] In some embodiments, the bead interacts with the probe. In some embodiments, the probe comprises a label. In some embodiments, the label comprises biotin. In some embodiments, the bead interacts with biotin. In some embodiments, the bead forms a conjugate

with the probe-bound target. In some embodiments, the bead is conjugated to the probe.

[00188] In some embodiments, detecting the presence or absence of the probe-bound target comprises detecting the probe-bound target or a portion thereof. In some embodiments, detecting the presence or absence of the probe-bound target comprises detecting the bead or a portion thereof. In some embodiments, detecting the presence or absence of the probe-bound target comprises detecting the labeled bead. In some embodiments, detecting the presence or absence of the probe-bound target comprises detecting an electrochemiluminescent tag. In some embodiments, the electrochemiluminescent tag comprises Tris(bipyridine)ruthenium(II) dichloride. In some embodiments, the electrochemiluminescent tag is Ruthenium (II) tris-bipyridine, N-hydroxysuccinimide. In some embodiments, detecting the presence or absence of the probe-bound target comprises detecting a SULFO TAG. In some embodiments, the detecting step comprises luminescence. In some embodiments, the detecting step comprises electrochemiluminescence.

[00189] In some embodiments, the method further comprises purification of the probe-bound target. In some embodiments, the probe-bound target is an unoccupied target. In some embodiments, the probe-bound target is a drug-occupied target. In another embodiment, purification of the probe-bound target comprises magnetic separation of probe-bound targets from non-probe-bound targets.

[00190] In some embodiments, the sample is a pre-treated sample, wherein the pre-treated sample is contacted with a drug prior to contact with the probe. In some embodiments, the sample is a non-treated sample, wherein the sample is not contacted with a drug prior to contact with the label.

[00191] In some embodiments, the probe comprises an agent. In some embodiments, the probe comprises an agent and a linker. In some embodiments, the probe comprises a label. In some embodiments, the probe comprises a label and a linker. In some embodiments, the agent is a BTK inhibitor. In some embodiments, the BTK inhibitor is a reversible BTK inhibitor. In some embodiments, the BTK inhibitor is an irreversible BTK inhibitor. In some embodiments, the BTK inhibitor is a selective, covalent BTK inhibitor. In some embodiment, the BTK inhibitor forms a covalent bond with a cysteine residue of a Bruton's tyrosine kinase (BTK). In some embodiments, the cysteine residue is cysteine 481. In some embodiments, the BTK inhibitor is selected from a list comprising LFM-A13, AVL-291, AVL-101, AVL-292, and ONO-WG-307. In some embodiments, the BTK inhibitor is ibrutinib. In some embodiments, the agent is an ITK inhibitor. In some embodiments, the agent is a BMX kinase inhibitor. In some embodiments, the agent is a TEC kinase inhibitor. In some embodiments, the agent is a BLK inhibitor.

[00192] In some embodiments, the agent is identical to the drug. For example, the drug and the agent can both be a BTK inhibitor (e.g., ibrutinib, AVL-292, ONO-WG-307). In some embodiments, the agent is similar to the drug. For example, the drug can be a BTK inhibitor and the agent can be a salt derivative of the BTK inhibitor. In some embodiments, the agent is different from the drug. For example, the drug can be ibrutinib and the agent can be AVL-292.

[00193] In some embodiments, the target is a receptor. In some embodiments, the target is a ligand. In some embodiments, the target is a kinase. In some embodiments, the kinase is BTK. In some instances, the kinase is ITK. In other embodiments, the kinase is BMX or BLK. In some instances, the kinase is TEC or TXK. In some embodiments, the kinase is HER1, HER2, HER3, or HER4. Alternatively, the kinase is JAK3.

[00194] In some embodiments, validating the drug comprises determining the efficacy of the drug on a target. In some embodiments, determining occupancy of the target by the drug comprises quantifying the presence or absence of probe-bound targets. In some embodiments, the drug is effective when the occupancy of the target is at least about 50%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, or 99%.

Diagnostics

[00195] Any of the methods, assays and systems can be used to inform therapeutic treatment and the over-all health care management of a subject informing method for determining a therapeutic regimen. In some embodiments, is a method for determining a therapeutic regimen comprising: (a) combining a sample comprising a target with a probe; (b) detecting the presence or absence of a probe-bound target; and (c) determining a therapeutic regimen based on the presence or absence of the probe-bound target.

[00196] Further disclosed herein is a method for determining efficacy of a test agent comprising: (a) combining a sample comprising a target with a probe; (b) detecting the presence or absence of a probe-bound target; and (c) determining efficacy of a test agent based on the presence or absence of the probe-bound target.

[00197] Further disclosed herein is a method for identifying drug responders comprising: (a) combining a sample comprising a target with a probe; (b) detecting the presence or absence of a probe-bound target; and (c) identifying drug responders based on the presence or absence of the probe-bound target.

[00198] Further disclosed herein is a method for identifying kinase modulators comprising: (a) combining a sample comprising a target with a probe; (b) detecting the presence or absence of a probe-bound target; and (c) identifying kinase modulators based on the presence or absence of the probe-bound target.

[00199] Disclosed herein is a method for determining drug resistance comprising: (a)

combining a sample comprising a target with a probe; (b) detecting the presence or absence of a probe-bound target; and (c) determining drug resistance based on the presence or absence of the probe-bound target.

[00200] *Samples*

[00201] In some embodiments, the methods, assays, and systems disclosed herein comprise contacting sample comprising a target with a probe. Suitable samples for use in any of the methods, assays, and systems disclosed herein comprise, but are not limited to, a whole blood sample, peripheral blood sample, lymph sample, tissue sample, tumor biopsy sample, bone marrow sample, or other bodily fluid sample. In some embodiments, the sample is a sample containing one or more cell types, or a lysate thereof, derived from a whole blood sample, peripheral blood sample, lymph sample, tissue sample, tumor biopsy sample, bone marrow sample, or other bodily fluid sample. Examples of bodily fluids include, but are not limited to, smears, sputum, biopsies, secretions, cerebrospinal fluid, bile, blood, lymph fluid, saliva, and urine. In some embodiments, cells of the sample are isolated from other components of the sample prior to use in the methods provided. In some embodiments, particular cell types of the sample are isolated from other cell types of the sample prior to use in the methods provided. For example, in some embodiments, peripheral blood mononuclear cells (PBMCs, e.g., lymphocytes, monocytes and macrophages) of a blood sample are isolated from other cell types of the blood sample prior to use in the methods provided. For example, in some embodiments, lymphocytes (e.g., B cells, T cells or NK cells) of the sample are isolated from other cell types of the sample prior to use in the methods provided. For example, in some embodiments, B cells of the sample are isolated from other cell types of the sample prior to use in the methods provided. In some embodiments, cells of the sample are lysed prior to use in the methods provided. For example, in some embodiments, cancer cells are isolated from normal cells of the sample prior to use in the methods provided.

[00202] Any of the samples disclosed herein comprises complex populations of cells, which can be assayed as a population, or separated into sub-populations. Such cellular and acellular samples can be separated by centrifugation, elutriation, density gradient separation, apheresis, affinity selection, panning, FACS, filtration, centrifugation with Hypaque, etc. By using antibodies specific for markers identified with particular cell types, a relatively homogeneous population of cells can be obtained. Alternatively, a heterogeneous cell population can be used.

[00203] Once a sample is obtained, it can be used directly, frozen, or maintained in appropriate culture medium for short periods of time. Methods to isolate one or more cells for use according to the methods of this invention are performed according to standard techniques and protocols well-established in the art.

[00204] In some embodiments, the sample is obtained from a subject. Such subject can be a human or a domesticated animal such as a cow, chicken, pig, horse, rabbit, dog, cat, or goat. In some embodiments, the cells used in the present invention are taken from a patient. Samples derived from an animal, e.g., human, can include, for example whole blood, sweat, tears, saliva, ear flow, sputum, lymph, bone marrow suspension, lymph, urine, saliva, semen, vaginal flow, cerebrospinal fluid, brain fluid, ascites, milk, secretions of the respiratory, intestinal or genitourinary tracts fluid, a lavage of a tissue or organ (e.g., lung) or tissue which has been removed from organs, such as breast, lung, intestine, skin, cervix, prostate, pancreas, heart, liver and stomach.

[00205] To obtain a blood sample, any technique known in the art can be used, e.g., a syringe or other vacuum suction device. A sample can be optionally pre -treated or processed prior to enrichment. Examples of pre-treatment steps include the addition of a reagent such as a stabilizer, a preservative, a fixant, a lysing reagent, a diluent, a drug, an anti-apoptotic reagent, an anti-coagulation reagent, an anti-thrombotic reagent, magnetic property regulating reagent, a buffering reagent, an osmolality regulating reagent, a pH regulating reagent, and/or a cross-linking reagent. For example, when a blood sample is obtained, a preservative such an anti-coagulation agent and/or a stabilizer can be added to the sample prior to enrichment.

[00206] A sample, such as a blood sample, can be analyzed under any of the methods, assays and systems disclosed herein within 1 week, 6 days, 5 days, 4 days, 3 days, 2 days, 1 day, 12 hrs, 6 hrs, 3 hrs, 2 hrs, or 1 hr from the time the sample is obtained.

[00207] In some embodiments, a sample can be combined with an enzyme or compound that selectively lyses one or more cells or components in the sample. For example, in a blood sample, platelets and/or enucleated red blood cells are selectively lysed to generate a sample enriched in nucleated cells. The cells of interest can subsequently be separated from the sample using methods known in the art.

[00208] When obtaining a sample from a subject (e.g., blood sample), the amount can vary depending upon subject size and the condition being screened. In some embodiments, up to 50, 40, 30, 20, 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1 mL of a sample is obtained. In some embodiments, 1-50, 2-40, 3-30, or 4-20 mL of sample is obtained. In some embodiments, more than 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 or 100 mL of a sample is obtained.

[00209] *Diseases and indications*

[00210] In some embodiments, any of the samples disclosed herein is obtained from a subject suffering from a disease or indication. In some embodiments, any of the samples disclosed herein is obtained from a subject suffering from a disease or indication mediated by a TEC family kinase. In some embodiments, the sample is from a subject suffering from an

autoimmune disease, an inflammatory disorder, or a proliferative disease, such as cancer. In some embodiments, the cancer is a solid tumor.

[00211] In some embodiments, the sample is from a subject suffering from a cancer. Cancers include, but are not limited to, sarcomas, carcinomas, and hematologic cancers. In some embodiments, the hematologic cancer is a leukemia, a lymphoma, or a myeloma.

[00212] In some embodiments, the cancer is a sarcoma. Sarcomas are cancers of the bone, cartilage, fat, muscle, blood vessels, or other connective or supportive tissue. Sarcomas include, but are not limited to, bone cancer, fibrosarcoma, chondrosarcoma, Ewing's sarcoma, malignant hemangioendothelioma, malignant schwannoma, bilateral vestibular schwannoma, osteosarcoma, soft tissue sarcomas (e.g., alveolar soft part sarcoma, angiosarcoma, cystosarcoma phylloides, dermatofibrosarcoma, desmoid tumor, epithelioid sarcoma, extraskeletal osteosarcoma, fibrosarcoma, hemangiopericytoma, hemangiosarcoma, Kaposi's sarcoma, leiomyosarcoma, liposarcoma, lymphangiosarcoma, lymphosarcoma, malignant fibrous histiocytoma, neurofibrosarcoma, rhabdomyosarcoma, and synovial sarcoma).

[00213] In some embodiments, the cancer is a carcinoma. Carcinomas are cancers that begin in the epithelial cells, which are cells that cover the surface of the body, produce hormones, and make up glands. By way of non-limiting example, carcinomas include breast cancer, pancreatic cancer, lung cancer, colon cancer, colorectal cancer, rectal cancer, kidney cancer, bladder cancer, stomach cancer, prostate cancer, liver cancer, ovarian cancer, brain cancer, vaginal cancer, vulvar cancer, uterine cancer, oral cancer, penile cancer, testicular cancer, esophageal cancer, skin cancer, cancer of the fallopian tubes, head and neck cancer, gastrointestinal stromal cancer, adenocarcinoma, cutaneous or intraocular melanoma, cancer of the anal region, cancer of the small intestine, cancer of the endocrine system, cancer of the thyroid gland, cancer of the parathyroid gland, cancer of the adrenal gland, cancer of the urethra, cancer of the renal pelvis, cancer of the ureter, cancer of the endometrium, cancer of the cervix, cancer of the pituitary gland, neoplasms of the central nervous system (CNS), primary CNS lymphoma, brain stem glioma, and spinal axis tumors. In some instances, the cancer is a skin cancer, such as a basal cell carcinoma, squamous, melanoma, nonmelanoma, or actinic (solar) keratosis. In some embodiments, the cancer is a pancreatic cancer, colon cancer, breast cancer, lung cancer, ovarian cancer, prostate cancer, thyroid cancer, bladder cancer, or proximal or distal bile duct carcinoma. In some embodiments, the cancer is a breast cancer.

[00214] In some instances, the cancer is a lung cancer. Lung cancer can start in the airways that branch off the trachea to supply the lungs (bronchi) or the small air sacs of the lung (the alveoli). Lung cancers include non-small cell lung carcinoma (NSCLC), small cell lung carcinoma, and mesothelioma. Examples of NSCLC include squamous cell carcinoma,

adenocarcinoma, and large cell carcinoma. In some embodiments, the mesothelioma is a cancerous tumor of the lining of the lung and chest cavity (pleura) or lining of the abdomen (peritoneum). In some instances, the cancer is a brain cancer, such as a glioblastoma.

[00215] In some embodiments, the cancer is a central nervous system (CNS) tumor. CNS tumors can be classified as gliomas or nongliomas. In some instances, the glioma is malignant glioma, high grade glioma, diffuse intrinsic pontine glioma. Examples of gliomas include astrocytomas, oligodendrogliomas (or mixtures of oligodendroglioma and astocytoma elements), and ependymomas. Astrocytomas include, but are not limited to, low-grade astrocytomas, anaplastic astrocytomas, glioblastoma multiforme, pilocytic astrocytoma, pleomorphic xanthoastrocytoma, and subependymal giant cell astrocytoma. Oligodendrogliomas include low-grade oligodendrogliomas (or oligoastrocytomas) and anaplastic oligodendriogliomas. Nongliomas include meningiomas, pituitary adenomas, primary CNS lymphomas, and medulloblastomas. In some instances, the cancer is a meningioma.

[00216] In some instances, the cancer is a leukemia. In some instances, the leukemia is an acute lymphocytic leukemia, acute lymphoblastic leukemia (ALL), precursor B-cell lymphoblastic leukemia, acute myelocytic leukemia (AML), acute promyelocytic leukemia (APL), chronic lymphocytic leukemia (CLL), chronic myelocytic leukemia (CML) or acute monocytic leukemia (AMoL). Additional types of leukemias include, but are not limited to hairy cell leukemia, chronic myelomonocytic leukemia, and juvenile myelomonocytic-leukemia.

[00217] In some instances, the cancer is a lymphoma. In some instances, the lymphoma is a Hodgkin's lymphoma. In other instances, the lymphoma is a non-Hodgkin's lymphoma (NHL). In some embodiments, the lymphoma is a B-cell NHL. A non-limiting list of the B-cell NHL includes Burkitt's lymphoma (e.g., Endemic Burkitt's Lymphoma and Sporadic Burkitt's Lymphoma), Cutaneous B-Cell Lymphoma, Cutaneous Marginal Zone Lymphoma (MZL), Diffuse Large Cell Lymphoma (DLBCL), Diffuse Mixed Small and Large Cell Lymphoma, Diffuse Small Cleaved Cell, Diffuse Small Lymphocytic Lymphoma, Extranodal Marginal Zone B-cell lymphoma, follicular lymphoma, Follicular Small Cleaved Cell (Grade 1), Follicular Mixed Small Cleaved and Large Cell (Grade 2), Follicular Large Cell (Grade 3), Intravascular Large B-Cell Lymphoma, Intravascular Lymphomatosis, Large Cell Immunoblastic Lymphoma, Large Cell Lymphoma (LCL), Lymphoblastic Lymphoma, MALT Lymphoma, Mantle Cell Lymphoma (MCL), immunoblastic large cell lymphoma, precursor B-lymphoblastic lymphoma, mantle cell lymphoma, chronic lymphocytic leukemia (CLL)/small lymphocytic lymphoma (SLL), extranodal marginal zone B-cell lymphoma-mucosa-associated lymphoid tissue (MALT) lymphoma, Mediastinal Large B-Cell Lymphoma, nodal marginal zone B-cell lymphoma, splenic marginal zone B-cell lymphoma, primary mediastinal B-cell lymphoma,

lymphoplasmocytic lymphoma, hairy cell leukemia, Waldenstrom's Macroglobulinemia, precursor B-cell lymphoblastic lymphoma, primary central nervous system (CNS) lymphoma, and AIDS-related lymphoma. Additional non-Hodgkin's lymphomas are contemplated within the scope of the present invention and apparent to those of ordinary skill in the art.

[00218] In some embodiments, the cancer is a T cell lymphoma. In some embodiments, the T cell lymphoma is extranodal T cell lymphoma, cutaneous T cell lymphomas (CTCL), peripheral T-cell lymphoma (PTCL), Sezary syndrome, mycosis fungoides, anaplastic large cell lymphoma, or angioimmunoblastic T cell lymphoma.

[00219] In some embodiments, the subject is suffering from an autoimmune disease, e.g., inflammatory bowel disease, arthritis, lupus, rheumatoid arthritis, psoriatic arthritis, osteoarthritis, Still's disease, juvenile arthritis, diabetes, myasthenia gravis, Hashimoto's thyroiditis, Ord's thyroiditis, Graves' disease Sjogren's syndrome, multiple sclerosis, Guillain-Barre syndrome, acute disseminated encephalomyelitis, Addison's disease, opsoclonus-myoclonus syndrome, ankylosing spondylitisis, antiphospholipid antibody syndrome, aplastic anemia, autoimmune hepatitis, coeliac disease, Goodpasture's syndrome, idiopathic thrombocytopenic purpura, optic neuritis, scleroderma, primary biliary cirrhosis, Reiter's syndrome, Takayasu's arteritis, temporal arteritis, warm autoimmune hemolytic anemia, Wegener's granulomatosis, psoriasis, alopecia universalis, Beh$et's disease, chronic fatigue, dysautonomia, endometriosis, interstitial cystitis, neuromyotonia, scleroderma, or vulvodynia.

[00220] In other embodiments, the subject is suffering from a heteroimmune condition or disease, e.g., graft versus host disease, transplantation, transfusion, anaphylaxis, allergy, type I hypersensitivity, allergic conjunctivitis, allergic rhinitis, or atopic dermatitis.

[00221] In some embodiments, the subject has an inflammatory disease, e.g., asthma, appendicitis, blepharitis, bronchiolitis, bronchitis, bursitis, cervicitis, cholangitis, cholecystitis, colitis, conjunctivitis, cystitis, dacryoadenitis, dermatitis, dermatomyositis, encephalitis, endocarditis, endometritis, enteritis, enterocolitis, epicondylitis, epididymitis, fasciitis, f!brositis, gastritis, gastroenteritis, hepatitis, hidradenitis suppurativa, laryngitis, mastitis, meningitis, myelitis myocarditis, myositis, nephritis, oophoritis, orchitis, osteitis, otitis, pancreatitis, parotitis, pericarditis, peritonitis, pharyngitis, pleuritis, phlebitis, pneumonitis, pneumonia, proctitis, prostatitis, pyelonephritis, rhinitis, salpingitis, sinusitis, stomatitis, synovitis, tendonitis, tonsillitis, uveitis, vaginitis, vasculitis, or vulvitis.

[00222] In further embodiments, the subject is suffering from a thromboembolic disorder, e.g., myocardial infarct, angina pectoris, reocclusion after angioplasty, restenosis after angioplasty, reocclusion after aortocoronary bypass, restenosis after aortocoronary bypass, stroke, transitory ischemia, a peripheral arterial occlusive disorder, pulmonary embolism, or deep venous

thrombosis.

[00223] In some embodiments, the subject is administered or has been administered one or more therapeutic agents for treatment of a disease or condition. In some embodiments, the subject is administered or has been administered a BTK inhibitor for treatment of a disease or condition. In some embodiments, the subject is administered or has been administered one or more therapeutic agents in addition to a BTK inhibitor for treatment of a disease or condition.

[00224] In some embodiments, the subject is administered or has been administered one or more chemotherapeutic agents for treatment of cancer. In some embodiments, the subject is administered or has been administered a BTK inhibitor for treatment of a cancer. In some embodiments, the subject is administered or has been administered one or more chemotherapeutic agents in addition to a BTK inhibitor for treatment of cancer. In some embodiments, the BTK inhibitor is ibrutinib.

Additional features of methods, assays, and systems

[00225] Disclosed herein are additional features of the methods, assays, and systems disclosed herein. The efficacy of the methods, assays and systems disclosed herein are about comparable to current protein occupancy methods, assays and systems (e.g., gel-based assays). In some embodiments, the efficacy of the methods, assays and systems are better than the current protein occupancy methods. In some instances, the methods, assays and systems provide improved specificity as compared to current protein occupancy methods. For example, the assay offers good specificity when signals for the negative control Jurkat lysates labeled with probe were at background levels for all lysate concentrations tested. In some instances, the specificity is at least about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, or 99%.

[00226] In some instances, the methods, assays, and systems disclosed herein provide improved sensitivity. In some instances, improved sensitivity can be determined from the amount of sample required. In some instances, the methods, assays, and systems allows for the use of at least about 2 times less lysate, at least about 3 times less lysate, at least about 4 times less lysate, at least about 5 times less lysate, at least about 6 times less lysate, at least about 7 times less lysate, at least about 8 times less lysate, at least about 9 times less lysate, or at least about 10 times less lysate than current methods. In some embodiments, about 2-10 times less lysate, about 3-7 times less lysate, about 3-6 times less lysate is used than current methods. For example, the improved sensitivity of the assay allows for use of 3-5 times less lysate than Western blot/ELISA, and to conserve precious samples.

[00227] The methods, assays and systems disclosed herein are straight-forward. The methods, assays and systems disclosed herein are quicker than current methods (e.g., Western blot). The methods, assays and systems disclosed herein can be completed in less than about 10 hours, less

than about 8 hours, less than about 7 hours, less than about 6 hours, less than about 5 hours, less than about 4 hours. Preferably, methods, assays and systems disclosed herein can be completed in less than about 2-7 hours, less than about 3-6 hours, less than about 3-5 hours.

[00228] The methods, assays and systems disclosed herein also provide for increased throughput. In some embodiments, throughput is increased by at least about 5%, at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 40%, at least about 50%, or at least about 60%.

[00229] The methods, assays and systems disclosed herein also provide for more uniform test conditions. For example, more samples can be run on a plate-based assay than on a single gel. Therefore, samples on a single plate will have uniform test conditions than samples on multiple gels. Furthermore, the uniformity of multiple plate-based assays is greater than the uniformity of multiple gels. In some instances, the uniformity of test conditions is at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, or at least about 90% greater.

[00230] In some embodiments, the methods, assays, and systems disclosed herein enable the use of less probe than current methods (e.g., gel-based formats). In some embodiments, at least less than about lOx, 20x, 30x, 40x, 50x, 60x, 70x less probe is used than current methods (e.g., gel-based formats). For example, the assay requires 40x less probe than the gel based format thereby offering savings on reagents.

[00231] In some embodiments, the methods, assays, and systems disclosed herein provide a large signal window. In some embodiments, the large signal window comprises 50:1, 60:1, 70:1, 80:1, 90:1, 100:1, 200:1, 300:1, 400:1, 500:1, 600:1, 700:1, 800:1, 900:1, 1000:1 with 9 μg per well of lysate,

[00232] In some embodiments, the methods, assays, and systems disclosed herein provide good reproducibility. In some embodiments, reproducibility is about less than about 10% CV, less than about 8% CV, less than about 6% CV, less than about 5% CV, less than about 4% CV, less than about 3% CV. Preferably, reproducibility is less than about 4-6% CV.

[00233] Techniques and suggestions to optimize the methods, assays, and systems disclosed herein include, but are not limited to imaging the plates in sectors, energizing the sectors once, and avoiding introducing bubbles in samples, detection antibody and read buffer solution. Further ways to optimize the methods, assays, and systems disclosed herein comprise keeping detection antibody stocks in the dark. However, working solutions do not need to be shielded from light. Furthermore, when adding small volumes, in the order of 25 μL, add to the bottom corner of the wells. In some embodiments, the methods, assays, and systems disclosed herein comprise shaking the plates during blocking, sample incubation, detection antibody incubation.

For optimization, do not leave assays in wash buffer or read buffer for extended periods of time. However, if additional time is required, leave in sample or detection antibody. Preferably, polypropylene plates and tubes are used for sample handling. Preferably, polystyrene for sample handling should be avoided. In some embodiments, samples can be vortexed and/or centrifuged prior to contact with the probe. Vortexing and/or centrifugation of the sample can ensure removal of any debris in sample. In some instances, plates can be blocked overnight at 4°C. In some instances, plates can be blocked for lhour at room temperature. If blocking overnight, allow the plate to equilibrate to room temp before proceeding with the assay.

[00234] In some embodiments, the methods, assays and systems disclosed herein comprise the use of a partial plate. In some instances, instructions for partial plate usage can be provided. In some instances, the methods, assays and systems disclosed herein comprise allowing plates to equilibrate to room temperature before opening the plate package. For the SI2400 MSD SECTOR IMAGER, the MSD plate is divided into 24 sectors (2 x 2 = 4 wells/sector -see Figure 20). Sectors can be energized once and as such when using partial plates, samples should be allocated to sectors. In some instances, volumes for all reagents are adjusted based on the portion of plate used. In some instances, unused sectors should be covered with a plate seal and kept dry during the course of the assay. In some instances, a plate seal should be removed prior to reading the plate.

EXAMPLES

EXAMPLE 1. Comparison of an anti-protein antibody coated plate and a streptavidin coated plate assay formats

[00235] The aim of the study was to convert an existing gel-based BTK occupancy assay to a plate based electrochemiluminescent assay to increase assay throughput. The purpose of the assay is to determine the relative amount of BTK that has not been bound by the covalent inhibitor (ibrutinib) hereafter referred to as the "drug" or ibrutinib. The drug binds to the active site of BTK and forms a disulfide bond with a cysteine residue. Compound 1-5 hereafter referred to as the "probe" consists of the ibrutinib linked to biotin via a long chain linker. In the gel-based assay, the probe is labeled with a fluorescent reporter. Labeling of samples labeled by the probe allows for the detection of BTK not occupied by drug. Two potential assay formats are shown in Figure 11.

[00236] <u>**Experiment 1**</u>

[00237] In this study, assay formats were tested for sensitivity, specificity, range, and to determine most suitable anti BTK Antibody .

[00238] **Samples**

[00239] *Positive control:* An aliquot of DOHH2 cell lysate (1 mg/mL) was inhibited with 1

μM ibrutinib then labeled with probe (1 μM).

[00240] *Negative controls:* Untreated DOHH2 and Jurkat cell lysates (lmg/mL) were labeled with probe (1 μM) and Untreated DOHH2 cell lysate

[00241] Materials used:

[00242] Standard Streptavidin plate (5-pack) Catalog #L15SA-2; Read Buffer T (50 mL) Catalog # R92TC-3, SULFO TAG Goat Anti-mouse (50 μg) Catalog# R32AC-5; SULFO TAG Goat Anti-rabbit (50 μg) Catalog# R32AB-5; SULFO TAG streptavidin (50 ug) Catalog# R32AD-5; MSD Standard Plates Catalog # L15XA-3; MSD Blocker A Catalog# R93AA-2; Protease inhibitor cocktail (e.g., Thermo/Pierce Halt™ Protease Inhibitor Cocktail EDTA free Cat # 87785 or Roche Complete Mini Protease inhibitor tablet, Cat # 1836170); Positive Control lysates from BTK expressing cell line (DOHH2); Negative Control lysates (Jurkat); ibrutinib (PCI); probe compound 1-5 (biotinylated probe)

[00243] BTK Antibodies:

[00244] The following antibodies were tested:

	Species	**Cat#**	**Vendor**	**Buffer**	**Conc.**
a-BTK	Mouse	611116	BD	BSA, glycerol, azide	250 μg/mL
a-BTK	Mouse	611117	BD	BSA, glycerol, azide	250 μg/mL
a-BTK	Rabbit	SAB4502936	Sigma	unknown	1 mg/mL

[00245] Solutions:

[00246] Blocking solution: 3% (w/v) MSD Blocker A in 1x Tris Wash buffer: 3g Blocker A + 100 mL 1x Tris wash Buffer. Store at 4°C for up to 14 days. Blocking solution can also be prepared in PBS-T

[00247] Wash Buffer: 1x MSD Tris wash buffer: 50 mL 10x Tris Wash buffer + 450 mL H20 (150 mM NaCl 50 mM Tris-HCl pH 7.5 0.02% Tween-20). PBS-T can also be used as a wash buffer

[00248] Capture Antibody dilution buffer: PBS w/o Ca2+ w/o Mg2+

[00249] Detection Antibody dilution buffer: 1% MSD Blocker A in 1x Tris Wash Buffer: 10 mL Blocking solution + 20 mL 1x Tris Wash Buffer or 10 mL Blocking solution + 20 mL PBS-T

[00250] Read Buffer: 1x MSD Read Buffer T: per plate 5 mL 4x Read Buffer T + 15 mL H20; 2x MSD Read Buffer T: per plate 10 mL 4x Read Buffer T + 10 mL H20

[00251] Cell lysates: Lysates were prepared by repeated freeze-thaw of cell pellets resuspended in PBS + protease inhibitors. PCI and probe labeling reactions were carried out in PBS-T + 1% BSA (assay buffer). Lysates were diluted in assay buffer + protease inhibitors.

[00252] Format 1: Streptavidin Detection

[00253] Figure 11A is a schematic of the Streptavidin detection assay (e.g., Assay Format 1). Briefly, the method comprises contacting a drug-treated sample comprising a target (e.g., a BTK kinase) with an anti-BTK antibody coated plated, wherein the BTK kinase is captured by the anti-BTK antibody. The BTK kinases captured by the anti-BTK antibody include drug-occupied BTK kinases and unoccupied BTK kinases. The captured BTK kinases are contacted with a probe. The probe comprises an agent that binds to the unoccupied BTK kinases. In this example, the probe is unable to bind to drug-occupied BTK kinases. The probe binds to the unoccupied BTK kinases to found a probe-bound BTK kinase. In this example, the probe comprises a label, which enables detection of the probe-bound BTK kinases. The probe-bound BTK kinases are detected by the addition of a labeled bead (e.g., SULFO TAG Streptavidin). The amount of probe-bound kinases is quantified by electrochemiluminescence. The quantification of the probe-bound kinases enables the determination of occupancy of the BTK kinase and efficacy of the drug. A more detailed protocol is disclosed herein.

[00254] Protocol - Assay Format 1

[00255] 1. Coat MSD Standard plate with 30 μL per well of anti-BTK capture antibody solution (diluted to 2 μg/mL in PBS). After addition of the Ab solution to the bottom corner of the wells, tap the plate to ensure that the solution distributes evenly across the bottom of each well. Seal with an adhesive plate seal and incubate O/N at 4°C. Do not shake the plate.

[00256] 2. Tap out plate and add 150 μï¸ per well blocking solution (3% [w/v] Blocker A). Seal and incubate with shaking 1h or more at RT.

[00257] 3. Wash plate 1x with ≥ 150 μL per well wash buffer. Tap dry.

[00258] 4. Add 30 μL of lysate according to the plate layout. Add lysate solution to the bottom corner of the wells. Seal and shake plate at 300-500 rpm 1 h at RT.

[00259] 5. Wash plate 3x with ≥ 150 μL per well wash buffer. Tap dry.

[00260] 6. Add 25 μï¸ per well of SULFO TAG streptavidin diluted to 0.5 μg/mL in 1% (w/v) Blocker A according to the plate layout. Seal and shake plate at 300-500 rpm 30-60 min at RT.

[00261] 7. Wash plate 3x with ≥ 150 μL per well 1x Tris wash buffer. Tap dry

[00262] 8. Add 150 μL per well 1x Read Buffer T (diluted in H20). Avoid bubbles - use reverse pipetting when adding Read Buffer. Read plate in SI2400

[00263] The plate layout and results are shown in Figure 12.

[00264] The signal of the positive control (DOHH2 + probe) titrates with the lysate concentration for all three capture antibodies tested. The highest signals were obtained using BD61 1116 and BD61 1117 anti-BTK capture antibodies. The BD61 1116 and BD61 1117 anti-BTK capture antibodies were comparable.

[00265] A maximum signal: background ratio for the positive control (DOHH2+ probe) of 125:1 was obtained with 1 μg/μL positive control lysate using the BD61 116 anti-BTK capture antibody.

[00266] Background was low for all concentrations of the DOHH2 (no probe) lysate tested indicating that there is no non-specific binding of SULFO TAG streptavidin to lysate proteins.

[00267] The positive control (DOHH2 + probe) lysate signals can be differentiated from the negative controls (DOHH2 + PCI + probe) and (Jurkat + probe) when using BD 611 116 (see Figure 13A) and BD61 1117 (see Figure 13B) anti-BTK capture antibodies but not when using the Sigma anti-BTK as capture antibody suggesting that the Sigma anti-BTK can bind other proteins in the cell lysates labeled by probe. The specificity ratios of positive :negative controls are shown in Table 1.

Table 1. Specificity ratios of positive: negative controls

Ratio	DOHH2 +Probe: Probe + PCI		
	Capture AB		
Lysate ug/ml	BD11116	BD1117	Sigma
1000	2.45	2.53	1.21
500	2.61	2.36	1.04
250	3.04	2.55	0.97
125	3.48	2.96	1.06
62.5	3.76	2.83	1.02
31.25	3.25	2.52	1.05
15.63	2.60	2.22	0.94
0	0.96	0.97	1.06

Ratio	DOHH2 +Probe: Jurkat + probe		
	Capture AB		
Lysate ug/ml	BD11116	BD1117	Sigma
1000	2.58	2.73	1.08
500	2.97	2.57	1.04
250	3.54	2.94	0.97
125	4.19	3.58	1.04
62.5	4.39	3.42	1.01
31.25	3.44	2.78	1.05
15.63	2.87	2.44	1.00
0	0.96	1.13	0.96

[00268] The Sigma anti-BTK antibody was not used in follow up experiments.

[00269] **Format 2: Streptavidin Capture**

[00270] Figures 1IB and 14A show schematics of the streptavidin-coated plate assay (e.g., Assay Format 2). Briefly, the method comprises blocking a MSD standard streptavidin plate with a probe or providing a probe-bound plate. In this example, the probe comprises a label and an agent, wherein the label (e.g., biotin) is captured by the streptavidin, thereby attaching the probe to the plate. A sample comprising a target (e.g., BTK) is applied to the plate. The target binds to or attaches to the probe via the agent (e.g., a drug such as a BTK inhibitor) to form a probe-bound compound. The probe-bound targets can be detected by a primary detection agent such as anti-target (e.g., anti-BTK) antibody. The primary detection reagent can be labeled and subsequently directly detected. As shown in Figure 1IB, the primary detection reagent is conjugated to a SULFO TAG to form a labeled primary detection reagent. However, as shown in Figure 14A, the primary detection reagent can be unlabeled. The method can further comprise

the addition of a secondary detection agent (e.g., anti-species antibody), as shown in Figure 14A. The secondary detection agent can be labeled (e.g., SULFO TAG anti-species antibody) and subsequently detected. The SULFO-labeled detection agents can be detected by electrochemiluminescence, which enables quantification of the probe-bound kinases. The quantification of the probe-bound kinases enables the determination of occupancy of the BTK kinase and efficacy of the drug. A more detailed protocol is disclosed herein.

[00271] Protocol - Assay Format 2

[00272] 1. Add 150 μL per well blocking solution (3% [w/v] Blocker A) to MSD standard streptavidin plate. Seal and incubate with shaking 1h or more at RT or block overnight at 4°C.

[00273] 2. Wash plate 1x with ≥ \50 μL per well wash buffer. Tap dry.

[00274] 3. Add *30* μï, of lysate according to the plate layout. Add lysate solution to the bottom corner of the wells. Seal and shake plate at 300-500 rpm 1h at RT.

[00275] 4. Wash plate 3x with ≥ 150 μL per well wash buffer. Tap dry.

[00276] 5. Add 25 μï, per well of anti-BTK antibody diluted to 0.5 μg/mL in 1% (w/v) Blocker A according to the plate layout. Seal and shake 1h at RT.

[00277] 6. Wash plate 3x with ≥ 150 μL per well wash buffer. Tap dry. Add 25 μL per well of SULFO TAG conjugated anti-species antibody diluted to 1 μg/mL in 1% (w/v) Blocker A according to the plate layout. Seal and shake plate 1h at RT.

[00278] 7. Wash plate 3x with ≥ 150 μL per well 1x Tris wash buffer. Tap dry

[00279] 8. Add 150 μL per well 1x Read Buffer T (diluted in H20). Avoid bubbles - use reverse pipetting when adding Read Buffer. Read plate in SI2400

Results

[00280] The plate layout and results are shown in Figure 14B-C.

[00281] The signal of the positive control (DOHH2 + probe) titrates with the lysate concentration when using BD6 11116 and BD6 11117 anti-BTK as detection antibodies up to 62 μg/mL lysate after which there is a hook effect possibly due to the presence of excess probe competing with probe-labeled BTK for the streptavidin surface. The hook occurs when final probe concentration in the sample is > 62 nM.

[00282] A maximum signal: background ratio for the positive control (DOHH2+ probe) of 240: 1 was obtained with 0.06 μg/μL positive control lysate using the BD61 116 anti-BTK detection antibody.

[00283] Background was low for all concentrations of the DOHH2 (no probe) lysate tested when using BD61 1116 and BD61 1117 anti-BTK as detection antibodies indicating that there is no non-specific binding of these antibodies to lysate proteins. In contrast, the Sigma anti-BTK antibody yielded high background signals for all conditions tested.

[00284] The positive control (DOHH2 + probe) lysate signals can be differentiated from the negative controls (DOHH2 + PCI + probe) and (Jurkat + probe) when using BD 611116 (see Figure 15A) and BD6 1111 7(see Figure 15B) anti-BTK detection antibodies but not when using the Sigma anti-BTK as detection antibody suggesting that the Sigma anti-BTK can bind other proteins in the cell lysates labeled by probe. Specificity ratios of positive:negative controls are shown in Table 2.

Table 2. Specificity ratios of positivemegative controls

Ratio DOHH2 +Probe: Probe + PCI

Lysate ug/ml	Detection AB BD11116	BD1117	Sigma
62.5	21.8	21.2	1.0
31.3	31.5	25.7	1.0
15.6	19.5	12.0	1.0
0.0	0.9	0.9	1.0

Ratio DOHH2 +Probe: Jurkat + probe

Lysate ug/ml	Detection AB BD11116	BD1117	Sigma
62.5	28.5	31.2	1.0
31.3	47.1	33.8	1.0
15.6	24.7	14.6	1.0
0.0	1.0	1.0	1.0

[00285] The Sigma anti-BTK antibody was not used in follow up experiments.

[00286] Signals for the negative controls are comparable to the signals from the 1 μM probe only wells.

[00287] The BD61 1116 and BD6 11117 anti-BTK detection antibodies were comparable.

[00288] Figure 16 shows a comparison of Assay format 1 (streptavidin-detection method) and Assay format 2 (streptavidin-capture method). As shown in Figure 16, Assay format 2 affords better sensitivity and better specificity than assay format.

[00289] **Experiment 2 - Optimizing probe concentration**

[00290] Assay format 2 (e.g., streptavidin-coated plate, streptavidin-capture method -see Figure 14A) was used for further optimization. Since a hook effect was observed at probe concentration > 62 nM and lysate concentration > 62 μg/mL, a checkerboard experiment was carried out to determine if the hook effect is linked to excess probe or excess protein concentration. Excess unconjugated probe can compete with BTK-bound probe for binding to the streptavidin surface once the binding capacity of the plate is reached.

[00291] **Aims:**

[00292] Determine optimal probe concentration required

[00293] Compare different probe: lysate ratios

[00294] **Samples**

[00295] Positive control: prepare DOHH2 lysate at 1 mg/mL in assay buffer.

[00296] Negative control: treat an aliquot of 1 mg/mL DOHH2 lysate with 1 μM PCI to have an excess of PCI so that BTK is maximally bound by PCI.

[00297] Dilute DOHH2 only and DOHH2 + PCI lysates to 100; 50; 25; 12.5; 6.25 and 3.12 µg/mL in assay buffer

[00298] Dilute probe to 10000; 2500; 625; 156; 39; 9 and 2 nM (lOOx concentration)

[00299] Add 1 µL lOOx probe to 100 µL diluted lysates in a 96 well polypropylene plate, incubate

[00300] **Protocol**

[00301] 1. Block MSD standard streptavidin plate with 150 µL per well 3% Blocker A 1h at RT or overnight at 4°C.

[00302] 2. Wash plate 1x with ≥ \50 µï, per well wash buffer. Tap dry.

[00303] 3. Add 50 µL of lysate according to the plate layout. Add lysate solution to the bottom corner of the wells. Seal and shake plate at 300-500 rpm 1h at RT.

[00304] 4. Wash plate 3x with ≥ 150 µL per well wash buffer. Tap dry.

[00305] 5. Add 25 µï, per well of BD61 1117 anti-BTK antibody diluted to 0.5 µg/mL in 1% (w/v) Blocker A. Seal and shake 1h at RT.

[00306] 6. Wash plate 3x with ≥ 150 µL per well wash buffer. Tap dry.

[00307] 7. Add 25 µL per well of SULFO TAG conjugated anti-mouse antibody diluted to 1 µg/mL in 1% (w/v) Blocker A. Seal and shake plate 1h at RT.

[00308] 8. Wash plate 3x with ≥ 150 µL per well 1x Tris wash buffer. Tap dry

[00309] 9. Add 150 µL per well 2x Read Buffer T (diluted in H20). Avoid bubbles - use reverse pipetting when adding Read Buffer. Read plate in SI2400

[00310] **Results:**

[00311] Results for the optimization experiment are shown in Figure 17.

[00312] Backgrounds are low for the negative control (DOHH2 + 1 µM PCI) samples up to 25 nM probe.

[00313] Above 25 nM probe, backgrounds increase. One possible explanation for this phenomenon is signal contribution from other proteins in the lysate also labeled with probe at high probe concentration as seen in the gel-based assay and/or signal contribution from the probe alone.

[00314] For the positive control (untreated DOHH2), signals increase with increasing probe concentration up to 25 nM, above which signals decrease or plateau when there is an excess of probe.

[00315] A final probe concentration of 25 nM is recommended.

[00316] **Experiment 3: Inhibition with Drug Titration**

[00317] **Aim:** Carry out inhibition experiment with titration series of PCI then label lysates with a final probe concentration of 25 nM probe.

[00318] **Samples:**

[00319] Positive control: DOHH2 lysate at 1 mg/mL in assay buffer.

[00320] Negative control: Jurkat lysate at 1 mg/mL in assay buffer.

[00321] Dilute lysates to 300; 150 and 75 µg/mL in assay buffer

[00322] Prepare a dilution series of PCI. The 100x PCI concentrations are: 100; 25; 6.25; 1.56; 0.39; 0.09 and 0.02 µM.

[00323] Treat DOHH2 lysates with PCI in a polypropylene plate e.g., 100 µL lysate + 1 µL 100x PCI solution

[00324] After the PCI inhibition add probe to all the samples to a final concentration of 25 nM (2 µL of 1.25 µM probe stock + 100 µL sample), incubate with shaking.

[00325] **Protocol:**

[00326] 1. Block MSD standard streptavidin plate with 150 µL per well 3% Blocker A 1h at RT or overnight at 4°C.

[00327] 2. Wash plate 1x with ≥ 150 µL per well wash buffer. Tap dry.

[00328] 3. Add 30 µL of lysate according to the plate layout. Add lysate solution to the bottom corner of the wells. Seal and shake plate 1h at RT.

[00329] 4. Wash plate 3x with ≥ 150 µL per well wash buffer. Tap dry.

[00330] 5. Add 25 µL per well of BD61 1117 anti-BTK antibody diluted to 0.5 µg/mL in 1% (w/v) Blocker A. Seal and shake 1h at RT.

[00331] 6. Wash plate 3x with ≥ 150 µL per well wash buffer. Tap dry. Add 25 µL per well of SULFO TAG conjugated anti-mouse antibody diluted to 1 µg/mL in 1% (w/v) Blocker A. Seal and shake plate 1h at RT.

[00332] 7. Wash plate 3x with ≥ 150 µL per well 1x Tris wash buffer. Tap dry

[00333] 8. Add 150 µL per well 1x Read Buffer T (diluted in H20). Avoid bubbles - use reverse pipetting when adding Read Buffer. Read plate in SI2400.

[00334] **Results:**

[00335] Results are shown in Figures 18-19

[00336] Signals of the negative control Jurkat lysates labeled with probe remained at background levels for all conditions tested.

[00337] A dose dependent decrease in signal was observed for positive control DOHH2 lysates treated with a titration series of PCI then labeled with 25 nM probe.

[00338] The PCI inhibition profile was comparable for all three concentrations of DOHH2 lysates tested (300, 150 and 75 µg/mL) and comparable to the reference gel based assay.

[00339] The assay offers a large signal window at all lysate concentrations tested:

Table 3. Signal ratio of 0: 1000 nM PCI

DOHH2 Lysate		Signal Window
ug/well		ug/mL
9	300	108
4.5	150	45
2.25	75	23

[00340] Assay reproducibility was very good with average %CV < 5%. The assay allowed for high specificity (good distinction from negative controls), sensitivity (use of less lysate compared to western blot methods (0.6 μg vs. 50 μg)) and efficiency (required less probe than the gel based formats (25 nM vs. 2.5 μM)).

EXAMPLE 2. Protein Occupancy Assay Protocol

[00341] An alternative protein occupancy assay protocol is provided.

[00342] 1. Add probe to the samples in assay buffer to a final concentration of 25 nM (2 μL of 1.25 μM probe stock + 100 μL sample), incubate with shaking.

[00343] 2. Block MSD standard streptavidin plate with 150 μL per well 3% Blocker A 1h at RT or overnight at 4°C.

[00344] 3. Wash plate 1x with ≥ 150 μL per well wash buffer. Tap dry.

[00345] 4. Add 30 μL of lysate per well. Add lysate solution to the bottom corner of the wells. Seal and shake plate 1h at RT.

[00346] 5. Wash plate 3x with ≥ 150 μL per well wash buffer. Tap dry.

[00347] 6. Add 25 μL per well of BD61 1117 or BD61 1116 anti-BTK antibody diluted to 0.5 μg/mL in 1% (w/v) Blocker A. Seal and shake 1h at RT.

[00348] 7. Wash plate 3x with ≥ 150 μL per well wash buffer. Tap dry. Add 25 μL per well of SULFO TAG conjugated anti-mouse antibody diluted to 1 μg/mL in 1% (w/v) Blocker A. Seal and shake plate 1h at RT.

[00349] 8. Wash plate 3x with ≥ 150 μL per well 1x Tris wash buffer. Tap dry

[00350] 9. Add 150 μL per well 1x Read Buffer T (diluted in H20). Avoid bubbles - use reverse pipetting when adding Read Buffer. Read plate in SI2400.

[00351] If desired, the following parameters can further be optimized (e.g., concentration of the anti-BTK detection antibody, concentration of the MSD SULFO TAG anti-mouse secondary detection antibody, or combining the anti-BTK detection antibody and the MSD SULFO TAG anti-mouse secondary detection antibody in a single incubation step).

EXAMPLE 3. Comparison of selected biotinylated probes in anti-protein antibody coated plate and a streptavidin coated plate assay formats

[00352] Selected biotinylated probes were tested for use in target occupancy assays using

either the anti-protein antibody coated plate or streptavidin coated plate assay formats. The following probes were tested:

[00353] Compound 1-1, MW = 808.01 (total- 80 mg)

[00354] Compound 1-2, MW = 793.38 (total- 60 mg)

[00355] Compound 1-3, MW = 780.98 (total- 32 mg)

[00356] Compound 1-4, MW = 766.95 (total- 27 mg)

[00357] Compound 1-5, MW = 1097.37 (total- 24 mg)

[00358] The structures of the probes tested are shown in Figure 21.

[00359] Stock solutions (5 **mM** and 10 **mM)** of the probes were prepared as follows. Compound 1-1, 1-2, 1-3, and 1-4: 2 or 4 mg probe in 500 µΐ DMSO and Compound 1-5: 12 or 24 mg in 2.2 ml DMSO, aliquots in 100 µΐ. For the assays 50x probe was prepared fresh the day of the assay: 1 µΐ of 5 mM probe to 39 µΐ PBS.

[00360] The following reagents were employed for the assays: BTK antibody: BD Biosciences 611 117; 2nd Antibody: Goat anti-mouse-HRP, Santa Cruz, SC-2302; Streptavidin-HRP, Thermo, Cat# 2 1130, 0.5 ml, 1-step Ultra TMB-ELISA Substrate, Thermo, cat# 34028, 250 ml; Stop solution: 0.16 M H2S04, Thermo, Cat# N600, 55 ml; Streptavidin coated plate: Thermo, cat# 15500, 5 plate; DOHH2 control lysate; Wash buffer: 0.05% PBST; 1% BSA.

[00361] A standard curve was performed according to the following protocol:

[00362] 1) Add 1 µΐ of 50x probe to 50 µΐ lysate (lug/ µΐ), incubate at 37C for 1 hr

[00363] 2) Block Streptavidin coated plate with 1% BSA at RT for 1 hr, wash w/ PBST, 3x

[00364] 3) Stop reaction by transfer samples onto ice for 10 min, then bring back to RT

[00365] 4) Make series dilution of probe labeled DOHH2 lysate with 1% BSA in an order of lug, 0.5µg, 0.25ug, 125ng, 62.5ng, 31.25ng, 15.6ng/µl to volume of 500 µΐ.

[00366] 5) Add 100 µΐ probe treated lysate to each well in triplicate

[00367] 6) Incubate at RT for 2 hrs

[00368] 7) Wash with PBST, 7x

[00369] 8) Add 2nd Ab-HRP, incubate at RT for 1 hr

[00370] 9) Wash with 200 µΐ PBST, 5x

[00371] 10) Add 100 µΐ TMB, incubate at RT for 15 min

[00372] 11) Add 50 µΐ stop solution

[00373] 12) Read at 450 nm

[00374] **Protocol Format 1: (Streptavidin coated plate)**

[00375] 1) Dilute lysate to desired concentration with PBS to final volume of 500 µΐ

[00376] 2) Add Biotinylated probe (final concentration 2.5 µM) to lysate, 37C for 1 hr

[00377] 3) Block streptavidin plate w/ 1% BSA, RT, 1 hr, wash w/ PBST 3x

[00378] 4) Stop reaction by transfer samples onto ice for 10 min, then bring back to RT

[00379] 5) Add lOOul treated lysate (in triplicate) to streptavidin plate, RT, 2 hr w/ rocking

[00380] 6) Wash with 200 µΐ PBST, 5x

[00381] 7) Add 100 µΐ 2nd Ab-HRP, incubate at RT for 1 hr

[00382] 8) Wash with 200 µΐ PBST, 3x

[00383] 9) Add 100 µI TMB, incubate at RT for 10 min

[00384] 10) Add 50 µΐ stop solution

[00385] 11) Read at 450 nm

[00386] **Protocol Format 2: (BTK antibody coated plate)**

[00387] 1) Coat 96 well- plate with 100 µΐ anti-BTK (1:1000 PBS), 4C, o/n

[00388] 2) Wash plate with PBST, 5x

[00389] 3) Block plate for lhr at RT with rocking, wash w/ PBST 3x

[00390] 4) Dilute lysate to desired concentration with PBS to final volume of 500 µΐ

[00391] 5) Add Biotinylated probe (final concentration 2.5 µM) to lysate, 37C for 1 hr

[00392] 6) Stop reaction by transfer samples onto ice for 10 min, then bring back to RT

[00393] 7) Add 100 µΐ treated lysate (in triplicate) to streptavidin plate, RT, 2 hr w/ rocking

[00394] 8) Wash with 200 µΐ PBST, 5x

[00395] 9) Add streptavidin-HRP, incubate at RT for 1 hr with rocking

[00396] 10) Wash with 200 µΐ PBST, 3x

[00397] 11) Add 100 µΐ TMB, incubate at RT for 15 min

[00398] 12) Add 50 µΐ stop solution

[00399] 13)Read at 450 nm

[00400] **Results:**

[00401] Exemplary results from the occupancy assays comparing the various probes are shown in Figure 22 for high and low probe concentrations for the streptavidin assay. The data demonstrated that Compound 1-5 exhibited the highest signal for binding to BTK out of the probes tested in the assay.

EXAMPLE 4. Comparison of kinase inhibitor activity of Compound 1-5 and parent compound ibrutinib

[00402] The ability of Compound 1-5 to inhibit TEC family kinases and homologous tyrosine kinases was assayed *in vitro* using a standard kinase inhibition assay performed by Nanosyn. The following kinases were assayed according the table 4 below:

Table 4.

Target	Vendor	Cat#	Lot	[Enzyme], nM	[ATP], μM	Inc time, hr
BLK	BPS	40401	111102	0.4	20	1
BMX	BPS	40402	111031-5	1.82	75	1
BTK	Millipore	14552	D7HN091U	0.95	20	1
ITK	Carna	08-181	10CBS-1259E	1	50	3
JAK3	INVITROGEN	PV3855	716673	2	2	3
LCK	INVITROGEN	P3043	850070F	0.4	50	1
SRC	INVITROGEN	P3044	26726B	3	25	1
TEC	Millipore	14-801	D8PN044U	4.6	12	1

[00403] Compounds were tested in singlicates using 12 pt (3x-dilutions) dose response format with top concentration of 10 mM or 1 mM.

[00404] Results are presented in Table 5. The data demonstrates that the Compound 1-5 probe exhibited an IC50 profile for TEC family kinase inhibition similar to the parent compound ibrutinib.

Table 5

Compound ID	BLK IC50 (μM)	BMX IC50 (μM)	ITK IC50 (μM)	JAK3 IC50 (μM)	LCK IC50 (μM)	SRC IC50 (μM)	TEC IC50 (μM)	BTK IC50 (μM)
Staurosporin	0.00213	0.0128	0.00569	0.000825	0.00227	0.00662	0.0129	0.0189
Ibrutinib	0.000362	0.00109	0.0623	0.0849	0.00745	0.0242	0.000643	0.000248
Compound I-5	0.00427	0.00557	0.198	4.87	0.00692	0.0363	0.00229	0.00102
Compound I-5	0.00366	0.00442	0.187	5.34	0.00599	0.0338	0.00171	0.000755

EXAMPLE 5. Development of Total BLK Protein Assays

[00405] The aim of this study was to demonstrate a method to screen antibodies against BLK to develop a sandwich immunoassay for quantifying total BLK in clinical samples on the MSD platform.

[00406] Materials used: MSD ELISA Conversion pack I (Catalog #K15A01-1) which contains: 96-well High Bind Plates, 96-well Standard Plates, SULFO-TAG™ NHS Ester, 150 nmoles, 4 Spin Columns, SULFO-TAG Labeled Streptavidin, 50 μg, SULFO-TAG Labeled Anti Mouse Antibody, 50 μg, SULFO-TAG Labeled Anti Rabbit Antibody, 50 μg, Blocker A Kit, 1 L, Blocker B, 2 g, Read Buffer T (4X), 200 mL.

[00407] The following BLK antibodies were tested:

Table 6

Cat. #	Vendor	Target	Species Reactivity	Host Species	Description	Formula-tion	Applica-tion tested	Conc.
AF2679	R & D	BLK	H,M,Rt	Goat	BLK Affinity Purified goat Polyclonal Ab	Lyoplilized from PBS solution	WB	1mg/ml
TA300020	Origene	BLK	H,M	Rabbit	Purified BLK Rabbit Polyclonal Antibody	PBS with Sodium Azide	WB	0.25mg/ml
H00000640-M02	Novus Biologicals	BLK	H,M,Rt	Mouse	IgG purified Mouse	PBS	WB, ELISA	
ab37830	Abcam	BLK	H,M	Rabbit	purified Rabbit Polyclonal	PBS with Sodium Azide	WB, ELISA, IHC	0.25mg/ml

[00408] Solutions:

[00409] Blocking solution: 3% (w/v) MSD Blocker A in 1x PBS: 3g Blocker A + 100 mL PBS. Store at 4°C for up to 14 days.

[00410] Wash Buffer: PBS-T (PBS + 0.05% Tween-20)

[00411] Capture Antibody dilution buffer: PBS w/o Ca2+ w/o Mg2+

[00412] Capture Antibody: Prepare 1 ml of 4μg/ml solution of each antibody in Capture Antibody dilution buffer

[00413] Detection Antibody dilution buffer: 1% MSD Blocker A in 1x PBS: 10 mL Blocking solution + 20 mL 1x PBS

[00414] Detection Antibody: Prepare 1-2ml of 2μg/ml solution of each antibody in 1% MSD Blocker A in 1x PBS

[00415] SULFO-TAG labeled Secondary Antibody: Prepare 3 ml each of 1μg/ml solution of anti-mouse and anti-rabbit SULFO-TAG antibody in 1% MSD Blocker A in 1x PBS

[00416] Read Buffer: 1x MSD Read Buffer T: per plate 5 mL 4x Read Buffer T + 15 mL H20 Mix by inversion, do not vortex.

[00417] Standards: Prepare a 10μg/ml stock solution of the recombinant BLK in 1% Blocker A. Then prepare the following dilutions for standard curve. Use a fresh tip for each dilution. Vortex well after each dilution.

[00418] Methods:

[00419] **<u>Experiment 1</u>**

[00420] Aims: Screen Antibodies to identify a suitable antibody pair for further assay development

[00421] Protocol:

[00422] 1. Solution coat one each of MSD high bind plate and Standard plate with 25 µï per well of the capture antibody solution (4µg/ml) as per the plate layout (step 1). Add antibody solution to the corner of the wells and gently tap the plate to disperse the liquid evenly, seal the plate and incubate O/N at 4°C without shaking.

[00423] 2. Tap out the plate next day and add 150 µï_ per well blocking solution (3% [w/v] Blocker A). Seal and incubate with shaking 1h or more at RT.

[00424] 3. Wash plate 1x with ≥ 150 µL per well PBS-T. Tap dry.

[00425] 4. Add 25 µL of recombinant protein diluted according to plate layout (step 2). Add solution to the bottom corner of the wells. Seal and shake plate at 300-500 rpm 1-2h at RT.

[00426] 5. Wash plate 3x with ≥ 150 µL per well PBS-T. Tap dry.

[00427] 6. Add 25 µL of detection antibody solution according to the plate layout (step 3). Add Ab solution to the bottom corner of the wells. Seal and shake plate at 300-500 rpm 1h at RT.

[00428] 7. Wash plate 3x with ≥ 150 µL per well PBS-T. Tap dry.

[00429] 8. Add 25 µL of SULFO-TAG anti-species detection Ab diluted to 1 µg/mL in detection antibody diluent according to the plate layout Step 3. Seal and shake plate at 300-500 rpm for 30 min at RT.

[00430] 9. Wash plate 3x with ≥ 150 µL per well PBS-T. Tap dry

[00431] 10. Add 150 µL per well 1x Read Buffer T (diluted in H20). Avoid bubbles - use reverse pipetting when adding Read Buffer. Read plate immediately in SI2400.

[00432] Results:

[00433] Raw signals for different antibody pairs are shown in Figure 23. Signal to background (S/B) for different antibody pairs are shown in Figure 24, the highest S/B ratio was observed for H00000640-M02. A nice titration of signal and S/B with protein concentration was observed for four antibody pairs for BLK. The antibody pair (AF2679 as capture and H00000640-MO2 as detect) and orientation with the highest S/B ratio was selected for further optimization of total BLK assay. For the same antibody pairs, higher S/B ratio was observed on standard plates.

[00434] **<u>Experiment 2</u>**

[00435] Aims:

[00436] 1) Develop assay conditions for BLK assay using AF2679 as capture and H00000640-M02 as detection antibody

[00437] 2) Test positive (DOHH2) and negative (Jurkat) cell lysates for BLK expression

[00438] Antibodies were conjugated to SULFO-TAG™ NHS Ester according to manufacturers instructions (Meso Scale Discovery).

[00439] Protocol:

[00440] 1. Solution coat MSD Standard plate with 25 µï per well of the capture antibody solution (4µg/ml) as per the plate layout . Add antibody solution to the corner of the wells and gently tap the plate to disperse the liquid evenly seal the plate and incubate O/N at 4°C without shaking. 2. Tap out the plate next day and add 150 µL per well blocking solution (5% [w/v] Blocker A). Seal and incubate with shaking 1h or more at RT.

[00441] 3. Wash plate 1x with ≥ \50 µL per well PBS-T. Tap dry.

[00442] 4. Add 25 µL of recombinant protein diluted according to plate layout. Add solution to the bottom corner of the wells. Seal and shake plate at 300-500 rpm l-2h at RT.

[00443] 5. Wash plate 3x with ≥ 150 µL per well PBS-T. Tap dry.

[00444] 6. Add 25 µL of detection antibody solution according to the plate layout . Add Ab solution to the bottom corner of the wells. Seal and shake plate at 300-500 rpm 1h at RT.

[00445] 7. Wash plate 3x with ≥ 150 µL per well PBS-T. Tap dry.

[00446] 8. Add 150 µL per well 1x Read Buffer T (diluted in H20) to plate 2. Avoid bubbles - use reverse pipetting when adding Read Buffer. Read plate immediately in SI2400.

[00447] **9.** Add 25 µL of SULFO-TAG anti-species detection Ab diluted to 1 µg/mL in detection antibody diluent according to the plate layout to plate 1. Seal and shake plate at 300-500 rpm for 30 min at RT.

[00448] 10. Wash plate 1 3x with ≥ 150 µL per well PBS-T. Tap dry.

[00449] 11. Add 150 µL per well 1x Read Buffer T (diluted in H20). Avoid bubbles - use reverse pipetting when adding Read Buffer. Read plate immediately in SI2400.

[00450] Results:

[00451] Raw signals for the assay are shown in Figure 25. Signal to background (S/B) for the assay is shown in Figure 26. The BLK assay demonstrated signal titration with protein concentration. The signal values for recombinant BLK protein using 1µg/ml capture antibody and 0^g/ml detection antibody are plotted in Figure 27. Positive to negative ratio for BLK in cell lysates is shown in table below:

Table 7

Capture (ug/ml)	0.5	1	2
10µg	1.49	1.52	1.91
5µg	1.35	1.27	1.45
2.5µg	1.05	1.15	1.17
Detect (ug/ml)	1		

[00452] A signal window of up to 46 fold was observed for BLK assay with 1μg/ml capture antibody (AF2679, R&D) and O^g/ml (H00000640-MO2, Novus Biologicals) detection antibody. The dynamic range of the assay appears to be ~3.51ogs. P/N ratio of ~2 fold for BLK was observed in cell lysates using partially optimized conditions.

EXAMPLE 6: ITK and BLK Protein Occupancy Assays

[00453] The aim of the study was to optimize the probe required for ITK and BLK occupancy assay on MSD Streptavidin plates and to demonstrate the assay performance using drug treatment of lysates. The purpose of the assay is to determine the relative amount of ΓTK and BLK that has not been bound by the covalent inhibitor hereafter referred to as the "drug". The "probe" consists of the drug linked to biotin via a long chain linker. Labeling of samples with the probe allows for the detection of ITK and BLK not occupied by drug. The assay format that has previously been successfully used with BTK is capturing the probe labeled protein on Streptavidin plates and detecting using anti-protein antibodies. A similar format was tested for ITK and BLK occupancy assays,

[00454] **Materials:**

[00455] Standard Streptavidin plate (5-pack) Catalog #L15SA-2, Read Buffer T (50 mL) Catalog # R92TC-3, SULFO-TAG Goat Anti-mouse (50 μg) Catalog# R32AC-5, SULFO-TAG Goat Anti-rabbit (50 μg) Catalog# R32AB-5, SULFO-TAG streptavidin (50 ug) Catalog# R32AD-5, MSD Blocker A Catalog# R93AA-2, Protease inhibitor cocktail (e.g., Thermo/Pierce Halt™ Protease Inhibitor Cocktail EDTA free Cat # 87785 or Roche Complete Mini Protease inhibitor tablet, Cat # 1836170), Positive Control lysates from ITK and BLK expressing cell line (DOHH2 and JURKAT), Negative Control lysates (THP-1 cells), BLK, ITK inhibitor drug ibrutinib "PCI"; Biotinylated probe Compound 1-5 "probe".

[00456] **Solutions:**

[00457] Blocking solution: 3% (w/v) MSD Blocker A in 1x Tris Wash buffer: 3g Blocker A + 100 mL 1x Tris wash Buffer. Store at 4°C for up to 14 days. Blocking solution may also be prepared in PBS-T

[00458] Wash Buffer: 1x MSD Tris wash buffer: 50 mL 10x Tris Wash buffer + 450 mL H20 (150 mM NaCl 50 mM Tris-HCl pH 7.5 0.02% Tween-20). PBS-T can also be used as a wash buffer

[00459] Detection Antibody dilution buffer: 1% MSD Blocker A in 1x Tris Wash Buffer: 10 mL Blocking solution + 20 mL 1x Tris Wash Buffer or 10 mL Blocking solution + 20 mL PBS-T

[00460] Read Buffer: 1x MSD Read Buffer T: per plate 5 mL 4x Read Buffer T + 15 mL

H20

[00461] Cell lysates: Lysates prepared by repeated freeze-thaw of cell pellets resuspended in PBS + protease inhibitors.

[00462] Assay Buffer: 1% blocker in Tris wash buffer + protease inhibitors

[00463] **Experiment 1 - Optimizing probe concentration**

[00464] **Aims:** Determine optimal probe concentration required and compare different probe: lysate ratios

[00465] **Samples:**

[00466] Positive control: DOHH2 and Jurkat cell lysates

[00467] Negative control: DOHH2 and Jurkat cells treated with luM PCI

[00468] Dilute DOHH2 only and DOHH2 + PCI lysates to 600; 300; 150; 75; 37.5;18.75ug/mL in assay buffer

[00469] Dilute probe to 6000; 1500; 375; 93.8; 23.4; 5.9 and 1.5 nM (50x concentration)

[00470] Add 1 µL IOOχ probe to 50 µL diluted lysates in a 96 well polypropylene plate, incubate

[00471] **Protocol:**

[00472] 1) Block MSD plates with 150 µï 3% Blocker A for 1-3h at RT with shaking at 900 rpm.

[00473] 2) Incubate titration of protein lysates with titration of probe in 50ul volume in a separate 96 well polypropylene plate for 1h with shaking.

[00474] 3) Wash MSD plate 2X with wash buffer.

[00475] 4) Transfer 30ul of probe lysate mix to MSD plates as per the plate layout and incubate for lh.

[00476] 5) Wash plate 3X with 150ul wash buffer.

[00477] 6) Add 25ul of anti-BLK or anti-ITK antibody diluted to 2ug/ml in 1% Blocker A. Incubate for 2h at RT with shaking at 900rpm.

[00478] 7) Wash plates 3X with 150 µï wash buffer.

[00479] 8) Add 25ul of SULFO-TAG labeled anti-rabbit antibody diluted to lug/ml in 1% Blocker A. Incubate for 1h at RT with shaking at 900rpm.

[00480] 9) Wash plates 3X with MSD wash buffer, add 150 µï IX Read Buffer and read immediately in sector Imager.

[00481] **Results:**

[00482] Results are presented in Figure 28 for the BLK and ITK probe assays. It was observed that the signal titrates with the protein concentration in lysate used for both BTK and ITK assays. The signal increases with increasing probe concentration for positive control lysates

and plateau beyond about 30 nM. The background signal is low for the negative control (Cell lysate +drug) samples, with very little increase at 120nM probe. A probe concentration of 50nM was recommended for further experiments.

[00483] **<u>Experiment 2; Inhibition with drug Titration Series</u>**

[00484] **Aim:** Carry out inhibition experiment with titration series of drug then label lysates with 50nM probe.

[00485] **Samples:**

[00486] Positive control: DOHH2 and Jurkat lysate at 1 mg/mL in assay buffer.

[00487] Negative control: THP-1 lysate at 1 mg/mL in assay buffer.

[00488] Dilute lysates to 500; 250 and 125 μg/mL in assay buffer

[00489] Prepare a dilution series of PCI. The 100x PCI concentrations are: 100; 25; 6.25; 1.56; 0.39; 0.09 μM; treat lysates with PCI in a polypropylene plate for 1h e.g., 100 μL lysate + 1 μL 100x PCI solution; after the PCI inhibition add probe to all the samples to a final concentration of 50nM, incubate for 1h at RT with shaking.

[00490] **Protocol:**

[00491] 1) Block MSD standard streptavidin plate with 150 μI_per well 3% Blocker A 1h at RT or overnight at 4°C.

[00492] 2) Wash plate 1x with ≥ 150 μL per well wash buffer. Tap dry.

[00493] 3) Add 30 μL of probe labeled lysate according to the plate layout. Add lysate solution to the bottom corner of the wells. Seal and shake plate 1h at RT.

[00494] 4) Wash plate 3x with ≥ 150 μï، per well wash buffer. Tap dry.

[00495] 5) Add 25 μL per well of anti-ITK antibody diluted to 2 μg/mL in 1% (w/v) Blocker A. Seal and incubate for 2h at RT with shaking at 900rpm.

[00496] 6) Add 25 μL per well of SULFO-TAG conjugated anti-species antibody diluted to 2 μg/mL in 1% (w/v) Blocker A. Seal and shake plate 1h at RT.

[00497] 7) Wash plate 3x with ≥ 150 μL per well 1x Tris wash buffer. Tap dry

[00498] 8) Add 150 μL per well 1x Read Buffer T (diluted in H20). Avoid bubbles - use reverse pipetting when adding Read Buffer. Read plate in SI2400.

[00499] **Results:**

[00500] Results for the ITK probe assay are presented in Figure 29 and in Table 8 below. The signal of negative control cell line, DOHH2, was observed at background levels at all concentrations of drug. The drug treated positive control, Jurkat cell lysates, demonstrated decrease in signal with increasing drug concentration. The % inhibition was irrespective of the lysate concentration used. The assay reproducibility was very good with average %CV < 5%.

Table 8

	ITK assay (Jurkat lysate)					
	500 µg/ml		250 µg/ml		125 µg/ml	
Drug (nM)	Av signal	%CV	Av signal	%CV	Av signal	%CV
untreated	1805	0.63	1098	3.5	596	2.1
DMSO	1721.5	4.15	1217	0.2	581.5	1.1
1.0	1720.5	4.81	1107	1.1	662	1.3
3.9	1696	0.67	814.5	2.0	422	2.3
15.6	1158	1.34	589	1.2	324	1.7
62.5	433.5	2.12	240	2.4	167.5	0.4
250	180.5	1.96	135.5	2.6	129	1.1
1000	155	0.00	134	2.1	135.5	13.0

[00501] **Experiment 3: Repeat of inhibition of ITK in PBMC lysates**

[00502] **Aim:** Carry out inhibition experiment with titration series of drug then label lysates with 50nM probe.

[00503] **Samples:**

[00504] Positive control: DOHH2, Jurkat and PBMC lysate at 1 mg/mL in assay buffer.

[00505] Negative control: THP- 1 lysate at 1 mg/mL in assay buffer.

[00506] Dilute lysates to 500; 250 and 125 µg/mL in assay buffer

[00507] Prepare a dilution series of PCI. The 100x PCI concentrations are: 100; 25; 6.25; 1.56; 0.39; 0.09 µM ; treat lysates with PCI in a polypropylene plate for 1h e.g., 100 µL lysate + 1 µL 100x PCI solution; after the PCI inhibition add probe to all the samples to a final concentration of 50nM, incubate for 1h at RT with shaking.

[00508] **Protocol:**

[00509] 1) Block MSD standard streptavidin plate with 150 µL per well 3% Blocker A 1h at RT or overnight at 4°C.

[00510] 2) Wash plate 1x with ≥ 150 µL per well wash buffer. Tap dry.

[00511] 3) Add 30 µL of probe labeled lysate according to the plate layout. Add lysate solution to the bottom corner of the wells. Seal and shake plate 1h at RT.

[00512] 4) Wash plate 3x with ≥ 150 µL per well wash buffer. Tap dry.

[00513] 5) Add 25 µL per well of anti-ITK antibody diluted to 2 µg/mL in 1% (w/v) Blocker A. Seal and incubate for 2h at RT with shaking at 900rpm.

[00514] 6) Wash plates 3X with 150 µl wash buffer.

[00515] 7) Add 25 µL per well of SULFO-TAG conjugated anti-species antibody diluted to 2 µg/mL in 1% (w/v) Blocker A. Seal and shake plate 1h at RT.

[00516] 8) Wash plate 3x with ≥ 150 µL per well 1x Tris wash buffer. Tap dry

[00517] 9) Add 150 µL per well 1x Read Buffer T (diluted in H20). Avoid bubbles - use reverse pipetting when adding Read Buffer. Read plate in SI2400.

[00518] Results:

[00519] Results for the ITK probe assay are presented in Figure 30 and in Table **9** below. A dose dependent decrease in signal of ITK was observed with PBMC lysates indicating inhibition of ITK by the drug in PBMC lysates. The reproducibility of ITK assay was very good again with %CV < 5%.

Table 9

	ITK inhibition (PBMC lysates)		
Drug (nM)	Av signal	%CV	% inhibition
Untreated	998	5	
DMSO	889	3	11
0.98	925	3	7
3.91	889	1	11
15.63	832	1	17
62.50	384	1	62
250	139	3	86
1000	106	5	89

EXAMPLE 7: ITK Protein Occupancy Assay

[00520] The ELISA SULFO-TAG ITK probe assay, based upon an electrochemical stimulation, will be used to determine the relative amount of ITK that has not been bound by ibrutinib. Ibrutinib binds to the active site of ITK and forms a disulfide bond with a cysteine residue (ITK-Cys442). Compound 1-5 is a probe that consists of ibrutinib linked to biotin via a long chain linker. The collected protein lysates are labeled with Compound 1-5 . Labeling of samples with the probe allows for the detection of ITK not occupied by drug. The probe conjugated with ITK (and un-conjugated probe) is captured by the Streptavidin (SA) plate that is subsequently incubated with mouse anti-ITK (BD#550503) and SULFO-TAG conjugated anti-mouse antibody (MSD, cat#R32AC-5). The SULFO-TAG labels emit light upon electrochemical stimulation initiated at the electrodes in each well and signal is measured. A larger the signal correlates to more unoccupied ITK sites of a sample while a lower signal correlates to more ibrutinib occupied ITK sites.

[00521] The baseline for ITK occupancy was set at the pre-dose Cyclel Day 1 sample and the percent of ITK occupancy at the prescribed time points was calculated by this baseline value. This percentage was used as the pharmacodynamic output and compared between different dose cohorts of patients. Thus the relationship between the ibrutinib dose cohort and ITK occupancy was defined.

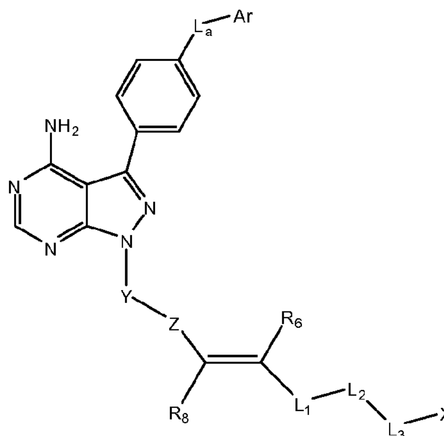
[00522] ITK occupancy in CLL patients on a phase II clinical trial of ibrutinib was determined. Samples were tested immediately prior to receiving ibrutinib and after eight days of daily oral administration (420mg/day). PBMC were collected, and lysed by freeze-thawing 4

times. After the final thaw, cells were centrifuged at 16,000g for 10 min at 4C to pellet insoluble material. Protease inhibitors were added to the protein lysates, and the protein lysates are labeled with a biotinylated derivative of drug, Compound 1-5, for 1 hr at RT and are added to a Streptavidin coated plate (MSD, cat #L15SA-2) that is blocked for 1 hr with blocking solution. After 1 hr of incubation, the plates were washed 3x and followed by an incubation of mouse anti-ITK (BD#550503) for another hr. The plate was washed 3x and incubated for 1 hr with SULFO-TAG conjugated anti-mouse antibody (MSD, cat#R32AC-5), washed, and read on a SI2400 for 3 min according to manufacturer's instructions. The data revealed between 40 and 80% of ITK is covalently bound by ibrutinib similar that achieved *in vitro*.

CLAIMS

What is claimed is:

1. A kit for determining drug target occupancy in a patient receiving a TEC family kinase inhibitor therapy, comprising a probe having the structure of Formula (II) comprising:



Formula (II);

wherein:

L_a is CH_2, O, NH or S;

Ar is optionally substituted aryl or optionally substituted heteroaryl;

Y is optionally substituted alkyl, optionally substituted heteroalkyl, optionally substituted cycloalkyl, optionally substituted heterocycloalkyl, optionally substituted aryl, or optionally substituted heteroaryl;

Z is C(O), OC(O), NHC(O), C(S), $S(0)_n$, $OS(0)_n$, $NHS(0)_n$, where n is 1 or 2;

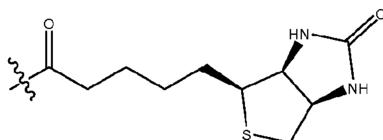
R_6 and R_8 are independently selected from H, optionally substituted alkyl, or optionally substituted heteroalkyl;

Li is optionally substituted alkyl or optionally substituted heteroalkyl;

L_2 is a bond, optionally substituted heterocycloalkyl, or -N(H)C(0)(CH $_2)_m$C(0)N(H), where m is 2-6;

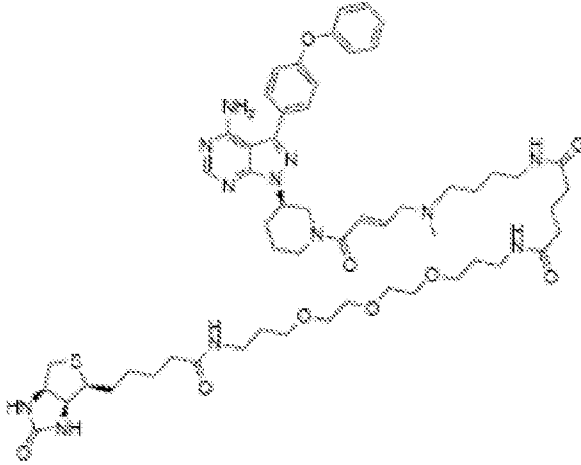
L_3 is optionally substituted alkyl or optionally substituted heteroalkyl; and X is a detectable label, wherein the probe binds to a TEC family kinase.

2. The kit of claim 1, wherein X is:



.

3. The kit of claim 1, wherein the probe has the structure of:



.

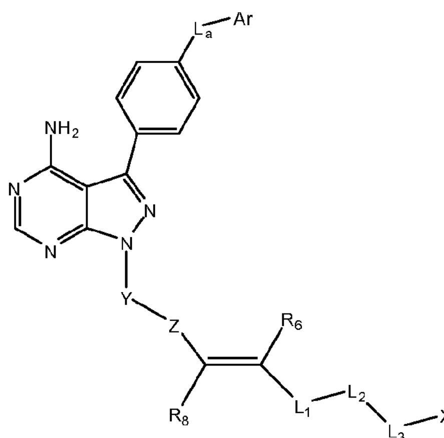
4. The kit of any of claims 1-3, wherein the probe binds to Bruton's tyrosine kinase (BTK), ITK, TEC, BMX, TXK or BLK.
5. The kit of any of claims 1-4, further comprising one or more solid supports selected from among a plate, a microplate, a bead or a plurality of beads.
6. The kit of claim 5, wherein the solid support is coated with a capture agent to form a coated solid support, wherein the capture agent binds to the probe.
7. The kit of claim 6, wherein the capture agent is streptavidin or an antibody.
8. The kit of any of claims 1-7, further comprising a primary detection agent, and optionally, a secondary detection agent that binds to the primary detection agent.
9. The kit of claim 8, wherein the primary detection agent or secondary detection agent comprises an antibody, a bead, a dye, a label, a tag, a fluorophore, or any combination thereof.
10. The kit of claim 9, wherein the primary detection agent is an anti-BTK antibody, an anti-ITK antibody, an anti-TEC antibody, an anti-TXK antibody, an anti-BMX antibody, or an anti-BLK antibody.
11. The kit of any of claims 8-10, wherein the primary or secondary detection agent is conjugated to an electrochemiluminescent tag.
12. A method for determining drug target occupancy in a patient receiving a TEC family kinase inhibitor therapy, comprising:

    (a) contacting a sample comprising a TEC family kinase with a probe to form a probe-bound kinase, wherein the sample is obtained from the patient following administration of at least one dose of an irreversible TEC family kinase inhibitor;

    (b) detecting the amount of probe-bound kinase in the sample; and

    (c) determining target occupancy of the TEC family kinase based on the amount of probe-bound kinase detected in the sample,

wherein the probe has the structure of Formula (II) comprising:



Formula (II);

wherein:

L_a is CH_2, O, NH or S;

Ar is optionally substituted aryl or optionally substituted heteroaryl;

Y is optionally substituted alkyl, optionally substituted heteroalkyl, optionally substituted cycloalkyl, optionally substituted heterocycloalkyl, optionally substituted aryl, or optionally substituted heteroaryl;

Z is C(O), OC(O), NHC(O), C(S), $S(0)_n$, $OS(0)_n$, $NHS(0)_n$, where n is 1 or 2;

R_6 and R$ are independently selected from H, optionally substituted alkyl, or optionally substituted heteroalkyl;

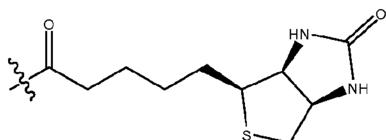
Li is optionally substituted alkyl or optionally substituted heteroalkyl;

L_2 is a bond, optionally substituted heterocycloalkyl, or -N(H)C(0)(CH $_2)_m$C(0)N(H), where m is 2-6;

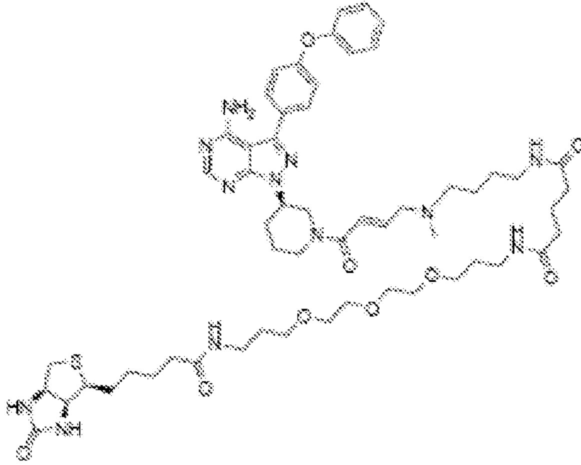
L_3 is optionally substituted alkyl or optionally substituted heteroalkyl; and

X is a detectable label.

13. The method of claim 12, wherein X is:



14. The method the method of claim 13, wherein the probe has the structure of:



.

15. The method of any of claims 12-14, wherein determining target occupancy comprises i) determining the number of binding sites not bound to the TEC family kinase inhibitor based on the amount of probe-bound kinase detected in the sample and ii) comparing said number to the total amount of active TEC family kinases in the sample.
16. The method the method of any of claims 12-15, further comprising determining or modifying a therapeutic regimen based on the target occupancy of the TEC family kinase.
17. A method for monitoring drug target occupancy in a patient receiving a TEC family kinase inhibitor therapy, comprising performing the method of any of claims 12-16 at two or more time points over the course of the therapy.
18. The method the method of any of claims 16-17, further comprising: i) increasing the dosage of the TEC family kinase inhibitor if the target occupancy is less than about 50%, ii) decreasing the dosage of the TEC family kinase inhibitor if the target occupancy is above at least about 70%, iii) maintaining the same therapeutic regimen of the TEC family kinase inhibitor or iv) discontinuing the therapeutic regimen.
19. The method of any of claims 12-18, further comprising determining the efficacy of the TEC family kinase inhibitor therapy based on the target occupancy, wherein the TEC family kinase inhibitor is i) effective when the occupancy of the TEC family kinase is at least about 70% or ii) ineffective when the occupancy of the TEC family kinase is less than about 50%.
20. The method of any of claims 12-19 above, wherein the TEC family kinase inhibitor an inhibitor of a Bruton's tyrosine kinase (BTK).
21. The method of any of claims 12-20, wherein the TEC family kinase inhibitor is ibrutinib.

22. The method of any of claims 12-21, further comprising capturing probe-bound kinase with a capture agent.
23. The method of claim 22, wherein the capture target is streptavidin or an antibody.
24. The method of claim 22 or 23, wherein the capture target is attached to a solid support.
25. The method claim 24, wherein the solid support is a plate, a microplate, a bead or a plurality of beads.
26. The method of any of claims 12-25, further comprising contacting the probe-bound kinase with a primary detection agent, and optionally, a secondary detection agent that binds to the primary detection agent.
27. The method of claim 26, wherein the primary detection agent or secondary detection agent comprises an antibody, a bead, a dye, a label, a tag, a fluorophore, or any combination thereof.
28. The method of any of claims 26-27 above, wherein the primary detection agent is an anti-BTK antibody, an anti-ITK antibody, an anti-TXK antibody, anti-TEC antibody, anti-BMX antibody, or anti-BLK antibody.
29. The method of any of claims 26-28, wherein the primary or secondary detection agent is conjugated to a chemiluminescent tag.
30. The method of any of claims 12-29, wherein patient is suffering from a cancer.
31. , The method of claim 30, wherein cancer is Hodgkin's lymphoma or a non-Hodgkin's lymphoma (NHL), chronic lymphocytic leukemia (CLL), small lymphocytic lymphoma (SLL), mantle cell leukemia (MCL), follicular lymphoma (FL), diffuse large B-cell lymphoma (DLBCL), Waldenstrom macroglobulinemia or multiple myeloma (MM).
32. The method of any of claims 12-31, wherein the sample is a blood sample, a lymph sample or tumor biopsy sample.

FIGURE 1

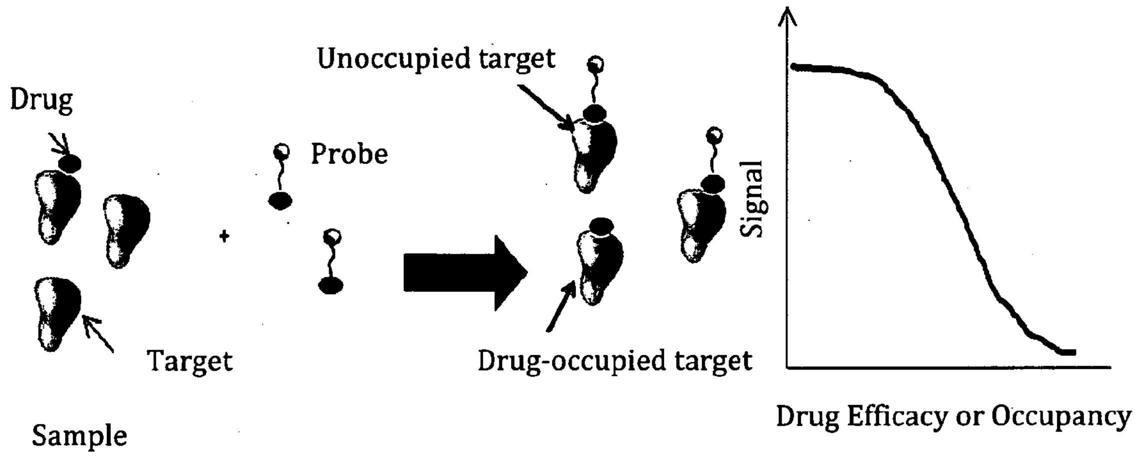
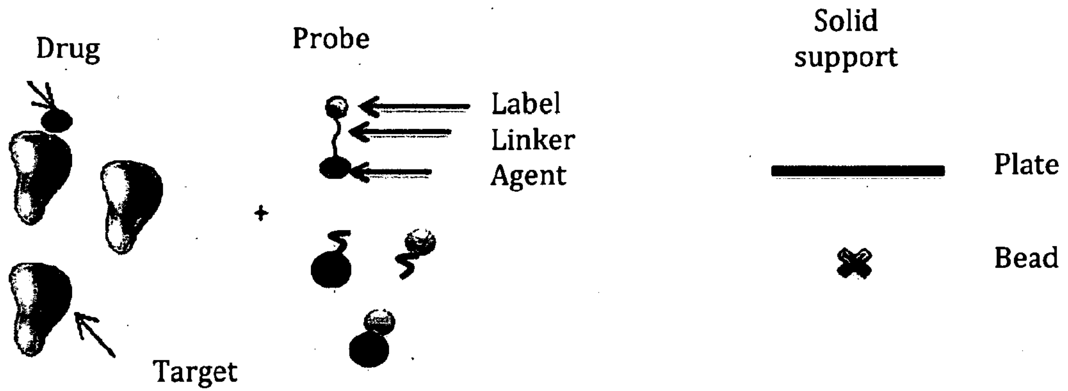


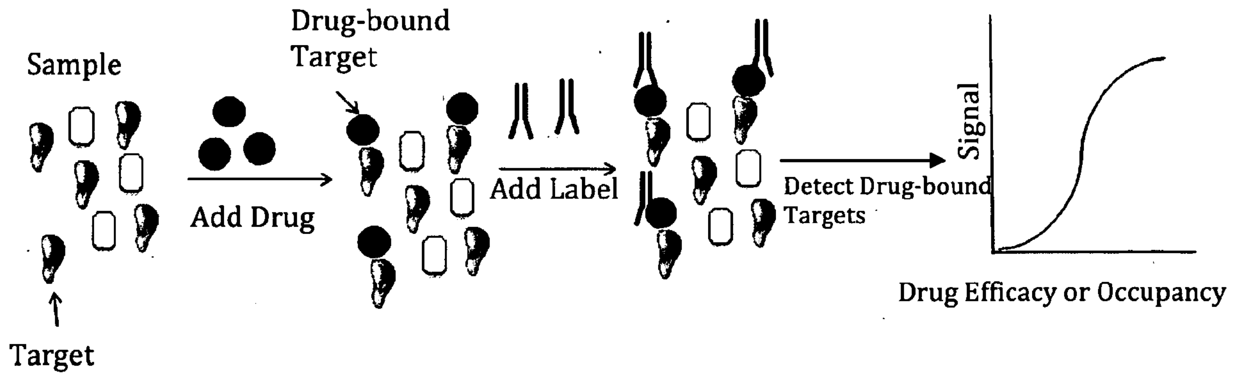
FIGURE 2



Kit Components:

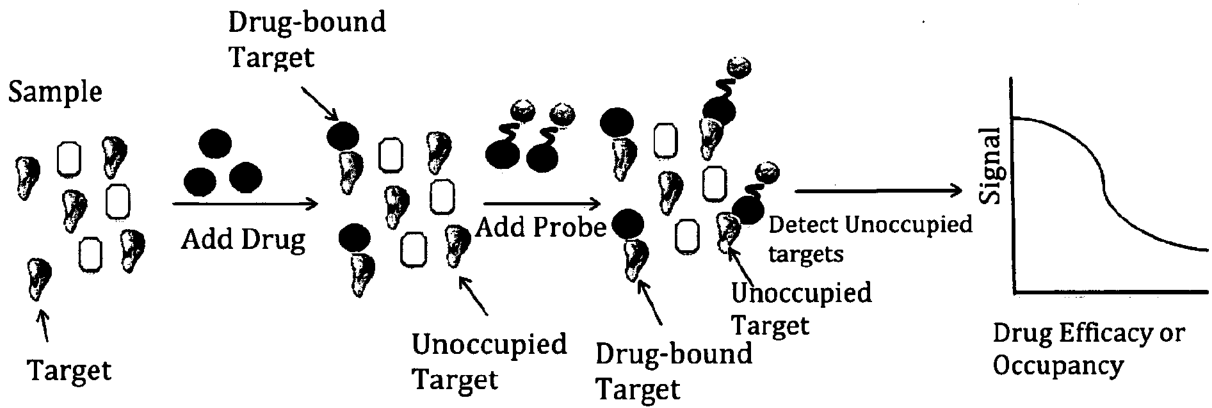
1. Drug (optional)
2. Target (optional)
3. Probe = agent, linker, label, or combination thereof
4. Solid support (optional)

FIGURE 3



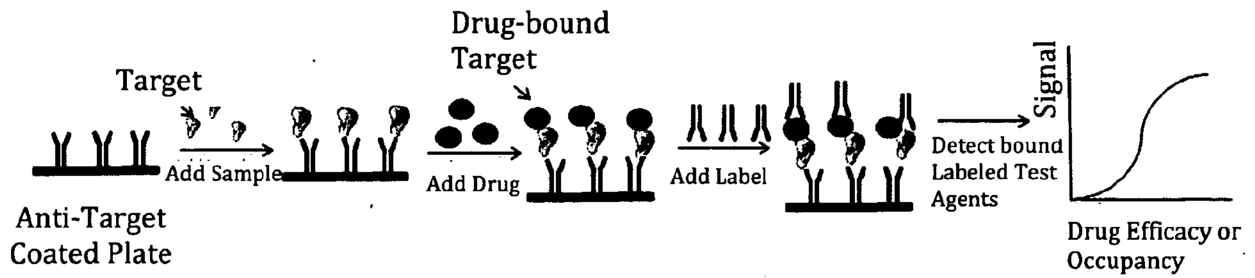
Drug efficacy or occupancy directly correlates to the signal intensity of the detected label (e.g., higher signal intensity = higher drug efficacy)

FIGURE 4



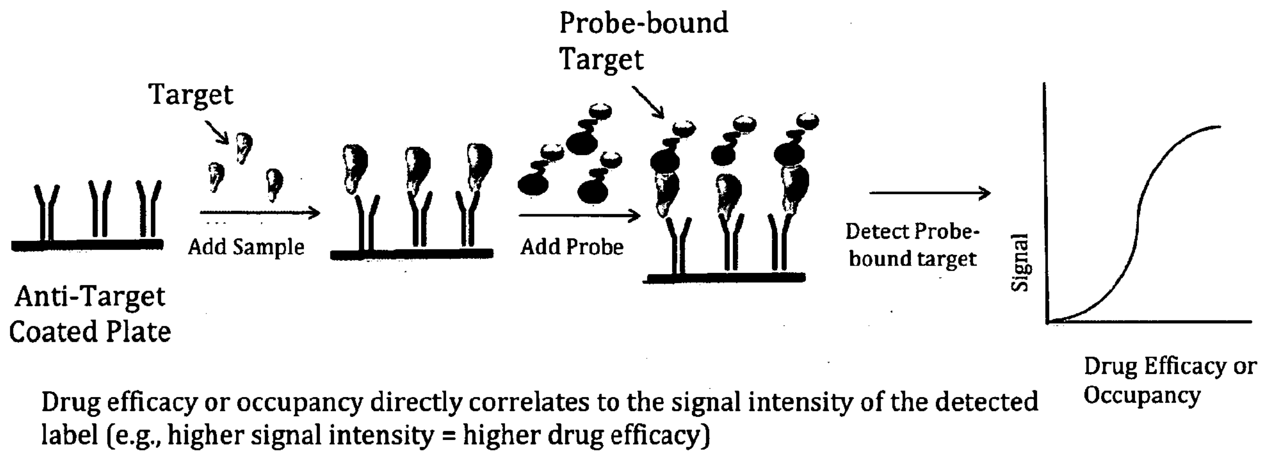
Drug efficacy or occupancy inversely correlates to the signal intensity of the detected label (e.g., higher signal intensity = higher drug efficacy)

FIGURE 5



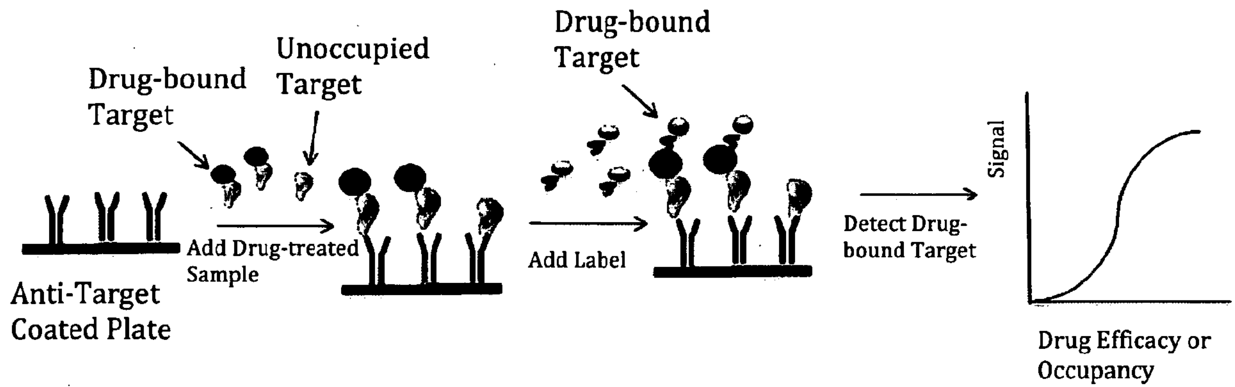
Drug efficacy or occupancy directly correlates to the signal intensity of the detected label (e.g., higher signal intensity = higher drug efficacy)

FIGURE 6



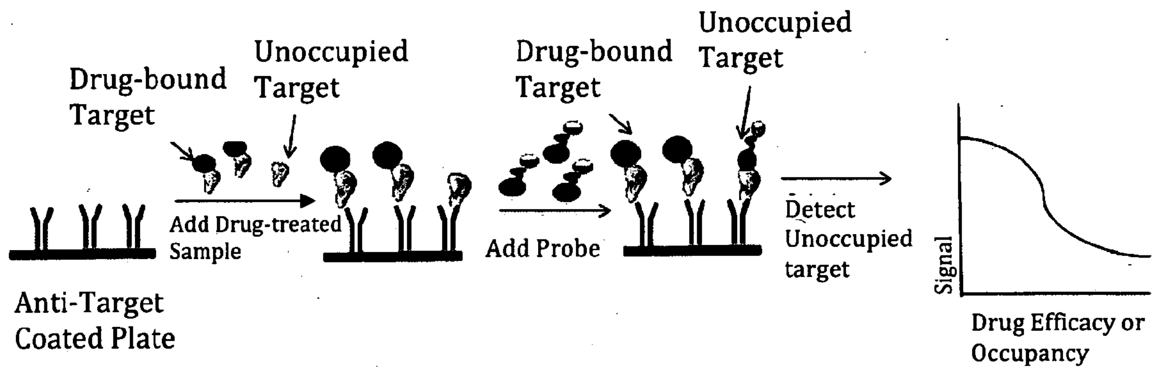
Drug efficacy or occupancy directly correlates to the signal intensity of the detected label (e.g., higher signal intensity = higher drug efficacy)

FIGURE 7



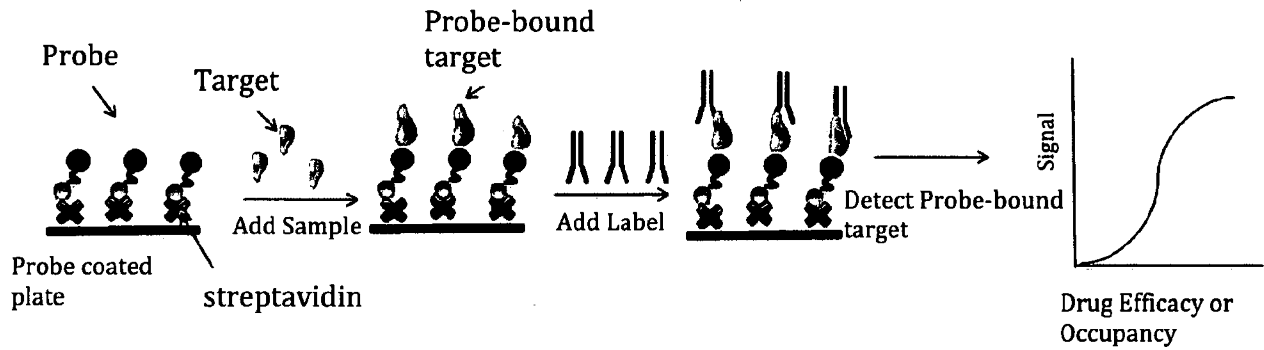
Drug efficacy or occupancy directly correlates to the signal intensity of the detected label (e.g., higher signal intensity = higher drug efficacy)

FIGURE 8



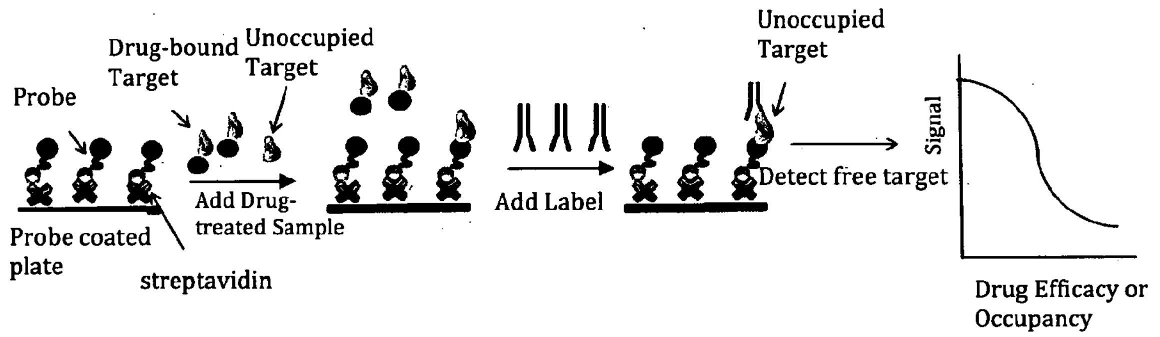
Drug efficacy or occupancy inversely correlates to the signal intensity of the detected label (e.g., higher signal intensity = higher drug efficacy)

FIGURE 9



Drug efficacy or occupancy directly correlates to the signal intensity of the detected label (e.g., higher signal intensity = higher drug efficacy)

FIGURE 10



Drug efficacy or occupancy inversely correlates to the signal intensity of the detected label (e.g., higher signal intensity = higher drug efficacy)

FIGURE 11

Two potential assay formats are shown below in which the assay specificity is conferred by a suitable anti-BTK antibody:

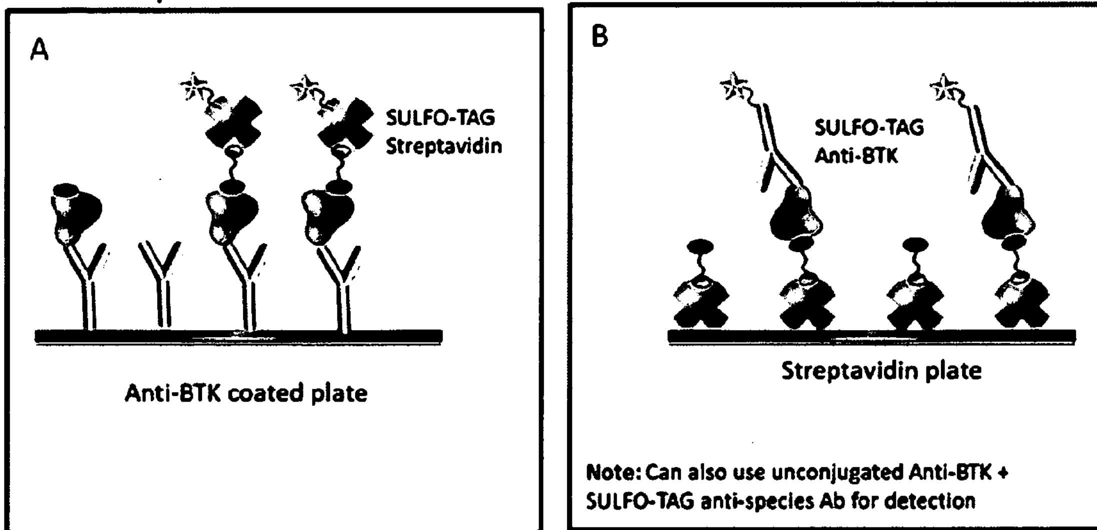


FIGURE 12

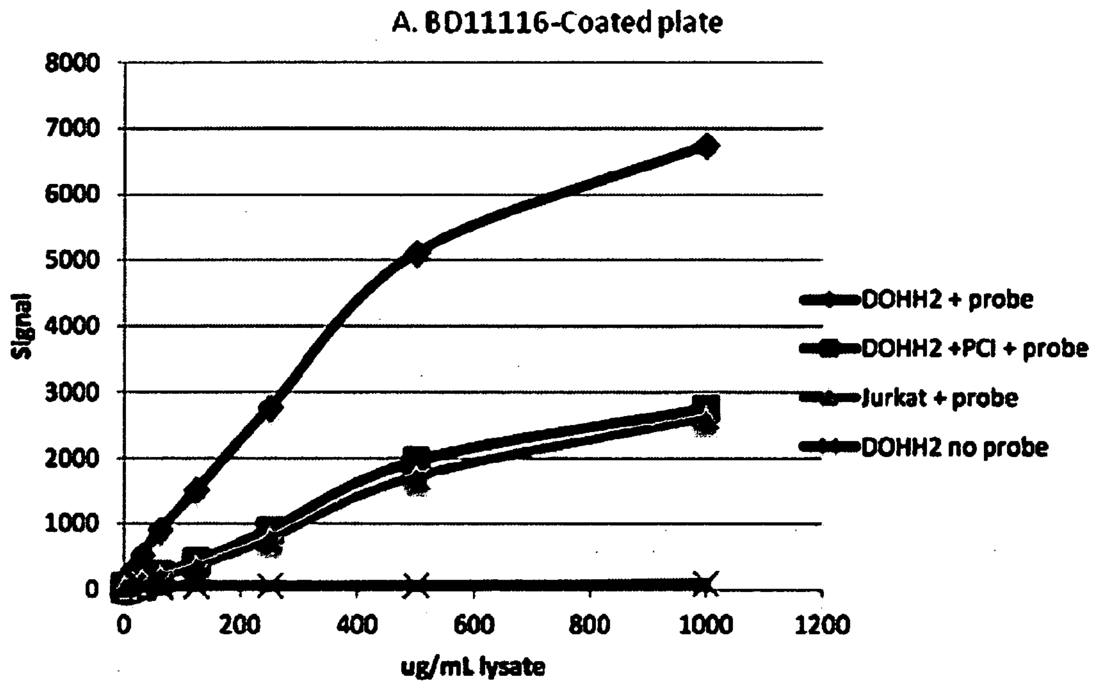
A. Plate layout

Lysate			DOHH2		Jurkat	DOHH2	DOHH2		Jurkat	DOHH2	DOHH2		Jurkat	DOHH2
			Probe	PCI+Probe	+ Probe	No Probe	Probe	PCI+Probe	+ Probe	No Probe	Probe	PCI+Probe	+ Probe	No Probe
ug/mL	ug/well		1	2	3	4	5	6	7	8	9	10	11	12
1000	30	A												
500	15	B												
250	7.5	C												
125	3.75	D												
62.5	1.875	E												
31.25	0.9375	F												
15.625	0.46875	G			1 uM probe only				1 uM probe only				1 uM probe only	
0	0	H												
Anti-BTK			BD Cat # 611116				BD Cat # 611117				Sigma			

B. Plate Results

	DOHH2		Jurkat	DOHH2	DOHH2		Jurkat	DOHH2	DOHH2		Jurkat	DOHH2
	+Probe	+PCI+probe	+ Probe	no probe	Probe	PCI+probe	+ Probe	no probe	Probe	PCI+probe	+ Probe	no probe
	1	2	3	4	5	6	7	8	9	10	11	12
A	6756	2756	2615	98	5225	2065	1912	88	2463	2043	2286	98
B	5112	1957	1723	69	3771	1598	1466	75	1565	1510	1500	73
C	2772	912	782	67	1959	769	666	66	662	683	683	66
D	1514	435	361	65	1088	368	304	60	318	301	307	62
E	914	243	208	52	673	238	197	55	192	189	191	63
F	526	162	153	52	378	150	136	59	133	127	127	58
G	307	118	107	1493	242	109	99	769	90	96	90	1097
H	54	56	56	1701	59	61	52	1125	55	52	57	1067
	BD11116 capture				BD11117 capture				Sigma capture			
Control	Positive	Negative	Negative	Negative	Positive	Negative	Negative	Negative	Positive	Negative	Negative	Negative

FIGURE 13



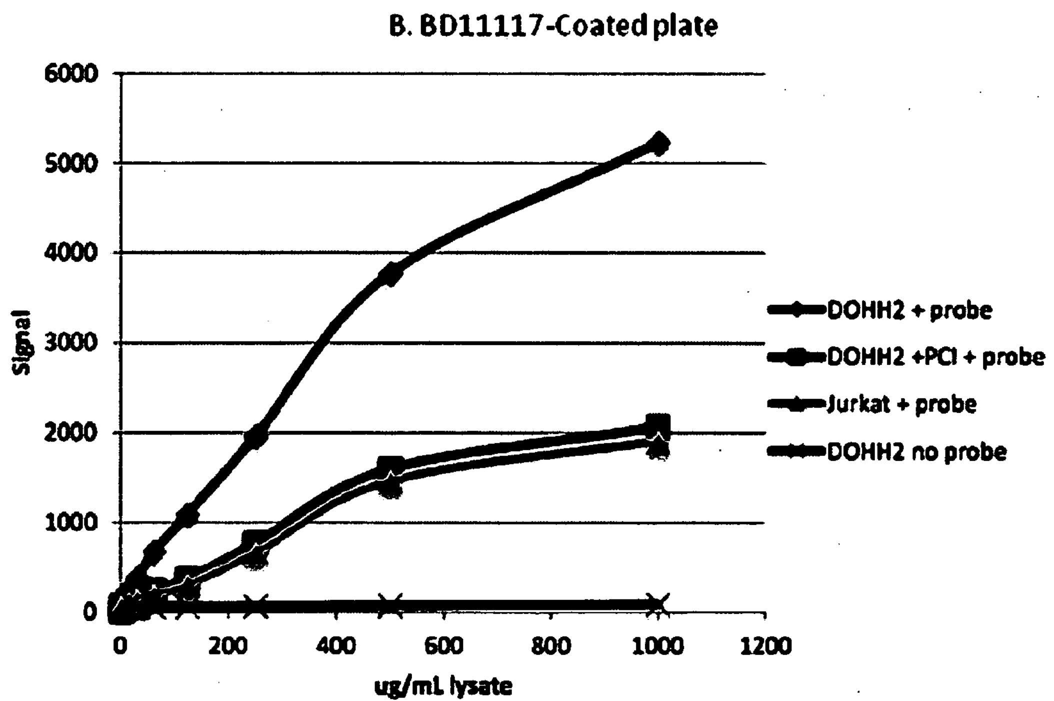
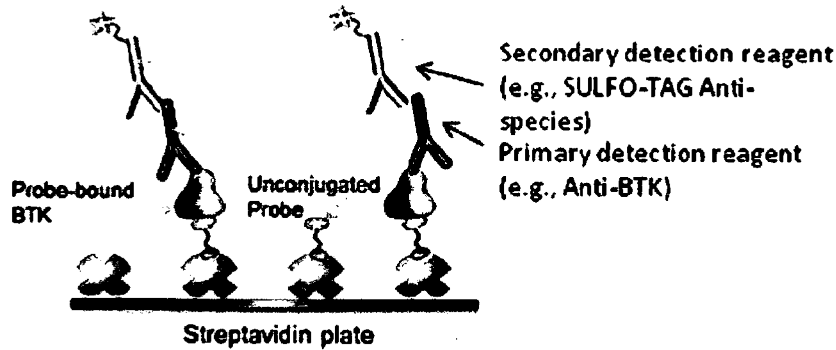


FIGURE 14

A. Assay overview



B. Plate layout

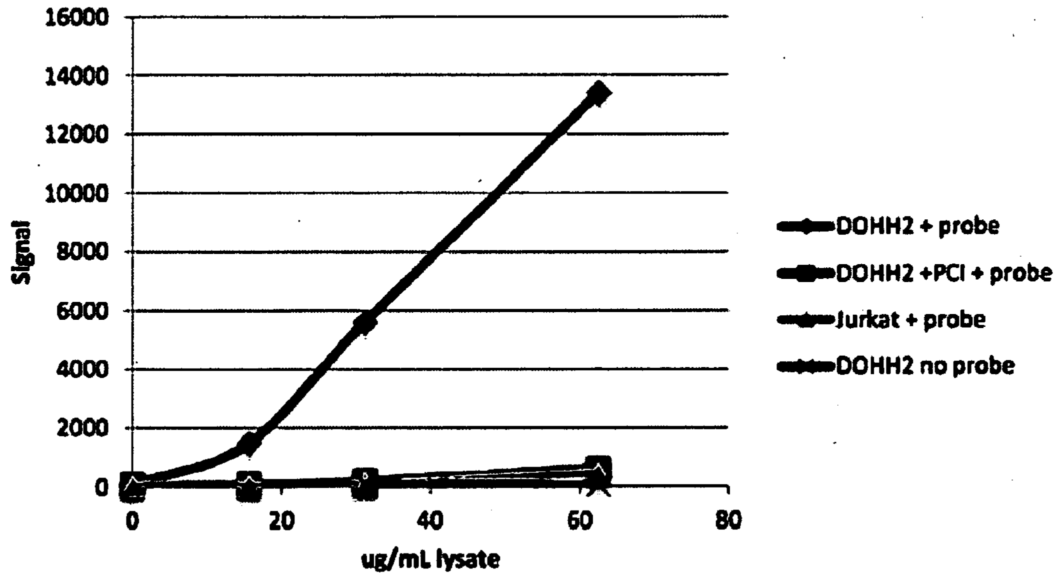
Lysate			DOHH2		Jurkat	DOHH2	DOHH2		Jurkat	DOHH2	DOHH2		Jurkat	DOHH2
			Probe	PCI+Probe	+ Probe	No Probe	Probe	PCI+Probe	+Probe	No Probe	Probe	PCI+Probe	+ Probe	No Probe
ug/mL	ug/well		1	2	3	4	5	6	7	8	9	10	11	12
1000	30	A												
500	15	B												
250	7.5	C												
125	3.75	D												
62.5	1.875	E												
31.25	0.9375	F												
15.625	0.46875	G				1 uM probe only				1 uM probe only				1 uM probe only
0	0	H												
Anti-BTK			BD Cat # 611116				BD Cat # 611117				Sigma			
Anti-species			SULFO-TAG anti-mouse								SULFO-TAG anti-rabbit			

C. Plate Results

Signal

Lysate			DOHH2		Jurkat	DOHH2	DOHH2		Jurkat	DOHH2	DOHH2		Jurkat	DOHH2
			Probe	+PCI+Probe	+ Probe	No Probe	Probe	PCI+Probe	+ Probe	No Probe	Probe	PCI+Probe	+ Probe	No Probe
ug/mL	ug/well		1	2	3	4	5	6	7	8	9	10	11	12
1000	30	A	2831	602	484	68	3326	794	693	132	3849	3761	4571	2075
500	15	B	3205	613	462	64	4077	770	614	119	3240	3014	3361	1796
250	7.5	C	3860	605	495	64	3206	770	608	109	2581	2683	2825	1659
125	3.75	D	4051	574	479	57	4498	717	574	100	2475	2451	2599	1707
62.5	1.875	E	13414	614	471	56	15004	709	481	95	2395	2392	2321	1608
31.25	0.9375	F	5601	178	119	62	5033	196	149	92	1963	1893	2050	2003
15.625	0.46875	G	1482	76	60	500	1502	125	103	541	1927	1919	1875	3292
0	0	H	56	63	58	727	90	101	91	531	1863	1857	1795	3224
a-BTK			BD11116 detection				BD11117 detection				Sigma detection			
Control			Positive	Negative	Negative	Negative	Positive	Negative	Negative	Negative	Positive	Negative	Negative	Negative

FIGURE 15



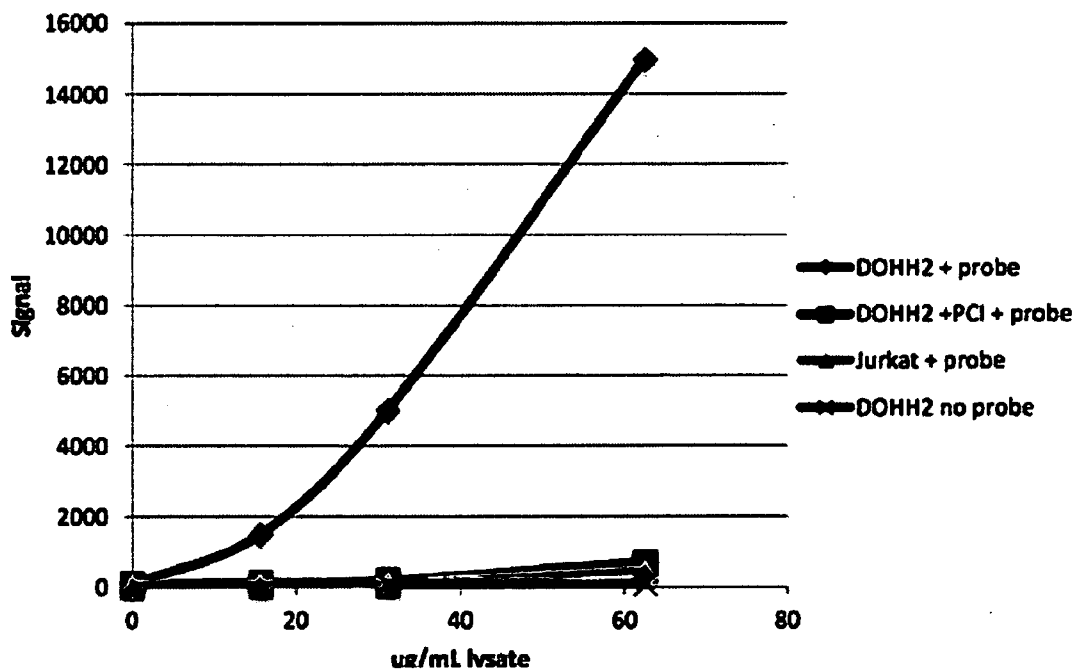
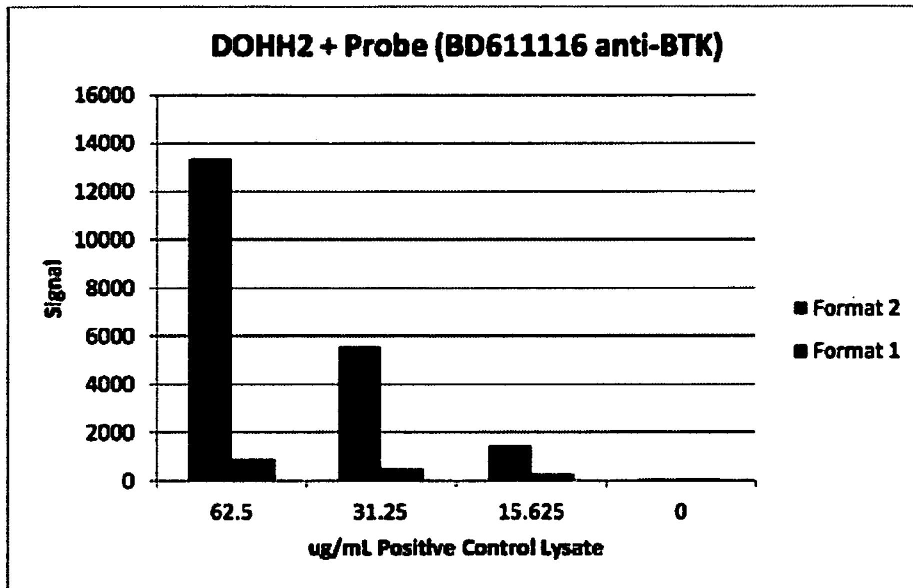


FIGURE 16



		Signal: Background (DOHH2 + Probe)					
Lysate		Format 1			Format 2		
ug/mL	ug/well	BD611116	BD611117	Sigma	BD611116	BD611117	Sigma
62.5	1.875	16.9	11.4	3.5	239.5	166.7	1.3
31.25	0.9375	9.7	6.4	2.4	100.0	55.9	1.1
15.625	0.46875	5.7	4.1	1.6	26.5	16.7	1.0
0	0	1.0	1.0	1.0	1.0	1.0	1.0

FIGURE 17

Experiment 2 Results

Signal

		DOHH2						DOHH2 + 1uM PCI						
		[illegible]	50.0	25.0	12.5	6.3	3.1	[illegible]	50.0	25.0	12.5	6.3	0.0	ug/mL lysate
Probe (nM)		1	2	3	4	5	6	7	8	9	10	11	12	
100	A	5981	895	520	723	397	382	732	523	505	427	417	379	
25	B	10743	1799	449	308	211	355	187	169	161	129	174	141	
6.25	C	1426	457	269	179	163	130	144	131	127	129	122	114	
1.563	D	285	240	144	131	130	133	139	140	124	128	110	110	
0.391	E	164	143	126	132	132	127	141	131	133	126	121	115	
0.098	F	149	135	141	131	131	123	134	139	125	124	120	110	
0.024	G	153	141	130	126	128	129	144	135	132	133	120	115	
0	H	141	140	135	114	120	116	130	125	130	122	111	120	

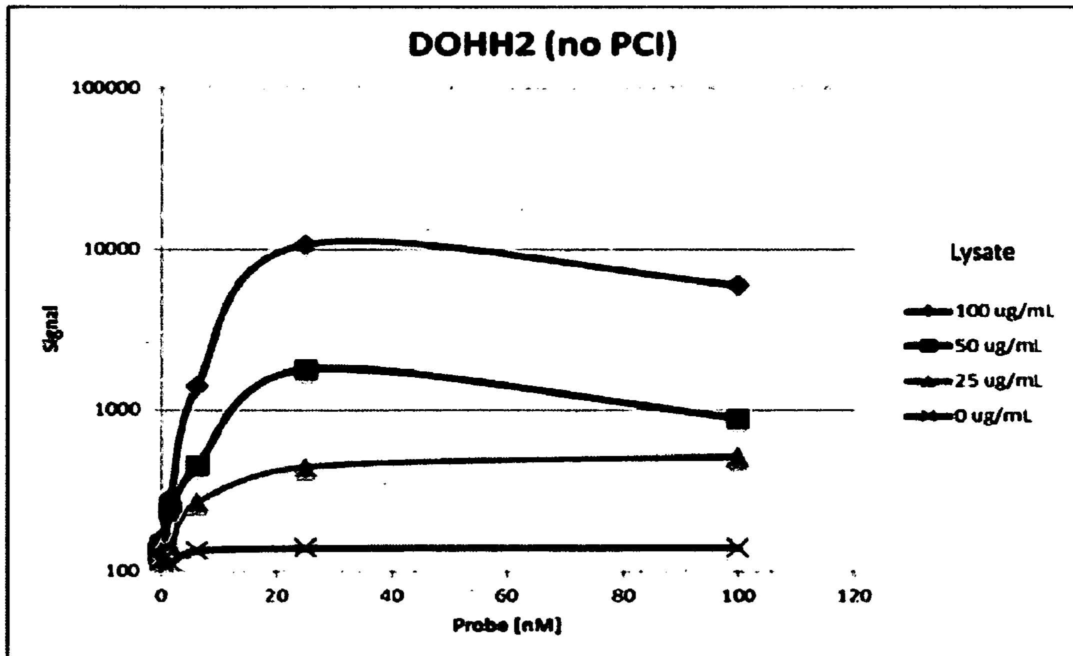


FIGURE 18

Experiment 3 Results

Signal

PCI nM	DOHH2 + 25 nM Probe						Jurkat					
	300 ug/mL		150 ug/mL		75 ug/mL		300 ug/mL		150 ug/mL		75 ug/mL	
1000	200	195	135	140	106	120	92	95	87	85	96	75
250	249	238	162	162	117	129	111	100	87	86	81	83
62.5	533	508	218	215	164	149	115	99	84	92	84	85
15.63	1904	1799	457	418	299	301	105	123	99	108	79	86
3.91	6333	5734	1212	1171	510	452	119	106	116	105	89	87
0.98	14386	13251	4104	3770	1652	1370	110	103	95	98	102	104
0.24	19283	18511	5644	5467	2059	1903	122	116	97	105	91	89
0	21299	21320	6648	5835	2540	2655	105	101	107	104	85	88

	DOHH2 + 25 nM Probe					
	300 ug/mL		150 ug/mL		75 ug/mL	
PCI nM	Av. Signal	%CV	Av. Signal	%CV	Av. Signal	%CV
1000	197.5	1.8	137.5	2.6	113.0	8.8
250	243.5	3.2	162.0	0.0	123.0	6.9
62.5	520.5	3.4	216.5	1.0	156.5	6.8
15.63	1851.5	4.0	437.5	6.3	300.0	0.5
3.91	6033.5	7.0	1191.5	2.4	481.0	8.5
0.98	13818.5	5.8	3937.0	6.0	1511.0	13.2
0.24	18897.0	2.9	5555.5	2.3	1981.0	5.6
0.00	21309.5	0.1	6241.5	9.2	2597.5	3.1

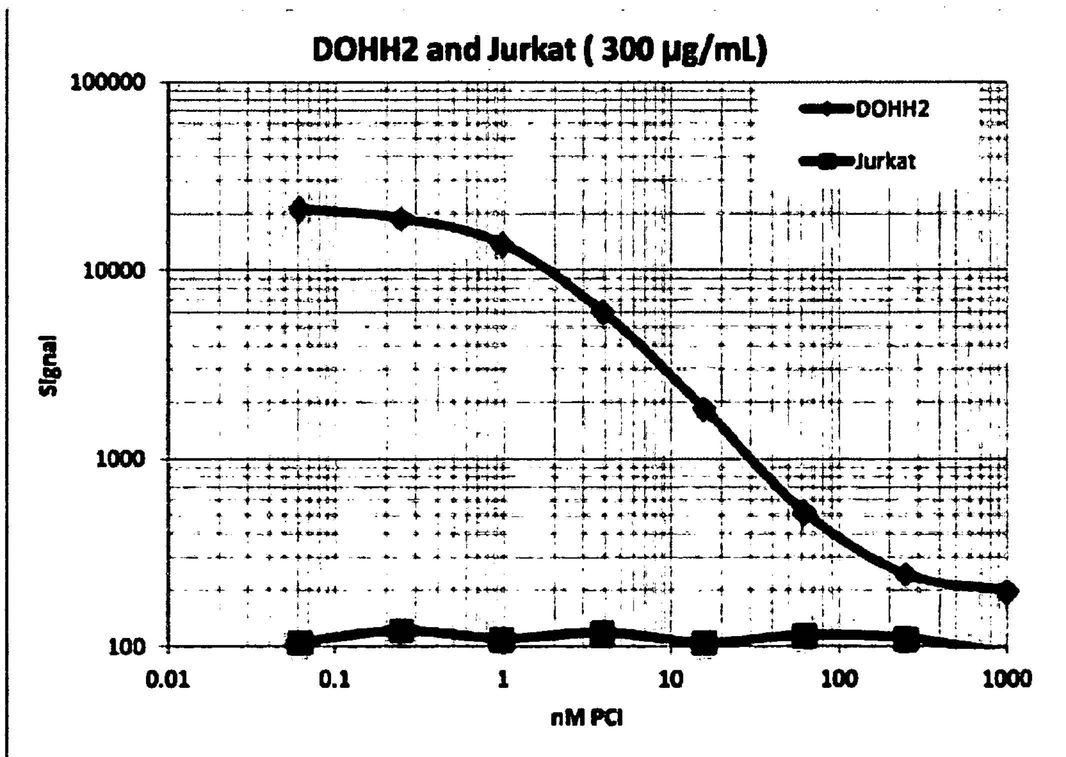
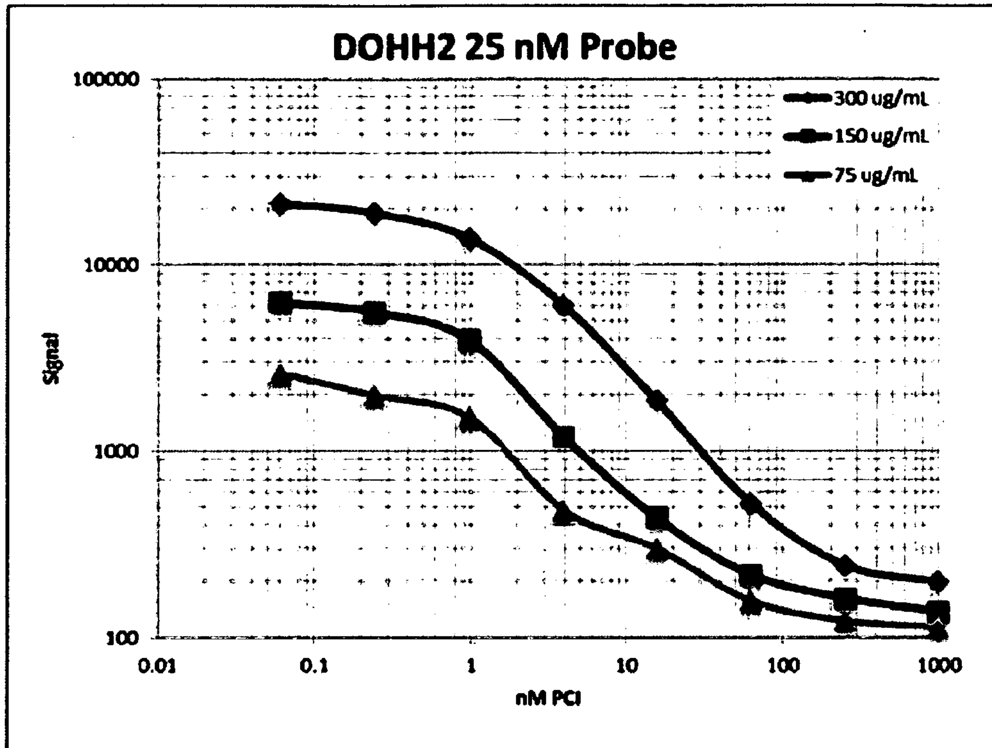


Figure 19



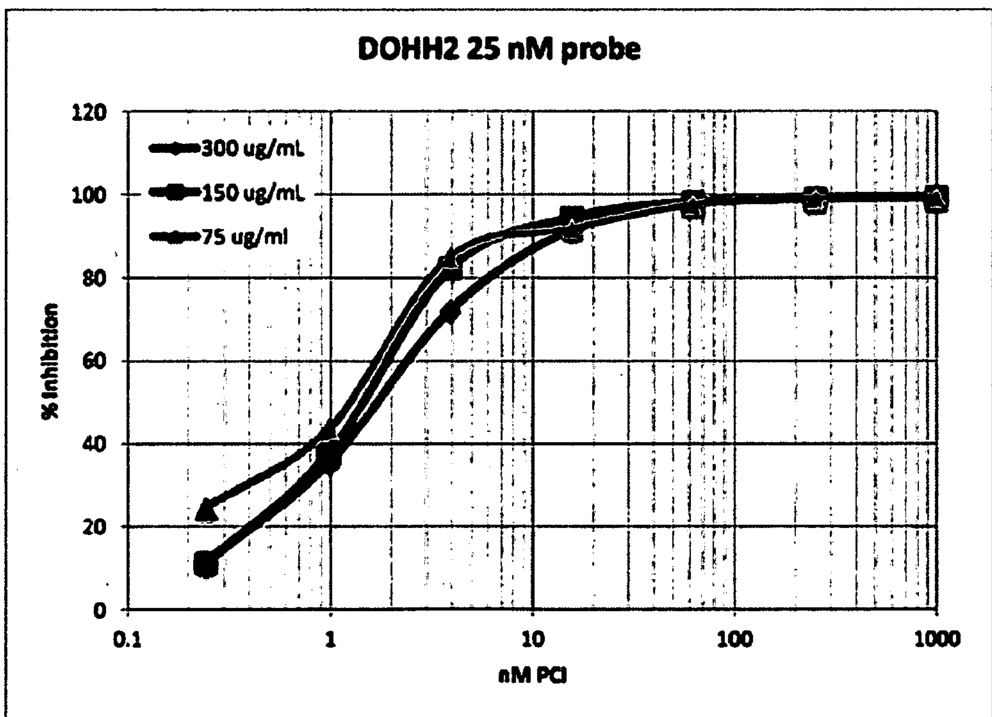


Figure 20

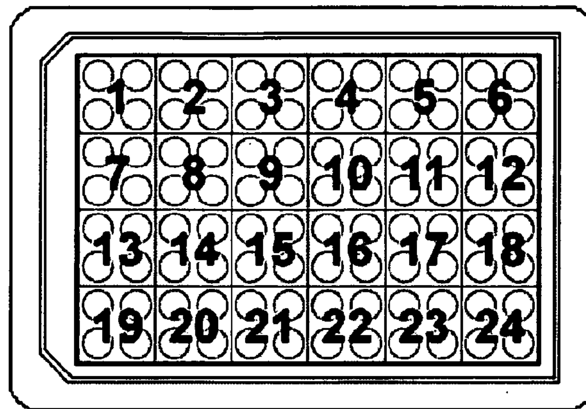


Figure 21

I-1

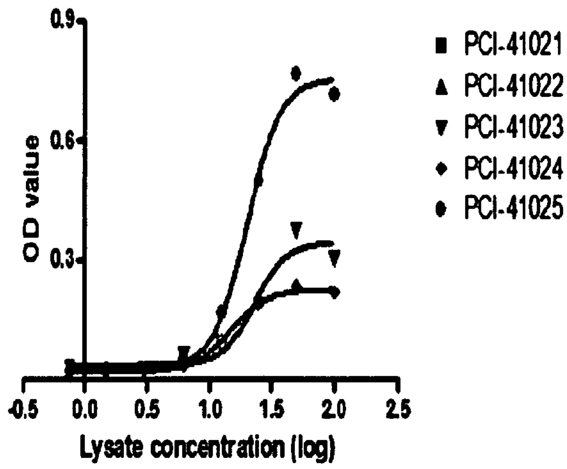
I-2

I-3

I-4

I-5

Figure 22



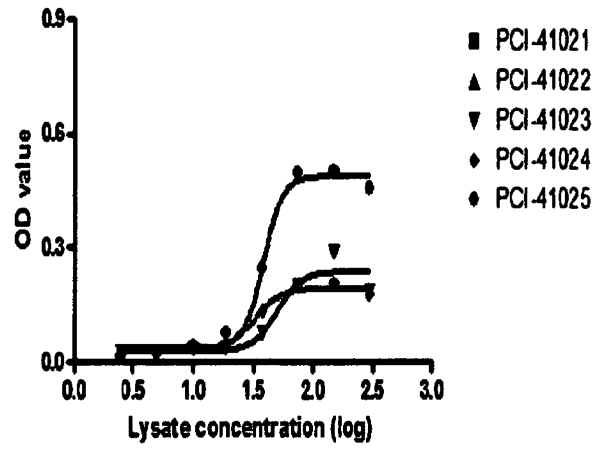


Figure 23

High Bind							
	1	2	3	4	5	6	7
detect	H00000640-M02	TA300020	ab37830	TA300020	ab37830	H00000640-M02	H00000640-M02
	BLK						
A	22542	19665	16462	869637	883322	386557	30515
B	6101	6220	4561	878666	882760	380678	13478
C	1880	3584	2384	918974	919245	388094	10437
D	958	3089	1945	856288	904215	392825	9930
E	767	3057	1946	878888	895960	373442	10341
F	709	2925	1835	884969	898635	393484	10086
G	726	2996	1809	900005	898054	379030	10436
H	714	2695	1664	878428	887059	381092	10164
capture Ab (4μg/ml)	AF2679			H00000640-M02		TA300020	ab37830
sec Ab	anti-mouse	anti-rabbit				anti-mouse	

Standard							
	1	2	3	4	5	6	7
detect	H00000640-M02	TA300020	ab37830	TA300020	ab37830	H00000640-M02	H00000640-M02
	BLK						
A	79754	70951	67410	980116	976332	821410	51642
B	20502	15013	13437	977669	973078	820543	18130
C	5858	4687	4421	974775	969697	760037	10510
D	2955	3108	2961	980337	975937	753261	9616
E	2186	2803	2591	980252	975122	743406	9620
F	2196	2724	2489	976511	974068	740798	9453
G	2038	2770	2466	952588	954001	750603	9775
H	1972	2857	2594	955273	953753	774825	10052
capture Ab (4μg/ml)	AF2679			H00000640-M02		TA300020	ab37830
sec Ab	anti-mouse	anti-rabbit				anti-mouse	

Figure 24

High Bind

detect	H00000640-M02	TA300020	ab37830	TA300020	ab37830	H00000640-M02	H00000640-M02
	BLK						
A	31.6	7.3	9.9	1.0	1.0	1.0	3.0
B	8.5	2.3	2.7	1.0	1.0	1.0	1.3
C	2.6	1.3	1.4	1.0	1.0	1.0	1.0
D	1.3	1.1	1.2	1.0	1.0	1.0	1.0
E	1.1	1.1	1.2	1.0	1.0	1.0	1.0
F	1.0	1.1	1.1	1.0	1.0	1.0	1.0
G	1.0	1.1	1.1	1.0	1.0	1.0	1.0
H	1	1	1	1	1	1	1
capture Ab (4µg/ml)	AF2679			H00000640-M02		TA300020	ab37830
sec Ab	anti-mouse	anti-rabbit				anti-mouse	

Standard

detect	H00000640-M02	TA300020	ab37830	TA300020	ab37830	H00000640-M02	H00000640-M02
	BLK						
A	40.4	24.8	26.0	1.0	1.0	1.1	5.1
B	10.4	5.3	5.2	1.0	1.0	1.1	1.8
C	3.0	1.6	1.7	1.0	1.0	1.0	1.0
D	1.5	1.1	1.1	1.0	1.0	1.0	1.0
E	1.1	1.0	1.0	1.0	1.0	1.0	1.0
F	1.1	1.0	1.0	1.0	1.0	1.0	0.9
G	1.0	1.0	1.0	1.0	1.0	1.0	1.0
H	1	1	1	1	1	1	1
capture Ab (4µg/ml)	AF2679			H00000640-M02		TA300020	ab37830
sec Ab	anti-mouse	anti-rabbit				anti-mouse	

Figure 25

	1	2	3	4	5	6	7	8	9	10	11	12
Capture	0.5µg/ml				1µg/ml				2µg/ml			
A	7363	7215	7911	862	13047	13953	13943	1078	21087	24093	25715	1505
B	2772	2634	3261	687	5019	5986	5897	782	9122	10709	10845	993
C	1093	1220	1526	463	2006	2384	2682	660	3277	3956	4375	767
D	498	634	913	580	902	1151	1510	707	1458	1800	2193	788
E	302	417	638	510	475	696	1102	618	787	1082	1528	686
F	258	365	770	441	364	545	1047	573	600	825	1175	655
G	233	308	554	179	312	510	889	156	473	720	1064	146
H	216	312	585	171	282	496	848	175	498	637	1065	152
Detect (µg/ml)	0.5	1	2	1	0.5	1	2	1	0.5	1	2	1
sec Ab (1µg/ml)	SULFO-TAG anti-mouse											

Figure 26

Capture	0.5μg/ml			1μg/ml			2μg/ml		
100000	34.1	23.1	13.5	46.3	28.1	16.4	42.3	37.8	24.1
33333	12.8	8.4	5.6	17.8	12.1	7.0	18.3	16.8	10.2
11111	5.1	3.9	2.6	7.1	4.8	3.2	6.6	6.2	4.1
3704	2.3	2.0	1.6	3.2	2.3	1.8	2.9	2.8	2.1
1235	1.4	1.3	1.1	1.7	1.4	1.3	1.6	1.7	1.4
412	1.2	1.2	1.3	1.3	1.1	1.2	1.2	1.3	1.1
137	1.1	1.0	0.9	1.1	1.0	1.0	0.9	1.1	1.0
0	1	1	1	1	1	1	1	1	1
Detect (μg/ml)	0.5	1	2	0.5	1	2	0.5	1	2

Figure 27

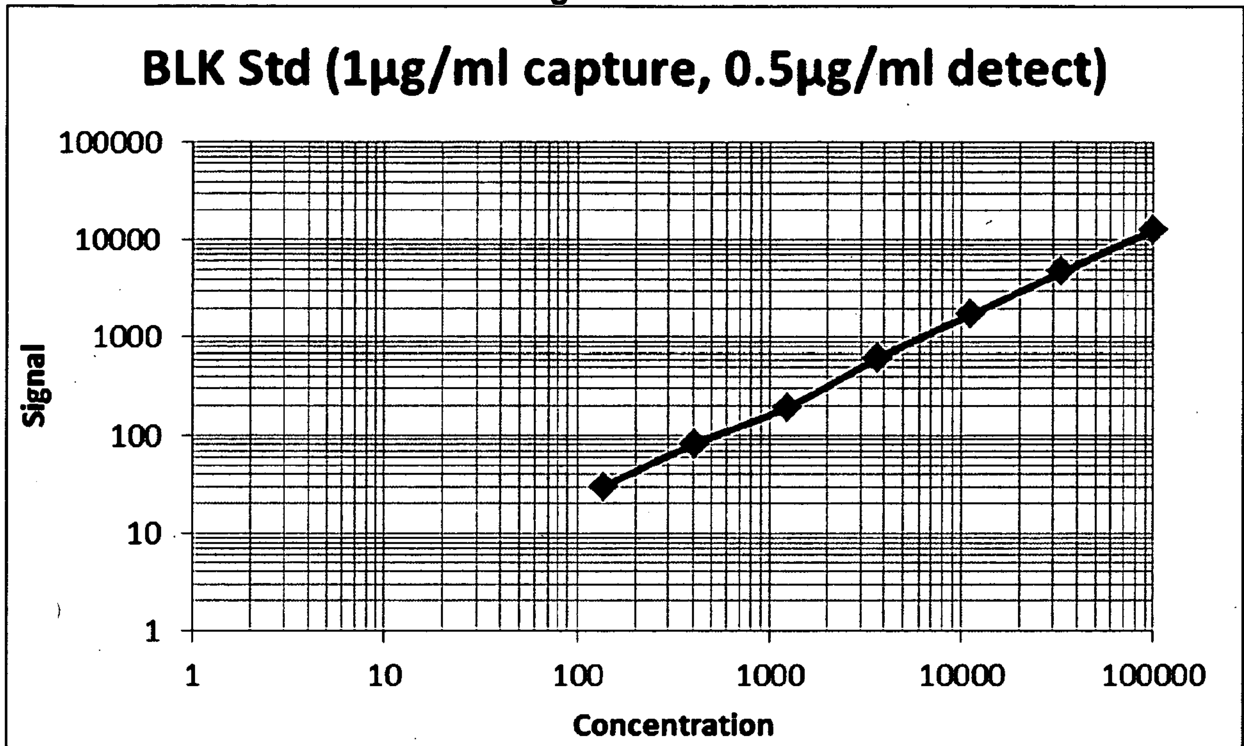
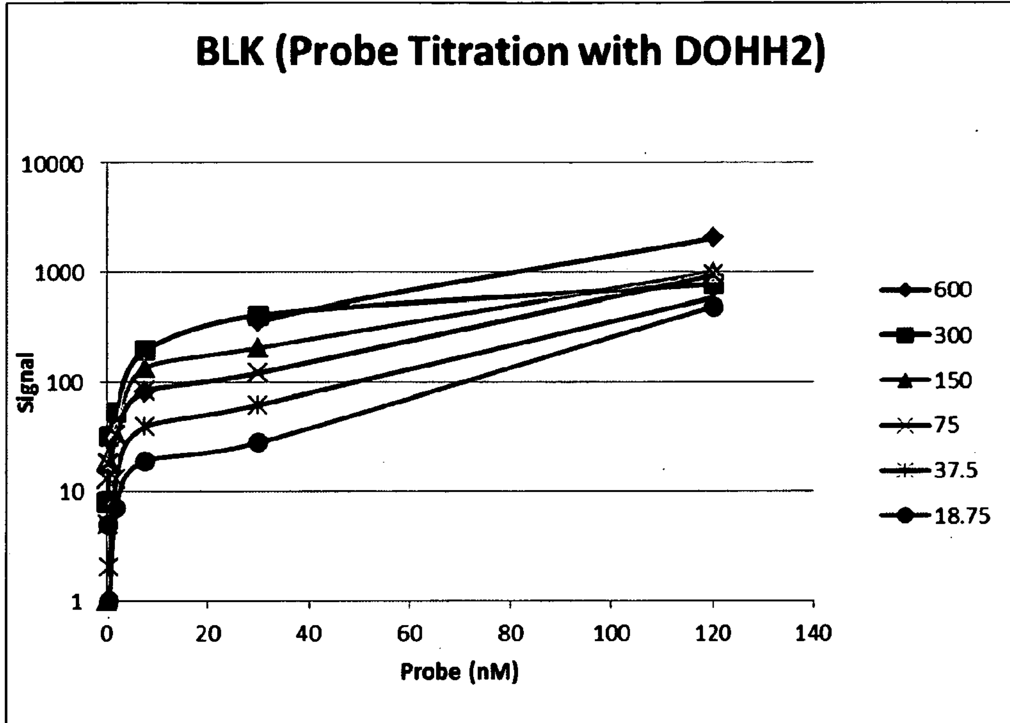


Figure 28

A



B

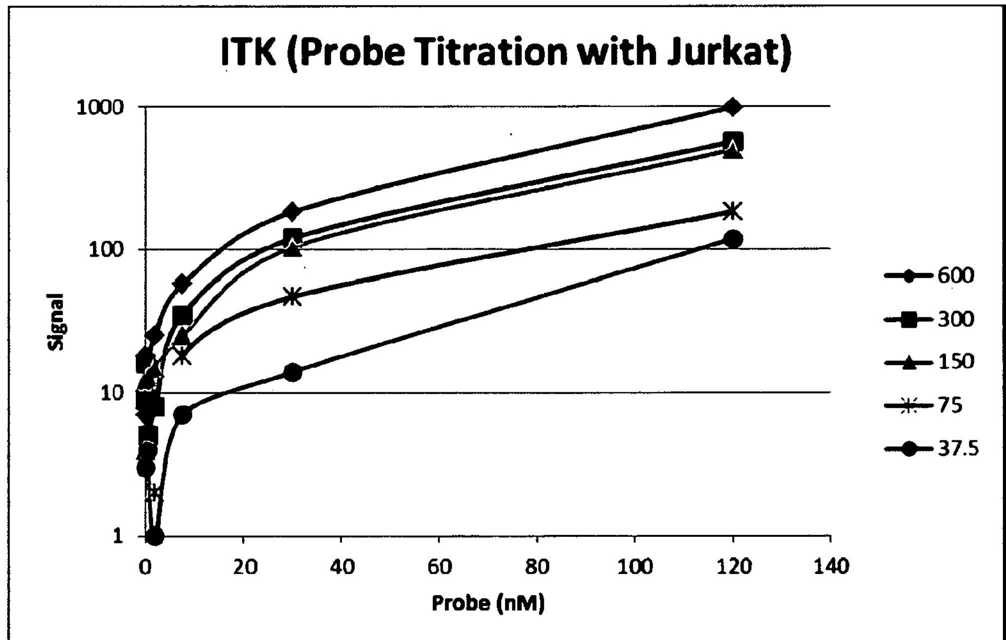
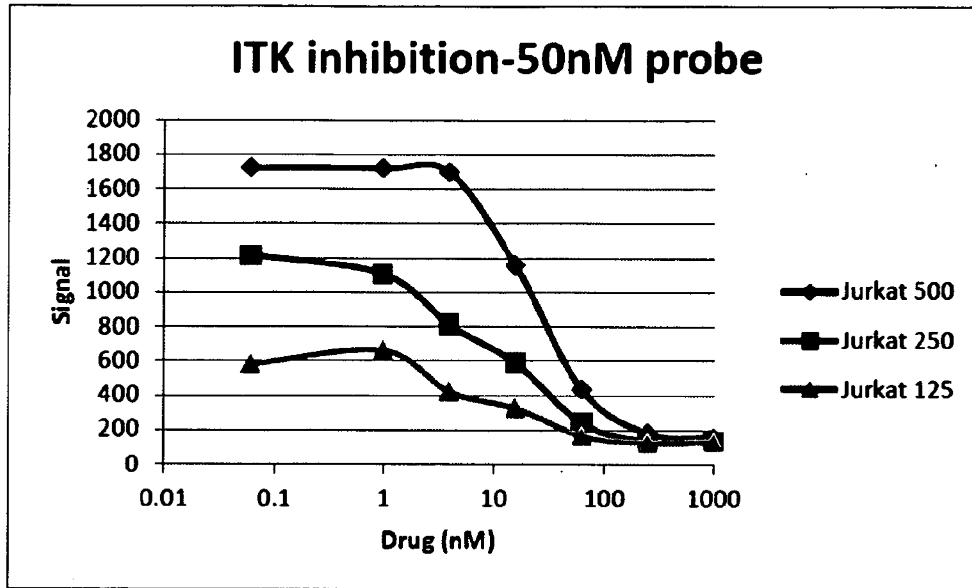


Figure 29

A



B

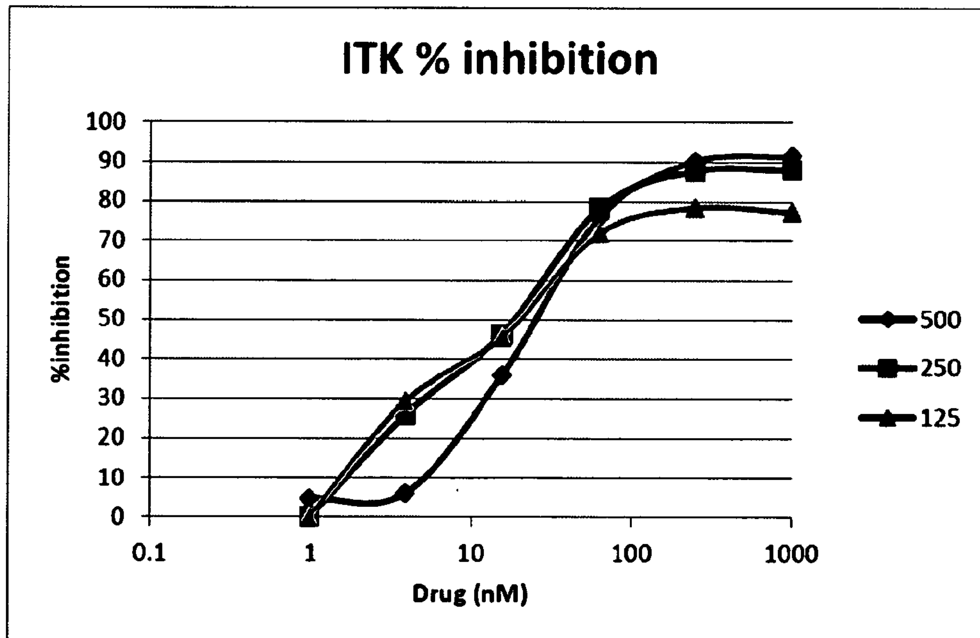
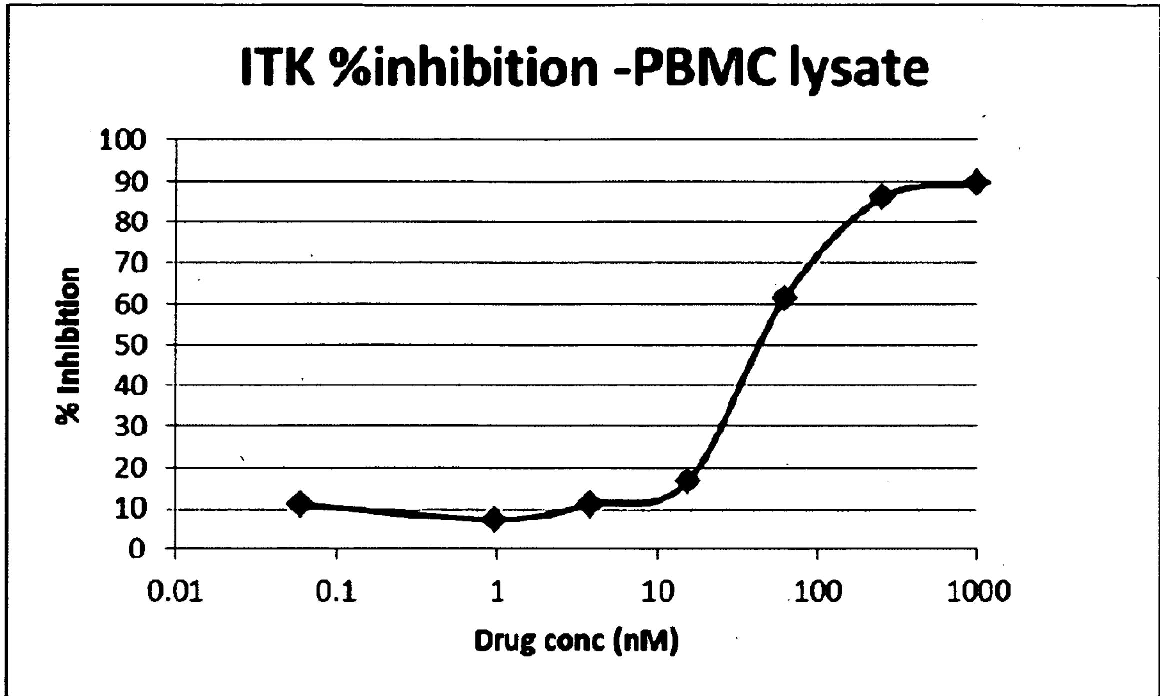


Figure 30



INTERNATIONAL SEARCH REPORT

International application No
PCT/US2013/064688

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07D403/12 A61K31/506 G01N33/68 G01N33/532
ADD.

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)
G01N A61K C07D

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal , BEILSTEIN Data , BIOSIS, CHEM ABS Data , EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	TJEERD BARF ET AL: "Irreversible Protein Kinase Inhibitors : Balancing the Benefits and Risks" JOURNAL OF MEDICINAL CHEMISTRY, vol . 55, no. 14, 26 July 2012 (2012-07-26) , pages 6243-6262, XP055096173 , ISSN: 0022-2623 , DOI: 10.1021/jm3003203 page 6255 - page 6256; figures 17,18	1,8-12, 20-23, 26-32

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* 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	Date of mailing of the international search report
20 January 2014	24/01/2014

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Cervigni, S

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2013/064688

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	YAN LOU ET AL: "Bruton's Tyrosine Kinase Inhibitors: Approaches to Potent and Selective Inhibition, Preclinical and Clinical Evaluation for Inflammatory Diseases and B Cell Malignancies", JOURNAL OF MEDICINAL CHEMISTRY, vol. 55, no. 10, 24 May 2012 (2012-05-24), pages 4539-4550, XP055080230, ISSN: 0022-2623, DOI: 10.1021/jm300035p page 13080; figure 1	1,8-12, 20-23, 26-32
X	L. A. HONIGBERG ET AL: "The Bruton tyrosine kinase inhibitor PCI-32765 blocks B-cell activation and is efficacious in models of autoimmune disease and B-cell malignancy", PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, vol. 107, no. 29, 6 July 2010 (2010-07-06), pages 13075-13080, XP55080251, ISSN: 0027-8424, DOI: 10.1073/pnas.1004594107 page 4543	1,8-12, 20-23, 26-32
X	WO 2010/123870 A1 (AVILA THERAPEUTICS INC [US]; SINGH JUSWINDER [US]; GHOSH SHOMIR [US]) 28 October 2010 (2010-10-28) the whole document page 119 - page 124	1,2, 4-13, 15-32

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2013/064688

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2010123870 AI	28-10-2010	AU 2010239444 AI	17-11-2011
		CA 2759655 AI	28-10-2010
		CN 102448462 A	09-05-2012
		EP 2421538 AI	29-02-2012
		JP 2012524123 A	11-10-2012
		KR 20120007055 A	19-01-2012
		SG 175291 AI	28-11-2011
		US 2010016296 AI	21-01-2010
		US 2011224432 AI	15-09-2011
		US 2013065892 AI	14-03-2013
		WO 2010123870 AI	28-10-2010

patsnap

专利名称(译)	tec系列激酶抑制剂治疗的辅助诊断		
公开(公告)号	EP2906556A1	公开(公告)日	2015-08-19
申请号	EP2013783762	申请日	2013-10-11
[标]申请(专利权)人(译)	环状药物公司		
申请(专利权)人(译)	Pharmacyclics的，INC.		
当前申请(专利权)人(译)	Pharmacyclics的，INC.		
[标]发明人	CHANG BETTY Y CHANG STELLA		
发明人	CHANG, BETTY, Y. CHANG, STELLA		
IPC分类号	C07D403/12 A61K31/506 G01N33/68 G01N33/532		
CPC分类号	A61K31/519 C12Q1/485 G01N33/57407 G01N33/94 C07D519/00 G01N33/57496 G01N33/581 G01N2333/912		
优先权	61/712675 2012-10-11 US		
外部链接	Espacenet		

摘要(译)

本发明提供了用于确定TEC家族激酶抑制剂对靶激酶的功效的方法，测定和系统。所述方法，测定和系统涉及通过TEC家族激酶抑制剂（例如BTK抑制剂）确定靶激酶的占有率。这种定量测量用于告知治疗处理和受试者的整体健康护理管理。例如，提供了用于诊断，预测和监测受益于TEC家族激酶抑制剂治疗的疾病或适应症的诊断试剂盒。在另一个实例中，还提供了用于鉴定TEC家族激酶抑制剂疗法的应答者，确定治疗方案和检测对TEC家族激酶抑制剂的抗性的诊断试剂盒。