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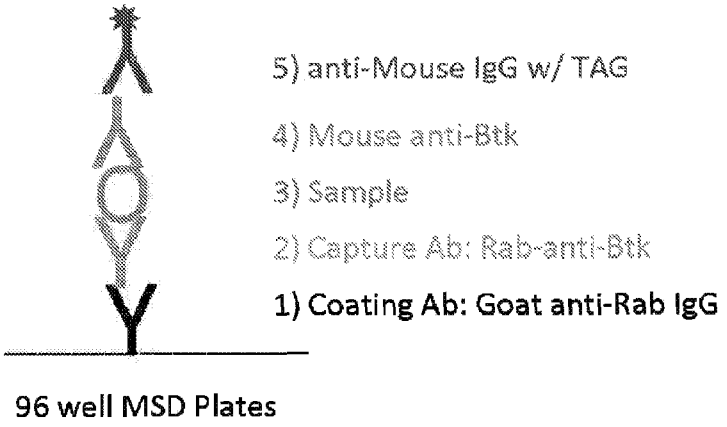
(54) Title: METHODS AND ASSAYS FOR QUANTIFICATION AND NORMALIZATION OF KINASE AND LIGAND BINDING

FIGURE 1



(57) Abstract: The invention provides methods and assay systems for both quantitative determination of a target, particularly a kinase, including a Tec family kinase, particularly BTK, and target occupancy, including the relative amount of available target associated with a target inhibitor or ligand, particularly wherein the target inhibitor or ligand is a covalent or irreversible kinase inhibitor, particularly including irreversible BTK inhibitor. The invention also relates to assays for determining target occupancy, normalized to total quantified target in a biological system or clinical sample, wherein target is quantified, normalized and occupancy determined using the same or a common assay format. In an aspect of the invention, biotinylated anti-BTK antibody and biotinylated BTK inhibitor probe are utilized in the assay format, on samples split for simultaneous or commensurate analysis.

METHODS AND ASSAYS FOR QUANTIFICATION AND NORMALIZATION OF KINASE AND LIGAND BINDING

CROSS-REFERENCE TO RELATED APPLICATION

[0001] This application claims the benefit of U.S. Provisional Application No. 62/092,942, filed December 17, 2014, which application is incorporated herein by reference in its entirety.

FIELD OF THE INVENTION

[0002] The present invention relates generally to a method and assay system for determination of the quantity of a target or enzyme, particularly a kinase, including a Tec family kinase, particularly Bruton's tyrosine kinase (BTK), and the relative amount of available target or enzyme associated with a target ligand or enzyme binder, particularly wherein the target ligand or enzyme binder is a covalent or irreversible kinase inhibitor, particularly including irreversible BTK inhibitor. The invention also relates to assays for determining target binding or occupancy, normalized to total quantified target in a biological system or clinical sample, wherein target is quantified, normalized and occupancy determined using the same or a common assay format.

BACKGROUND OF THE INVENTION

[0003] Protein kinases are a large family of conserved enzymes that transfer a γ-phosphate group from ATP to amino acid residues, including tyrosine, serine and threonine, in a ubiquitous mechanism for cellular signal transduction. Protein kinase inhibitors (PKIs) had historically been reversible ATP competitive inhibitors, however, given the high sequence conservation of the ATP-binding site, achieving a high degree of kinase selectivity is challenging. Irreversible inhibitors or covalent kinase inhibitors that react specifically with the enzyme and/or change it via covalent modification are attractive alternatives. Many such inhibitors target the highly nucleophilic thiol group of cysteine residues, which are found in and around the ATP-binding site. A series of irreversible inhibitors have advanced to clinical trials that target Cys797 conserved in EGFR, Her2 and Her4. A number of non-EGFR family kinases possess a cysteine

at a comparable position to the Cys797 of EGFR. These include members of the Tec family of kinases (BMX, BTK (Btk), ITK, TEC and TXK), Scr family member BLK, MKKa7 kinase, and JAK3 kinase (Liu, Q et al (2013) Chemistry & Biology 20:146-159).

[0004] Bruton's tyrosine kinase (Btk), a member of the Tec family of non-receptor tyrosine kinases, plays an essential role in the B-cell signaling pathway linking cell surface B-cell receptor (BCR) stimulation to downstream intracellular responses. It is required for the normal development and function of B-lymphocytes in humans and mice as evidenced by mutations in the Btk gene that result in the X-linked agammaglobulinemia (XLA) phenotype in humans and a less severe X-linked immunodeficiency phenotype (xid) in mice. *See, e.g.,* D. A. Fruman, et al., (2000), *Immunity* 13:1-3. Btk is expressed in all hematopoietic cells types except T lymphocytes and natural killer cells, and participates in a number of TLR and cytokine receptor signaling pathways including lipopolysaccharide (LPS) induced TNF-α production in macrophages, suggesting a general role for Btk in immune regulation.

[0005] Btk contains an amino-terminal pleckstrin homology (PH) domain, followed by a Tec homology (TH) domain, regulatory Src homology (SH3, SH2) domains, and a C-terminal kinase (SH1) domain. In unstimulated B cells, Btk is localized to the cytoplasm where it is catalytically inactive, presumably due to a tertiary conformation arising from intramolecular interactions between the kinase domain and the SH2 and/or SH3 domains that block access of substrates to the active site. After BCR stimulation, Btk is recruited to the cell membrane via interactions between the N-terminal PH domain and cell membrane phosphoinositides. Membrane-associated Btk is then phosphorylated at Tyr 551 in the activation loop by Src family kinases. Subsequent Btk auto-phosphorylation at Tyr 223 stabilizes the active conformation and fully activates Btk kinase activity. Activated Btk phosphorylates phospholipase (PLCγ), initiating calcium mobilization and generating diacylglycerol (DAG) as secondary signals, eventually leading to transcriptional activation and amplification of BCR stimulation.

[0006] Small molecule Btk inhibitors have been developed with anticipated therapeutic benefit in the treatment of lymphoma and autoimmune diseases. A potent irreversibly acting small molecule inhibitor of Btk, PCI-32765, has demonstrated promising clinical activity in an ongoing phase I study in patients with B-cell NHL. PCI-32765 inhibits BCR signaling downstream of Btk, selectively blocks B-cell activation, and is efficacious in animal models of arthritis, lupus, and B-cell lymphoma (Honigberg LA et al. (2010) PNAS 107(20):13075-13080). Irreversible inhibitor PCI-32765 is one of a series of Btk inhibitors that bind covalently to

cysteine residue Cys-481 in the active site leading to irreversible inhibition of Btk enzymatic activity (Pan Z et al (2007) Chem Med Chem 2:58-61).

[0007] Potent and selective kinase inhibitors, particularly irreversible inhibitors, are highly desirable as potential specific therapeutics and as probes to pharmacologically interrogate kinase biology. Assessing potency and inhibition of irreversible or covalent inhibitors necessitates an accurate and efficient determination of the relative amount of bound inhibitor to the total amount of enzyme target. There is an increasing need for integrated target and physiology based preclinical drug discovery approaches that allow for determination and evidence of target engagement and subsequent effects on *in vivo* efficacy and toxicology. One approach has been to use target site occupancy assays to understand the optimal dosing regimen to achieve a desired therapeutic effect and minimize side effects. Traditionally, these target site occupancy assays have relied on the use of radioisotoically labeled tracers, detected by positron emission tomography (PET), single photon emission computer tomography (SPECT) or liquid scintillation spectroscopy, to drive the analysis of structure-activity-relationship within a drug-discovery effort. Although this approach has proven to be very useful and can provide conclusive evidence of target engagement, it can often be very expensive and not allow for the degree of throughput that is often required in early preclinical discovery efforts. Alternative approaches utilize labeled drug or ligand normalized to cumbersome protein target determinations, particularly utilizing distinct formats that are not directly comparable.

[0008] There is a need for rapid, simple, dependable, and accurate assays and methods to determine the amount of bound inhibitor, particularly relative to the total or available amount of target, including in a clinical setting on clinical samples.

[0009] The citation of references herein shall not be construed as an admission that such is prior art to the present invention.

SUMMARY OF THE INVENTION

[00010] In accordance with the present invention, assay methods and assay systems are provided for quantification of a protein or enzyme target and target occupancy, particularly for a covalent or irreversible inhibitor of the target. In accordance with the methods and assays of the invention, a single, common format is utilized and serves as the basis for both quantification of target and for determination of target occupancy. The methods and assays of the invention provide for use of a common set of reagents for determination and evaluation of target quantification and target occupancy. The methods and assays of the invention allow for standardization and eliminate the need for multiple formats or evaluation protocols.

[00011] The invention provides a method or assay to determine target occupancy and quantitate total target of a kinase, particularly a Tec family kinase, wherein target occupancy is determined utilizing a target occupancy probe and total target is quantitated using an anti-target specific binding member, wherein the target occupancy probe and anti-target specific binding member are tagged with a moiety for capture by the same capture moiety binder. In accordance with the method, a target containing sample is obtained and split or separated for each of (a) target occupancy determination and (b) total target determination. In accordance with the method or assay, each of (a) target occupancy determination and (b) total target determination is conducted comprising the steps of: (i) contacting a sample with target occupancy probe in (a) and anti-target specific binding member in (b), each tagged with a same capture moiety or a capture moiety that binds to the same capture binder, to form an occupancy probe-target complex in (a) and a specific binding member-target complex in (b); (ii) capturing the complex from (a) and (b) with a capture moiety binder coated solid support; and (iii) detecting the presence and amount of captured occupancy probe-target complex in (a) and specific binding member-target complex in (b).

[00012] The invention provides a method or assay to determine target occupancy and quantitate total target of a kinase, particularly a non-receptor tyrosine kinase, particularly a Tec family kinase, comprising:

(i) obtaining a sample and splitting or aliquoting the sample for (a) target occupancy determination and (b) total target determination;

(ii) contacting the sample for (a) with a capture moiety tagged target occupancy probe and the sample for (b) with capture moiety tagged anti-target specific binding member, wherein the capture moiety in (a) and (b) is the same or is capable of binding to the same capture moiety binder, and wherein a target occupancy probe-target complex is formed in (a) and a specific binding member-target complex is formed in (b);

(iii) incubating each of (a) and (b) with a capture moiety binder coated solid support under conditions wherein binding of the target complex in (a) and (b) to the solid support occurs; and

(iv) detecting the target complex in (a) and (b) by contacting the target complex bound solid support with a primary anti-target specific binding member and determining the amount of target complex in (a) and (b).

[00013] In a particular aspect of the method and assay, target is quantified by comparison to a standard curve generated using recombinant target protein, wherein the standard curve is obtained simultaneously or approximately the same time as the samples are analyzed.

[00014] In an aspect of the method and assay, the primary anti-target specific binding member is an antibody, particularly a labeled antibody. In an aspect, in (iv) the target complex bound solid support is contacted with a secondary tagged or labeled antibody that recognizes or binds the primary anti-target specific binding member for detection.

[00015] In an aspect of the method and assay, the solid support is washed between steps (iii) and (iv). In an aspect, the solid support is washed in (iv) after primary anti-target specific binding member is contacted and prior to contacting with a secondary tagged or labeled antibody that recognizes or binds the primary anti-target specific binding member.

[00016] In aspects of the method and assay, the sample is a cell lysate, particularly a lysate of PBMCs. In an aspect, the samples for (a) target occupancy determination and (b) total target determination are processed side by side, simultaneously or in series. In an aspect the target is BTK, the target occupancy probe is a labeled or biotinylated BTK inhibitor, the anti-target specific binding member is an anti-BTK monoclonal antibody. In an aspect, the capture moiety is biotin and the capture moiety binder is avidin, streptavidin or neutravidin. In an aspect the anti-BTK monoclonal antibody is a biotinylated anti-BTK antibody.

[00017] The invention provides a method or assay to determine BTK target occupancy and quantitate total target of BTK in a sample, comprising:

(i) obtaining a sample and splitting or aliquoting the sample for (a) target occupancy determination and (b) total target determination;

(ii) contacting the sample for (a) with a capture moiety tagged BTK occupancy probe and the sample for (b) with capture moiety tagged anti-BTK antibody, wherein the capture moiety in (a) and (b) is biotin or is capable of binding to streptavidin or avidin, and wherein a BTK occupancy probe-BTK complex is formed in (a) and an anti-BTK antibody-BTK complex is formed in (b);

(iii) incubating each of (a) and (b) with a streptavidin or avidin coated solid support under conditions wherein binding of the BTK occupancy probe-BTK complex in (a) and the anti-BTK antibody-BTK complex in (b) to the solid support occurs; and

(iv) detecting the BTK occupancy probe-BTK complex in (a) and the anti-BTK antibody-BTK complex in (b) by contacting the complex bound solid support with a primary anti-BTK antibody and determining the amount of complex in (a) and (b).

[00018] In an aspect of the method or assay the BTK occupancy probe is a labeled covalent or irreversible inhibitor of BTK. In an aspect of the method or assay the BTK occupancy probe is labeled ibrutinib, particularly biotinylated ibrutinib, particularly having the structure of Formula IV.

[00019] It is a further object of the present invention to provide a method and associated assay system for screening substances such as drugs, agents and the like, potentially effective as competitive or irreversible inhibitors of a Tec family kinase, including a BTK kinase, wherein the ability of a drug, agent or the like to bind BTK is accessed by determining target BTK occupancy of a drug, agent or the like, either directly by utilizing labeled or tagged drug, agent or the like as target occupancy probe in assays of the invention, or by determining occupancy of said drug, agent or the like by assessing unbound target BTK available for target occupancy probe binding in the presence of said drug, agent or the like.

[00020] The invention further provides a test kit for the demonstration of the quantity of target and target occupancy of inhibitor, particularly BTK target and BTK inhibitor comprising:

(a) a predetermined amount of BTK-occupancy probe such as biotinylated ibrutinib and a predetermined amount of biotinylated anti-BTK antibody;

(b) a solid support coated with streptavidin or avidin;

(c) other reagents; and

(d) directions for use of said kit.

[00021] It is to be understood that the methods and assays described herein are not limited to the particular methodology, protocols, cell lines, constructs, and reagents described herein and in some embodiments will 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 assays described herein, which will be limited only by the appended claims.

[00022] Other objects and advantages will become apparent to those skilled in the art from a review of the following description which proceeds with reference to the following illustrative drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

[00023] FIGURE 1 depicts a sandwich ELISA for total BTK, utilizing a first coating antibody (for example goat anti-rabbit IgG), followed by a capture antibody (for example rabbit anti-target BTK). Sample is then applied, an anti-target antibody (for example mouse anti-BTK) is added, and then a secondary antibody which is tagged or detectable and recognizes or binds to the anti-target antibody (for example anti-mouse IgG with a TAG) is introduced for detection.

[00024] FIGURE 2A-2D depicts assay results using sandwich ELISA as depicted in Figure 1 for BTK in an MSD format. Results are tabulated in A and C and standard curves are shown in

B and D. A and B provide results with 1 μg/ml capture antibody; C and D provide results with 0.5 μg/ml capture antibody. Capture antibody was Rabbit anti-BTK monoclonal antibody (Abcam Cat No. 32555, C-term peptide). Detection antibody was anti-BTK monoclonal antibody (BD Bioscience Cat No 611117, N-term rec protein 2-172 aa). BTK protein was from BPS Bioscience (Cat No 40405; full length, GST tagged).

[00025] FIGURE 3A-3C provides sandwich ELISA assay results for recombinant BTK versus DoHH2 cell lysate protein. A tabulates results with recombinant BTK. B graphs recombinant BTK protein results. Cell lysate results are tabulated in C. Cap-Ab-1 refers to 1 μg/ml capture antibody; Cap-Ab-2 refers to 0.5 μg/ml capture antibody.

[00026] FIGURE 4A-4C depicts sandwich ELISA MSD format for cell lysates (DoHH2 cells). Assay results using higher amounts of coating antibody 2 μg/ml and 4 μg/ml, capture antibody are shown in A and B respectively. C graphs results with 0.5 μg/ml, 1 μg/ml, 2 μg/ml, and 4 μg/ml coating antibody.

[00027] FIGURE 5 depicts an alternative sandwich ELISA format without coating antibody (goat anti-rabbit IgG). The capture antibody (rabbit or mouse anti-BTK antibody) on the plates recognizes target (e.g. BTK). Sample is then applied, followed by mouse or rabbit anti-target (e.g. BTK) antibody, and then secondary anti-mouse or rabbit IgG with a tag or HRP as signal for detection.

[00028] FIGURE 6 depicts protein PAGE gel stained with Coomassie to visualize proteins. Control lane of protein markers is first, followed by 5 lanes of various commercially available recombinant BTK protein samples. Lane 1:Invitrogen (Cat No. PV3363; Full length, His Tagged); Lane 2: Millipore (Cat No. 14-552; Full length, His Tagged); Lane 3: BPS Bioscience (Cat No. 40405; Full length, GST Tagged) MW = 106 kDa; Lane 4: Janssen (BTK – WT, 15ul diluted to 50ul PBS); Lane 5: Janssen (BTK – C481S, 15ul diluted to 50ul PBS). Protein concentration (0.1mg/ml). 10ul protein + 10ul PBS + 10ul Reducer + 10ul 4X NuPAGE Sample Buffer are mixed, 12ul of the mixture loaded per well, and the gel Pierce Commassie Stained (ON @ RT).

[00029] FIGURE 7 depicts an experimental protocol for a total BTK quantification ELISA using a capture antibody only, as shown in Figure 5. Each box depicts a step in the protocol. First, 96 well high protein binding plates (Pierce/MSD) are utilized. Antibody at 2 μg/ml is coated onto the plates in PBS overnight at 4°C. The plates are then washed and blocked in 3%BSA (250 μl) for 1-2 hours at room temperature (RT). Either recombinant BTK (R-BTK) or cell lysates are added in 1%BSA (100 μl) and incubated overnight at 4°C. Primary anti-BTK

antibody is added in 1%BSA (100 µl) for 2 hours at RT, followed by secondary antibody in 1% BSA (100 µl) for 1 hour at RT. Detection reagent (100 µl) is added and the plates are read.

[00030] FIGURE 8A-8D depicts results of the capture antibody ELISA method in standard (STD) (A and C) and meso-scale discovery (MSD) (B and D) formats. A and B provide results with recombinant BTK protein, C and D provide results with cell lysates. The capture antibody utilized was anti-BTK rabbit monoclonal antibody (CST Cat No 8547) and detection antibody was anti-BTK mouse monoclonal antibody (BD Cat No 611117).

[00031] FIGURE 9A-9D depicts results of the capture antibody ELISA method in standard (STD) (A and C) and meso-scale discovery (MSD) (B and D) formats. A and B provide results with recombinant BTK protein, C and D provide results with cell lysates. The capture antibody utilized was anti-BTK rabbit polyclonal antibody (Abcam Cat No 8547) and detection antibody was anti-BTK mouse monoclonal antibody (BD Cat No 611117). No BTK protein was captured and quantified in the cell lysates.

[00032] FIGURE 10A-10D depicts results of the capture antibody ELISA method in standard (STD) (A and C) and meso-scale discovery (MSD) (B and D) formats. A and B provide results with recombinant BTK protein, C and D provide results with cell lysates. The capture antibody utilized was anti-BTK mouse monoclonal antibody (BD Cat No 611117) and detection antibody was anti-BTK rabbit polyclonal antibody (Abcam Cat No 8547). Almost no BTK protein was captured and quantified in the cell lysates.

[00033] FIGURE 11A-11D depicts results of the capture antibody ELISA method in standard (STD) (A and C) and meso-scale discovery (MSD) (B and D) formats. A and B provide results with recombinant BTK protein, C and D provide results with cell lysates. The capture antibody utilized was anti-BTK mouse monoclonal antibody (BD Cat No 611117) and detection antibody was biotinylated anti-BTK rabbit monoclonal antibody (CST Cat No 12624S). Some BTK protein was captured and quantified in the cell lysates.

[00034] FIGURE 12 depicts a novel and simplified sandwich ELISA format without coating or capture antibody. In this new assay, sample is mixed with biotinylated anti-target antibody (e.g. biotinylated mouse or rabbit anti-BTK antibody) allowed to bind to plates and then detected using anti-target antibody (e.g. mouse or rabbit anti-BTK antibody) and a secondary labeled or tagged antibody (e.g. anti-mouse or anti-rabbit antibody with tag or HRP).

[00035] FIGURE 13 depicts an experimental protocol for a total BTK quantification ELISA using a biotinylated anti-target antibody, as shown in Figure 12. Each box depicts a step in the protocol. The assay provides target (e.g. BTK) occupancy data (normalized to total target (BTK)) and quantification of total target (BTK). The assay is suitable for recombinant target

(BTK) or cell lysate samples. A sample for analysis is split and a portion is run through a target occupancy assay using biotinylated target occupancy probe (biotin-probe), while the other portion is used for a total target (BTK) assay using biotinylated anti-target antibody (e.g. biotin-anti-BTK). The biotin-probe complex (target occupancy probe-target complex) and the biotin-anti-BTK complex (anti-target specific binding member-target complex) are captured using streptavidin coated plates. Detection is then accomplished, for example using streptavidin HRP or a tag. Total BTK is determined and total occupancy data is derived by normalizing bound biotin-probe to total BTK.

[00036] FIGURE 14A-14B provides results of standard (STD) format ELISA for target (BTK) occupancy using (A) recombinant BTK (Rec-BTK) and (B) cell lysates using biotinylated BTK target occupancy probe (biotin ibrutinib/41025). Protein concentrations for the cell lysate studies range from 0 to 1500 μg/ml.

[00037] FIGURE 15A-15D provides results of standard (STD) format ELISA for total target (BTK) using (A) and (C) recombinant BTK (Rec-BTK) and (B) and (D) cell lysates using biotinylated anti-target (anti-BTK) antibody (biotinylated rabbit anti-BTK monoclonal antibody, Cell Signal Technology, Cat No 12624S). Protein concentrations for the cell lysate studies range from 0 to 1500 μg/ml (B) and 0 to 200 μg/ml (D).

[00038] FIGURE 16A-16B provides results of MSD format ELISA for target (BTK) occupancy using (A) recombinant BTK (Rec-BTK) and (B) cell lysates using biotinylated BTK target probe (biotin ibrutinib/41025). Protein concentrations for the cell lysate studies range from 0 to 1500 μg/ml.

[00039] FIGURE 17A-17D provides results of MSD format ELISA for total target (BTK) using (A) and (C) recombinant BTK (Rec-BTK) and (B) and (D) cell lysates using biotinylated anti-target (anti-BTK) antibody (biotinylated rabbit anti-BTK monoclonal antibody, Cell Signal Technology, Cat No 12624S). Protein concentrations for the cell lysate studies range from 0 to 1500 μg/ml (B) and 0 to 200 μg/ml (D).

[00040] FIGURE 18A-18B provides results of STD format ELISA for target (BTK) occupancy using (A) recombinant BTK (Rec-BTK) and (B) cell lysates using biotinylated BTK target occupancy probe (biotin ibrutinib/41025). Protein concentrations for the cell lysate studies range from 0 to 4000 μg/ml. The results of each assay are tabulated below the graphs.

[00041] FIGURE 19A-19B provides results of MSD format ELISA for target (BTK) occupancy using (A) recombinant BTK (Rec-BTK) and (B) cell lysates using biotinylated BTK target occupancy probe (biotin ibrutinib/41025). Protein concentrations for the cell lysate studies range from 0 to 4000 μg/ml. The results of each assay are tabulated below the graphs.

[00042] FIGURE 20 provides results of total target (BTK) ELISA STD format using biotinylated anti-target (anti-BTK) antibody for (A) recombinant BTK (Rec-BTK) and (B) cell lysates. Protein concentrations for the cell lysate studies range from 0 to 4000 μg/ml. The results of each assay are tabulated below the graphs.

[00043] FIGURE 21 provides results of total target (BTK) ELISA MSD format using biotinylated anti-target (anti-BTK) antibody for (A) recombinant BTK (Rec-BTK) and (B) cell lysates. Protein concentrations for the cell lysate studies range from 0 to 4000 μg/ml. The results of each assay are tabulated below the graphs.

[00044] FIGURE 22 depicts BTK quantification strategies and three methods denoted Method 1, Method 2, and Method 3. Method 1 corresponds to the present novel method. Cell lysates are split initially and side by side assays run for BTK occupancy using a target occupancy probe (e.g. Biotin-probe) and for total BTK determination using an anti-target specific binding member (e.g. Biotin anti-BTK antibody). Alternative Method 2 and Method 3 are depicted wherein lysates are screened for target occupancy (e.g. BTK), the supernatant collected, transferred, and assessed for total target using anti-target antibody (e.g. anti-BTK).

[00045] FIGURE 23 depicts the steps of Method 1.

[00046] FIGURE 24 provides assay results and BTK occupancy, free BTK and standard curve for patient #1 with Method 1. Patient was administered inhibitor (ibrutinib; PCI-32765) 420 mg daily dose and BTK target occupancy was determined on Day 1 (vehicle dose), Day 2, Day 8, Day 15, Day 29 and at the end of the study.

[00047] FIGURE 25 provides assay results and BTK occupancy, free BTK and standard curve for patient #2 with Method 1. Patient was administered inhibitor (ibrutinib; PCI-32765) 420 mg daily dose and BTK target occupancy was determined on Day 1 (vehicle dose), Day 8, Day 15 and Day 29.

[00048] FIGURE 26 provides assay results and BTK occupancy, free BTK and standard curve for patient #3 with Method 1. Patient was administered inhibitor (ibrutinib; PCI-32765) 420 mg daily dose and BTK target occupancy was determined on Day 1 (vehicle dose), Day 2, Day 8, Day 15 and Day 29.

[00049] FIGURE 27 provides assay results and BTK occupancy, free BTK and standard curve for patient #4 with Method 1. Patient was administered inhibitor (ibrutinib; PCI-32765) 420 mg daily dose and BTK target occupancy was determined on Day 1 (vehicle dose), Day 2, Day 8, Day 15, Day 29 and at the end of the study.

[00050] FIGURE 28 depicts the steps of Method 2. Lysates are screened for BTK occupancy and the supernatants transferred for separate analysis to determine total BTK in the recovered supernatants.

[00051] FIGURE 29 provides assay results and BTK occupancy, free BTK and standard curve for patient #1 with Method 2. Patient was administered inhibitor (ibrutinib; PCI-32765) 420 mg daily dose and BTK target occupancy determined on Day 1 (vehicle), Day 2, Day 8, Day 15, Day 29 and at the end of the study.

[00052] FIGURE 30 provides assay results and BTK occupancy, free BTK and standard curve for patient #2 with Method 2. Patient was administered inhibitor (ibrutinib; PCI-32765) 420 mg daily dose and BTK target occupancy was determined on Day 1 (vehicle), Day 8, Day 15 and Day 29.

[00053] FIGURE 31 provides assay results and BTK occupancy, free BTK and standard curve for patient #3 with Method 2. Patient was administered inhibitor (ibrutinib; PCI-32765) 420 mg daily dose and BTK target occupancy determined on Day 1 (vehicle), Day 2, Day 8, Day 15 and Day 29.

[00054] FIGURE 32 provides assay results and BTK occupancy, free BTK and standard curve for patient #4 with Method 2. Patient was administered inhibitor (ibrutinib; PCI-32765) 420 mg daily dose and BTK target occupancy determined on Day 1 (vehicle), Day 2, Day 8, Day 15, Day 29 and at the end of the study.

[00055] FIGURE 33 depicts the steps of Method 3. Lysates are screened for BTK occupancy and the supernatants transferred for separate analysis to determine total BTK in the recovered supernatants using anti-BTK pre-coated plates.

[00056] FIGURE 34 provides assay results and BTK occupancy, free BTK and standard curve for patient #1 with Method 3. Patient was administered inhibitor (ibrutinib; PCI-32765) 420 mg daily dose and BTK target occupancy determined on Day 1 (vehicle), Day 2, Day 8, Day 15, Day 29 and at the end of the study.

[00057] FIGURE 35 provides assay results and BTK occupancy, free BTK and standard curve for patient #2 with Method 3. Patient was administered inhibitor (ibrutinib; PCI-32765) 420 mg daily dose and BTK target occupancy determined on Day 1 (vehicle), Day 8, Day 15and Day 29.

[00058] FIGURE 36 provides assay results and BTK occupancy, free BTK and standard curve for patient #3 with Method 3. Patient was administered inhibitor (ibrutinib; PCI-32765) 420 mg daily dose and BTK target occupancy determined on Day 1 (vehicle), Day 2, Day 8, Day 15 and Day 29.

[00059] FIGURE 37 provides assay results and BTK occupancy, free BTK and standard curve for patient #4 with Method 3. Patient was administered inhibitor (ibrutinib; PCI-32765) 420 mg daily dose and BTK target occupancy determined on Day 1 (vehicle), Day 2, Day 8, Day 15, Day 29 and at the end of the study.

[00060] FIGURE 38 provides a comparison of patient results across Methods 1, 2, and 3 and compares same to BTK occupancy evaluated in a separate study with patients administered an 820 mg ibrutinib dose and where occupancy was determined by relative amount of free unbound BTK in a time course study.

DETAILED DESCRIPTION

[00061] In accordance with the present invention there may be employed conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Sambrook et al, "Molecular Cloning: A Laboratory Manual" (1989); "Current Protocols in Molecular Biology" Volumes I-III [Ausubel, R. M., ed. (1994)]; "Cell Biology: A Laboratory Handbook" Volumes I-III [J. E. Celis, ed. (1994))]; "Current Protocols in Immunology" Volumes I-III [Coligan, J. E., ed. (1994)]; "Oligonucleotide Synthesis" (M.J. Gait ed. 1984); "Nucleic Acid Hybridization" [B.D. Hames & S.J. Higgins eds. (1985)]; "Transcription And Translation" [B.D. Hames & S.J. Higgins, eds. (1984)]; "Animal Cell Culture" [R.I. Freshney, ed. (1986)]; "Immobilized Cells And Enzymes" [IRL Press, (1986)]; B. Perbal, "A Practical Guide To Molecular Cloning" (1984).

[00062] Therefore, if appearing herein, the following terms shall have the definitions set out below.

[00063] The terms "Bruton's tyrosine kinase", "BTK", "Btk" may be used herein interchangeably, and as used throughout the present application and claims refer to a non-receptor tyrosine kinase, which is a member of the Tec family of non-receptor tyrosine kinases. BTK plays a role in the B-cell signaling pathway, particularly linking cell surface B-cell receptor (BCR) stimulation to downstream intracellular responses.

[00064] "Tec family kinases" are a family of related kinases which are non-receptor tyrosine kinases characterized by a pleckstrin homology (PH) domain and a proline rich region. Kinases that are recognized members of the Tec family include particularly BTK (Bruton's tyrosine kinase), BMX (BMX non-receptor tyrosine kinase), ITK (also denoted EMT, TSK; IL-2 inducible T cell kinase), TEC (tyrosine protein kinase) and TXK (also denoted RLK; tyrosine protein kinase).

[00065] An "irreversible binder" refers to a ligand or binder which associates with a target or receptor includes wherein noncovalent binding is so tight that the ligand or binder does not dissociate for a very long period of time (sometimes days). In such cases, the association is effectively irreversible and does not reach equilibrium (or equal binding and dissociation) within the relevant time frame.

[00066] An "irreversible inhibitor" is an inhibitor that binds to an enzyme or target, usually reacting with the enzyme and causing chemical changes to the active sites of enzymes. An irreversible inhibitor's effects cannot be reversed. A main role of irreversible inhibitors includes modifying key amino acid residues needed for enzymatic activity. Irreversible inhibitors are covalently or noncovalently bound to the target enzyme and dissociate very slowly from the enzyme. Irreversible inhibitors often form strong covalent bonds with an enzyme or target. These inhibitors may act at, near, or remote from the active site and they may not be displaced by the addition of excess substrate. Irreversible inhibitors can modify the basic structure of the enzyme/target such that the enzyme activity is impaired or the enzyme ceases to work.

[00067] A "covalent inhibitor" refers to an inhibitor that directly and covalently binds to its target or enzyme. A covalent inhibitor is an inhibitor that acts via a covalent mechanism of action. A covalent inhibitor achieves a sustained duration of inhibition.

[00068] In contrast to an irreversible or covalent binder, a "reversible binder" binds through a non-covalent binding process, wherein the ligand can both bind to and dissociate from the receptor. Equilibrium is reached when the time following mixing the binder and receptor or target to which it binds is long compared to the time for 50% binding and dissociation. A "reversible inhibitor" Reversible inhibitors can bind to enzymes through weak non-covalent interactions such as ionic bonds, hydrophobic interactions, and hydrogen bonds. Because reversible inhibitors do not form any chemical bonds or reactions with the enzyme, they are formed rapidly and can be easily removed; thus the enzyme and inhibitor complex is rapidly dissociated in contrast to irreversible inhibition.

[00069] The term "target occupancy" relates to what percentage of target, such as enzymes, receptors, ion channels, transporters are occupied by a drug, ligand, binder etc. In order to determine the target occupancy, it is necessary to relate (i) the amount of a target that is interacting with or bound by a target binder, including a drug, ligand, inhibitor to (ii) the total amount of a target in a given milieu, sample, physiological system.

[00070] The term "capture moiety" as used herein refers to a part or group of a molecule that is capable of being specifically bound by another molecule or entity, particularly wherein the another molecule or entity is attached or linked to a solid support. For example, useful capture

moieties include affinity labels for which specific and selective ligands are available (e.g., biotin with avidin or streptavidin, glutathione with GST), haptens and proteins for which antisera or monoclonal antibodies are available, nucleic acid molecules with a sequence complementary to a target, and peptides for which specific and selective ligands are available (e.g., histidine tag with Ni). The solid support can be, for example, a filter, a plate, a membrane, a chromatographic resin, or a bead.

[00071] The term "specific binding member" refers to a member of a pair of molecules which have binding specificity for one another. The members of a specific binding pair may be naturally derived or wholly or partially synthetically produced. Examples of types of specific binding pairs are antigen-antibody, hormone-hormone receptor, receptor-ligand, enzyme-substrate. This application is particularly concerned with antigen-antibody type binding pairs. In a particular aspect, a specific binding pair is an antibody, binding fragment thereof, or a ligand that binds a molecule without altering the molecule.

[00072] An "antibody" is any immunoglobulin, including antibodies and fragments thereof, that binds a specific epitope. The term "antibody" describes an immunoglobulin whether natural or partly or wholly synthetically produced. The term also covers any polypeptide or protein having a binding domain which is, or is homologous to, an antibody binding domain. CDR grafted antibodies are also contemplated by this term. The term encompasses polyclonal, monoclonal, and chimeric antibodies, the last mentioned are described in further detail in U.S. Patent Nos. 4,816,397 and 4,816,567. The term "antibody(ies)" includes a wild type immunoglobulin (Ig) molecule, generally comprising four full length polypeptide chains, two heavy (H) chains and two light (L) chains, or an equivalent Ig homologue thereof (e.g., a camelid nanobody, which comprises only a heavy chain); including full length functional mutants, variants, or derivatives thereof, which retain the essential epitope binding features of an Ig molecule, and including dual specific, bispecific, multispecific, and dual variable domain antibodies; Immunoglobulin molecules can be of any class (e.g., IgG, IgE, IgM, IgD, IgA, and IgY), or subclass (e.g., IgG1, IgG2, IgG3, IgG4, IgA1, and IgA2). Also included within the meaning of the term "antibody" are any "antibody fragment".

[0001] An "antibody fragment" means a molecule comprising at least one polypeptide chain that is not full length, including (i) a Fab fragment, which is a monovalent fragment consisting of the variable light (VL), variable heavy (VH), constant light (CL) and constant heavy 1 (CH1) domains; (ii) a F(ab')2 fragment, which is a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; (iii) a heavy chain portion of an Fab (Fd) fragment, which consists of the VH and CH1 domains; (iv) a variable fragment (Fv) fragment,

which consists of the VL and VH domains of a single arm of an antibody, (v) a domain antibody (dAb) fragment, which comprises a single variable domain (Ward, E.S. et al., Nature 341, 544-546 (1989)); (vi) a camelid antibody; (vii) an isolated complementarity determining region (CDR); (viii) a Single Chain Fv Fragment wherein a VH domain and a VL domain are linked by a peptide linker which allows the two domains to associate to form an antigen binding site (Bird et al, Science, 242, 423-426, 1988; Huston et al, PNAS USA, 85, 5879-5883, 1988); (ix) a diabody, which is a bivalent, bispecific antibody in which VH and VL domains are expressed on a single polypeptide chain, but using a linker that is too short to allow for pairing between the two domains on the same chain, thereby forcing the domains to pair with the complementarity domains of another chain and creating two antigen binding sites (WO94/13804; P. Holliger et al Proc. Natl. Acad. Sci. USA 90 6444-6448, (1993)); and (x) a linear antibody, which comprises a pair of tandem Fv segments (VH-CH1-VH-CH1) which, together with complementarity light chain polypeptides, form a pair of antigen binding regions; (xi) multivalent antibody fragments (scFv dimers, trimers and/or tetramers (Power and Hudson, J Immunol. Methods 242: 193-204 9 (2000)); (xii) a minibody, which is a bivalent molecule comprised of scFv fused to constant immunoglobulin domains, CH3 or CH4, wherein the constant CH3 or CH4 domains serve as dimerization domains (Olafsen T et al (2004) Prot Eng Des Sel 17(4):315-323; Hollinger P and Hudson PJ (2005) Nature Biotech 23(9):1126-1136); and (xiii) other non-full length portions of heavy and/or light chains, or mutants, variants, or derivatives thereof, alone or in any combination.

[0002] As antibodies can be modified in a number of ways, the term "antibody" should be construed as covering any specific binding member or substance having a binding domain with the required specificity. Thus, this term covers antibody fragments, derivatives, functional equivalents and homologues of antibodies, including any polypeptide comprising an immunoglobulin binding domain, whether natural or wholly or partially synthetic. Chimeric molecules comprising an immunoglobulin binding domain, or equivalent, fused to another polypeptide are therefore included. Cloning and expression of chimeric antibodies are described in EP-A-0120694 and EP-A-0125023 and U.S. Patent Nos. 4,816,397 and 4,816,567.

[0003] An "antibody combining site" is that structural portion of an antibody molecule comprised of heavy and light chain variable and hypervariable regions that specifically binds antigen.

[0004] The phrase "antibody molecule" in its various grammatical forms as used herein contemplates both an intact immunoglobulin molecule and an immunologically active portion of an immunoglobulin molecule.

[00073] Exemplary antibody molecules are intact immunoglobulin molecules, substantially intact immunoglobulin molecules and those portions of an immunoglobulin molecule that contains the paratope, including those portions known in the art as Fab, Fab', $F(ab')_2$ and F(v), which portions are preferred for use in the therapeutic methods described herein. Fab and $F(ab')_2$ portions of antibody molecules are prepared by the proteolytic reaction of papain and pepsin, respectively, on substantially intact antibody molecules by methods that are well-known. See for example, U.S. Patent No. 4,342,566 to Theofilopolous et al. Fab' antibody molecule portions are also well-known and are produced from $F(ab')_2$ portions followed by reduction of the disulfide bonds linking the two heavy chain portions as with mercaptoethanol, and followed by alkylation of the resulting protein mercaptan with a reagent such as iodoacetamide. An antibody containing intact antibody molecules is preferred herein.

[00074] The phrase "monoclonal antibody" in its various grammatical forms refers to an antibody having only one species of antibody combining site capable of immunoreacting with a particular antigen. A monoclonal antibody thus typically displays a single binding affinity for any antigen with which it immunoreacts. A monoclonal antibody may therefore contain an antibody molecule having a plurality of antibody combining sites, each immunospecific for a different antigen; e.g., a bispecific (chimeric) monoclonal antibody.

[00075] The term “alkyl,” by itself or as part of another molecule means, unless otherwise stated, a straight or branched chain, or cyclic hydrocarbon radical, or combination thereof. In some embodiments, the alkyl chain is fully saturated, mono- or polyunsaturated. In other embodiments, the alkyl chain includes di- and multivalent radicals, having the number of carbon atoms designated (*i.e.* C_1-C_{10} means one to ten carbons). In other embodiments, examples of saturated hydrocarbon radicals include, but are not limited to, groups such as methyl, ethyl, n-propyl, isopropyl, n-butyl, t-butyl, isobutyl, sec-butyl, cyclohexyl, (cyclohexyl)methyl, cyclopropylmethyl, homologs and isomers of, for example, n-pentyl, n-hexyl, n-heptyl, n-octyl, and the like. An unsaturated alkyl group is one having one or more double bonds or triple bonds. In a further embodiment, examples of unsaturated alkyl groups include, but are not limited to, vinyl, 2-propenyl, crotyl, 2-isopentenyl, 2-(butadienyl), 2,4-pentadienyl, 3-(1,4-pentadienyl), ethynyl, 1- and 3-propynyl, 3-butynyl, and the higher homologs and isomers. In yet further embodiments, the term “alkyl,” unless otherwise noted, includes those derivatives of alkyl defined in more detail herein, such as “heteroalkyl”, “haloalkyl” and “homoalkyl”.

[00076] The term “carbonyl” as used herein refers to a group containing a moiety selected from the group consisting of -C(O)-, -S(O)-, $-S(O)_2-$, and –C(S)-, including, but not limited to, groups containing a least one ketone group, and/or at least one aldehyde group, and/or at least

one ester group, and/or at least one carboxylic acid group, and/or at least one thioester group. Such carbonyl groups include ketones, aldehydes, carboxylic acids, esters, and thioesters. In some embodiments, such groups are a part of linear, branched, or cyclic molecules.

[00077] The term "chemiluminescent group," as used herein, refers to a group which emits light as a result of a chemical reaction without the addition of heat. By way of example only, luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) reacts with oxidants like hydrogen peroxide (H_2O_2) in the presence of a base and a metal catalyst to produce an excited state product (3-aminophthalate, 3-APA).

[00078] The term "chromophore," as used herein, refers to a molecule which absorbs light of visible wavelengths, UV wavelengths or IR wavelengths.

[00079] The term "detectable label," as used herein, refers to a label which is observable using analytical techniques including, but not limited to, fluorescence, chemiluminescence, electron-spin resonance, ultraviolet/visible absorbance spectroscopy, mass spectrometry, nuclear magnetic resonance, magnetic resonance, and electrochemical methods.

[00080] The term "dye," as used herein, refers to a soluble, coloring substance which contains a chromophore.

[00081] The term "fluorophore," as used herein, refers to a molecule which upon excitation emits photons and is thereby fluorescent.

[00082] In some embodiments, the term "label," as used herein, refers to a substance which is incorporated into a compound and is readily detected, whereby its physical distribution is detected and/or monitored.

[00083] The term "subject" or "patient" as used herein, refers to an animal which is the object of treatment, observation or experiment. In one embodiment the subject or patient is a mammal including, but not limited to, a human.

[00084] The term "probe" refers to a compound or molecule for the detection of a target. The probe may comprise an agent, a linker, a label, or any combination thereof. In one instance, the probe may comprise an agent and a linker, or an agent and a label, or a label. In another instance, the probe comprises an agent and a label.

[00085] In some embodiments, the term "substituents" also referred to as "non-interfering substituents" refers to groups which are used to replace another group on a molecule. Such groups include, but are not limited to, halo, C_1-C_{10} alkyl, C_2-C_{10} alkenyl, C_2-C_{10} alkynyl, C_1-C_{10} alkoxy, C_5-C_{12} aralkyl, C_3-C_{12} cycloalkyl, C_4-C_{12} cycloalkenyl, phenyl, substituted phenyl, toluolyl, xylenyl, biphenyl, C_2-C_{12} alkoxyalkyl, C_5-C_{12} alkoxyaryl, C_5-C_{12} aryloxyalkyl, C_7-C_{12} oxyaryl, C_1-C_6 alkylsulfinyl, C_1-C_{10} alkylsulfonyl, -$(CH_2)_m$-O-(C_1-C_{10} alkyl) wherein m is from 1

to 8, aryl, substituted aryl, substituted alkoxy, fluoroalkyl, heterocyclic radical, substituted heterocyclic radical, nitroalkyl, -NO_2, -CN, -NRC(O)-(C_1-C_{10} alkyl), -C(O)-(C_1-C_{10} alkyl), C_2-C_{10} alkthioalkyl, -C(O)O-(C_1-C_{10} alkyl), -OH, -SO_2, =S, -COOH, -NR_2, carbonyl, -C(O)-(C_1-C_{10} alkyl)-CF_3, -C(O)-CF_3, -C(O)NR_2, -(C_1-C_{10} aryl)-S-(C_6-C_{10} aryl), -C(O)-(C_6-C_{10} aryl), -$(CH_2)_m$-O-$(CH_2)_m$-O-(C_1-C_{10} alkyl) wherein each m is an integer from 1 to 8, -C(O)NR_2, -C(S)NR_2, -SO_2NR_2, -NRC(O)NR_2, -NRC(S)NR_2, salts thereof, and the like. In some embodiments, each R group in the preceding list includes, but is not limited to, H, alkyl or substituted alkyl, aryl or substituted aryl, or alkaryl. Where substituent groups are specified by their conventional chemical formulas, written from left to right, they equally encompass the chemically identical substituents that would result from writing the structure from right to left; for example, -CH_2O- is equivalent to –OCH_2-.

[00086] In some embodiments, and by way of example only, substituents for alkyl and heteroalkyl radicals (including those groups referred to as alkylene, alkenyl, heteroalkylene, heteroalkenyl, alkynyl, cycloalkyl, heterocycloalkyl, cycloalkenyl, and heterocycloalkenyl) includes, but is not limited to: -OR, =O, =NR, =N-OR, -NR_2, -SR, -halogen, -SiR_3, -OC(O)R, -C(O)R, -CO_2R, -$CONR_2$, -$OC(O)NR_2$, -NRC(O)R, -$NRC(O)NR_2$, -$NR(O)_2R$, -NR-$C(NR_2)$=NR, -S(O)R, -$S(O)_2R$, -$S(O)_2NR_2$, -$NRSO_2R$, -CN and –NO_2. In further embodiments, each R group in the preceding list includes, but is not limited to, hydrogen, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, including but not limited to, aryl substituted with 1-3 halogens, substituted or unsubstituted alkyl, alkoxy or thioalkoxy groups, or aralkyl groups. In some embodiments when two R groups are attached to the same nitrogen atom, they are combined with the nitrogen atom to form a 5-, 6-, or 7-membered ring. In other embodiments for example, -NR_2 includes, but is not be limited to, 1-pyrrolidinyl and 4-morpholinyl.

[00087] In other embodiments and by way of example, substituents for aryl and heteroaryl groups include, but are not limited to, -OR, =O, =NR, =N-OR, -NR_2, -SR, -halogen, -SiR_3, -OC(O)R, -C(O)R, -CO_2R, -$CONR_2$, -$OC(O)NR_2$, -NRC(O)R, -$NRC(O)NR_2$, -$NR(O)_2R$, -NR-$C(NR_2)$=NR, -S(O)R, -$S(O)_2R$, -$S(O)_2NR_2$, -$NRSO_2R$, -CN, –NO_2, -R, -N_3, -$CH(Ph)_2$, fluoro(C_1-C_4)alkoxy, and fluoro(C_1-C_4)alkyl, in a number ranging from zero to the total number of open valences on the aromatic ring system. In a further embodiment, each R group in the preceding list includes, but is not limited to, hydrogen, alkyl, heteroalkyl, aryl and heteroaryl.

[00088] In some embodiments, the assay is performed on a sample obtained from a subject that has been administered a TEC family kinase inhibitor, particularly a BTK kinase inhibitor. 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 or the BTK inhibitor.

[00089] A "replicon" is any genetic element (e.g., plasmid, chromosome, virus) that functions as an autonomous unit of DNA replication *in vivo*; i.e., capable of replication under its own control.

[00090] A "vector" is a replicon, such as plasmid, phage or cosmid, to which another DNA segment may be attached so as to bring about the replication of the attached segment.

[00091] A "DNA molecule" refers to the polymeric form of deoxyribonucleotides (adenine, guanine, thymine, or cytosine) in its either single stranded form, or a double-stranded helix. This term refers only to the primary and secondary structure of the molecule, and does not limit it to any particular tertiary forms. Thus, this term includes double-stranded DNA found, *inter alia*, in linear DNA molecules (e.g., restriction fragments), viruses, plasmids, and chromosomes. In discussing the structure of particular double-stranded DNA molecules, sequences may be described herein according to the normal convention of giving only the sequence in the 5' to 3' direction along the nontranscribed strand of DNA (i.e., the strand having a sequence homologous to the mRNA).

[00092] An "origin of replication" refers to those DNA sequences that participate in DNA synthesis.

[00093] A DNA "coding sequence" is a double-stranded DNA sequence which is transcribed and translated into a polypeptide *in vivo* when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxyl) terminus. A coding sequence can include, but is not limited to, prokaryotic sequences, cDNA from eukaryotic mRNA, genomic DNA sequences from eukaryotic (e.g., mammalian) DNA, and even synthetic DNA sequences. A polyadenylation signal and transcription termination sequence will usually be located 3' to the coding sequence.

[00094] Transcriptional and translational control sequences are DNA regulatory sequences, such as promoters, enhancers, polyadenylation signals, terminators, and the like, that provide for the expression of a coding sequence in a host cell.

[00095] A "promoter sequence" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. For purposes of defining the present invention, the promoter sequence is bounded at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable

above background. Within the promoter sequence will be found a transcription initiation site (conveniently defined by mapping with nuclease S1), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase. Eukaryotic promoters will often, but not always, contain "TATA" boxes and "CAT" boxes. Prokaryotic promoters contain Shine-Dalgarno sequences in addition to the -10 and -35 consensus sequences.

[00096] An "expression control sequence" is a DNA sequence that controls and regulates the transcription and translation of another DNA sequence. A coding sequence is "under the control" of transcriptional and translational control sequences in a cell when RNA polymerase transcribes the coding sequence into mRNA, which is then translated into the protein encoded by the coding sequence.

[00097] The term "oligonucleotide," as used herein in referring to the probe of the present invention, is defined as a molecule comprised of two or more ribonucleotides, preferably more than three. Its exact size will depend upon many factors which, in turn, depend upon the ultimate function and use of the oligonucleotide.

[00098] The term "primer" as used herein refers to an oligonucleotide, whether occurring naturally as in a purified restriction digest or produced synthetically, which is capable of acting as a point of initiation of synthesis when placed under conditions in which synthesis of a primer extension product, which is complementary to a nucleic acid strand, is induced, i.e., in the presence of nucleotides and an inducing agent such as a DNA polymerase and at a suitable temperature and pH. The primer may be either single-stranded or double-stranded and must be sufficiently long to prime the synthesis of the desired extension product in the presence of the inducing agent. The exact length of the primer will depend upon many factors, including temperature, source of primer and use of the method. For example, for diagnostic applications, depending on the complexity of the target sequence, the oligonucleotide primer typically contains 15-25 or more nucleotides, although it may contain fewer nucleotides.

[00099] The primers herein are selected to be "substantially" complementary to different strands of a particular target DNA sequence. This means that the primers must be sufficiently complementary to hybridize with their respective strands. Therefore, the primer sequence need not reflect the exact sequence of the template. For example, a non-complementary nucleotide fragment may be attached to the 5' end of the primer, with the remainder of the primer sequence being complementary to the strand. Alternatively, non-complementary bases or longer sequences can be interspersed into the primer, provided that the primer sequence has sufficient complementarity with the sequence of the strand to hybridize therewith and thereby form the template for the synthesis of the extension product.

[000100] As used herein, the terms "restriction endonucleases" and "restriction enzymes" refer to bacterial enzymes, each of which cut double-stranded DNA at or near a specific nucleotide sequence.

[000101] A cell has been "transformed" by exogenous or heterologous DNA when such DNA has been introduced inside the cell. The transforming DNA may or may not be integrated (covalently linked) into chromosomal DNA making up the genome of the cell. In prokaryotes, yeast, and mammalian cells for example, the transforming DNA may be maintained on an episomal element such as a plasmid. With respect to eukaryotic cells, a stably transformed cell is one in which the transforming DNA has become integrated into a chromosome so that it is inherited by daughter cells through chromosome replication. This stability is demonstrated by the ability of the eukaryotic cell to establish cell lines or clones comprised of a population of daughter cells containing the transforming DNA. A "clone" is a population of cells derived from a single cell or common ancestor by mitosis. A "cell line" is a clone of a primary cell that is capable of stable growth *in vitro* for many generations.

[000102] Two DNA sequences are "substantially homologous" when at least about 75% (preferably at least about 80%, and most preferably at least about 90 or 95%) of the nucleotides match over the defined length of the DNA sequences. Sequences that are substantially homologous can be identified by comparing the sequences using standard software available in sequence data banks, or in a Southern hybridization experiment under, for example, stringent conditions as defined for that particular system. Defining appropriate hybridization conditions is within the skill of the art. See, e.g., Maniatis et al., *supra*; DNA Cloning, Vols. I & II, *supra*; Nucleic Acid Hybridization, *supra*.

[000103] Two amino acid sequences are "substantially homologous" when at least about 70% of the amino acid residues (preferably at least about 80%, and most preferably at least about 90 or 95%) are identical, or represent conservative substitutions.

[000104] A "heterologous" region of the DNA construct is an identifiable segment of DNA within a larger DNA molecule that is not found in association with the larger molecule in nature. Thus, when the heterologous region encodes a mammalian gene, the gene will usually be flanked by DNA that does not flank the mammalian genomic DNA in the genome of the source organism. Another example of a heterologous coding sequence is a construct where the coding sequence itself is not found in nature (e.g., a cDNA where the genomic coding sequence contains introns, or synthetic sequences having codons different than the native gene). Allelic variations or naturally-occurring mutational events do not give rise to a heterologous region of DNA as defined herein.

[000105] The phrase "pharmaceutically acceptable" refers to molecular entities and compositions that are physiologically tolerable and do not typically produce an allergic or similar untoward reaction, such as gastric upset, dizziness and the like, when administered to a human.

[000106] The term "standard hybridization conditions" refers to salt and temperature conditions substantially equivalent to 5 x SSC and 65°C for both hybridization and wash. However, one skilled in the art will appreciate that such "standard hybridization conditions" are dependent on particular conditions including the concentration of sodium and magnesium in the buffer, nucleotide sequence length and concentration, percent mismatch, percent formamide, and the like. Also important in the determination of "standard hybridization conditions" is whether the two sequences hybridizing are RNA-RNA, DNA-DNA or RNA-DNA. Such standard hybridization conditions are easily determined by one skilled in the art according to well known formulae, wherein hybridization is typically 10-20°C below the predicted or determined T_m with washes of higher stringency, if desired.

[000107] As used herein, "pg" means picogram, "ng" means nanogram, "ug" or "µg" mean microgram, "mg" means milligram, "ul" or "µl" mean microliter, "ml" means milliliter, "l" means liter.

[000108] Determining and monitoring the percentage of enzymes, receptors, ion channels or transporters that are occupied by a prospective drug (e.g. agent, ligand, binder), or the percentage that must be occupied by a drug(s) to trigger therapeutic effects in patients, or by drug(s) to induce physiological effects in humans or animals has become an important aspect of designing, evaluating, and validating target inhibitors, particularly for covalent or irreversible target inhibitors. Measurements of target occupancy can help assure proper dosing and targeting of compounds in preclinical and clinical drug development as well as in basic research. Target occupancy generalizations can be especially important in establishing initial dosing recommendations for the many new drug targets provided by genomic and proteomic initiatives, where little data is available on their functional responses.

[000109] Positron Emission Tomography (PET) and Single Photon Emission Computed Tomography (SPECT), along with data from an array of preclinical methodologies, have been utilized to provide answers to the question of target occupancy and its correlation to therapeutic effects, including side effects. These traditional target site occupancy assays (PET, SPECT and also liquid scintillation spectroscopy), which have relied on the use of radioisotopically labeled tracers, have also been utilized to drive the analysis of structure-activity-relationship within a drug-discovery effort. Although these approaches have proven useful and can provide conclusive

evidence of target engagement, they are very expensive and do not allow for the degree of throughput that is often required in early preclinical discovery efforts.

[000110] Other assays and approaches determine target engagement or occupancy relatively, including by assessing target ligand binding directly or indirectly and relatively determining an increase or decrease in the amount of target probe bound. For example, target engagement or occupancy of BTK inhibitors have been determined relatively, including by assessing target probe binding directly or indirectly and relatively determining an increase or decrease in the amount of target probe bound. WO2008054827 and US2008214501 describe BTK activity probes comprising a BTK inhibitor moiety, a reporter moiety, and a linker moiety linking the inhibitor moiety to the reporter moiety. BTK inhibitors have been evaluated using signal based relative determinations wherein more or less probe is bound or detected, with relative amounts of probe occupied target, unoccupied target or drug occupied target altering the overall amount of signal in a relative fashion, but not using quantitative determination. For example, Chang et al WO2014/059368 describes Tec family drug efficacy or occupancy determinations based on increasing or decreasing signal intensity. However, quantitative or normalized target occupancy is not determined in a single, comparable format. These prior art methods provide relative information but cannot be readily utilized to quantitatively assess bound target, or to compare bound target across studies, including comparing one candidate inhibitor versus another by assessing target occupancy, or evaluating quantitative amount of target occupancy to compare affinity and selectivity for a target. Candidate inhibitors in research evaluations in animals or cells, in clinical samples, even in clinical samples from the same patient under different conditions or at different times, cannot be dependably or quantitatively compared.

[000111] The methods and assay systems of the present invention enable quantitative determination of total target and target occupancy in a single and simple format, replacing relative occupancy determinations and also costly and time consuming tests and formats, particularly multiple formats which cannot be cross-compared. The present methods provide simple and standardized alternatives to cumbersome methods that have, for example, utilized sandwich assays (such as ELISA) combined with Western blotting and manual quantification (Pan, Z et al (2007) Chem Med Chem 2:58-61; De Rooji, MF et al (2012) Blood 119:2590-2594; Honigberg, LA et al ((2007) Blood 110:Abstract 1592).

[000112] In its primary aspect, the present invention relates to a novel method and assay system for determining both the amount of a given target and the relative amount of ligand bound to target, or target occupied by a ligand. In a preferred aspect of the invention method and assay system, each determination is conducted on a sample utilizing a same or comparable format,

wherein standard curves and standardized reagents are utilized in both determinations, and occupied target can be directly normalized to quantitated target. Utilizing the same or comparable format permits direct comparison of the results and quantitative determinations and avoids application of multiple techniques or formats and extrapolation or assumptions across distinct formats. The assay is completed using a single format and assay basis.

[000113] Thus, in accordance with the method and assays of the invention, target occupancy and total target are evaluated and determined in a single comparable format. The basis for the single and comparable format is utilization of a same capture moiety on both the target occupancy probe and on the anti-target specific binding member. Utilization of the same capture moiety enables cross comparison and same format reagents.

[000114] The invention provides a method or assay to determine target occupancy and quantitate total target of a kinase, particularly a non-receptor tyrosine kinase, particularly a Tec family kinase, wherein target occupancy is determined utilizing a target occupancy probe and total target is quantitated using an anti-target specific binding member, wherein the target occupancy probe and anti-target specific binding member are tagged with a moiety for capture by the same capture moiety binder. In accordance with the method, a target containing sample is obtained and split or aliquoted for each of (a) target occupancy determination and (b) total target determination. In accordance with the method or assay, each of (a) target occupancy determination and (b) total target determination is conducted comprising the steps of: (i) contacting a sample with target occupancy probe in (a) and anti-target specific binding member in (b), each tagged with a same capture moiety or a capture moiety that binds to the same capture binder, to form an occupancy probe-target complex in (a) and a specific binding member-target complex in (b); (ii) capturing the complex from (a) and (b) with a capture moiety binder coated solid support; and (iii) detecting the presence and amount of captured occupancy probe-target complex in (a) and specific binding member-target complex in (b).

[000115] The determined amount of occupancy probe-target complex can be normalized to the amount of specific binding member-target complex to provide quantitative determination of target occupancy. The determined target occupancy amount can be a direct occupancy value, for example wherein the target inhibitor being evaluated is used as the target occupancy probe and is directly labeled with the capture moiety. In an alternative aspect of the method and assay, the determined occupancy amount can be an indirect occupancy value, for example wherein a target inhibitor to be evaluated is combined with target prior to step (i) and then a sample is contacted with target occupancy probe, wherein the amount of captured occupancy probe-target complex indicates the remaining target which is unoccupied by the target inhibitor being evaluated. In

such an alternative method, target occupancy of the target inhibitor being evaluated is the inverse of the amount of captured occupancy probe-target complex, for example the amount of target that is not captured corresponds to the target occupancy of the inhibitor being evaluated. In an aspect of the invention, it should be noted that the use of target site specific target occupancy probe allows for the screening, identification, and comparison of alternative potential or lead target site specific inhibitors. Thus, the availability of a common format and quantitative target occupancy evaluations permits the comparison of candidate target site specific inhibitors using a standardized and quantitative system.

[000116] The invention provides a method or assay to determine target occupancy and quantitate total target of a kinase, particularly a non-receptor tyrosine kinase, particularly a Tec family kinase, comprising:

(i) obtaining a sample and splitting or aliquoting the sample for (a) target occupancy determination and (b) total target determination;

(ii) contacting the sample for (a) with a capture moiety tagged target occupancy probe and the sample for (b) with capture moiety tagged anti-target specific binding member, wherein the capture moiety in (a) and (b) is the same or is capable of binding to the same capture moiety binder, and wherein a target occupancy probe-target complex is formed in (a) and a specific binding member-target complex is formed in (b);

(iii) incubating each of (a) and (b) with a capture moiety binder coated solid support under conditions wherein binding of the target complex in (a) and (b) to the solid support occurs; and

(iv) detecting the target complex in (a) and (b) by contacting the target complex bound solid support with a primary anti-target specific binding member and determining the amount of target complex in (a) and (b).

[000117] In a particular aspect of the method and assay, target is quantified by comparison to a standard curve generated using recombinant target protein, wherein the standard curve is obtained simultaneously or approximately the same time as the samples are analyzed.

[000118] In an aspect of the method and assay, the primary anti-target specific binding member is an antibody, particularly a labeled antibody. In an aspect, in (iv) the target complex bound solid support is contacted with a secondary tagged or labeled antibody that recognizes or binds the primary anti-target specific binding member for detection.

[000119] In an aspect of the method and assay, the solid support is washed between steps (iii) and (iv). In an aspect, the solid support is washed in (iv) after primary anti-target specific binding member is contacted and prior to contacting with a secondary tagged or labeled antibody that recognizes or binds the primary anti-target specific binding member.

[000120] In an aspect of the method and assay, the target occupancy probe and anti-target specific binding member are directly captured to a solid support, for example by direct binding to a plate, filter, or bead. The plate, filter, or bead is preferably coated or pre-coated with capture binder. In an aspect the plate, filter, or bead is coated or pre-coated with streptavidin. The bead may be a magnetic bead. 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. A solid support may comprise a bead, plate, an array or a bead attached to 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. Examples of beads include streptavidin beads, agarose beads, magnetic beads, Dynabeads®, MACS® microbeads, antibody conjugated beads, protein A conjugated beads, protein G conjugated beads, protein A or G conjugated beads, protein L conjugated beads, oligo-dT conjugated beads, silica beads, silica-like beads, anti-biotin microbead, anti-fluorochrome microbead, and BcMag™ CarboxyTerminated Magnetic Beads.

[000121] Suitable samples for any of the methods and assays provided 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. The sample may contain one or more cell types, or a lysate thereof, derived from a whole blood sample, peripheral blood sample, peripheral blood mononuclear cell (PBMC) 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. Cells of the sample may be isolated from other components of the sample prior to use in the methods provided. Particular cell types of the sample may be isolated from other cell types of the sample prior to use in the methods provided. 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. B cells of the sample may be isolated from other cell types of the sample prior to use in the methods provided. Cells of the sample may be lysed prior to use in the methods provided. In some embodiments, cancer cells may be isolated from normal cells of the sample prior to use in the methods provided. A sample may comprise 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, using antibodies specific for markers identified with particular cell types. Alternatively, a heterogeneous cell population can be used. 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.

Target and Target Inhibitors

[000122] In one aspect, the target is an enzyme, particularly a kinase, particularly an EGFR or non-EGFR kinase. The target is particularly a non-EGFR kinase and may be a member of the Tec family of kinases (BMX, BTK (Btk), ITK, TEC and TXK), Scr family member BLK, MKKa7 kinase, or a JAK3 kinase. In a particularly preferred aspect, target is a Tec family kinase and is BMX, BTK, ITK, TEC or TXK. In a particular embodiment target is BTK.

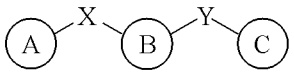
[000123] Target inhibitor is particularly a specific inhibitor, particularly inhibiting a kinase family, such as particularly a Tec family kinase inhibitor, and may be a non specific Tec family inhibitor, a preferential inhibitor against one or more member of the Tec family of kinases or may be a specific kinase inhibitor. The target inhibitor may be a BMX specific inhibitor, a BTK specific inhibitor, an ITK specific inhibitor, a TEC specific inhibitor or a TXK specific inhibitor. The target inhibitor is particularly a BTK inhibitor. The target inhibitor is particularly a covalent inhibitor. The target inhibitor is particularly an irreversible inhibitor.

[000124] In a particular aspect, the target inhibitor and/or target occupancy probe is directed against a cysteine (Cys) conserved in the ATP-binding site of the target kinase. In a particular embodiment, the target inhibitor is directed against Cys481 in BTK, or the comparable Cys in one or more of the Tec family kinases.

[000125] In an aspect of the invention, the target inhibitor is utilized as target occupancy probe. In a particular aspect of the invention, the target inhibitor or target occupancy probe is a Tec family kinase inhibitor. In a particular aspect of the invention, the target inhibitor or target occupancy probe is a Tec family kinase inhibitor, and is capable of inhibiting one or more of BMX, BTK, ITK, TEC and TXK. In a particular aspect of the invention, the target inhibitor or target occupancy probe is a BTK inhibitor. In a particular aspect of the invention, the target inhibitor or target occupancy probe is a BMX inhibitor. In a particular aspect of the invention, the target inhibitor or target occupancy probe is an ITK inhibitor. In a particular aspect of the invention, the target inhibitor or target occupancy probe is a TEC inhibitor. In a particular aspect of the invention, the target inhibitor or target occupancy probe is a TXK inhibitor. Several

BTK inhibitors, particularly irreversible and covalent inhibitors are in development and being evaluated clinically for various indications, including chronic lymphocytic leukemia (CLL), small lymphocytic lymphoma (SLL), and other relapsed or refractory B cell lymphomas (D'Cruz OJ and Uckun, FM (2013) OncoTargets Ther 6:161-176). One such inhibitor is ibrutinib/PCI-32765, (1-[(3*R*)-3-[4-amino-3-(4-phenoxyphenyl)pyrazolo[3,4-d]pyrimidin-1-yl]piperidin-1-yl]prop-2-en-1-one), which is an orally active, small-molecule inhibitor that forms an irreversible bond with cysteine-481 in the active site of BTK and inhibits BTK phosphorylation on Tyr223 (Honigsberg LA et al (2010) PNAS 107(29):13075-13080;). Other BTK inhibitors include AVL-101 and AVL-294 (Avila Therapeutics), Dasatinib/Sprycel or Dasatinib plus fludarabine, LFM-A13, ONO-WG-307, GDC-0834 (D'Cruz OJ and Uckun FM (2013) OncoTargets and Therapy 6:161-176).

[000126] In a preferred aspect of the invention the target occupancy probe is an irreversible inhibitor and is a Tec family inhibitor. In a preferred aspect, the target occupancy probe is a BTK inhibitor. The target occupancy probe may comprise an inhibitor portion or moiety and a reporter or capture moiety, wherein the reporter or capture moiety may particularly correspond to the capture moiety. Tec kinase family activity probes, particularly BTK activity probes which may be suitable as components of the target occupancy probe are described in US2008214501 and in WO2008054827, incorporated herein by reference. In one aspect the target occupancy probe comprises a probe of Formula (I) comprising:



Formula (I);

wherein:

A is a Tec family inhibitor moiety, particularly a Btk inhibitor moiety;

X and Y are independently selected from the group consisting of:

a bond, -O(C=O)-, -NR^a(C=O)-, -NR^a-,  , -O-, -S-, -S-S-, -O-NR^a-, -O(C=O)O-, -O(C=O)NR^a, -NR^a(C=O)NR^a-, -N=CR^a-, -S(C=O)-, -S(O)-, and -S(O)$_2$-;

wherein  forms a N-containing heterocycle;

B is absent or is a linker moiety;

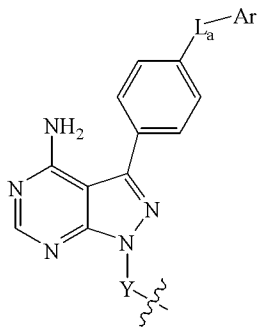
C is a reporter or capture moiety; and

R^a is hydrogen or alkyl.

[000127] In an embodiment, the B linker moiety is absent and the reporter or capture moiety may be directly linked to the inhibitor moiety. In a particular embodiment, the C is a capture moiety.

[000128] In one embodiment, the moiety comprising an irreversible inhibitor is derived from an irreversible inhibitor of Btk. In some embodiments, such irreversible inhibitors of Btk should possess at least one of the following characteristics: potency, selectively and cell permeability. In further embodiments, such irreversible inhibitors of Btk possess at least two of the aforementioned characteristics, and in further embodiments, at least all of the aforementioned characteristics.

[000129] In one embodiment, the target occupancy probe comprises a Btk inhibitor moiety is derived from a Btk inhibitor having the structure of Formula (II):



Formula (II)

wherein:

L_a is CH_2, O, NH or S;

Ar is a substituted or unsubstituted aryl, or a substituted or unsubstituted heteroaryl; and

Y is an optionally substituted group selected from among alkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, aryl, and heteroaryl.

[000130] In some embodiments, L_a is CH_2, O, or NH. In other embodiments, L_a is O or NH. In yet other embodiments, L_a is O.

[000131] In other embodiments, Ar is a substituted or unsubstituted aryl. In yet other embodiments, Ar is a 6-membered aryl or heteroaryl. In some other embodiments, Ar is phenyl.

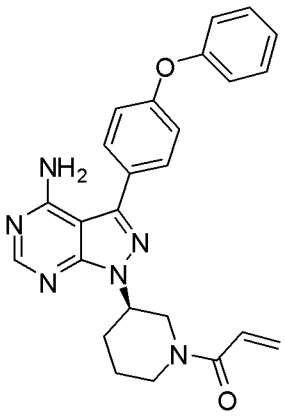
[000132] In some embodiments, Y is an optionally substituted group selected from among alkyl, heteroalkyl, cycloalkyl, and heterocycloalkyl. In other embodiments, Y is an optionally substituted group selected from among C_1-C_6alkyl, C_1-C_6heteroalkyl, 4-, 5-, 6-, or 7-membered cycloalkyl, and 4-, 5-, 6-, or 7-membered heterocycloalkyl. In yet other embodiments, Y is an optionally substituted group selected from among C_1-C_6alkyl, C_1-C_6heteroalkyl, 5- or 6-membered cycloalkyl, and 5- or 6-membered heterocycloalkyl containing 1 or 2 N atoms. In

some other 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 4-, 5-, 6-, or 7-memebered cycloalkyl ring; or Y is a 4-, 5-, 6-, or 7-membered heterocycloalkyl ring. In some embodiments, Y is pyrrolidinyl, piperdinyl, or cyclohexyl.

[000133] In some embodiments, the Btk inhibitor moiety is derived from a compound selected from among: 1-(3-(4-amino-3-(4-phenoxyphenyl)-1H-pyrazolo[3,4-d]pyrimidin-1-yl)piperidin-1-yl)prop-2-en-1-one; (E)-1-(3-(4-amino-3-(4-phenoxyphenyl)-1H-pyrazolo[3,4-d]pyrimidin-1-yl)piperidin-1-yl)but-2-en-1-one; 1-(3-(4-amino-3-(4-phenoxyphenyl)-1H-pyrazolo[3,4-d]pyrimidin-1-yl)piperidin-1-yl)sulfonylethene; 1-(3-(4-amino-3-(4-phenoxyphenyl)-1H-pyrazolo[3,4-d]pyrimidin-1-yl)piperidin-1-yl)prop-2-yn-1-one; 1-(4-(4-amino-3-(4-phenoxyphenyl)-1H-pyrazolo[3,4-d]pyrimidin-1-yl)piperidin-1-yl)prop-2-en-1-one; N-((1s,4s)-4-(4-amino-3-(4-phenoxyphenyl)-1H-pyrazolo[3,4-d]pyrimidin-1-yl)cyclohexyl)acrylamide; 1-((R)-3-(4-amino-3-(4-phenoxyphenyl)-1H-pyrazolo[3,4-d]pyrimidin-1-yl)pyrrolidin-1-yl)prop-2-en-1-one; 1-((S)-3-(4-amino-3-(4-phenoxyphenyl)-1H-pyrazolo[3,4-d]pyrimidin-1-yl)pyrrolidin-1-yl)prop-2-en-1-one; 1-((R)-3-(4-amino-3-(4-phenoxyphenyl)-1H-pyrazolo[3,4-d]pyrimidin-1-yl)piperidin-1-yl)prop-2-en-1-one; 1-((S)-3-(4-amino-3-(4-phenoxyphenyl)-1H-pyrazolo[3,4-d]pyrimidin-1-yl)piperidin-1-yl)prop-2-en-1-one; and (E)-1-(3-(4-amino-3-(4-phenoxyphenyl)-1H-pyrazolo[3,4-d]pyrimidin-1-yl)piperidin-1-yl)-4-(dimethylamino)but-2-en-1-one.

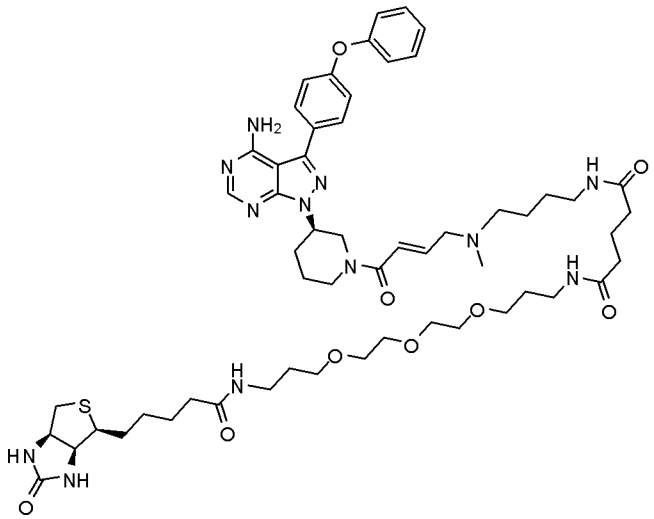
[000134] In another embodiment, the linker moiety is selected from a bond, a polymer, a water soluble polymer, optionally substituted alkyl, optionally substituted heteroalkyl, optionally substituted heterocycloalkyl, optionally substituted cycloalkyl, optionally substituted heterocycloalkylalkyl, optionally substituted heterocycloalkylalkenyl, optionally substituted aryl, optionally substituted heteroaryl, and optionally substituted heterocycloalkylalkenylalkyl. In some embodiments, the linker moiety is an optionally substituted heterocycle. In other embodiments, the heterocycle is selected from aziridine, oxirane, episulfide, azetidine, oxetane, pyrroline, tetrahydrofuran, tetrahydrothiophene, pyrrolidine, pyrazole, pyrrole, imidazole, triazole, tetrazole, oxazole, isoxazole, oxirene, thiazole, isothiazole, dithiolane, furan, thiophene, piperidine, tetrahydropyran, thiane, pyridine, pyran, thiapyrane, pyridazine, pyrimidine, pyrazine, piperazine, oxazine, thiazine, dithiane, and dioxane. In some embodiments, the heterocycle is piperazine. In further embodiments, the linker moiety is optionally substituted with halogen, CN, OH, NO_2, alkyl, S(O), and $S(O)_2$. In other embodiments, the water soluble polymer is a PEG group.

[000135] In a particular embodiment, the target occupancy probe BTK inhibitor moiety is ibrutinib and has the formula 1-[(3*R*)-3-[4-amino-3-(4-phenoxyphenyl)pyrazolo[3,4-d]pyrimidin-1-yl]piperidin-1-yl]prop-2-en-1-one). Ibrutinib structure is as follows:



FORMULA (III)

[000136] In a particular aspect the target occupancy probe is ibrutinib linked to biotin. In another particular aspect the target occupancy probe comprises the following structure:



PCI-41025

Chemical Formula: $C_{56}H_{80}N_{12}O_9S$
Exact Mass: 1096.59
Molecular Weight: 1097.37

FORMULA (IV)

Capture Moiety

[000137] In accordance with the method and assays of the invention, the target occupancy probe and anti-target specific binding member are each tagged with a same capture moiety or a capture moiety that binds to the same capture binder. The capture moiety may be captured by binding or interacting directly or indirectly to a solid support. In a preferred aspect, the target

probe and anti-target specific binding member are captured directly to a solid support, such as a plate, filter, or a bead. The plate, filter, or bead may preferably be coated with the capture binder. Binding and affinity between the capture moiety and capture binder should be significant and stable, in order that capture can be efficient and sufficiently quantitative. Small capture moieties are preferred, particularly wherein the capture moieties do not interfere or minimally interfere with the target inhibitor and/or anti-target specific binding member binding to the target. Particularly, the capture moiety needs to be suitable and applicable for attachment to small molecules, such as the inhibitor moieties described herein. For example, the capture moiety need be readily attachable, directly or via a linker, to such small molecules as ibrutinib, without interfering with or altering ibrutinib binding or association with target, e.g. BTK.

[000138] In a particularly preferred aspect of the invention, the capture moiety is biotin. In a particularly preferred aspect of the invention, the capture binder is a biotin binder and may be avidin, streptavidin, or neutravidin.

[000139] Biotin is a vitamin (Vitamin H, Vitamin B7, Coenzyme R) that is present in small amounts in all living cells and is critical for a number of biological processes including cell growth and the citric acid cycle. Because biotin is relatively small, it can be conjugated to many proteins and other molecules without significantly altering their biological activity. The highly specific interaction of biotin-binding proteins with biotin make it a useful tool in assay systems designed to detect and target biological analytes. Many proteins, such as antibodies, can be labeled with several biotin tags, each able to be bound by a biotin-binding protein. An optimized biotin-to-probe ratio can greatly increase the signal output of a detection system making it possible to create very sensitive assays.

[000140] The avidin-biotin complex is the strongest known non-covalent interaction ($K_d = 10^{-15}$ M) between a protein and ligand. The bond formation between biotin and avidin is very rapid, and once formed, is unaffected by extremes of pH, temperature, organic solvents and other denaturing agents. These features of biotin and avidin (features that are shared by streptavidin and NeutrAvidin) are useful for purifying or detecting proteins conjugated to either component of the interaction. Streptavidin is a tetrameric biotin-binding protein that is isolated from *Streptomyces avidinii* and has a mass of 60,000 Daltons. An alternative to streptavidin includes deglycosylated avidin, commercially available as NeutrAvidin (Thermo-Scientific).

Anti-Target Specific Binding Member

[000141] Suitable anti-target specific binding members applicable in the instant methods and assays include antibodies and fragments thereof that bind to the target. In a particular aspect an

anti-target specific binding member is an antibody or binding fragment thereof specifically directed against the target, and which does not react with or bind other targets, including related family targets, (e.g., having a binding affinity K_i > 1 μM or > 10 μM, or at least 100 fold or 1000 fold more binding affinity with the target that it specifically binds). Thus for example, in an aspect of the invention the anti-target specific binding member is an anti-BTK antibody that is specific for BTK, particularly human BTK, and that does not or does not significantly recognize or bind another Tec family member or kinase (e.g., having a binding affinity K_i > 1 μM or > 10 μM, or at least 100 fold or 1000 fold more binding affinity with BTK).

[000142] In another embodiment of the invention the anti-target specific binding member is an antibody specifically directed against a Tec kinase family member which is not BTK. In another embodiment of the invention the anti-target specific binding member is an antibody specifically directed against Tec kinase. In a further embodiment of the invention the anti-target specific binding member is an antibody specifically directed against ITK kinase. In another aspect of the invention the anti-target specific binding member is an antibody specifically directed against BMX kinase. In another embodiment of the invention the anti-target specific binding member is an antibody specifically directed against TXK kinase.

[000143] In accordance with an embodiment of the invention, a monoclonal anti-BTK antibody is utilized in determining total target BTK. The anti-BTK may be a commercially available BTK antibody, particularly a monoclonal antibody recognizing human BTK and not cross reacting with Tec kinase or other Tec kinase family members.

[000144] Exemplary anti-BTK antibodies for use in the methods of the invention include anti-BTK monoclonal antibody (BD Bioscience Cat No 611117, N-term rec protein 2-172 aa), rabbit anti-BTK monoclonal antibody (Abcam Cat No. 32555, C-term peptide), anti-BTK rabbit mAb (CST Cat No. 12624S, N-term peptide). Preferred anti-BTK antibody is monoclonal anti-BTK antibody. Preferred anti-BTK antibody is biotinylated anti-BTK rabbit mAb (Cell Signal Technology (CST) Cat No. 12624S, N-term peptide). Alternatively, a new or novel BTK antibody, including monoclonal BTK antibody, may be generated utilizing methods and approaches well known and available to one of skill in the art.

[000145] The antibody or fragment thereof can be tagged directly or indirectly with the capture moiety. In a particular aspect the antibody or fragment is directly tagged with the capture moiety. Antibodies tagged with capture moieties may be commercially available and utilized. As provided herein, for example, biotin labeled anti-BTK antibody for use in the invention may be commercially obtained.

[000146] Panels of monoclonal antibodies produced against target peptides, including Tec family member kinase peptides, can be screened for various properties; i.e., isotype, epitope, affinity, etc. High affinity antibodies are also particularly useful. Preferably, the anti-target antibody used in the diagnostic methods of this invention is an affinity purified polyclonal antibody. More preferably, the antibody is a monoclonal antibody (mAb). In addition, it is preferable for the anti-target antibody molecules used herein be in the form of Fab, Fab', $F(ab')_2$ or F(v) portions of whole antibody molecules.

[000147] A monoclonal antibody useful in practicing the present invention can be produced by initiating a monoclonal hybridoma culture comprising a nutrient medium containing a hybridoma that secretes antibody molecules of the appropriate antigen specificity. The culture is maintained under conditions and for a time period sufficient for the hybridoma to secrete the antibody molecules into the medium. The antibody-containing medium is then collected. The antibody molecules can then be further isolated by well-known techniques.

[000148] Media useful for the preparation of these compositions are both well-known in the art and commercially available and include synthetic culture media, inbred mice and the like. An exemplary synthetic medium is Dulbecco's minimal essential medium (DMEM) supplemented with 4.5 gm/l glucose, 20 mm glutamine, and 20% fetal calf serum. An exemplary inbred mouse strain is Balb/c.

[000149] Methods for producing monoclonal anti-target antibodies are also well-known in the art. Typically, the present target or a peptide analog is used either alone or conjugated to an immunogenic carrier, as the immunogen in the before described procedure for producing anti-target monoclonal antibodies. The hybridomas are screened for the ability to produce an antibody that immunoreacts with the target peptide and target kinase.

[000150] The presence of target in cells can be ascertained by the usual immunological procedures applicable to such determinations. A number of useful procedures are known. Three such procedures which are especially useful utilize either the target labeled with a detectable label, antibody Ab_1 labeled with a detectable label, or antibody Ab_2 labeled with a detectable label. The procedures may be summarized by the following equations wherein the asterisk indicates that the particle is labeled, and "~" stands for the target:

A. $\sim^* + Ab_1 = \sim^* Ab_1$

B. $\sim + Ab^* = \sim Ab_1^*$

C. $\sim + Ab_1 + Ab_2^* = \sim Ab_1 Ab_2^*$

[000151] The procedures and their application are all familiar to those skilled in the art and accordingly may be utilized within the scope of the present invention. The "competitive"

procedure, Procedure A, is described in U.S. Patent Nos. 3,654,090 and 3,850,752. Procedure C, the "sandwich" procedure, is described in U.S. Patent Nos. RE 31,006 and 4,016,043. Still other procedures are known such as the "double antibody," or "DASP" procedure. In each instance, the target forms complexes with one or more antibody(ies) or binding partners and one member of the complex is labeled with a detectable label. The fact that a complex has formed and, if desired, the amount thereof, can be determined by known methods applicable to the detection of labels. It will be seen from the above, that a characteristic property of Ab_2 is that it will react with Ab_1. This is because Ab_1 raised in one mammalian species has been used in another species as an antigen to raise the antibody Ab_2. For example, Ab_2 may be raised in goats using rabbit antibodies as antigens. Ab_2 therefore would be anti-rabbit antibody raised in goats. For purposes of this description and claims, Ab_1 will be referred to as a primary or anti-target antibody, and Ab_2 will be referred to as a secondary or anti-Ab_1 antibody.

[000152] Detection means and labels are employed in the methods and assays of the invention so as to detect the target complexes and may include radioactive elements, enzymes, chemicals which fluoresce when exposed to ultraviolet light, and others. A number of fluorescent materials are known and can be utilized as labels. These include, for example, fluorescein, rhodamine, auramine, Texas Red, AMCA blue and Lucifer Yellow. A particular detecting material is anti-rabbit antibody prepared in goats and conjugated with fluorescein through an isothiocyanate. The radioactive label can be detected by any of the currently available counting procedures. The preferred isotope may be selected from ^{3}H, ^{14}C, ^{32}P, ^{35}S, ^{36}Cl, ^{51}Cr, ^{57}Co, ^{58}Co, ^{59}Fe, ^{90}Y, ^{125}I, ^{131}I, and ^{186}Re. Enzyme labels are likewise useful, and can be detected by any of the presently utilized colorimetric, spectrophotometric, fluorospectrophotometric, amperometric or gasometric techniques. The enzyme is conjugated to the selected particle by reaction with bridging molecules such as carbodiimides, diisocyanates, glutaraldehyde and the like. Many enzymes which can be used in these procedures are known and can be utilized. The preferred are peroxidase, ß-glucuronidase, ß-D-glucosidase, ß-D-galactosidase, urease, glucose oxidase plus peroxidase and alkaline phosphatase.

[000153] Examples of labels include, but are not limited to, chemical, biochemical, biological, colorimetric, enzymatic, fluorescent, luminescent labels, chemiluminescent labels, and electrochemiluminescent labels. The label may be 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. The label may be a chemical label. Examples of chemical labels can include, but are not limited to, biotin and radioisotopes (e.g., iodine, carbon, phosphate, hydrogen). In some embodiments, the methods, assays and kits disclosed herein comprise a biological label. Biological labels comprise metabolic labels, including, but not limited to, bioorthogonal azide-modified amino acids, sugars, and other compounds. Enzymatic labels can include, but are not limited to horseradish peroxidase (HRP), alkaline phosphatase (AP), glucose oxidase, and b-galactosidase. In some embodiments, the enzymatic label is luciferase. A fluorescent label may be 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-Xrhodamine (ROX™), LIZ™, VIC™, NED™, PET™, SYBR, PicoGreen, RiboGreen. The fluorescent label may be a green fluorescent protein (GFP), red fluorescent protein (RFP), yellow fluorescent protein, phycobiliproteins (e.g., allophycocyanin, phycocyanin, phycoerythrin, and phycoerythrocyanin).

[000154] In one aspect, the method and assay determines the target occupancy of the Tec family kinase inhibitor in the target occupancy probe or the probe.

[000155] In another aspect, the method and assay determines the target occupancy of a compound other than the probe, such as an unlabeled compound that is suspected of occupying the target when in contact with the target. In such cases, the compound can be in contact with the target in the sample prior to the sample being in contact with the probe. For example, in a sample obtained from a patient who has been administered a compound, the compound may occupy the target after administration, and the occupancy can be determined by the methods and

assays described herein. The contacting of the target in the sample with the compound may occur after obtaining the sample by, e.g., adding the compound to the sample. For example, a compound may be added in vitro to a sample containing a recombinant kinase before the sample is contacted with the probe.

[000156] When subjecting such samples to the methods and assays described above, the target occupancy probe will bind to target sites not already bound by the compound and the target site occupancy of the compound is indirectly measured. With more compound occupying the target, less target occupancy probe is bound to target and captured and detected in the assay. In such an instance, target occupancy of the compound being assessed is inversely proportional to the amount of labeled target probe detected. Less target occupancy probe indicates that more compound is bound and occupying target.

[000157] In some embodiments, a method or assay is provided to determine target occupancy by a compound suspected of occupying the target, and quantitate total target of a Tec family kinase target in a sample, comprising:

(i) obtaining a sample and splitting or aliquoting the sample for (a) target occupancy determination and (b) total target determination, wherein the target in the sample has been in contact with the compound;

(ii) contacting the sample for (a) with a capture moiety tagged target occupancy probe and the sample for (b) with a capture moiety tagged anti-target specific binding member, wherein the capture moiety in (a) and (b) is the same or is capable of binding to the same capture moiety binder, and wherein a target occupancy probe-target complex is formed in (a) and a specific binding member-target complex is formed in (b);

(iii) incubating each of (a) and (b) with a capture moiety binder coated solid support under conditions wherein binding of the target complex in (a) and (b) to the solid support occurs; and

(iv) detecting the target complex in (a) and (b) by contacting the target complex bound solid support with a primary anti-target specific binding member, optionally further contacting the target complex bound solid support with a secondary tagged or labeled antibody that recognizes or binds the primary anti-target specific binding member for detection; and determining the amount of target complex in (a) and (b) to determine the amount of target occupancy by the target occupancy probe and the amount of total target;

(v) determining the target occupancy by the compound based on the amount of target occupancy by the target occupancy probe and the amount of total target.

[000158] The capture moiety tagged target occupancy probe, capture moiety tagged anti-target specific binding member, capture moiety binder coated solid support, primary anti-target specific

binding member, secondary tagged or labeled antibody, assay and determination methods, etc. are as described in details herein. The compound suspected of occupying the target can be any compound of interest for the determination, such as compounds in a screening experiment for determining potential binding with one or more targets, compounds in *in vivo* experiments, or clinical uses for determining their target occupancy when administered to a subject.

[000159] In some embodiments, the compound is a known BTK inhibitor, such as those described in US Patents 7,514,444, 7,732,454, 7,825,118, 7,960,396, 8,236,812, 8,377,946, 8,501,724, 8,563,568, 8,883,435, 8,940,725, 9,073,947, 9,133,134, and 9,138,436, US Patent Applications US2015/0094319, US2014/0107151, US2014/0144099, US2014/0155385, US2014/0155406, US2014/0221333, US2014/0221398, US/2014/0303161, US2014/0364410, US2015/0174128, and US US2015/0158865, and International Patent Applications WO2014/022390, WO2015/048689, WO2015/134210, WO2015/095099, WO2015/095102, and WO2015/002894, each and all incorporated herein by reference. In some embodiments, the compound is ibrutinib, ACP-196, AVL-101, AVL-294, BGB-3111, Dasatinib/Sprycel or Dasatinib plus fludarabine, LFM-A13, ONO-WG-307, or GDC-0834.

[000160] In a further embodiment of this invention, commercial test kits suitable for use by a medical specialist may be prepared to determine the quantity of target, quantity of available target, or target occupancy prior to, during, or after treatment with a kinase inhibitor, particularly an irreversible Tec kinase inhibitor, particularly an irreversible BTK inhibitor. In accordance with the methods and assays discussed above, such kits will contain at least the tagged anti-target specific binding member, for instance an antibody specific thereto, and directions, of course, depending upon the method selected, e.g., ELISA and standard or MSD format, and the like. The kits may also contain peripheral reagents such as buffers, stabilizers, etc.

[000161] Accordingly, a test kit may be prepared for the demonstration of the quantity of target and target occupancy of inhibitor, particularly BTK and BTK inhibitor comprising:

(a) a predetermined amount of BTK-occupancy probe such as biotinylated ibrutinib and a predetermined amount of biotinylated anti-BTK antibody;

(b) a solid support coated with streptavidin or avidin; and

(c) directions for use of said kit.

[000162] In some embodiments, the kits further comprises one or more other reagents such as buffers, stabilizers, etc., used in assays such as ELISA.

[000163] The invention may be better understood by reference to the following non-limiting Examples, which are provided as exemplary of the invention. The following examples are

presented in order to more fully illustrate the preferred embodiments of the invention and should in no way be construed, however, as limiting the broad scope of the invention.

EXAMPLE 1

Development of Total BTK ELISA for Quantification and Normalization

[000164] Experiments were undertaken to evaluate and develop a format and system for quantitating or determining the total amount of a kinase target. Sandwich ELISA was evaluated with a first coating antibody, a second capture antibody, anti-target antibody and a labeled antibody binding the anti-target antibody. The assay was based on the meso scale discovery (MSD) platform and is depicted in FIGURE 1. Tec family kinase member BTK was utilized as target for assay format development.

[000165] Rabbit anti-BTK monoclonal antibody (Abcam Cat No. 32555, C-term peptide) was used as capture antibody. Detection antibody was anti-BTK monoclonal antibody (BD Bioscience Cat No 611117, N-term rec protein 2-172 aa). BTK protein from BPS Bioscience (Cat No 40405; full length, GST tagged) was used as target.

[000166] The MSD format assay was evaluated using 1 µg/ml capture Ab and 0.5 µg/ml capture Ab. The tabulated results and standard curves are shown in FIGURE 2. The assay was repeated using BTK protein from cell lysates and compared (FIGURE 3). The assay, however, failed to quantitate BTK protein in DoHH2 cell lysates.

[000167] The assay was repeated on DoHH2 cell lysates using higher amounts of coating antibody, particularly 0.5 µg, 1 µg, 2 µg and 3 µg coating Ab (FIGURE 4), but again failed due to the weak signal detected for cellular BTK protein.

[000168] Sandwich ELISA was next evaluated on both standard and MSD formats for total BTK determination. In this instance, coating Ab goat anti-rabbit IgG was eliminated and anti-BTK Rabbit or mouse antibody (Ab) was used as capture Ab. The assay design is depicted in FIGURE 5. A series of commercially available BTK recombinant proteins were evaluated. Recombinant BTK available from each of Invitrogen (Cat No PV3363, full length, His tagged), Millipore (Cat No 14-552, full length, His tagged), BPS Bioscience (Cat No 40405, full length, GST tagged, MW=106kDa), Janssen (BTK-WT, 15µl diluted to 50 µl PBS) and Janssen (BTK-C481S) were evaluated in a purity check using SDS-PAGE and Pierce Coumassie total protein staining (FIGURE 6). For each commercial preparation, 10 µl protein (at concentration 0.1mg/ml) was combined with 10 µl PBS, 10 µl reducer and 10 µl 4X NuPAGE Sample Buffer, 12 µl loaded per well, and the gel stained with Pierce Coumassie overnight at room temperature. The Invitrogen BTK protein was purer than the other proteins by Coumassie staining.

[000169] An Experimental Protocol for a total BTK quantification ELISA is depicted in FIGURE 7. Briefly, 96 well high protein binding plates (Pierce/MSD) are utilized. Capture antibody at 2 µg/ml is coated onto the plates in PBS overnight at 4°C. The plates are then washed and blocked in 3%BSA (250 µl) for 1-2 hours at room temperature (RT). Either recombinant BTK (R-BTK) (Invitrogen) or cell lysates are added in 1%BSA (100 µl) and incubated overnight at 4°C. Primary anti-BTK antibody is added in 1%BSA (100 µl) for 2 hours at RT, followed by secondary antibody in 1% BSA (100 µl) for 1 hour at RT. Detection reagent (100 µl) is added and the plates are read. The results of assay studies using Anti-BTK Rabbit mAb as capture antibody (CST Cat No 8547, N term peptide) and Anti-BTK mAb (BD Cat No 611117, N term rec protein 2-172) as detection antibody are depicted in FIGURE 8. Both DoHH2 and Jurkat cells (Jurkat cells lack BTK and serve as a negative control) were evaluated, as was recombinant BTK in both a standard (STD) and meso-scale discovery (MSD) format. BTK in the cell lysates peaked and flattened at a maximum luminescence.

[000170] Using an alternative capture antibody (Abcam Cat No 32555, C term peptide) did not provide better results, and in fact failed to quantify BTK in DoHH2 cell lysates (FIGURE 9). Switching out the capture and detection antibodies to use BD Cat No 611117 as capture antibody and Abcam Cat No 32555 as detection antibody did not give successful results and similarly failed to quantify BTK in cell lysates (FIGURE 10).

[000171] Thus, a series of alternative anti-BTK antibodies were evaluated as capture and detection antibodies in sandwich ELISAs for quantification of total BTK, particularly in cell lysates, and failed.

[000172] The capture and detection antibodies were again switched out and the luminescence scale was adjusted. BD Cat No 611117 Anti-BTK mAb was used as capture antibody and Biotin anti-BTK rabbit monoclonal antibody RmAb (CST Cat No 12624S, N term peptide) as detection antibody with improved results (FIGURE 11). A combination of biotinylated anti-BTK antibody and streptavidin-HRP antibody enhances the sensitivity of detection.

EXAMPLE 2

[000173] In view of the failures attendant to the assay methods tested in Example 1, a novel method was developed. The novel assay method is suitable for either standard (STD) or the Meso-Scale Discovery (MSD) formats. The principles are depicted visually in FIGURE 12. The use of both a coating antibody and a capture antibody were eliminated from the assay. Instead, for total BTK assay, the sample is mixed with tagged anti-BTK antibody (for example biotinylated anti-BTK antibody) wherein the tagged BTK antibody is tagged with a capture

moiety that facilitates direct attachment to the plates via the capture moiety (tag). In this instance, biotin is used for capture moiety and facilitates binding to streptavidin coated plates. Then, bound BTK - which is bound by virtue of direct association of the tagged or labeled anti-BTK antibody with the assay plate or surface - is detected. Detection is accomplished for example by addition of another anti-BTK antibody, which recognizes and binds to the bound BTK on the assay plate or surface. In the exemplary format depicted in FIGURE 12, the detection antibody is mouse anti-BTK antibody. Detection can be direct, wherein the detection antibody is directly labeled, or facilitated by a secondary antibody that recognizes the detection antibody, for example anti-mouse antibody with a tag or HRP or other direct label.

[000174] The experimental strategy for a method and assay system to quantitate total target and target occupancy is presented in FIGURE 13. The strategy is shown for BTK, particularly for determining total BTK quantification in absolute amounts and BTK target occupancy data normalized to total BTK. The strategy permits determination of total BTK and BTK target occupancy using a single simple system. Biotin is utilized as a capture moiety on both the anti-BTK antibody used for total BTK quantification, and on the target occupancy probe used to determine BTK target occupancy. Total BTK and BTK target occupancy probe-target complexes are captured using streptavidin plates and then detection proceeds, for example using streptavidin HRP or tag, for example using labeled detection antibody.

[000175] BTK target occupancy was determined in the assay ELISA using standard format. The biotinylated probe used in this study for determining BTK occupancy is biotinylated irreversible BTK inhibitor ibrutinib/41025. BTK occupancy is evaluated in cell lysates using biotin-ibrutinib as target occupancy probe (DoHH2 cells and BTK negative Jurkat cells as control) and also with recombinant BTK (Rec-BTK) from a commercial source, particularly Invitrogen BTK (FIGURE 14). Similarly, total BTK is determined in standard ELISA format using biotinylated anti-BTK antibody against recombinant BTK (Invitrogen) and cell lysates (DoHH2 and Jurkat cells as control) (FIGURE 15). BTK target occupancy and total BTK were similarly assessed using biotinylated-probe irutinib and biotinylated anti-BTK antibody in the MSD format (FIGURES 16 and 17).

[000176] The occupancy and total BTK assays using biotinylated probe in the occupancy assay and biotinylated anti-BTK antibody for total BTK were repeated in the standard and MSD formats using higher protein concentrations for the cell lysates (FIGURES 18-21) with successful results.

[000177] These BTK target occupancy studies evaluated direct occupancy of an inhibitor probe, wherein the biotin labeled target inhibitor served as target occupancy probe and was incubated

and directly monitored and evaluated for occupancy. Alternatively, the target occupancy probe can be added to a sample or incubated in cells wherein an unlabeled inhibitor is already present. In such an instance, the target occupancy probe will bind to target sites not already bound by unlabeled inhibitor and the target site occupancy of unlabeled inhibitor is indirectly measured. With more unlabeled inhibitor occupying the target, less target occupancy probe is bound to target and captured and detected in the assay. In such an instance, target occupancy of the inhibitor being assessed is inversely proportional to the amount of labeled target probe detected. Less target occupancy probe indicates more unlabeled target inhibitor is bound and occupying target.

[000178] In conclusion, a simple assay system has been designed and developed to detect and quantify Tec family kinase, for example BTK, and to simultaneously or serially, using the same assay format and overlapping reagents, determine target occupancy of a target inhibitor or ligand. This assay system eliminates the cumbersome current method that involves determination of inhibitor bound target using one format, such as ELISA, combined with an alternative and dissimilar format for quantification of total target, such as Western blotting and manual quantification of total target. Determination of both target (for example BTK) occupancy and its normalization to quantified total target (BTK) present in cellular samples or cell lysates can be done simultaneously using standard curves generated using recombinant BTK protein or cell lysate BTK. The assay is reproducible, yields reliable data and the sensitivity/detection limit is < 1 ng/ml of total BTK. The assay works in both standard (STD) and MSD format.

[000179] PROTOCOLS

[000180] An exemplary detailed combined assay protocol for the occupancy and total BTK ELISA-based assay can be performed according to the following procedure. This procedure is provided for illustrative purposes and particular aspects or reagents may be altered or substituted by one of skill and knowledge in the art. The protocol will determine the amount of BTK probe (e.g., 41025) binding to the target active site that is not already occupied by inhibitor (e.g. ibrutinib) and also to quantify the amount of total BTK present in the samples. The BTK probe used in the exemplary assay is ibrutinib linked to biotin as shown above in Formula (IV).

[000181] OCCUPANCY ASSAY PROTOCOL:

Reagents (Assay reagents are mostly from Meso Scale Discovery - Rockville, MD (denoted MSD))

[000182] Materials:

Standard Streptavidin plate (SA plate) (5-pack) Catalog #L15SA-2 (MSD)
Read Buffer T (50 mL) Catalog # R92TC-3 (MSD)

SULFO-TAG Goat Anti-mouse (50 μg) Catalog# R32AC-5 (MSD)
MSD Blocker A Catalog# R93AA-2 (MSD)
Tris Wash Buffer Catalog # R61TX-2 (MSD)
Protease inhibitor: Sigma, Catalog # P8340
Mouse-anti-human Btk anti body BD Biosciences, Catalog # 611117
Positive Control lysates from BTK expressing cell line (DOHH2)
Negative Control lysates (Jurkat)
Blocking buffer: 3% MSD Blocker A in 1x Tris Wash buffer (3 g Blocker A + 100 ml 1x Wash)
Dilution buffer: 1% MSD blocker A in 1x Tris Wash buffer
Wash buffer: 1x MSD Tris wash buffer (diluted from 10x stock in H_2O; 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.02% Tween-20; PBS-T can also be used)
Read buffer: 1x MSD read buffer (diluted from 10x stock in H_2O)
Probe 41025: Prepare 20X solution in dilution buffer from 100 uM stock (5ul/ml to give a final concentration of 25 nM)
Note: SA plate and all reagents should be at RT before use. Make Ab dilutions right before use.
Primary (1^o) Ab: Mouse anti-BTK mAb, 1:1000 dilution in dilution buffer
Secondary (2^o) Ab: anti-mouse– SULFO-TAG – 1:500 dilution in dilution buffer

Cell lysates: prepare by repeated freeze-thaw of cell pellets resuspended in DPBS + protease inhibitors (PI); alternatively, cell lysates can be prepared by brief sonication.
Prepare lysate to 50 ug/ul (300 ul/ml) in dilution buffer – add 40-50 ul/well for assay
ON a separate regular plate, add 95 ul/well diluted samples + add 5ul of 20X diluted probe, mix and shake for 1 hr, RT

[000183] Procedure:

1. Make sure SA plate and reagents are at RT before starting
2. Block SA plate w/ 250 ul/well blocking buffer, 1 hr RT, shaking
3. Wash plate w/ 250 ul/well Tris wash buffer, 2x, tap dry
4. Add 50 ul/well pre-incubated samples above (treated cell lysates + probe) in duplicate and shake the plates for 1 hr, RT
5. Wash plate w/ 250 ul/well Tris wash buffer, 3x, tap dry
6. Add 50 ul/well diluted 1^o Ab, 1 hr RT, shaking
7. Wash plate w/ 250 ul/well Tris wash buffer, 3x, tap dry
8. Add 50 ul/well 2^o Ab-SULFO-TAG, covered, 1 hr RT, shaking
9. Wash plate w/ 250 ul/well Tris wash buffer, 3x, tap dry
10. Add 150 ul/well of 1x read buffer (4x stock diluted in H_2O); set-up machine to step 11d before adding read buffer
11. Read plate in SI2400 immediately
    a. Open MSD program, click machine icon in toolbar
    b. Select 96-well, check return plate, check read 1 plate, (check partial plate and highlight wells)
    c. Click Run
    d. Select read each spot, title the file, select folder to save in
    e. Click OK to read plate

[000184] TOTAL BTK QUANTIFICATION ASSAY PROTOCOL:

[000185] Materials:

Standard Streptavidin plate (5-pack) Catalog #L15SA-2 (MSD)

Read Buffer T (50 mL) Catalog # R92TC-3 (MSD)
SULFO-TAG Goat Anti-mouse (50 μg) Catalog# R32AC-5 (MSD)
MSD Blocker A Catalog# R93AA-2 (MSD)
Tris Wash Buffer Catalog # R61TX-2 (MSD)
Protease inhibitor: Sigma, Catalog # P8340
Recombinant His-BTK WT protein (Full Length); Invitrogen, Catalog # PV3363
Biotinylated anti-BTK RmAb, Cell Signal Technology, Catalog # 12624
Mouse-anti-human Btk anti body BD Biosciences, Catalog # 611117
Positive Control lysates from BTK expressing cell line (DOHH2)
Negative Control lysates (Jurkat)
Blocking buffer: 3% MSD Blocker A in 1x Tris Wash buffer (3 g Blocker A + 100 ml 1x Wash)
Dilution buffer: 1% MSD blocker A in 1x Tris Wash buffer
Wash buffer: 1x MSD Tris wash buffer (diluted from 10x stock in H_2O; 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.02% Tween-20; PBS-T can also be used)
Read buffer: 1x MSD read buffer (diluted from 10x stock in H_2O)
Note: SA plate and all reagents should be at RT before use. Make Ab dilutions right before use.
Capture antibody: Biotinylated Rabbit anti-BTK antibody, 1-10 dilution buffer (50ul/500ul)
Primary (1^o) Ab: Mouse anti-BTK mAb, 1:1000 dilution in dilution buffer
Secondary (2^o) Ab: anti-mouse– SULFO-TAG – 1:500 dilution in dilution buffer

Cell lysates: prepare by repeated freeze-thaw of cell pellets resuspended in DPBS + protease inhibitors (PI). Alternatively, cell lysates can be prepared by brief sonication
Prepare lysate to 50 ug/ul (300 ul/ml) in dilution buffer – add 40-50 ul/well for assay
ON a separate regular plate, add 95 ul/well diluted samples + add 5ul of 1-10 diluted capture antibody, mix and shake for 1 hr, RT
Similarly to create standard curve for quantification, serially dilute recombinant BTK protein (3 fold) starting from 50ng/ml. Take 95ul of pure protein solution and then add 5ul of 1-10 diluted capture antibody, mix and shake for 1 hr, RT (this needs to be done simultaneously with cell lysate experiment). Once the standard curve is generated, use the linear equation to determine the amount of BTK present in cell lysates (unknown sample).

[000186] Procedure:

12. Make sure SA plate and reagents are at RT before starting
13. Block SA plate w/ 250 ul/well blocking buffer, 1 hr RT, shaking
14. Wash plate w/ 250 ul/well Tris wash buffer, 2x, tap dry
15. Add 50 ul/well pre-incubated samples above (treated cell lysates + biotinylated antibody) in duplicate and shake the plates for 1 hr, RT (Follow same steps for recombinant BTK protein)
16. Wash plate w/ 250 ul/well Tris wash buffer, 3x, tap dry
17. Add 50 ul/well diluted 1^o Ab, 1 hr RT, shaking
18. Wash plate w/ 250 ul/well Tris wash buffer, 3x, tap dry
19. Add 50 ul/well 2^o Ab-SULFO-TAG, covered, 1 hr RT, shaking
20. Wash plate w/ 250 ul/well Tris wash buffer, 3x, tap dry

21. Add 150 ul/well of 1x read buffer (4x stock diluted in H2O); set-up machine to step 11d before adding read buffer

22. Read plate in SI2400 immediately
    a. Open MSD program, click machine icon in toolbar
    b. Select 96-well, check return plate, check read 1 plate, (check partial plate and highlight wells)
    c. Click Run
    d. Select read each spot, title the file, select folder to save in
    e. Click OK to read plate

[000187] DATA ANALYSIS:

1. BTK Occupancy = Provides amount of BTK occupancy by the drug (based on protein normalization)
2. BTK ELISA = Provides total BTK protein present in samples in terms of RLU (relative light units) which can be used to normalize the occupancy data to give accurate occupancy values.
3. Rec BTK Standard Curve = Used to determine the total amount of BTK in terms of pg/ml in samples
4. Percent Occupancy = Take Pre-dose (or vehicle) occupancy as 0% and obtain the % occupancy for other dosed samples (multiply by 100)
5. Amount of BTK (pg/ml) = Multiply the amount of BTK (pg/ml) found by linear equation with RLU of Occupancy and divide it by RLU of Total BTK
6. BTK Occupancy Data / Total BTK ELISA data = Normalized BTK Occupancy by the drug
7. Normalized BTK Occupancy Data – Total BTK ELISA data = Total of free BTK unoccupied
8. Total of free BTK unoccupied / BTK amount derived from standard curve (recombinant BTK protein) = Free BTK quantification values in terms of pg/ml or ng/ml

EXAMPLE 3

[000188] The new BTK Occupancy and Total BTK Assay was validated using clinical samples and compared to data derived from other methods. The novel assay and method was evaluated on (human) clinical samples from patients treated with BTK inhibitor to determine BTK occupancy and Total BTK. Patient samples were evaluated from a phase 2 clinical study trial (NCT01478581; Study of the Bruton's Tyrosine Kinase Inhibitor in Subjects with Relapsed or Relapsed and Refractory Multiple Myeloma). The patients (Cohort 1) were administered ibrutinib (PCI-32765) 420 mg per day and evaluated.

[000189] Three methods were utilized and results compared. The method strategies are depicted in FIGURE 22. The first method (Method 1) (FIGURE 22 and 23) corresponds to the novel method provided herein, wherein sample cell lysates are split and used directly to

determine, on the one hand, ligand (BTK) occupancy using labeled probe and, on the other hand, total BTK using labeled anti-BTK antibody, with comparison against a BTK standard curve based on recombinant BTK protein. The same format, such as MSD ELISA, is used for all BTK determinations in Method 1. In Methods 2 and 3, cell lysates are first incubated with labeled probe for BTK occupancy determination. The supernatant is then recovered, collected and transferred for separate analysis of total BTK using anti-BTK antibody (Method 3, FIGURE 22 and 33) or biotinylated anti-BTK antibody (Method 2, FIGURE 22 and 28).

[000190] Peripheral blood mononuclear cell samples (PBMCs) from 4 patients, denoted patient #1, patient #2, patient #3 and patient #4 were evaluated to determine BTK occupancy and total BTK using Methods 1, 2 and 3. Each patient received 420 mg ibrutinib daily and were evaluated on Day 1 (vehicle dose) and on each of inhibitor dosing Day 2, Day 8, Day 15 and Day 29, at the end of the study, as indicated in the figures. The results of Method 1 (FIGURE 23) assays for Patient #1, #2, #3 and #4 are shown in FIGURES 24, 25, 26 and 27, respectively. Results are provided for linear standard curve, % BTK occupancy and amount of free BTK. Occupancy correlates against free BTK with Method 1. The results of Method 2 (FIGURE 28) assays for Patient #1, #2, #3 and #4 are shown in FIGURES 29, 30, 31 and 32, respectively. The results of Method 3 (FIGURE 33) assays for Patient #1, #2, #3 and #4 are shown in FIGURES 34, 35, 36 and 37, respectively.

[000191] Patient sample data across the methods are compared in FIGURE 38. Also in FIGURE 38, patient sample results are shown for comparison from previous samples results from patients dosed with a single 840 mg dose of ibrutinib and followed for up to 8 days (200 hours) (Chaturvedi, S et al (2014, September 22) AACR Hematological Malignancies, Poster Session B, B18). Figure 38 results demonstrate that the instant novel method results are reliable and sensitive and are consistent with other methods, without the requirement for multiple distinct formats, sample transfer between formats, and possible errors due to sample handling and transfer across formats or for separate assessments.

[000192] This invention may be embodied in other forms or carried out in other ways without departing from the spirit or essential characteristics thereof. The present disclosure is therefore to be considered as in all aspects illustrate and not restrictive, the scope of the invention being indicated by the appended Claims, and all changes which come within the meaning and range of equivalency are intended to be embraced therein.

[000193] Various references are cited throughout this Specification, each of which is incorporated herein by reference in its entirety.

WHAT IS CLAIMED IS:

1. A method or assay to determine target occupancy and quantitate total target of a Tec family kinase target in a sample, comprising:
(i) obtaining a sample and splitting or aliquoting the sample for (a) target occupancy determination and (b) total target determination;
(ii) contacting the sample for (a) with a capture moiety tagged target occupancy probe and the sample for (b) with a capture moiety tagged anti-target specific binding member, wherein the capture moiety in (a) and (b) is the same or is capable of binding to the same capture moiety binder, and wherein a target occupancy probe-target complex is formed in (a) and a specific binding member-target complex is formed in (b);
(iii) incubating each of (a) and (b) with a capture moiety binder coated solid support under conditions wherein binding of the target complex in (a) and (b) to the solid support occurs; and
(iv) detecting the target complex in (a) and (b) by contacting the target complex bound solid support with a primary anti-target specific binding member and determining the amount of target complex in (a) and (b).

2. The method or assay of claim 1 wherein the target complex bound solid support in (iv) is contacted with a secondary tagged or labeled antibody that recognizes or binds the primary anti-target specific binding member for detection.

3. The method or assay of claim 1 wherein the Tec family target kinase is BTK.

4. The method or assay of claim 1 wherein the target is quantified by comparison to a standard curve generated using recombinant Tec family kinase target protein, wherein the standard curve is obtained simultaneously or approximately the same time as the samples are analyzed.

5. The method or assay of claim 1 or 2 wherein the anti-target specific binding member is a monoclonal antibody specific for the Tec family kinase.

6. The method or assay of claim 1 or 2 wherein the capture moiety is biotin and the capture moiety binder is streptavidin.

7. The method or assay of claim 1 wherein the sample is a cell lysate of PBMCs.

8. The method or assay of claim 1 wherein the samples for (a) target occupancy determination and (b) total target determination are processed side by side, simultaneously or in series.

9. The method or assay of claim 1 wherein the target occupancy probe is a labeled irreversible Tec family kinase inhibitor.

10. The method or assay of claim 1 wherein the target occupancy probe is labeled ibrutinib.

11. The method or assay of claim 9, wherein the target occupancy determination (a) determines the target occupancy of the Tec family kinase inhibitor in the target occupancy probe.

12. The method or assay of claim 9, wherein the target occupancy determination (a) determines the occupancy of an unlabeled compound that has been in contact with the target in the sample before step (ii) contacting of the sample with the target occupancy probe, and optionally before step (i).

13. A method or assay to determine BTK target occupancy and quantitate total target of BTK in a sample, comprising:
(i) obtaining a sample and splitting or aliquoting the sample for (a) BTK target occupancy determination and (b) BTK total target determination;
(ii) contacting the sample for (a) with a capture moiety tagged BTK occupancy probe and the sample for (b) with capture moiety tagged anti-BTK antibody, wherein the capture moiety in (a) and (b) is biotin or is capable of binding to streptavidin or avidin, and wherein a BTK

occupancy probe-BTK complex is formed in (a) and an anti-BTK antibody-BTK complex is formed in (b);

(iii) incubating each of (a) and (b) with a streptavidin or avidin coated solid support under conditions wherein binding of the BTK occupancy probe-BTK complex in (a) and the anti-BTK antibody-BTK complex in (b) to the solid support occurs; and

(iv) detecting the BTK occupancy probe-BTK complex in (a) and anti-BTK antibody-BTK complex in (b) by contacting the complex bound solid support with a primary anti-BTK antibody and determining the amount of complex in (a) and (b).

14. The method or assay of claim 13 wherein the target complex bound solid support in (iv) is contacted with a secondary tagged or labeled antibody that recognizes or binds the primary anti-BTK antibody for detection.

15. The method or assay of claim 13 wherein the target is quantified by comparison to a standard curve generated using recombinant BTK protein, wherein the standard curve is obtained simultaneously or approximately the same time as the samples are analyzed.

16. The method or assay of claim 13 wherein the BTK occupancy probe is a biotinylated irreversible BTK inhibitor.

17. The method or assay of claim 13 wherein the irreversible BTK inhibitor is ibrutinib.

18. The method or assay of claim 16, wherein the BTK target occupancy determination (a) determines the BTK target occupancy of the BTK inhibitor in the BTK occupancy probe.

19. The method or assay of claim 16, wherein the BTK target occupancy determination (a) determines the BTK occupancy of an unlabeled compound that has been in contact with BTK in the sample before step (ii) contacting of the sample with the BTK occupancy probe, and optionally before step (i).

20. The method or assay of claim 1 or 13 wherein the solid support is a streptavidin coated plate.

21. A method or assay to determine target occupancy by a compound suspected of occupying the target, and to quantitate total target of a Tec family kinase target in a sample, comprising:

(i) obtaining a sample and splitting or aliquoting the sample for (a) target occupancy determination and (b) total target determination, wherein the target in the sample has been in contact with the compound;

(ii) contacting the sample for (a) with a capture moiety tagged target occupancy probe and the sample for (b) with a capture moiety tagged anti-target specific binding member, wherein the capture moiety in (a) and (b) is the same or is capable of binding to the same capture moiety binder, and wherein a target occupancy probe-target complex is formed in (a) and a specific binding member-target complex is formed in (b);

(iii) incubating each of (a) and (b) with a capture moiety binder coated solid support under conditions wherein binding of the target complex in (a) and (b) to the solid support occurs; and

(iv) detecting the target complex in (a) and (b) by contacting the target complex bound solid support with a primary anti-target specific binding member, optionally further contacting the target complex bound solid support with a secondary tagged or labeled antibody that recognizes or binds the primary anti-target specific binding member for detection; and determining the amount of target complex in (a) and (b) to determine the amount of target occupancy by the target occupancy probe and the amount of total target;

(v) determining the target occupancy by the compound based on the amount of target occupancy by the target occupancy probe and the amount of total target.

FIGURE 1

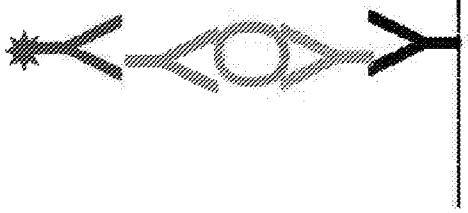
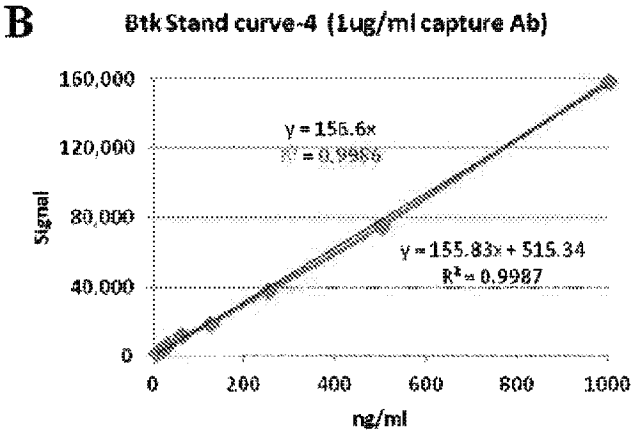

5) anti-Mouse IgG w/ TAG
4) Mouse anti-Btk
3) Sample
2) Capture Ab: Rab-anti-Btk
1) Coating Ab: Goat anti-Rab IgG
96 well MSD Plates

FIGURE 2

A

	Cap-Ab-1			
ng/ml	Signal			Average signal
1000	160722	157708	157080	158503
500	79100	76312	69447	74953
250	37841	36948	37818	37536
125	18873	18438	19995	19102
62.5	14621	9951	11597	12056
31.3	6293	8375	6072	6913
15.6	4116	2911	3106	3378
7.8	2540	1932	1871	2114

B



C

ng/ml	Cap-Ab-2			Average signal
1000	155753	146054	140973	147593
500	74280	71455	66588	70774
250	36520	36260	33447	35409
125	19956	19073	18031	19020
62.5	10511	9837	9636	9995
31.3	5691	5818	5114	5541
15.6	3212	3127	2785	3041
7.8	1752	1809	1688	1750

D

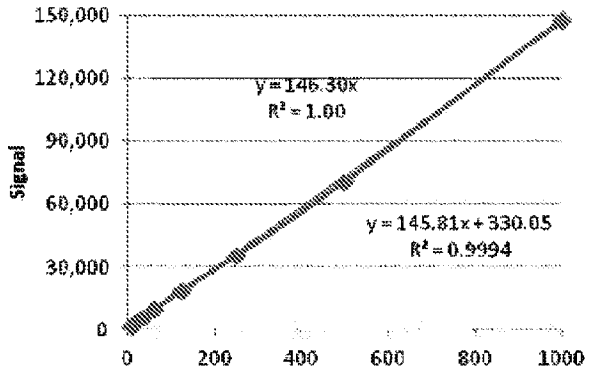


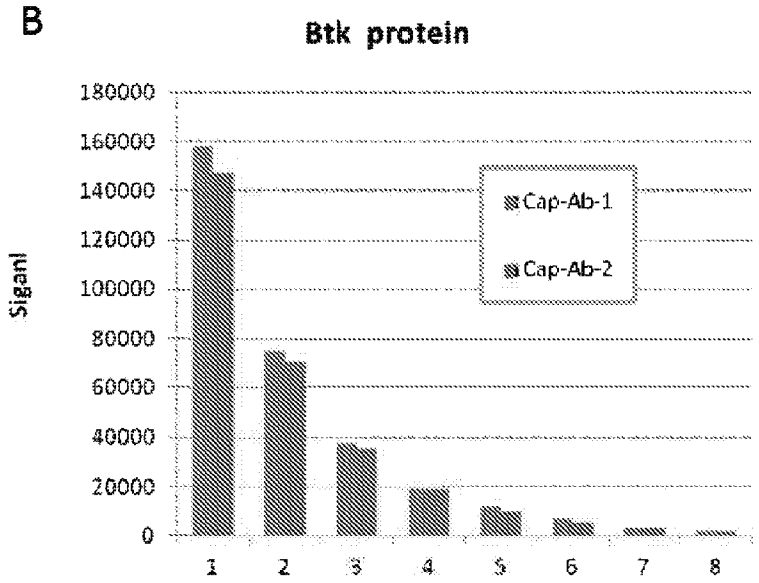
FIGURE 3

A

Btk protein

ng/ml	Cap-Ab-1	Cap-Ab-2
1000	158503	147593
500	74953	70774
250	37536	35409
125	19102	19020
62.5	12056	9995
31.3	6913	5541
15.6	3378	3041
7.8	2114	1750

B



C DOHH2 protein

	Cap Ab-1	Cap Ab-2
DOHH2	Signal	Signal
500 ug	981	807
300 ug	639	568
150ug	517	483

FIGURE 4

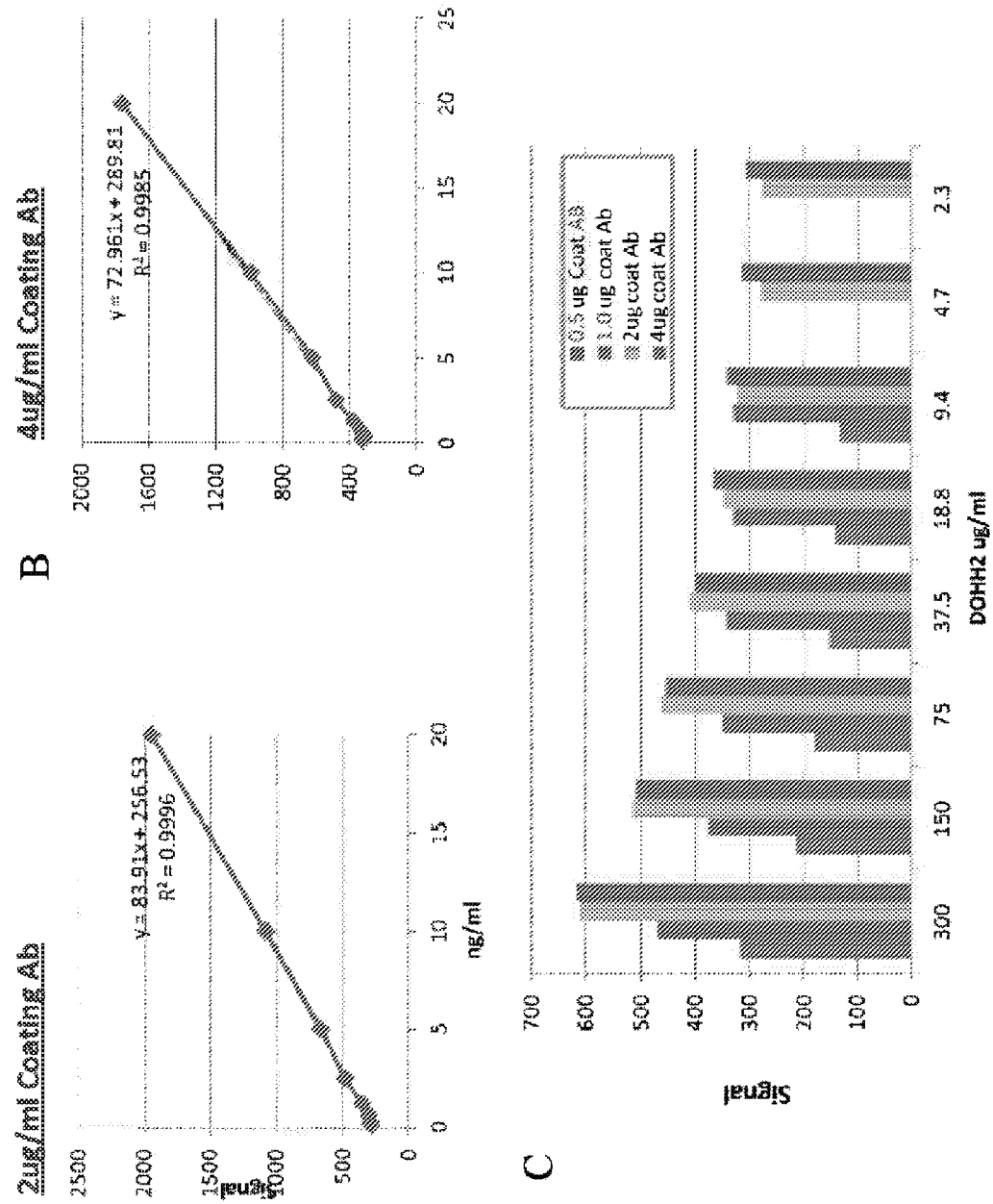

A
2ug/ml Coating Ab
y = 83.91x + 256.53
R² = 0.9996
Signal
ng/ml
B
4ug/ml Coating Ab
y = 72.961x + 289.81
R² = 0.9985
C
0.5 ug Coat Ab
1.0 ug coat Ab
2ug coat Ab
4ug coat Ab
Signal
DOHH2 ug/ml

FIGURE 5

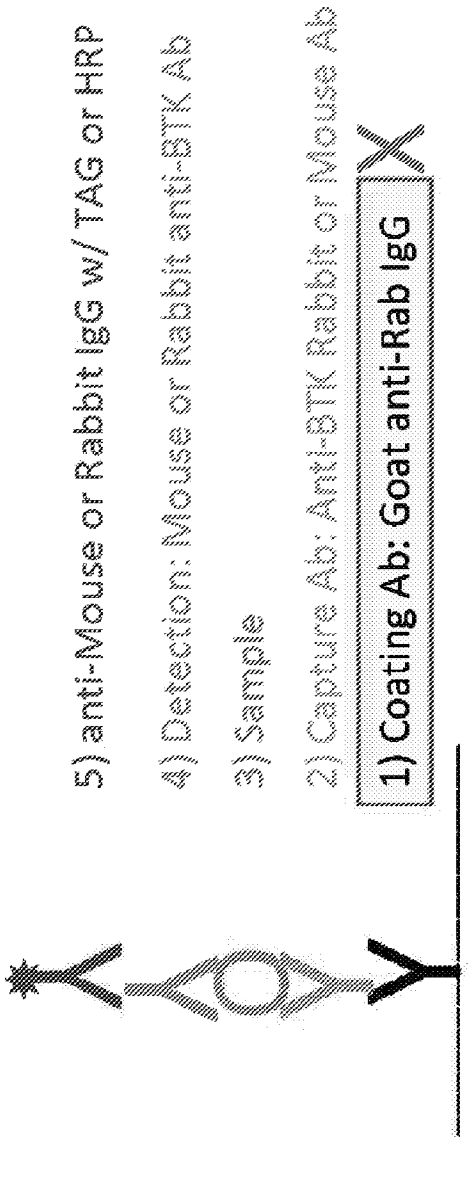

5) anti-Mouse or Rabbit IgG w/ TAG or HRP
4) Detection: Mouse or Rabbit anti-BTK Ab
3) Sample
2) Capture Ab: Anti-BTK Rabbit or Mouse Ab
1) Coating Ab: Goat anti-Rab IgG
96 well STD and MSD Plates

FIGURE 6

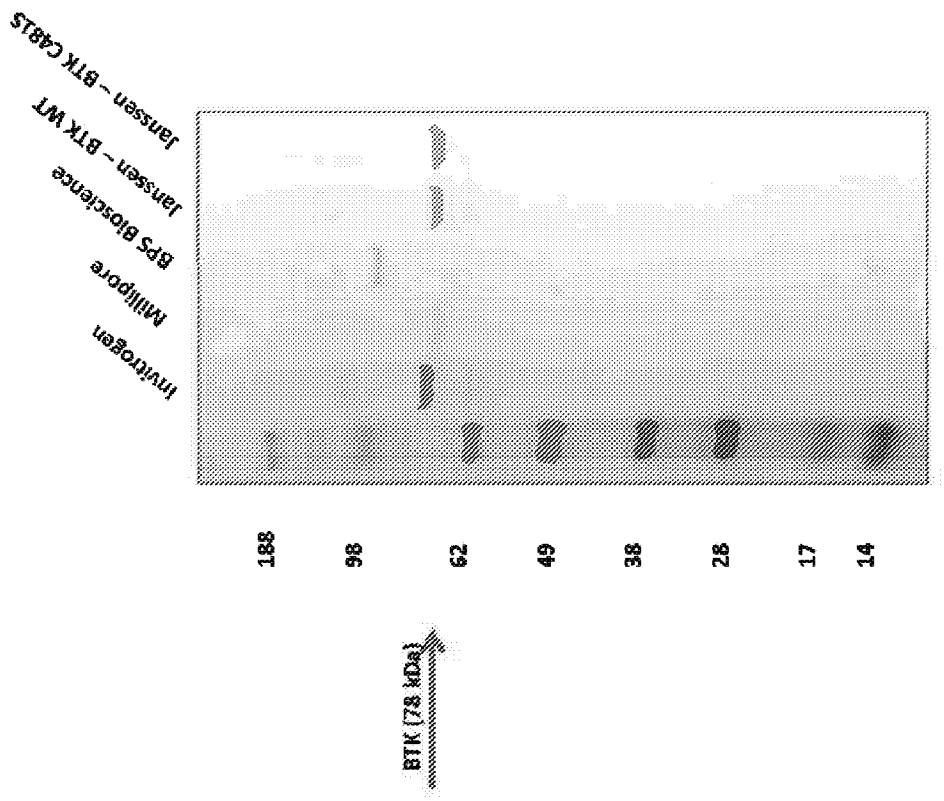

Janssen – BTK C481S
Janssen – BTK WT
BPS Bioscience
Millipore
Invitrogen
188
98
62
49
38
28
17
14
BTK (78 kDa)

FIGURE 7

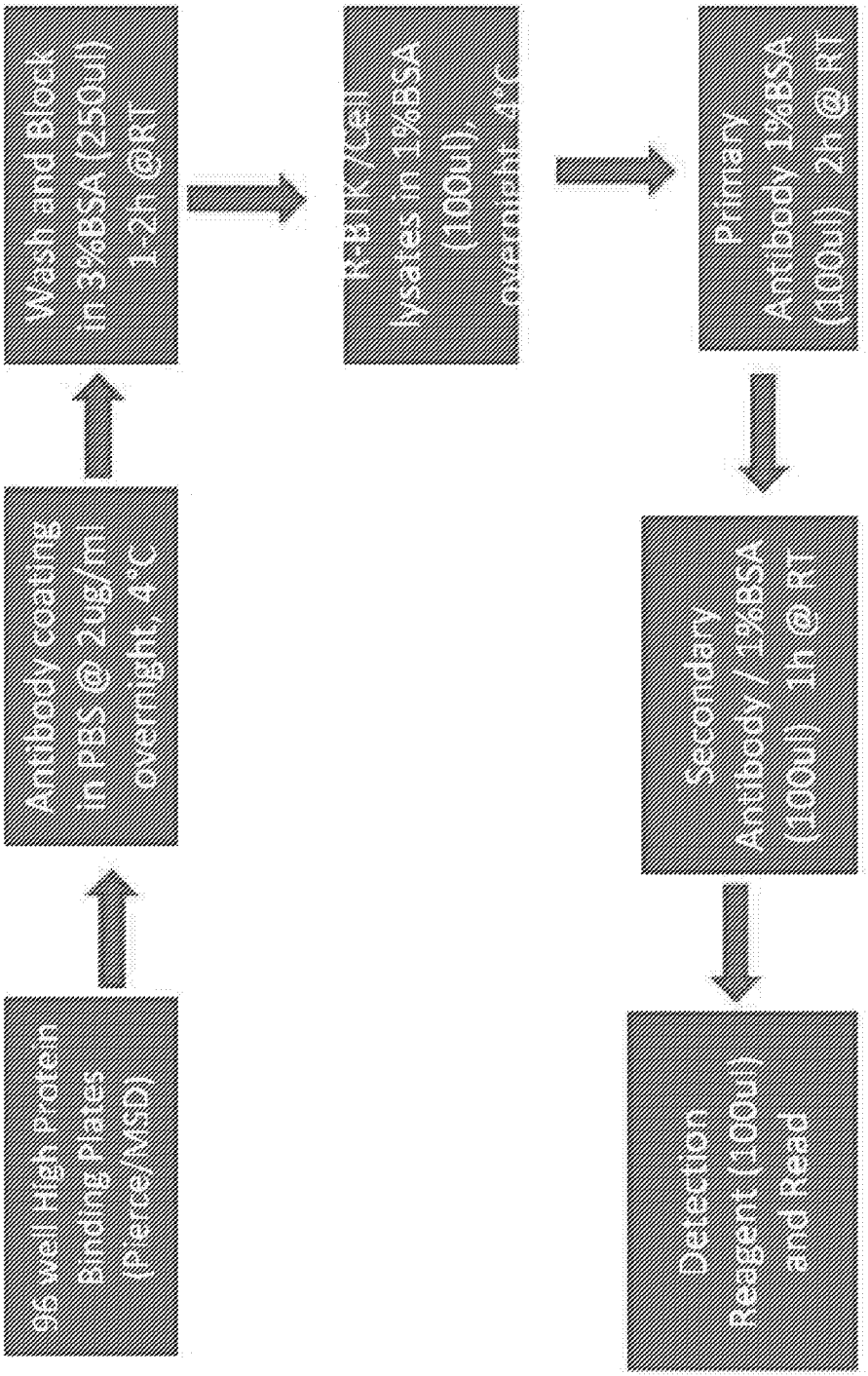
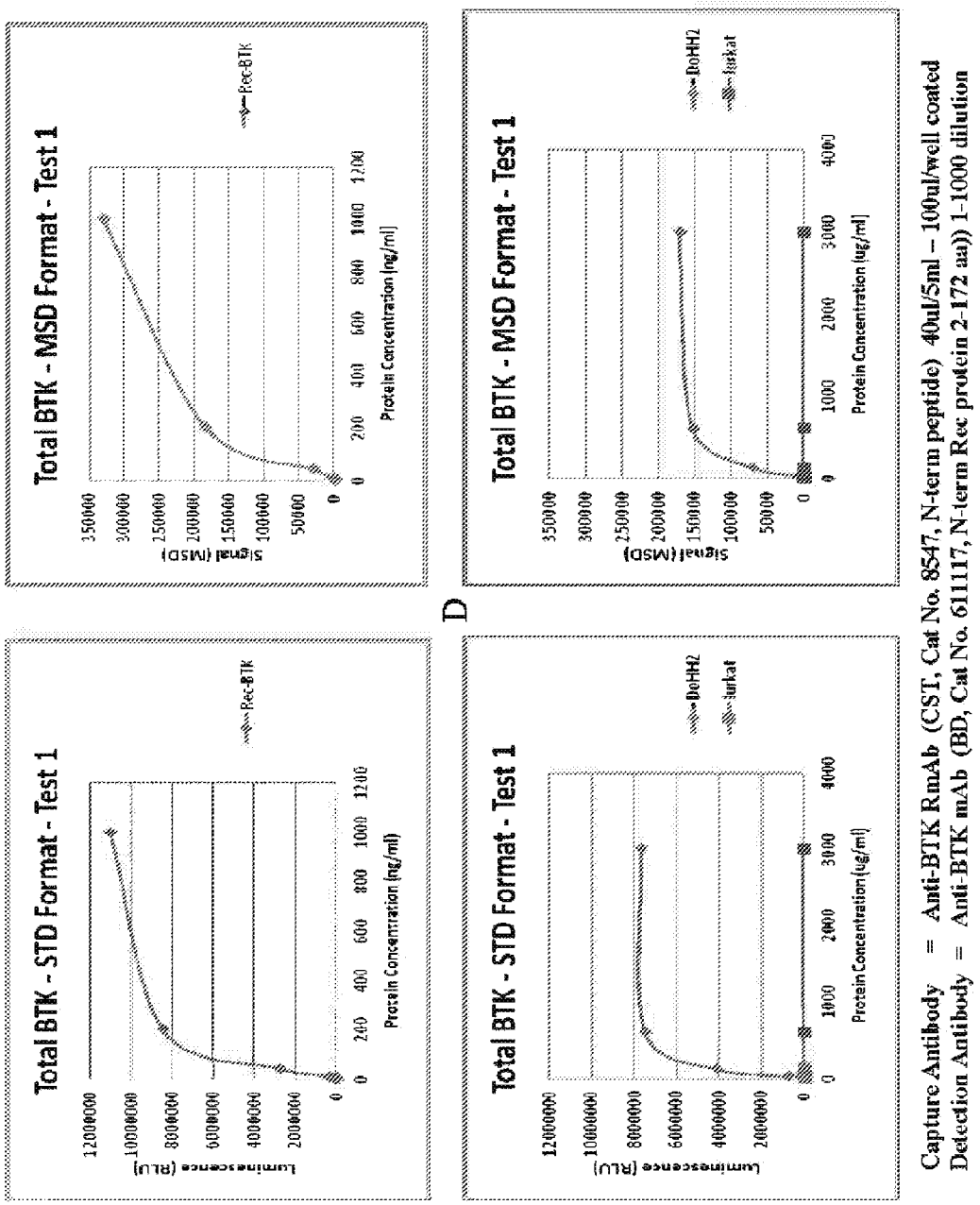
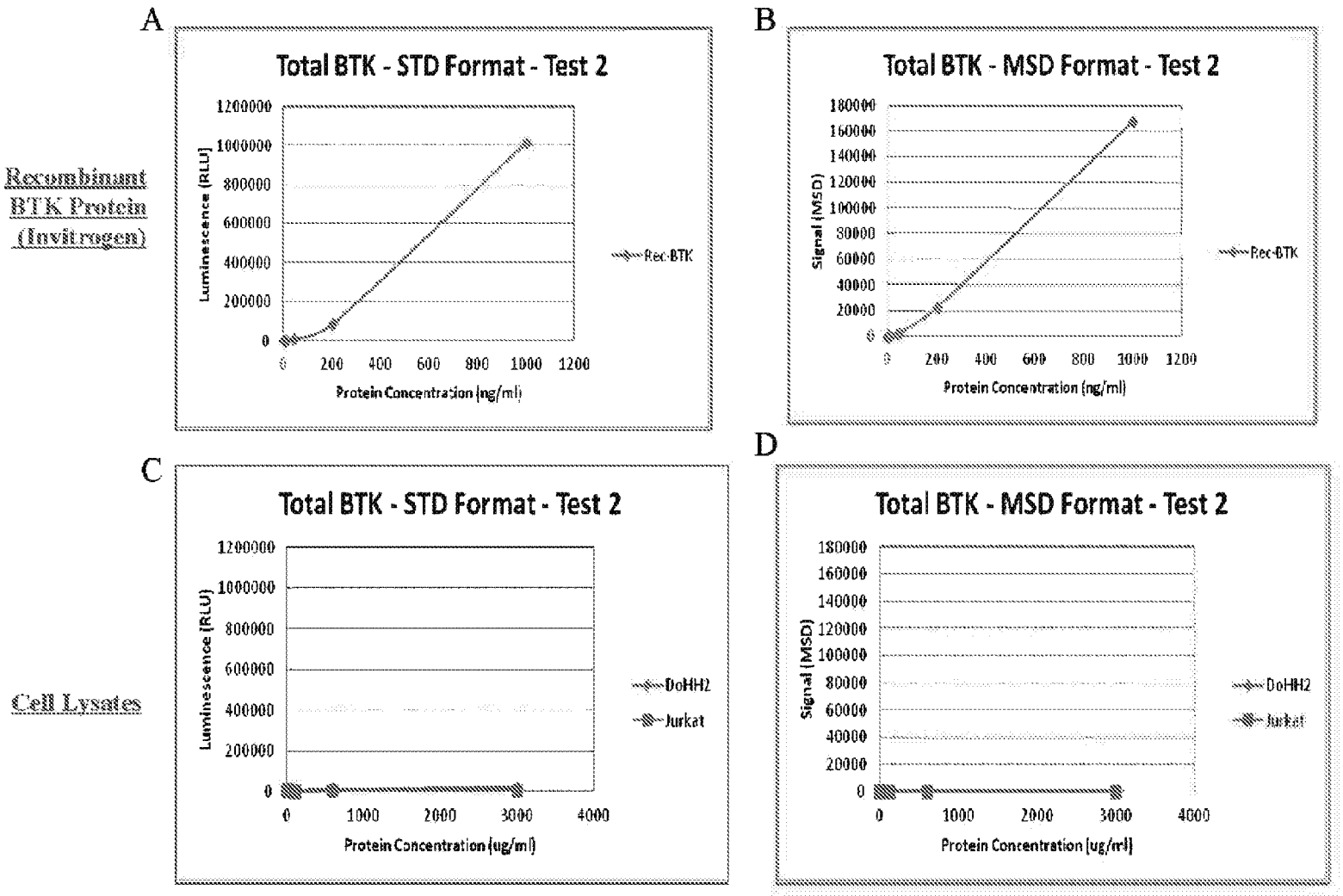

96 well High Protein Binding Plates (Pierce/MSD)
Antibody coating in PBS @ 2ug/ml overnight, 4°C
Wash and Block in 3%BSA (250ul) 1-2h @RT
Kinase/Cell lysates in 1%BSA (100ul), overnight, 4°C
Primary Antibody 1%BSA (100ul) 2h @ RT
Secondary Antibody / 1%BSA (100ul) 1h @ RT
Detection Reagent (100ul) and Read

FIGURE 8



Capture Antibody = Anti-BTK RmAb (CST, Cat No. 8547, N-term peptide) 40ul/5ml – 100ul/well coated
Detection Antibody = Anti-BTK mAb (BD, Cat No. 611117, N-term Rec protein 2-172 aa)) 1-1000 dilution

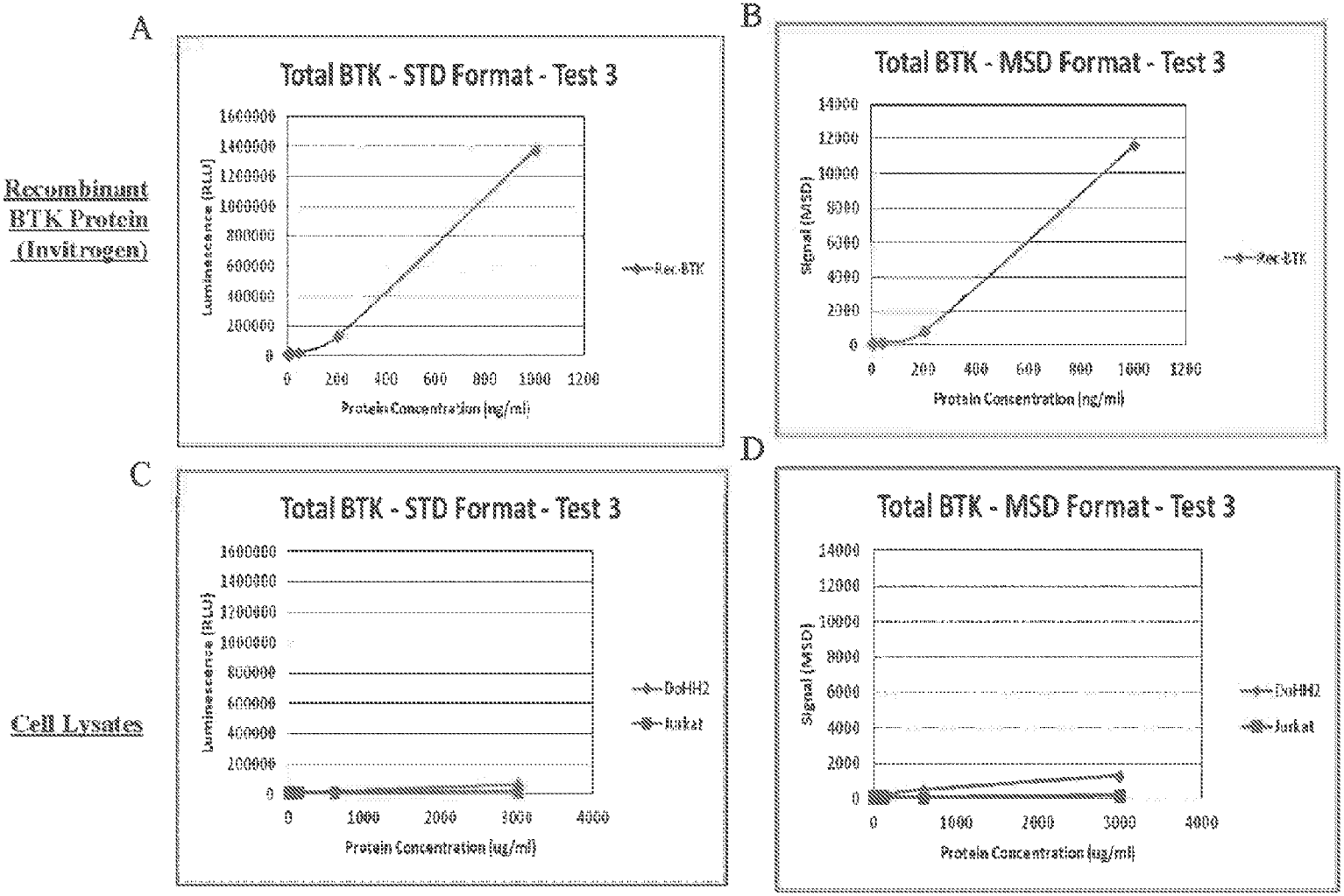
FIGURE 9



Capture Antibody = Anti-BTK RpAb (Abcam, Cat No.32555, C-term peptide) 40ul/5ml – 100ul/well coated
Detection Antibody = Anti-BTK mAb (BD, Cat No. 611117, N-term Rec protein 2-172 aa) 1-1000 dilution

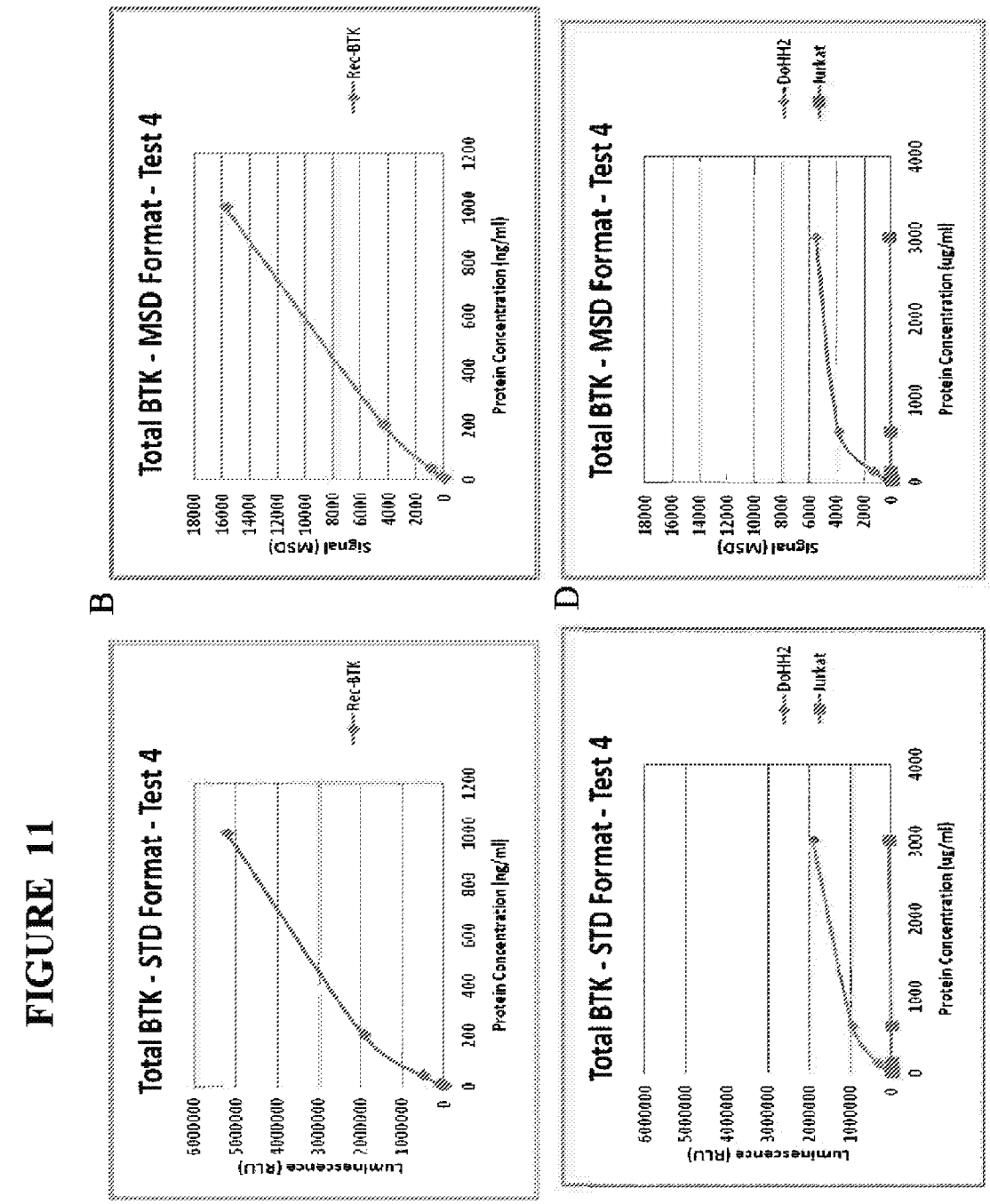
WO 2016/100593 PCT/US2015/066244

FIGURE 10



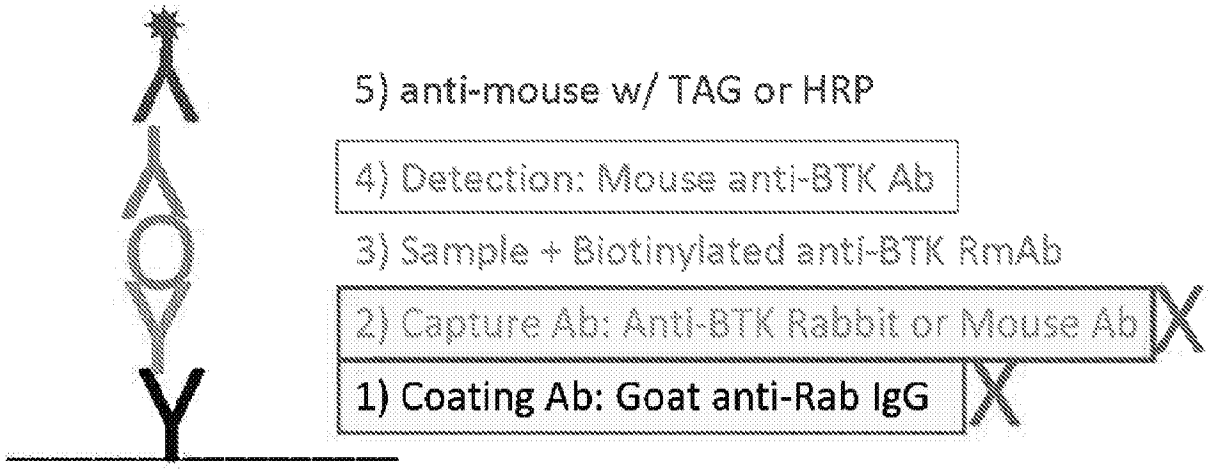
Capture Antibody = Anti-BTK mAb (BD, Cat No. 611117, N-term Rec Protein 2-172 aa) 40ul/5ml -- 100ul/well
Detection Antibody = Anti-BTK RpAb (Abcam, Cat No. 32555, C-term peptide) 1-500 dilution

FIGURE 11



Capture Antibody = Anti-BTK mAb (BD, Cat No. 611117, N-term Rec protein 2-172 aa)) 40ul/5ml – 100ul/well coated
Detection Antibody = Biotin Anti-BTK RmAb (CST, Cat No. 12624S, N-term peptide) 1-1000 dilution

FIGURE 12



96 well STD and MSD Plates

FIGURE 13

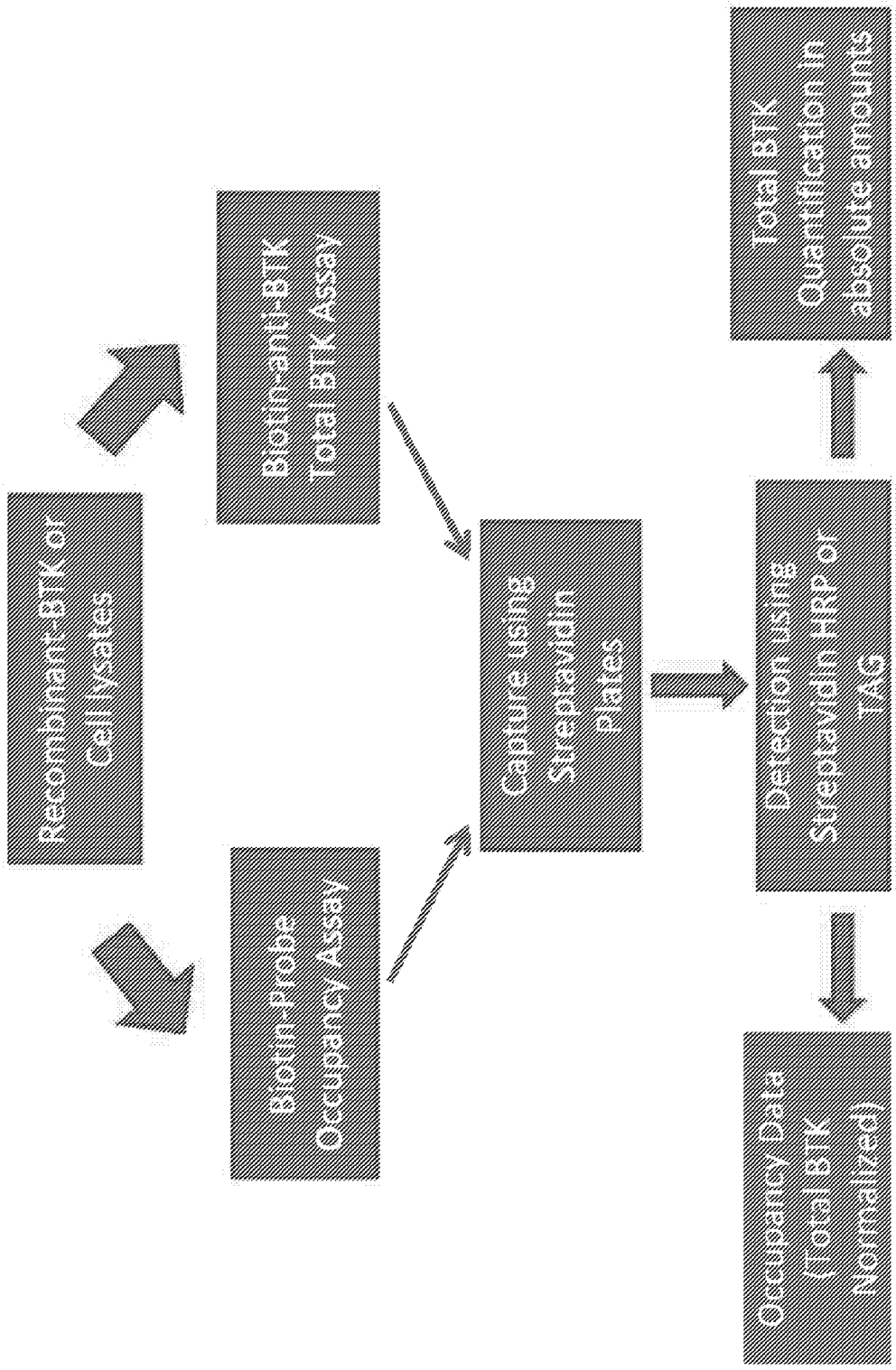
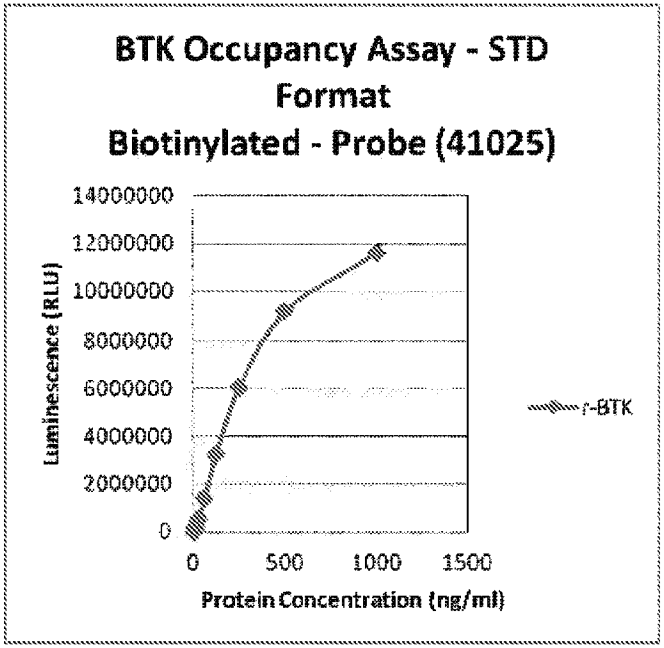

Recombinant-BTK or Cell lysates
Biotin-anti-BTK Total BTK Assay
Biotin-Probe Occupancy Assay
Capture using Streptavidin Plates
Detection using Streptavidin HRP or TAG
Total BTK Quantification in absolute amounts
Occupancy Data (Total BTK Normalized)

FIGURE 14

A Rec-BTK (Invitrogen)



B Cell Lysates

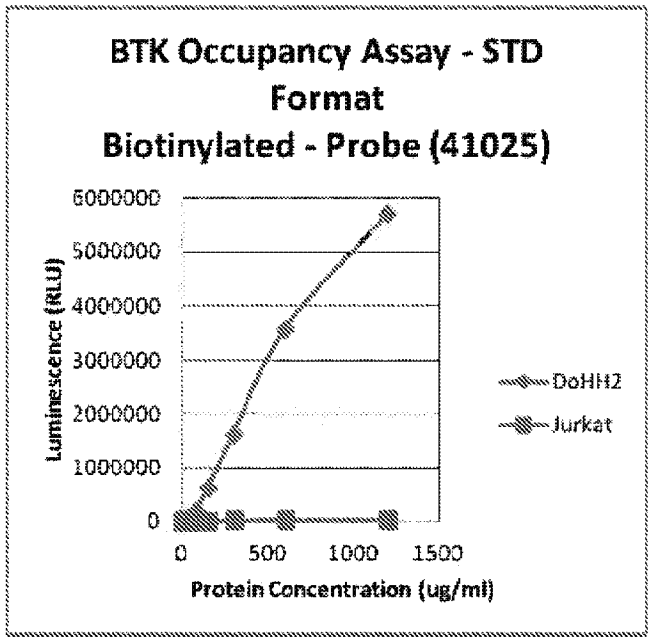


FIGURE 15

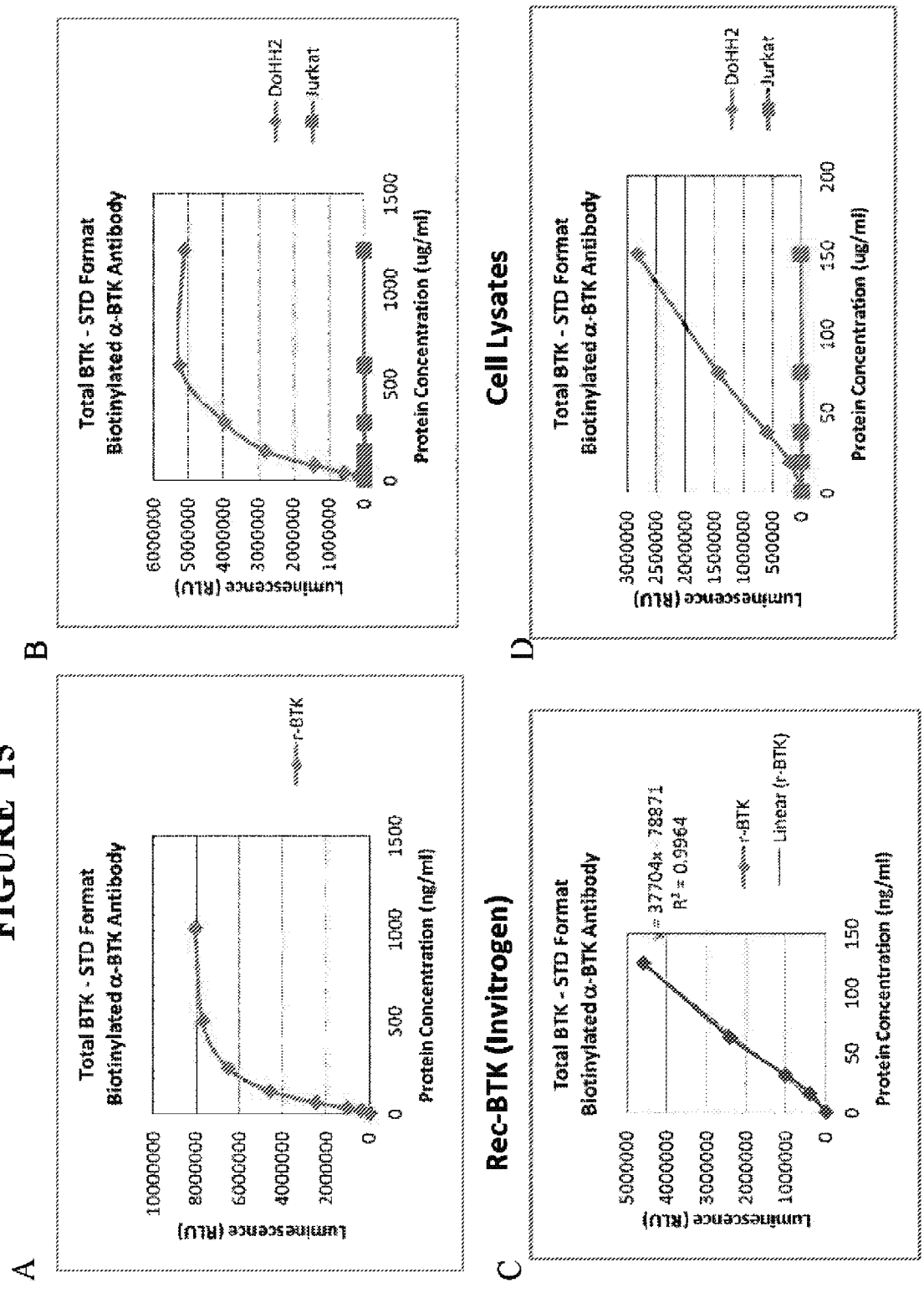
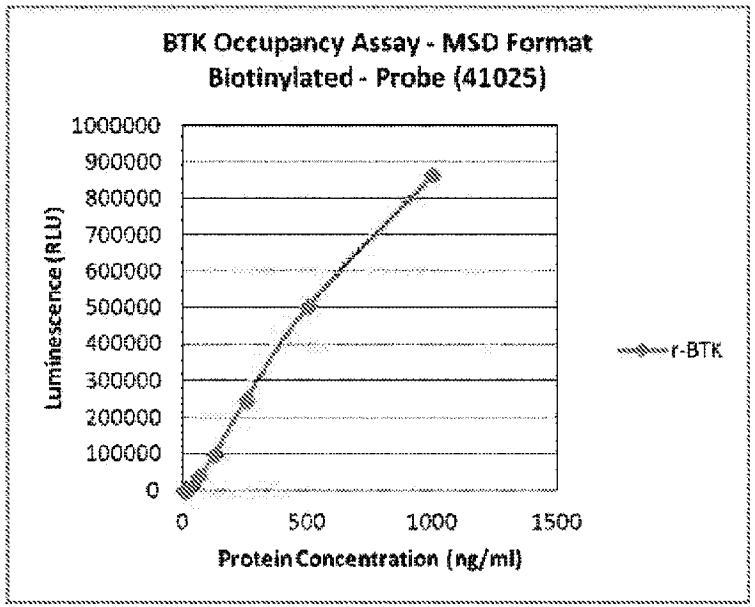

A
Total BTK - STD Format
Biotinylated α-BTK Antibody
r-BTK
Luminescence (RLU)
Protein Concentration (ng/ml)
B
Total BTK - STD Format
Biotinylated α-BTK Antibody
DoHH2
Jurkat
Luminescence (RLU)
Protein Concentration (ug/ml)
C
Rec-BTK (Invitrogen)
Total BTK - STD Format
Biotinylated α-BTK Antibody
y = 37704x - 78871
R² = 0.9964
r-BTK
Linear (r-BTK)
Luminescence (RLU)
Protein Concentration (ng/ml)
D
Cell Lysates
Total BTK - STD Format
Biotinylated α-BTK Antibody
DoHH2
Jurkat
Luminescence (RLU)
Protein Concentration (ug/ml)

FIGURE 16

A

Rec-BTK (Invitrogen)



B

Cell Lysates

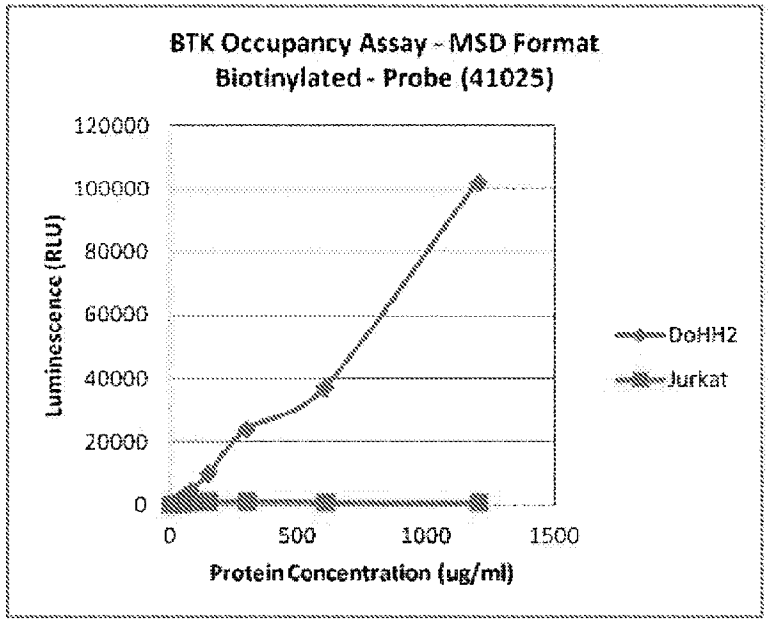


FIGURE 17

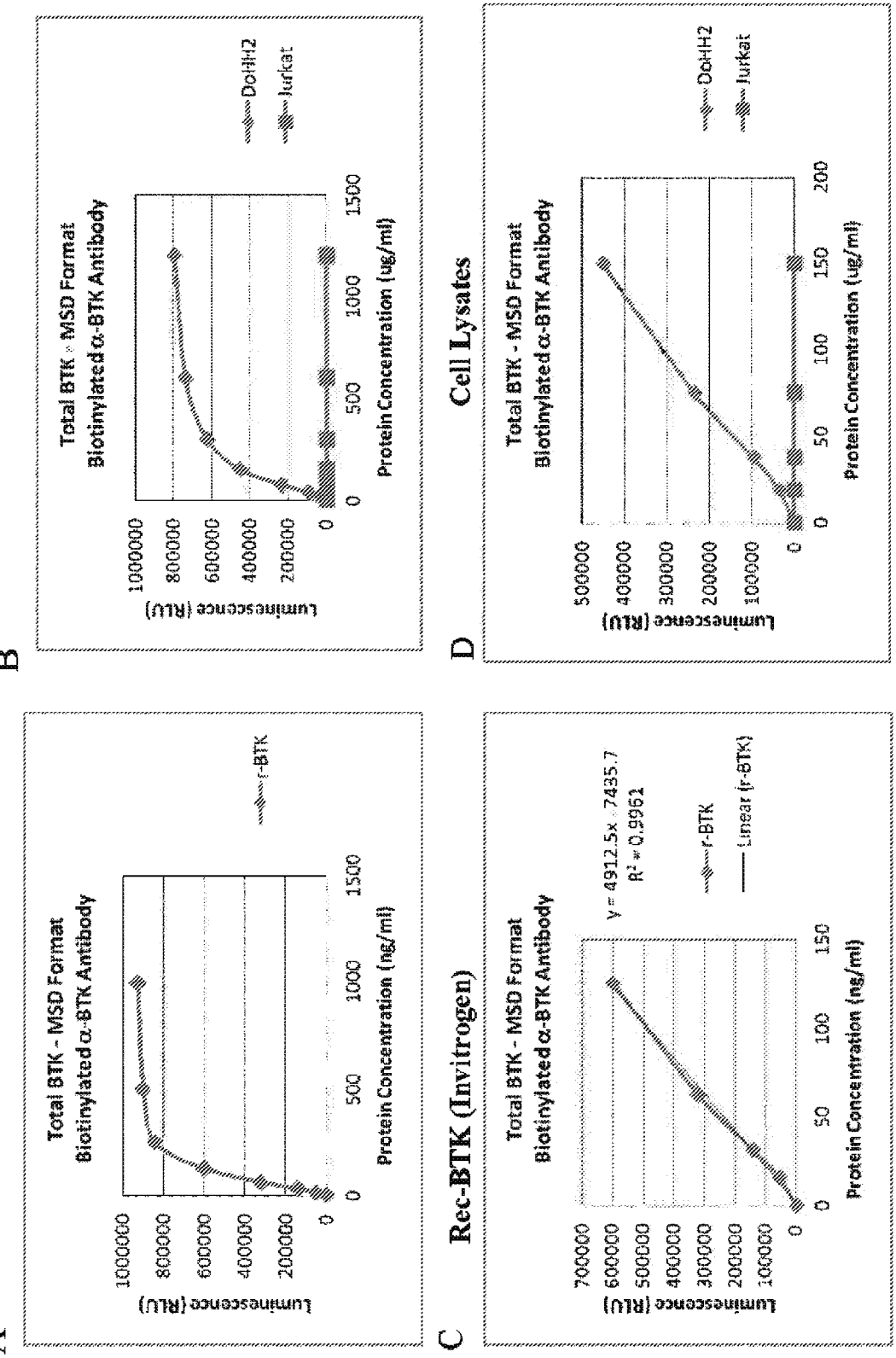
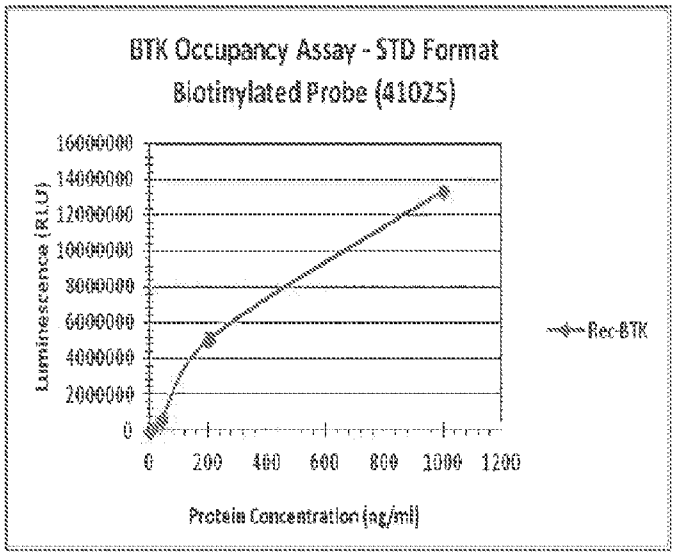

A
Total BTK - MSD Format
Biotinylated α-BTK Antibody
Luminescence (RLU)
Protein Concentration (ng/ml)
r-BTK
B
Total BTK - MSD Format
Biotinylated α-BTK Antibody
Luminescence (RLU)
Protein Concentration (ug/ml)
DoHH2
Jurkat
C
Rec-BTK (Invitrogen)
Total BTK - MSD Format
Biotinylated α-BTK Antibody
y = 4912.5x + 7435.7
R² = 0.9962
r-BTK
Linear (r-BTK)
Luminescence (RLU)
Protein Concentration (ng/ml)
D
Cell Lysates
Total BTK - MSD Format
Biotinylated α-BTK Antibody
Luminescence (RLU)
Protein Concentration (ug/ml)
DoHH2
Jurkat

FIGURE 18

A

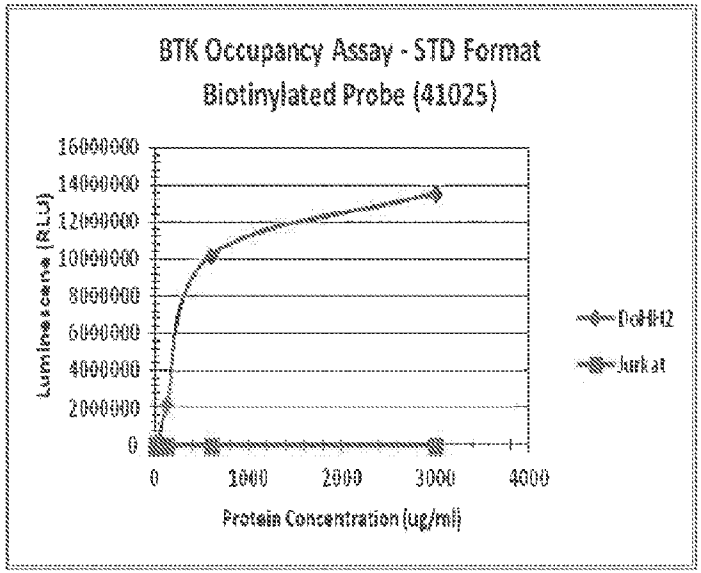
Rec-BTK (Invitrogen)



ng/ml	Rec-BTK
1000	13409666
200	5079063
40	671343
8	46576
1.6	3811
0.32	1370
0.064	1110
0	734

B

Cell Lysates

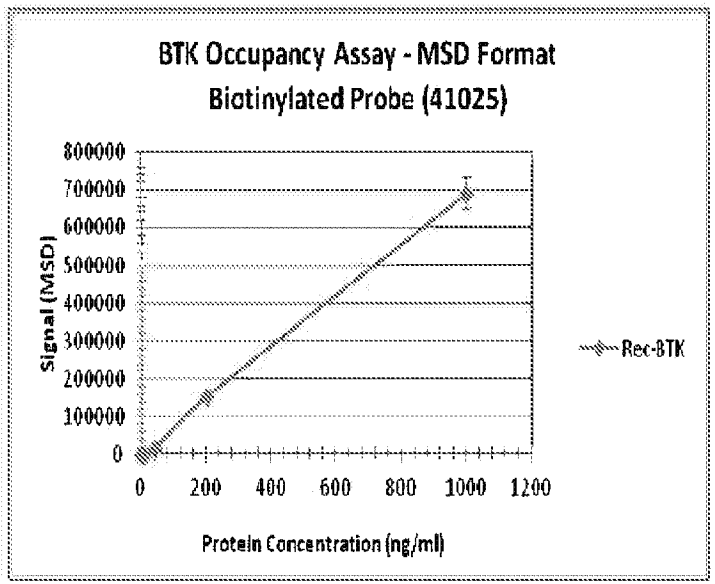


ug/ml	DoHH2	Jurkat
3000	13564371	11360
600	10218347	7141
120	2264295	4817
24	163891	3025
4.8	6303	1824
0.96	1363	1467
0.192	1058	1253
0	967	961

FIGURE 19

A

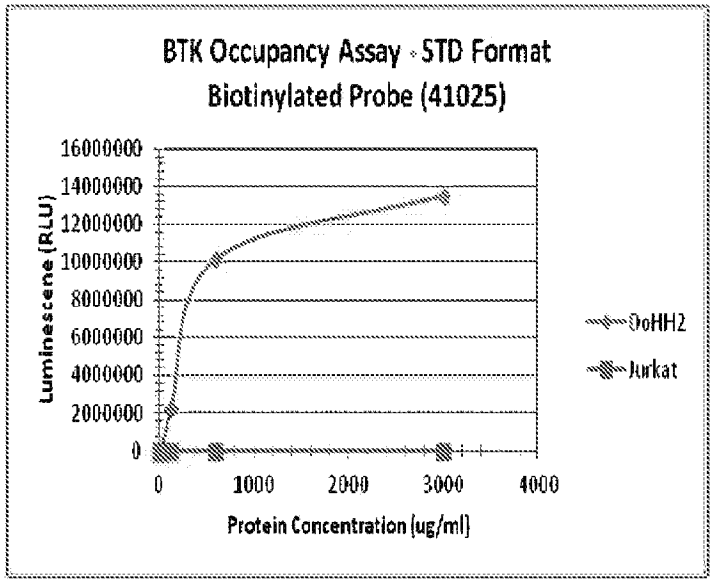
Rec-BTK (Invitrogen)



	Rec-BTK
1000	693271
200	151638
40	15333
8	1263
1.6	189
0.32	106
0.064	104
0	98
ng/ml	

B

Cell Lysates

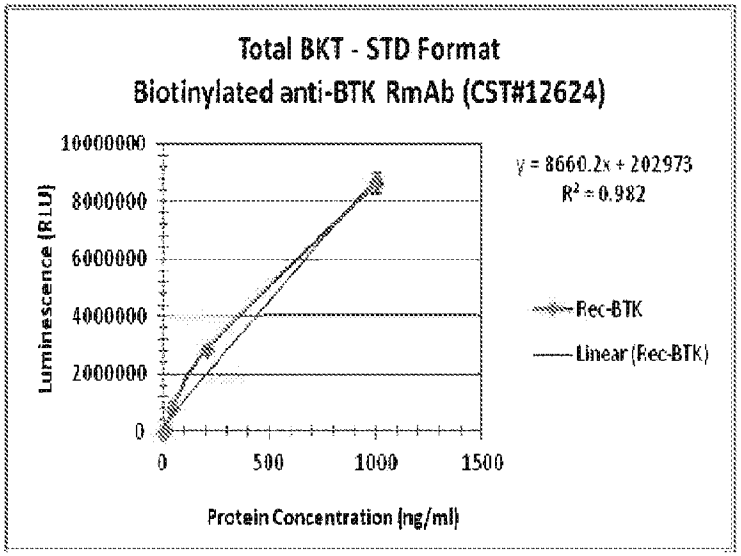


	DoHH2	Jurkat
3000	591796	247
600	226208	238
120	32574	179
24	2365	126
4.8	237	104
0.96	114	94
0.192	107	95
0	99	94
ug/ml		

FIGURE 20

A

Rec-BTK (Invitrogen)

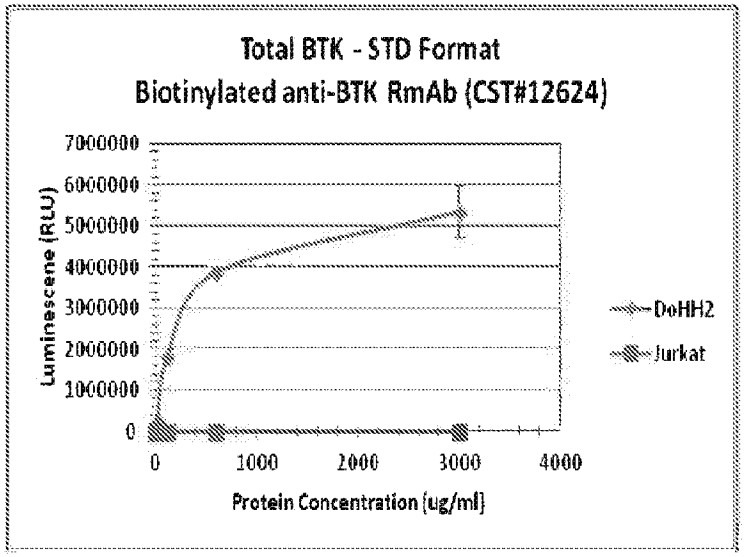


	Rec-BTK
1000	8669518
200	2858208
40	821952
8	90011
1.6	5174
0.32	1863
0.064	1117
0	1065

ng/ml

B

Cell Lysates



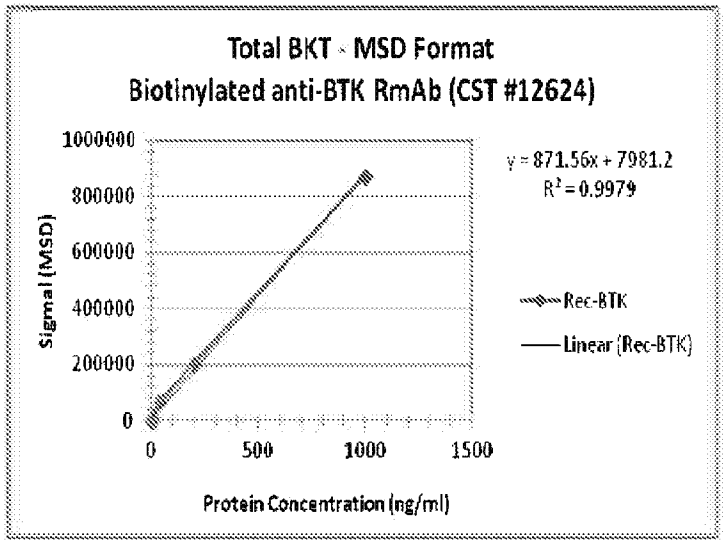
	DoHH2	Jurkat
3000	5351108	3843
600	3875866	3330
120	1833404	3025
24	240906	1889
4.8	10581	1487
0.96	1558	1162
0.192	896	1143
0	1318	844

ug/ml

FIGURE 21

A

Rec-BTK (Invitrogen)

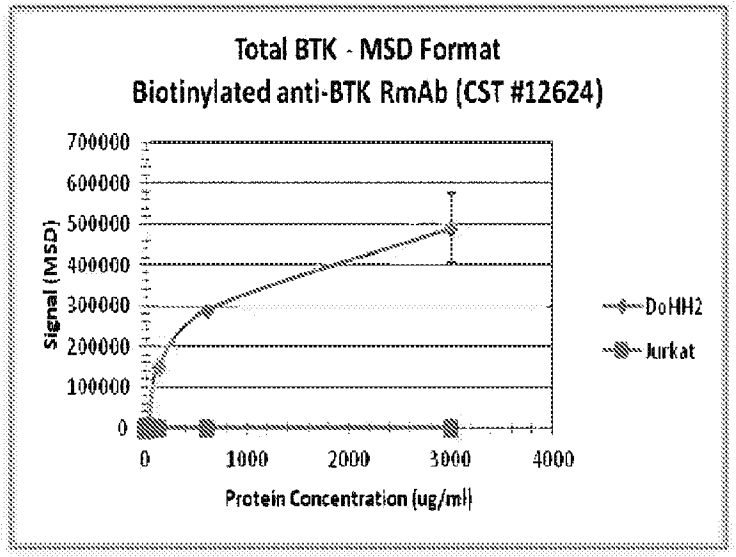


	Rec-BTK
1000	874264
200	204559
40	65355
8	8096
1.6	759
0.32	132
0.064	79
0	49

ng/ml

B

Cell Lysates



	DoHH2	Jurkat
3000	491568	241
600	291112	132
120	151600	66
24	19393	58
4.8	1350	51
0.96	142	53
0.192	58	52
0	47	53

ug/ml

FIGURE 22

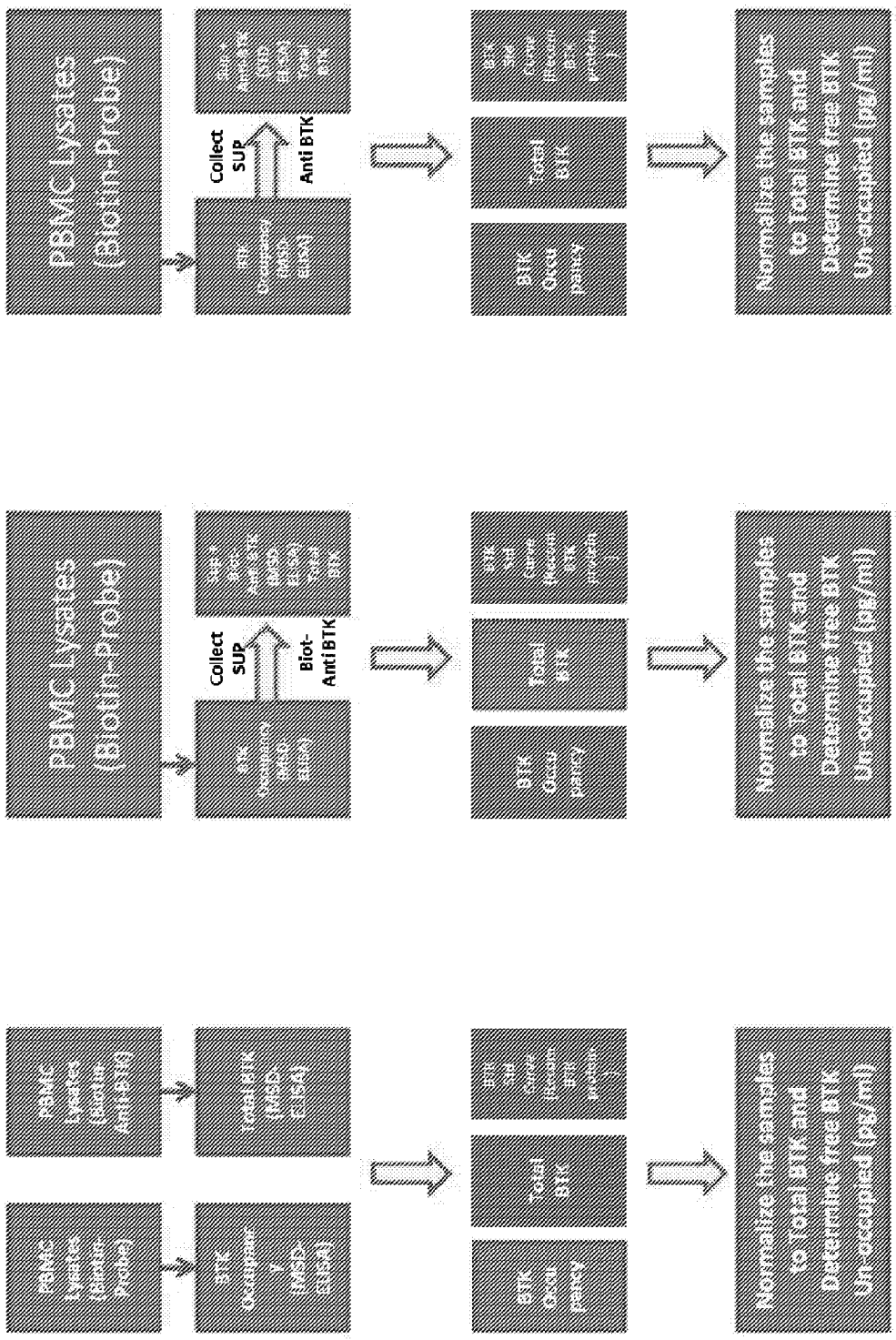

Method -1
PBMC Lysates (Biotin-Probe)
BTK Occupancy (MSD-ELISA)
PBMC Lysates (Anti BTK)
Total BTK (MSD-ELISA)
BTK Occupancy
Total BTK
Normalize the samples to Total BTK and Determine free BTK
Method -2
PBMC Lysates (Biotin-Probe)
BTK Occupancy (MSD-ELISA)
Collect SUP
Biot-Anti BTK
Total BTK (MSD-ELISA)
BTK Occupancy
Total BTK
Normalize the samples to Occ BTK and Determine free BTK
Method -3
PBMC Lysates (Biotin-Probe)
BTK Occupancy (MSD-ELISA)
Collect SUP
Anti BTK
Total BTK (MSD-ELISA)
BTK Occupancy
Total BTK
Normalize the samples to Total BTK and Determine free BTK

FIGURE 23
BTK Quantification: METHOD-1

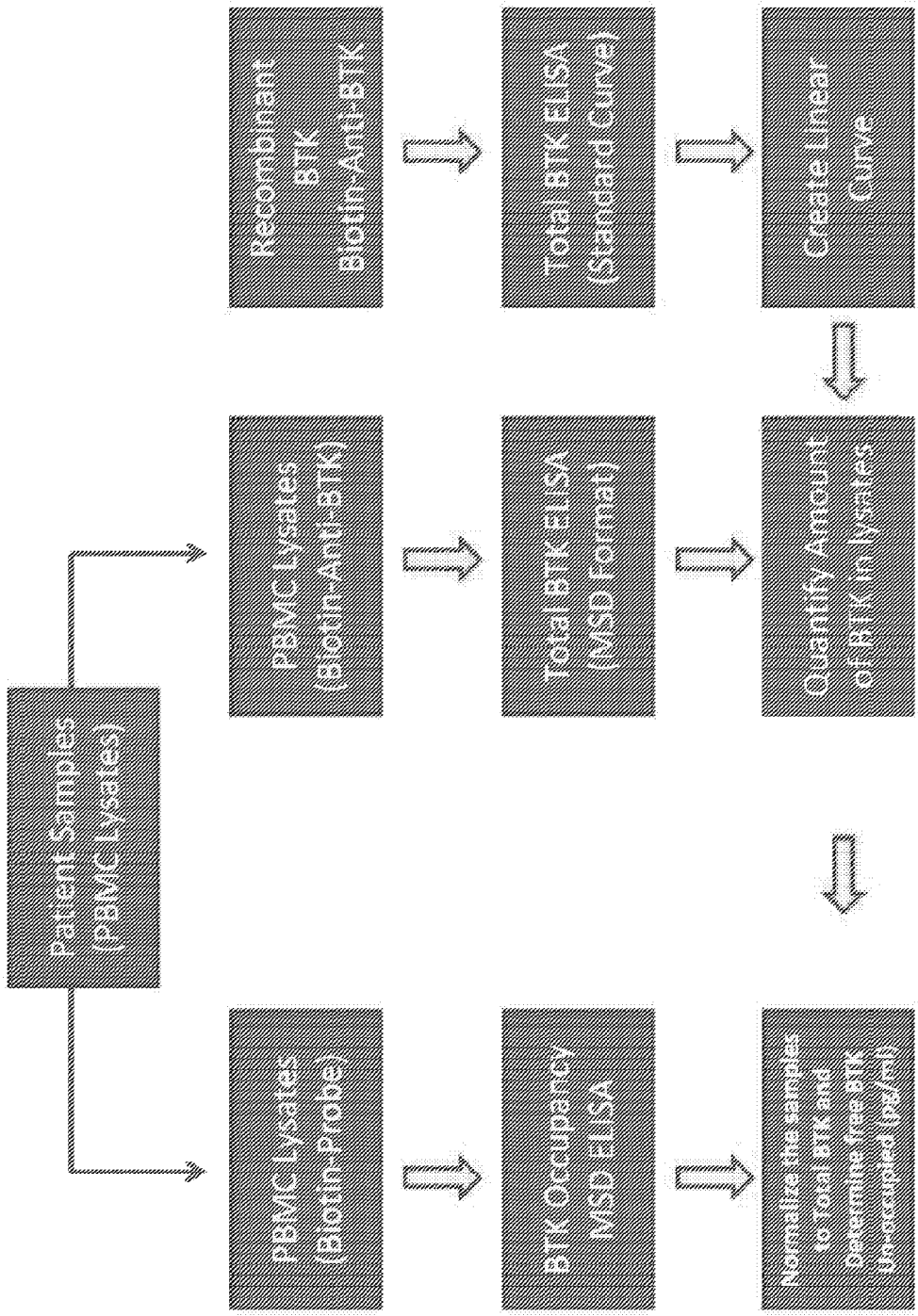


FIGURE 24

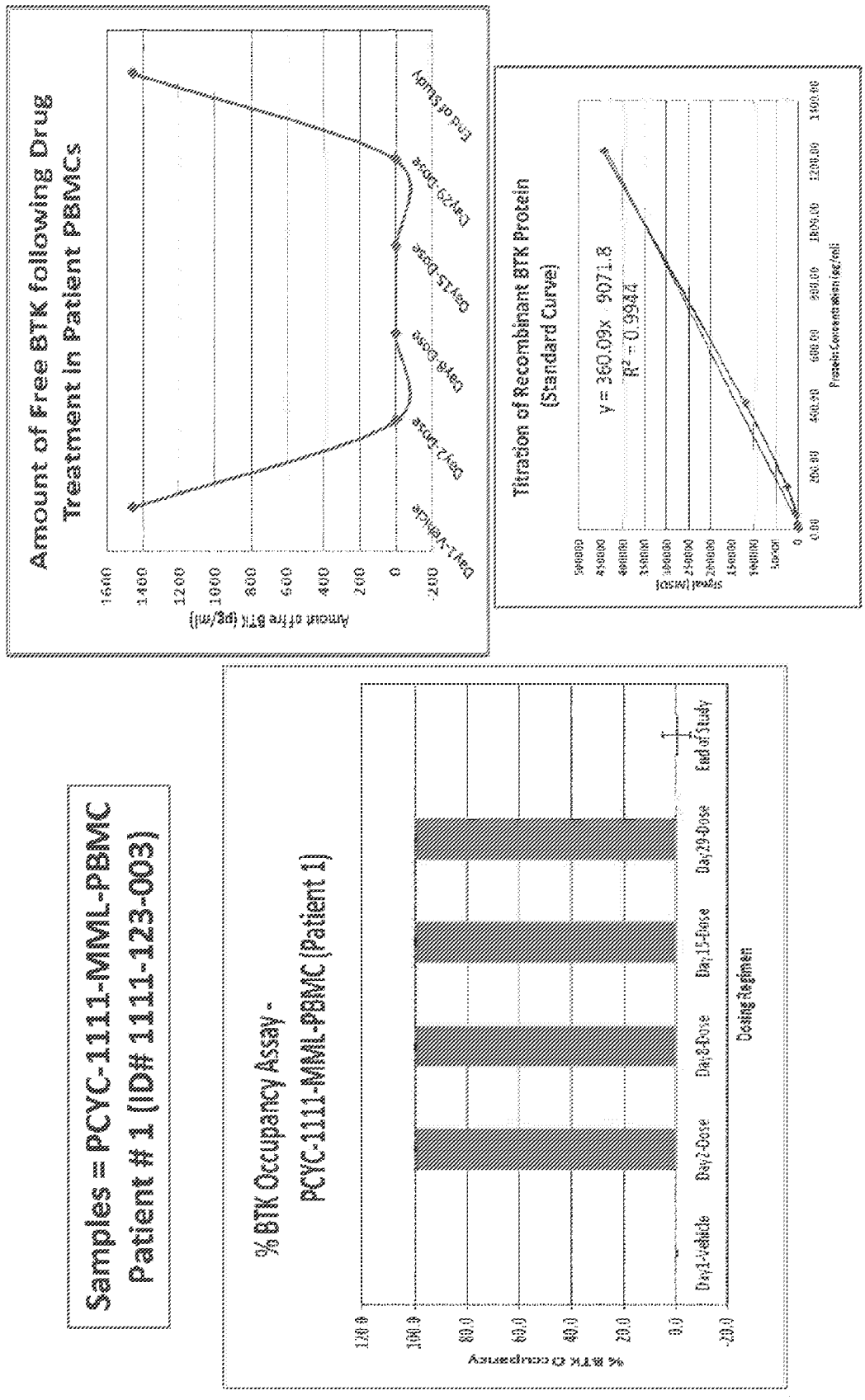

Samples = PCYC-1111-MML-PBMC
Patient # 1 (ID# 1111-123-003)
% BTK Occupancy Assay -
PCYC-1111-MML-PBMC (Patient 1)
% BTK Occupancy
Dosing Regimen
Day1-Vehicle
Day2-Dose
Day8-Dose
Day15-Dose
Day29-Dose
End of Study
Amount of Free BTK following Drug
Treatment in Patient PBMCs
Amount of Free BTK (pg/ml)
Titration of Recombinant BTK Protein
(Standard Curve)
y = 360.09x - 907.3
R² = 0.9944
Signal (Mean)
Protein Concentration (pg/well)

FIGURE 2S

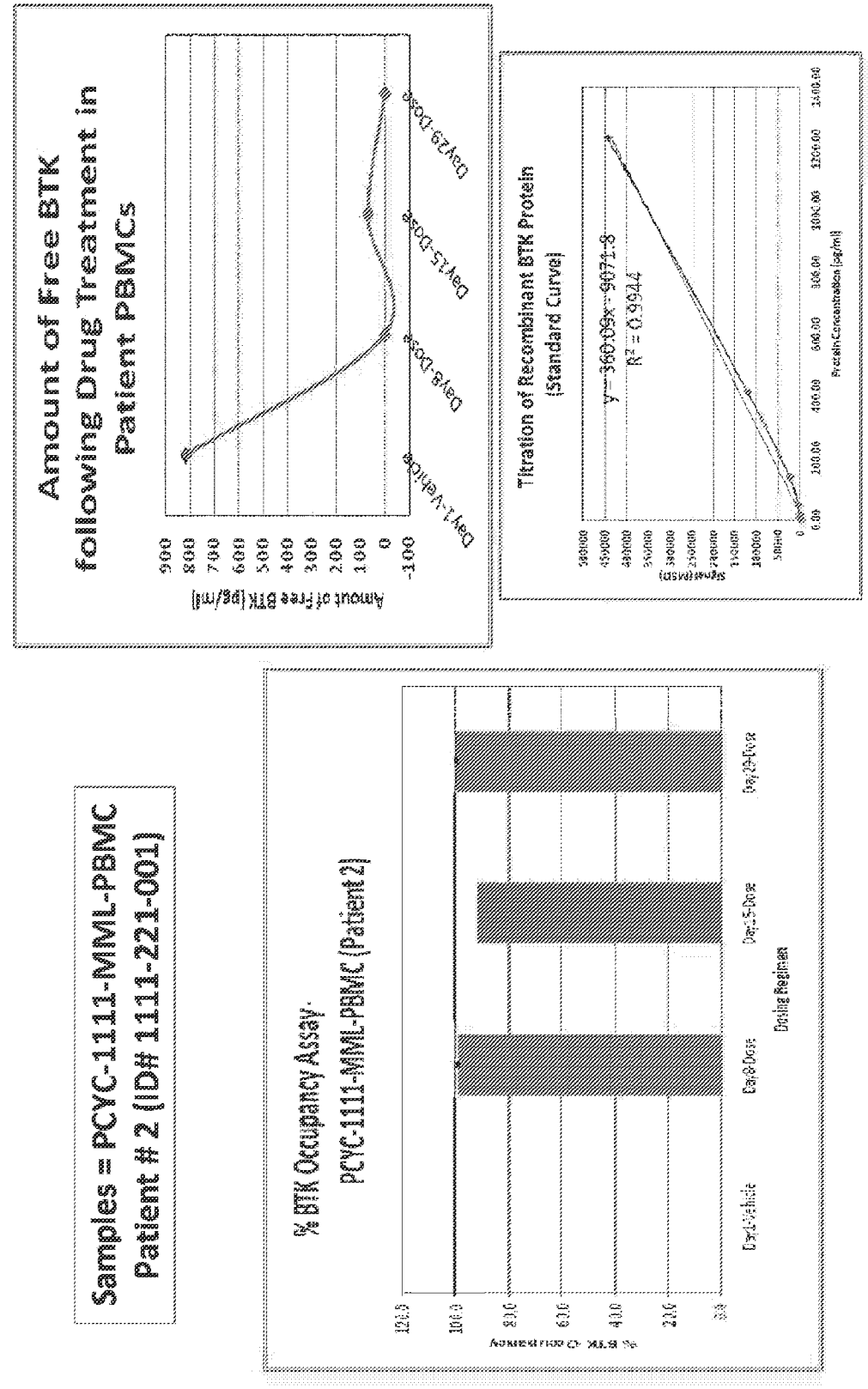

Samples = PCYC-1111-MML-PBMC
Patient # 2 (ID# 1111-221-001)
% BTK Occupancy Assay
PCYC-1111-MML-PBMC (Patient 2)
Dosing Regimen
Amount of Free BTK
following Drug Treatment in
Patient PBMCs
Amount of Free BTK (pg/ml)
Titration of Recombinant BTK Protein
(Standard Curve)
y = 360.09x - 9071.8
R² = 0.9944

FIGURE 26

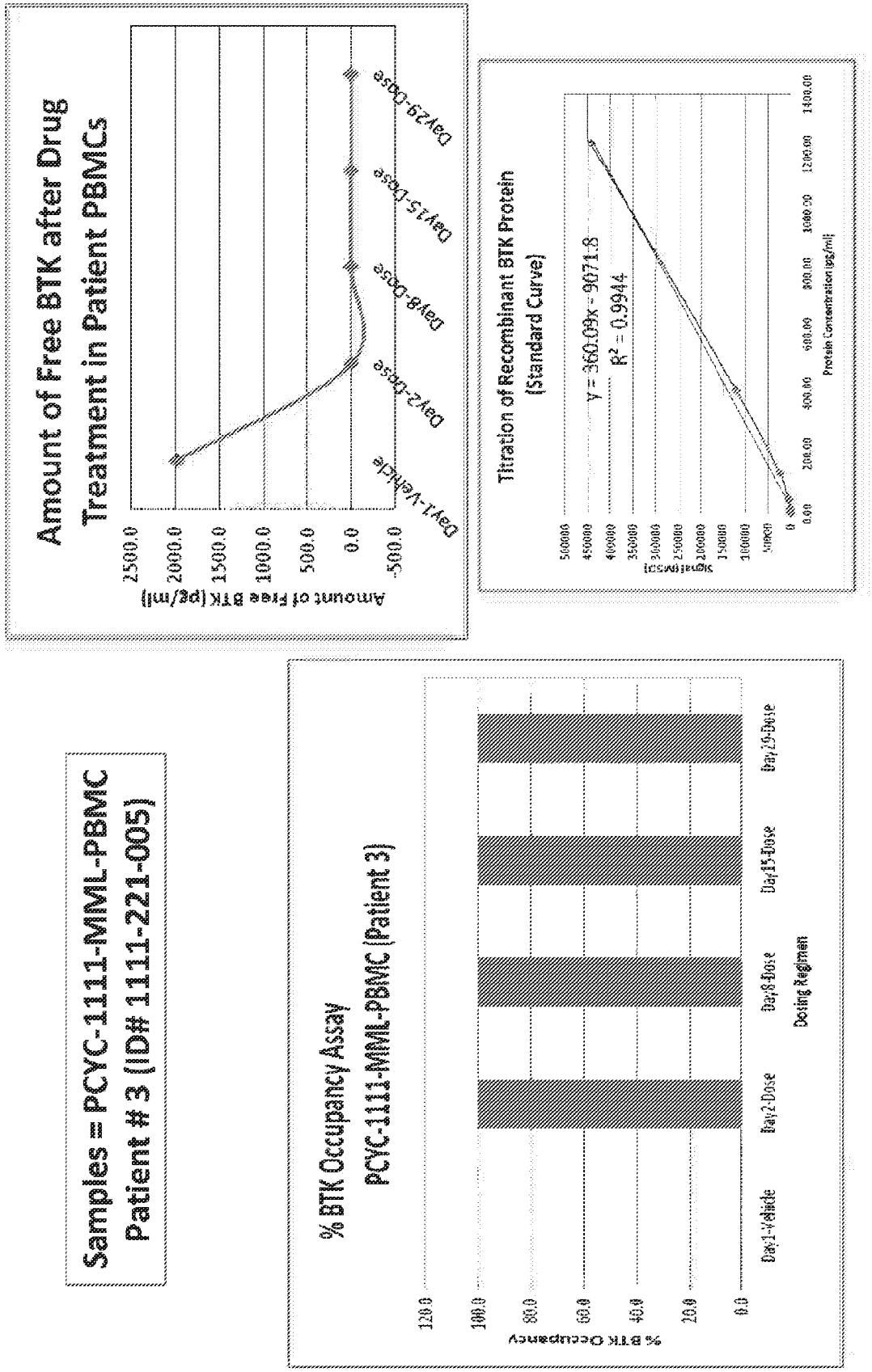

Samples = PCYC-1111-MML-PBMC
Patient # 3 (ID# 1111-221-005)
Amount of Free BTK after Drug
Treatment in Patient PBMCs
Amount of Free BTK (pg/ml)
2500.0
2000.0
1500.0
1000.0
500.0
0.0
-500.0
Day1-Vehicle
Day2-Dose
Day8-Dose
Day15-Dose
Day29-Dose
Titration of Recombinant BTK Protein
(Standard Curve)
y = 360.09x - 9071.8
R² = 0.9944
Signal (mean)
Protein Concentration (pg/ml)
% BTK Occupancy Assay
PCYC-1111-MML-PBMC (Patient 3)
% BTK Occupancy
120.0
100.0
80.0
60.0
40.0
20.0
0.0
Day1-Vehicle
Day2-Dose
Day8-Dose
Day15-Dose
Day29-Dose
Dosing Regimen

FIGURE 27

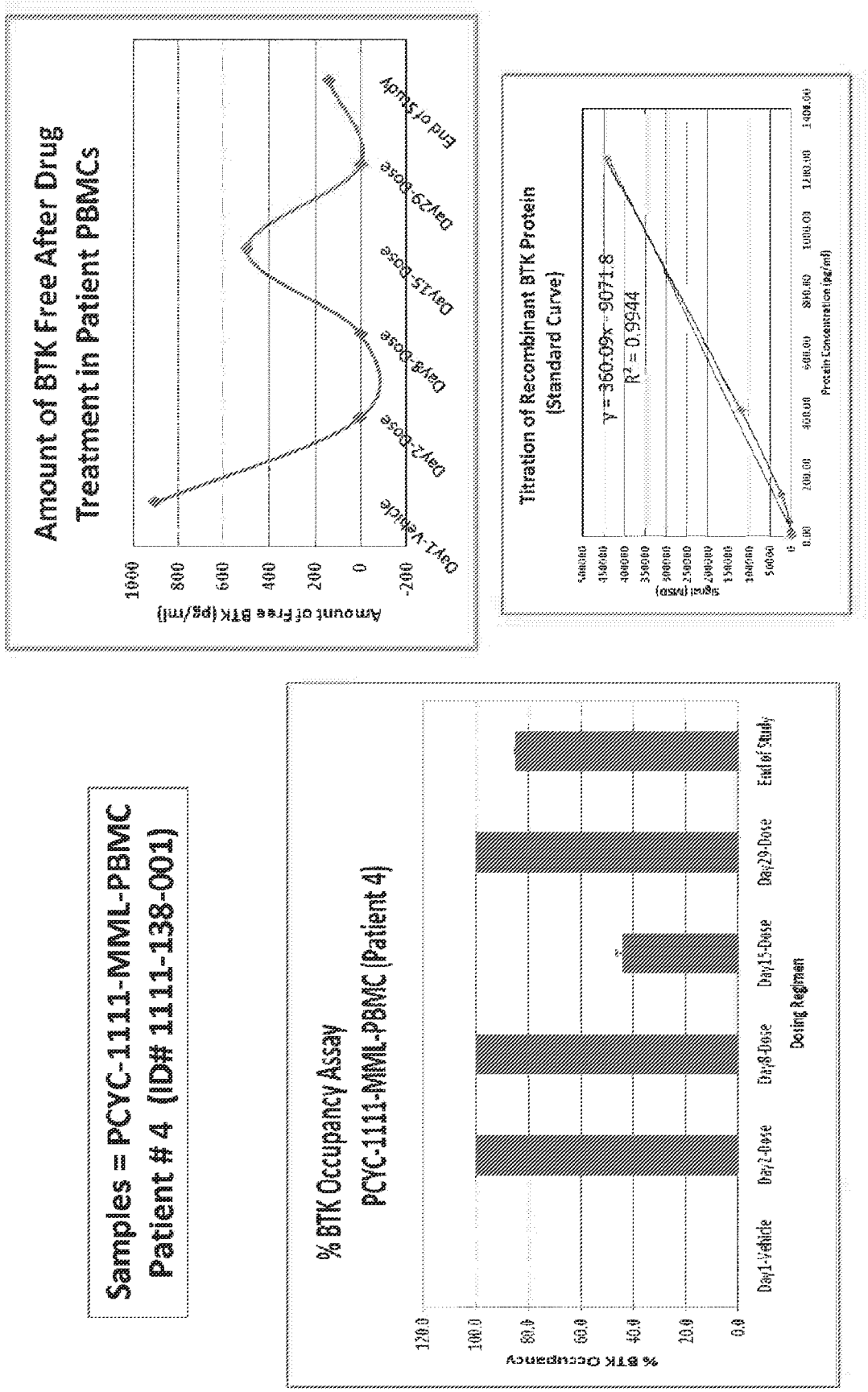

Samples = PCYC-1111-MML-PBMC
Patient # 4 (ID# 1111-138-001)
% BTK Occupancy Assay
PCYC-1111-MML-PBMC (Patient 4)
% BTK Occupancy
120.0
100.0
80.0
60.0
40.0
20.0
0.0
Day1-Vehicle
Day2-Dose
Day8-Dose
Day15-Dose
Day29-Dose
End of Study
Dosing Regimen
Amount of BTK Free After Drug
Treatment in Patient PBMCs
Amount of Free BTK (pg/ml)
1000
800
600
400
200
0
-200
Day1-Vehicle
Day2-Dose
Day8-Dose
Day15-Dose
Day29-Dose
End of Study
Titration of Recombinant BTK Protein
(Standard Curve)
y = 360.09x - 9071.8
R² = 0.9944
Signal (MSD)
Protein Concentration (pg/ml)

FIGURE 28
BTK Quantification: METHOD-2

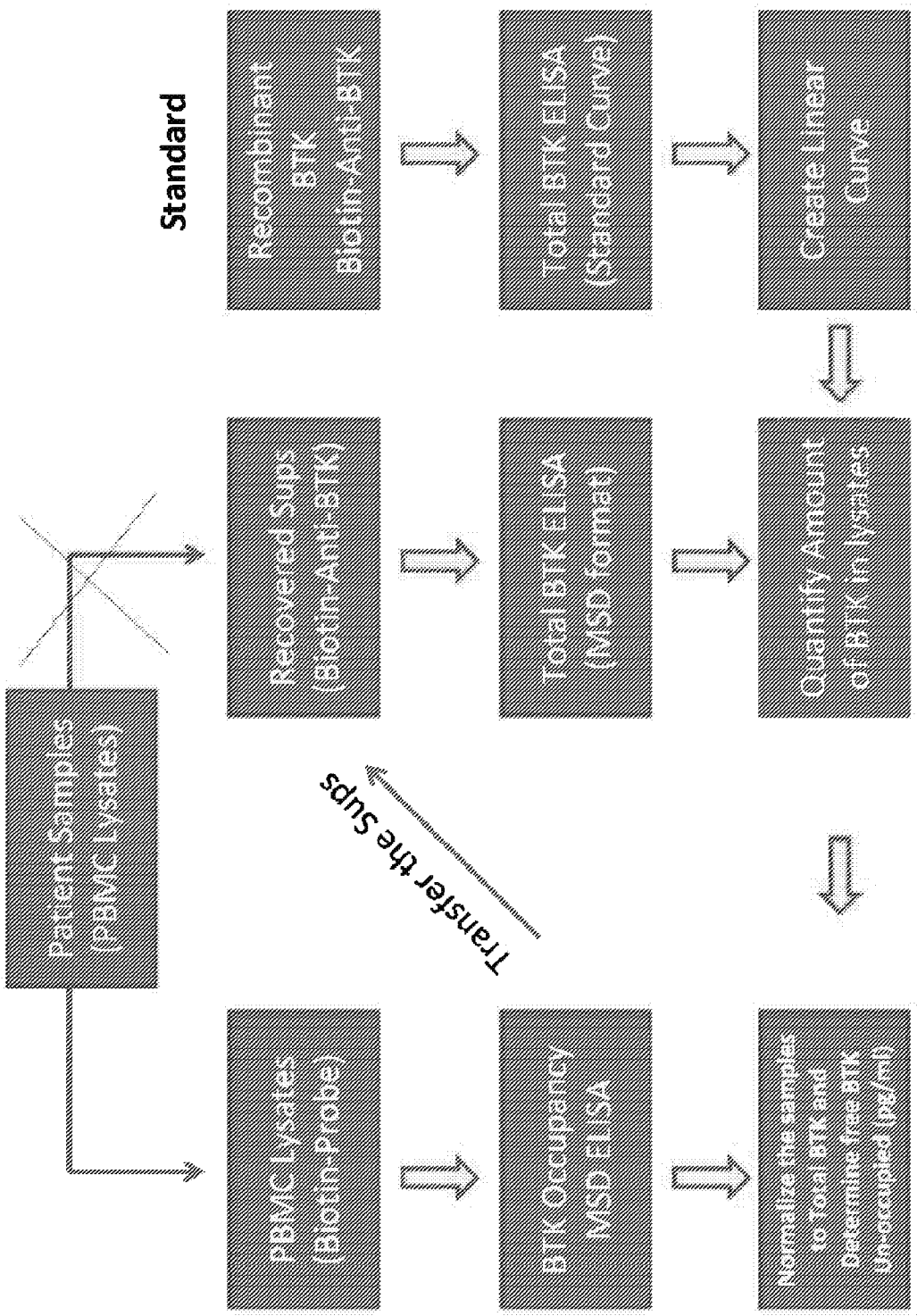


FIGURE 29

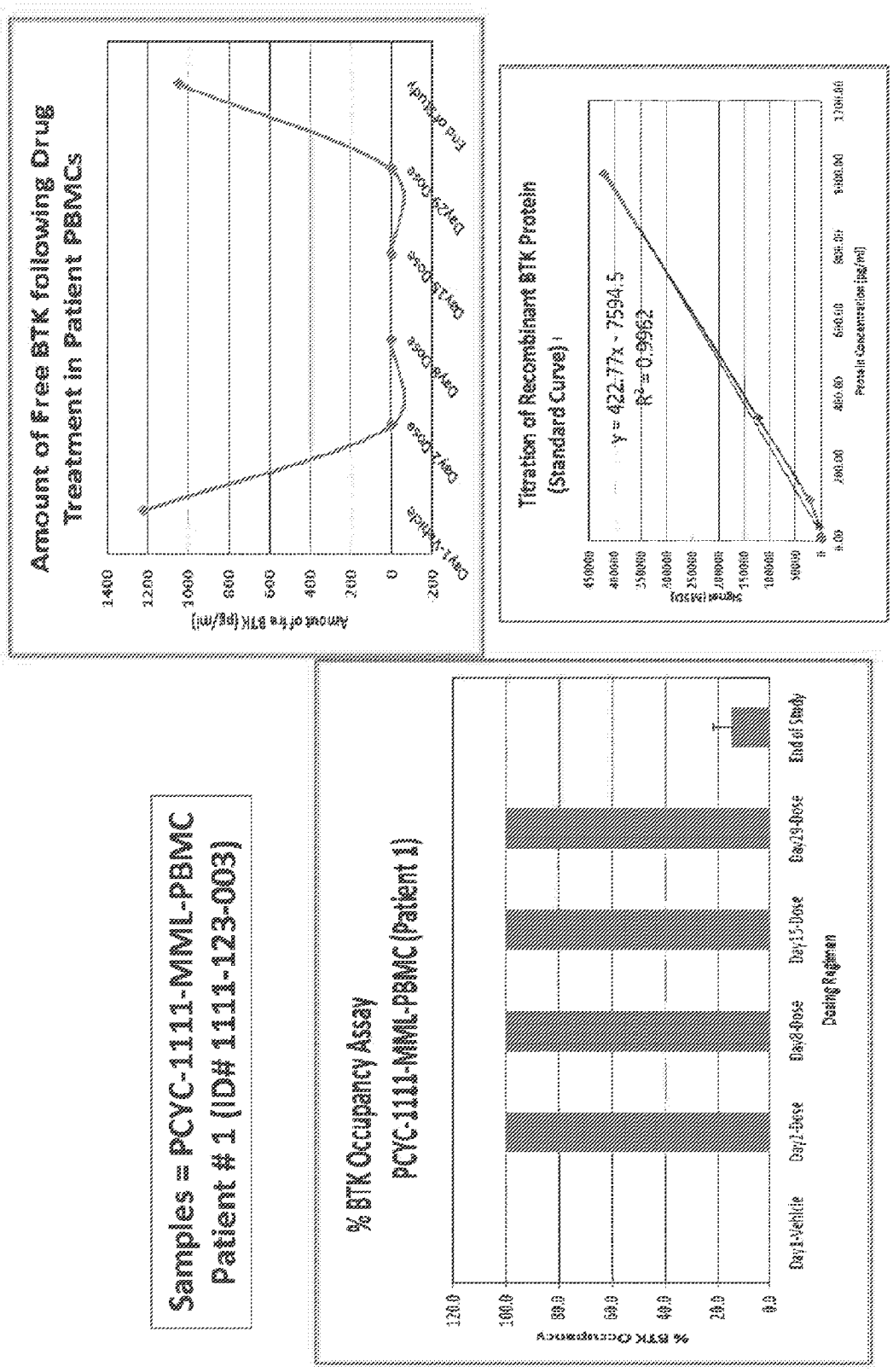

Samples = PCYC-1111-MML-PBMC
Patient # 1 (ID# 1111-123-003)
Amount of Free BTK following Drug Treatment in Patient PBMCs
Amount of free BTK (pg/ml)
Day1-Predose
Day2-Dose
Day8-Dose
Day15-Dose
Day29-Dose
End of Study
Titration of Recombinant BTK Protein (Standard Curve)
y = 422.77x - 7594.5
R² = 0.9962
Protein Concentration (pg/ml)
% BTK Occupancy Assay
PCYC-1111-MML-PBMC (Patient 1)
% BTK Occupancy
Dosing Regimen

FIGURE 30

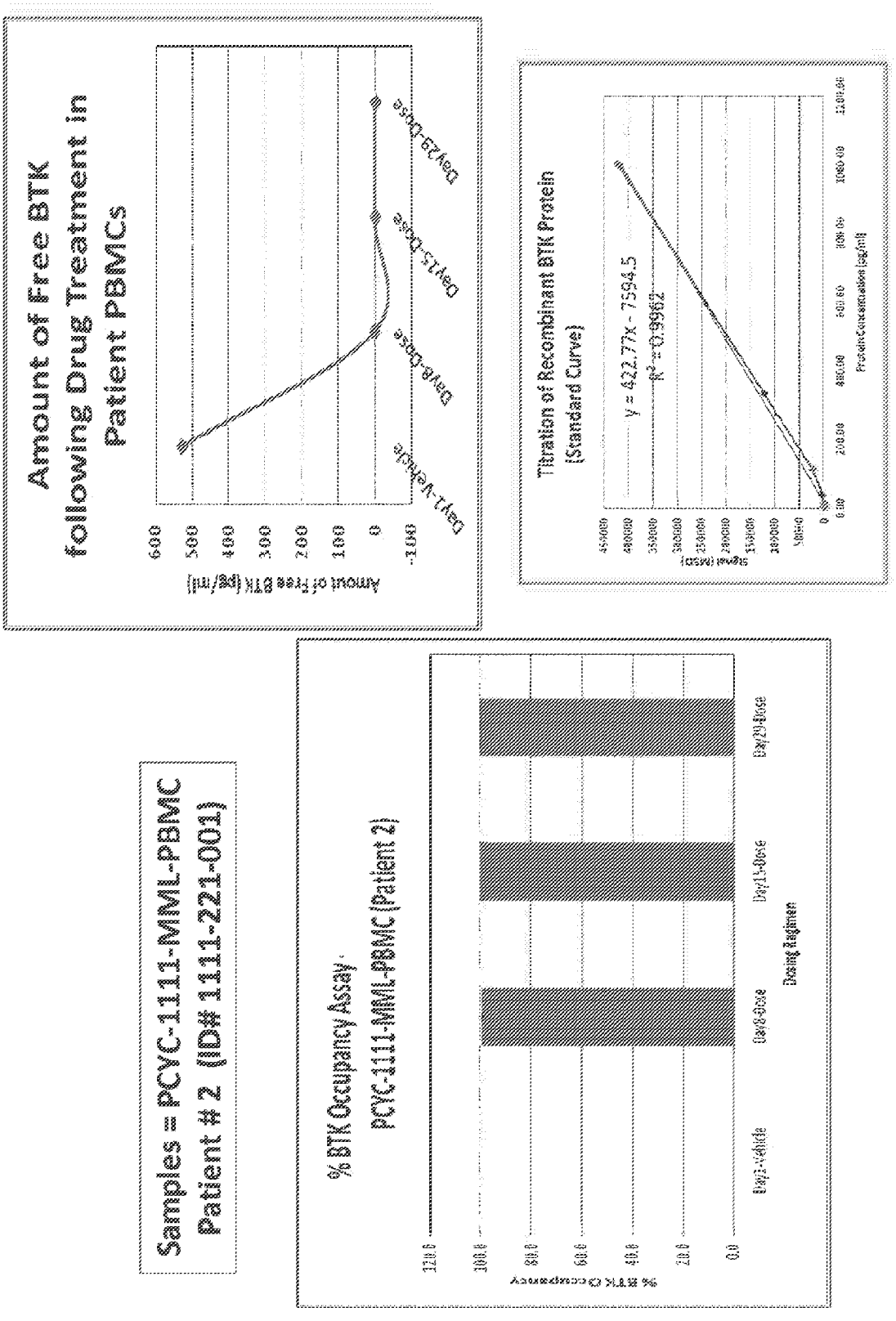

Samples = PCYC-1111-MML-PBMC
Patient # 2 (ID# 1111-221-001)
% BTK Occupancy Assay
PCYC-1111-MML-PBMC (Patient 2)
% BTK Occupancy
120.0
100.0
80.0
60.0
40.0
20.0
0.0
Day1-vehicle
Day8-Dose
Day15-Dose
Day29-Dose
Dosing Regimen
Amount of Free BTK following Drug Treatment in Patient PBMCs
Amount of Free BTK (pg/mL)
600
500
400
300
200
100
0
-100
Day1-Vehicle
Day8-Dose
Day15-Dose
Day29-Dose
Titration of Recombinant BTK Protein (Standard Curve)
y = 422.77x - 7594.5
R² = 0.9962

FIGURE 31

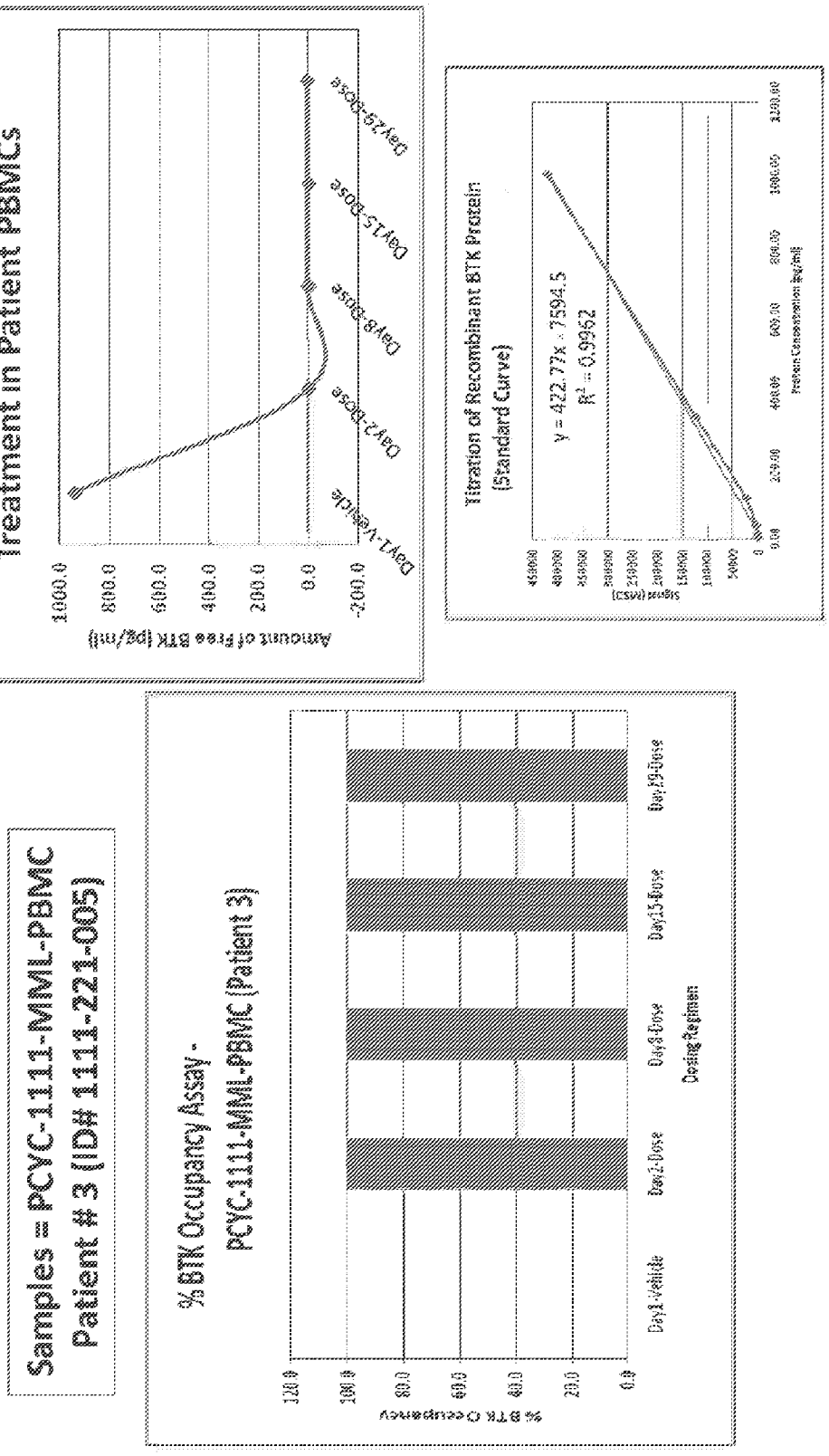

Samples = PCYC-1111-MML-PBMC
Patient # 3 (ID# 1111-221-005)
% BTK Occupancy Assay -
PCYC-1111-MML-PBMC (Patient 3)
% BTK Occupancy
120.0
100.0
80.0
60.0
40.0
20.0
0.0
Day1-Vehicle
Day2-Dose
Day8-Dose
Day15-Dose
Day29-Dose
Dosing Regimen
Amount of Free BTK after Drug
Treatment in Patient PBMCs
Amount of Free BTK (pg/ml)
1000.0
800.0
600.0
400.0
200.0
0.0
-200.0
Day1-Vehicle
Day2-Dose
Day8-Dose
Day15-Dose
Day29-Dose
Titration of Recombinant BTK Protein
(Standard Curve)
y = 422.77x - 7594.5
R² = 0.9962

FIGURE 32

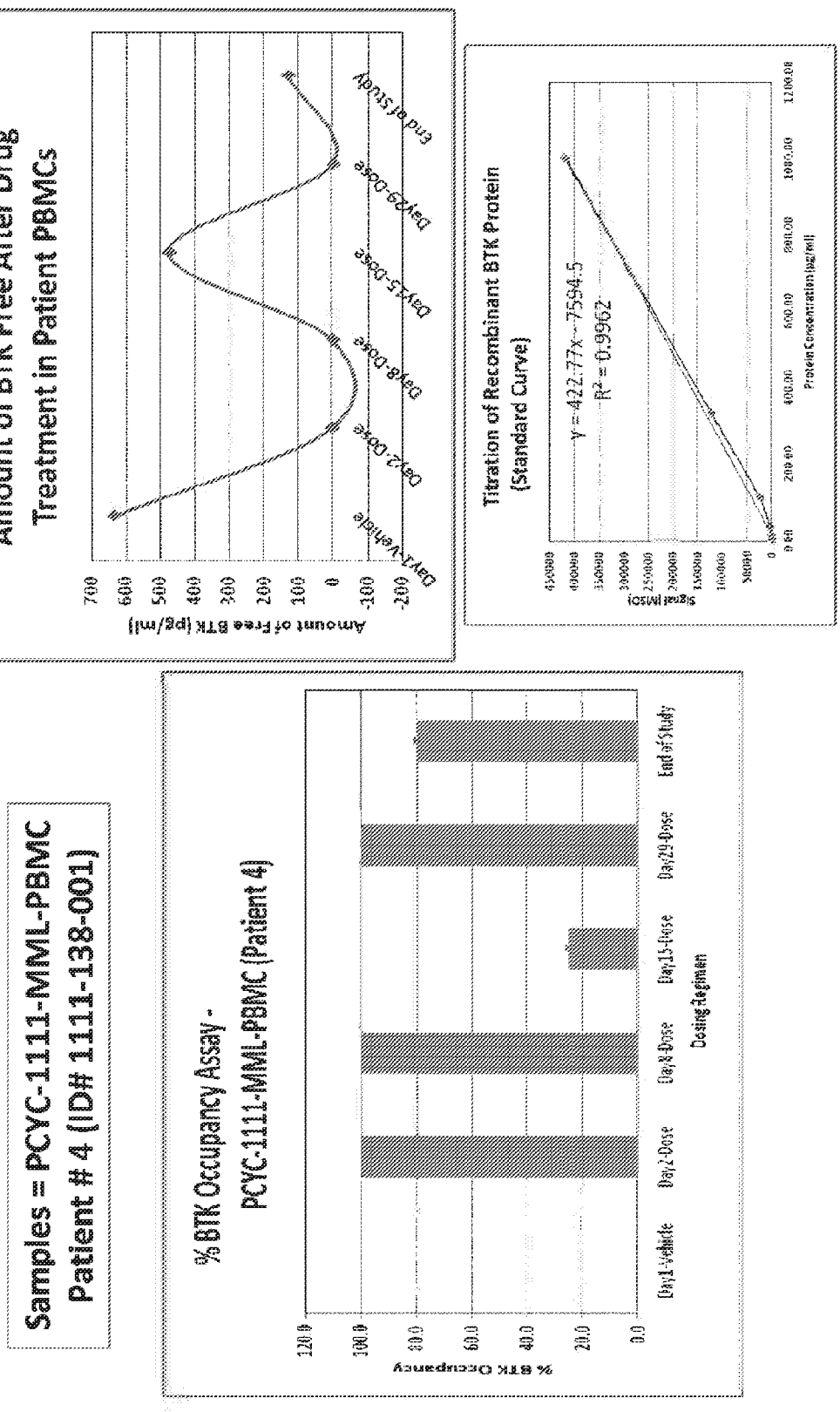

Samples = PCYC-1111-MML-PBMC
Patient # 4 (ID# 1111-138-001)
% BTK Occupancy Assay -
PCYC-1111-MML-PBMC (Patient 4)
% BTK Occupancy
120.0
100.0
80.0
60.0
40.0
20.0
0.0
Day1-Vehicle
Day2-Dose
Day8-Dose
Day15-Dose
Day29-Dose
End of Study
Dosing Regimen
Amount of BTK Free After Drug
Treatment in Patient PBMCs
Amount of Free BTK (pg/ml)
700
600
500
400
300
200
100
0
-100
-200
Day1-Vehicle
Day2-Dose
Day8-Dose
Day15-Dose
Day29-Dose
End of Study
Titration of Recombinant BTK Protein
(Standard Curve)
y = 422.77x - 7594.5
R² = 0.9962
Signal (M/z)
Protein Concentration (pg/well)

FIGURE 33

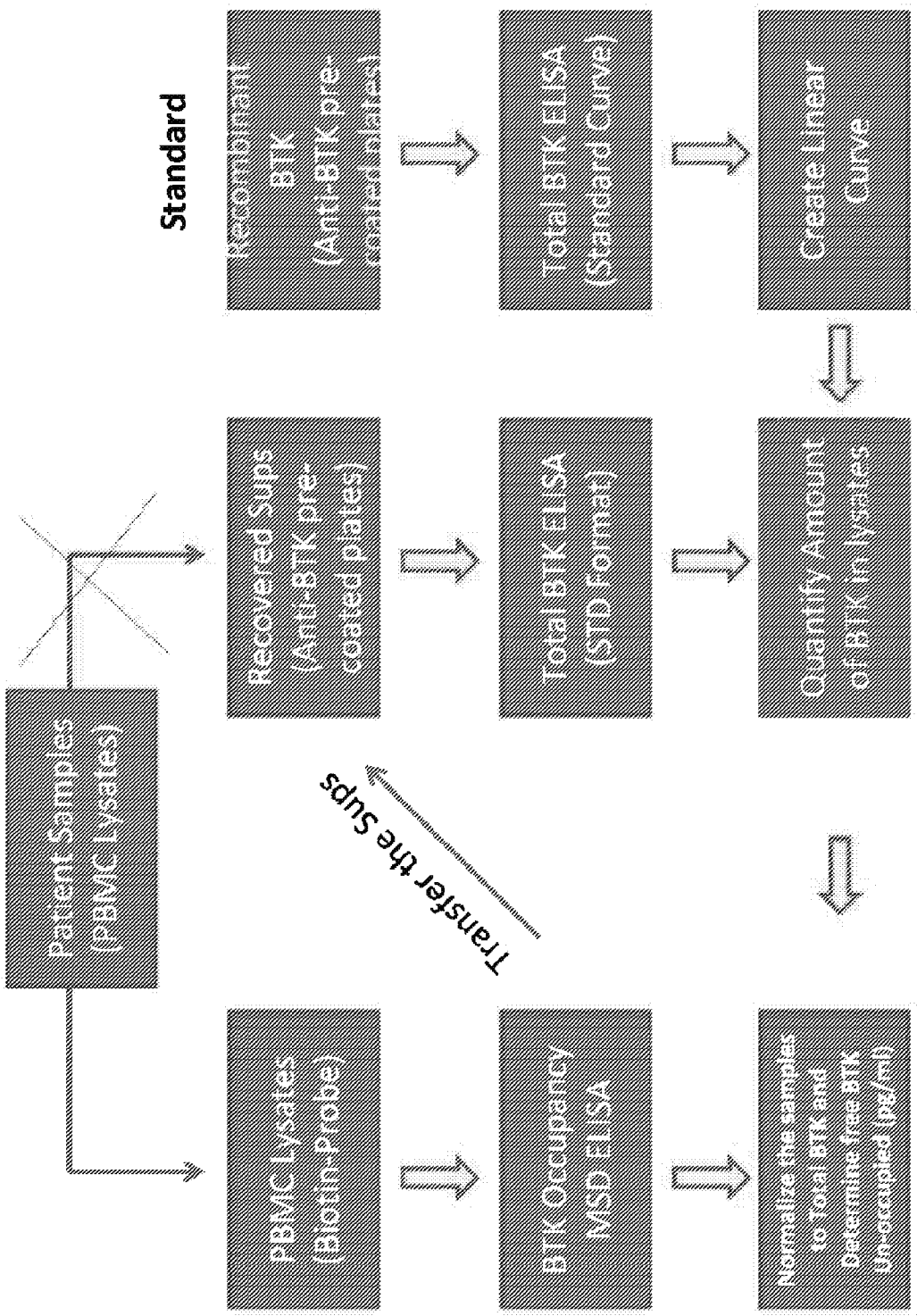
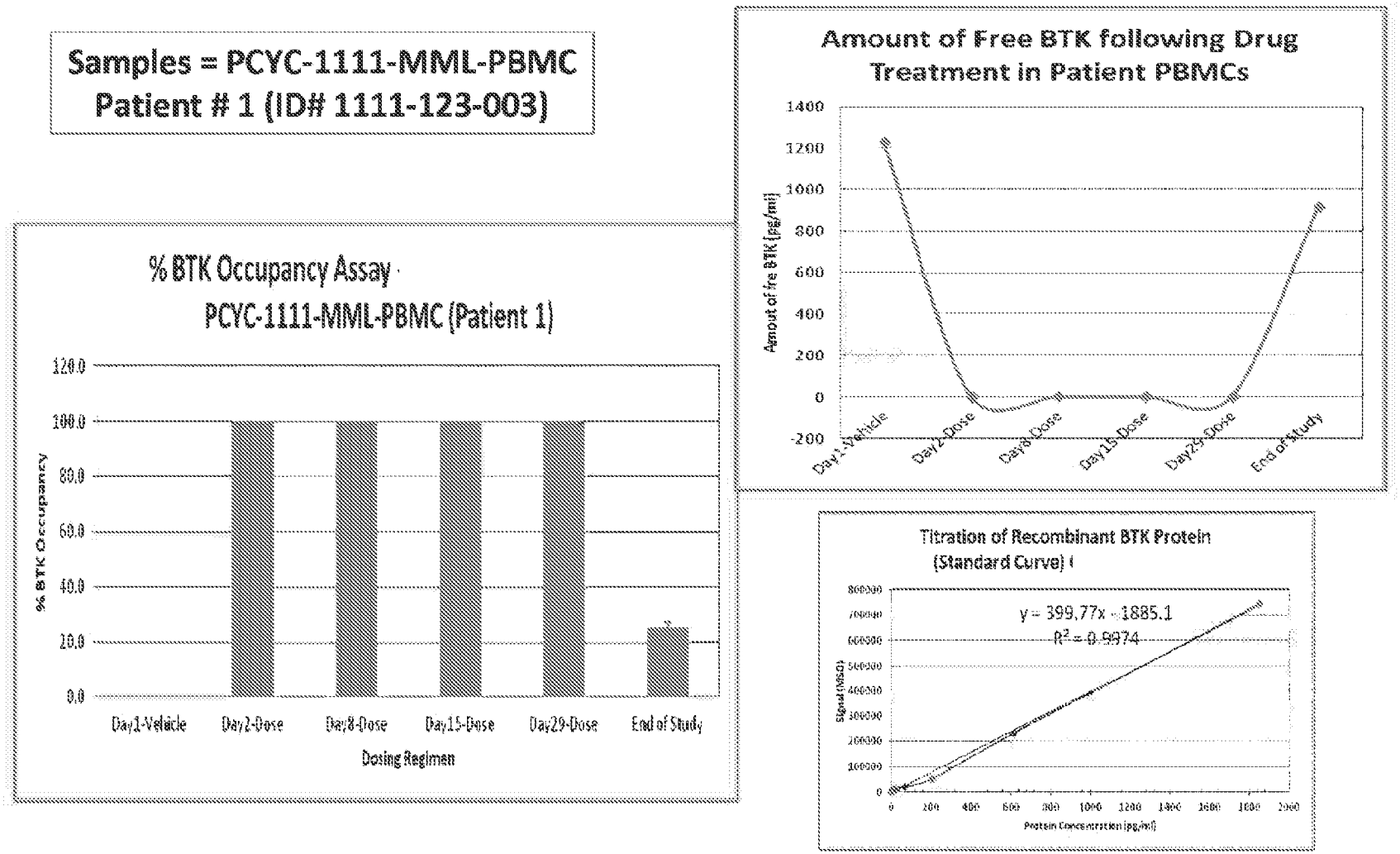

Standard
Recombinant BTK (Anti-BTK pre-coated plates)
Total BTK ELISA (Standard Curve)
Create Linear Curve
Patient Samples (PBMC Lysates)
Recovered Sups (Anti-BTK pre-coated plates)
Total BTK ELISA (SIB format)
Quantify Amount of BTK in lysates
Transfer the Sups
PBMC Lysates (Biotin-Probe)
BTK Occupancy MSD ELISA
Normalize the samples to Total BTK and Determine free BTK Unoccupied (pg/ml)

FIGURE 34



WO 2016/100593 PCT/US2015/066244

FIGURE 35

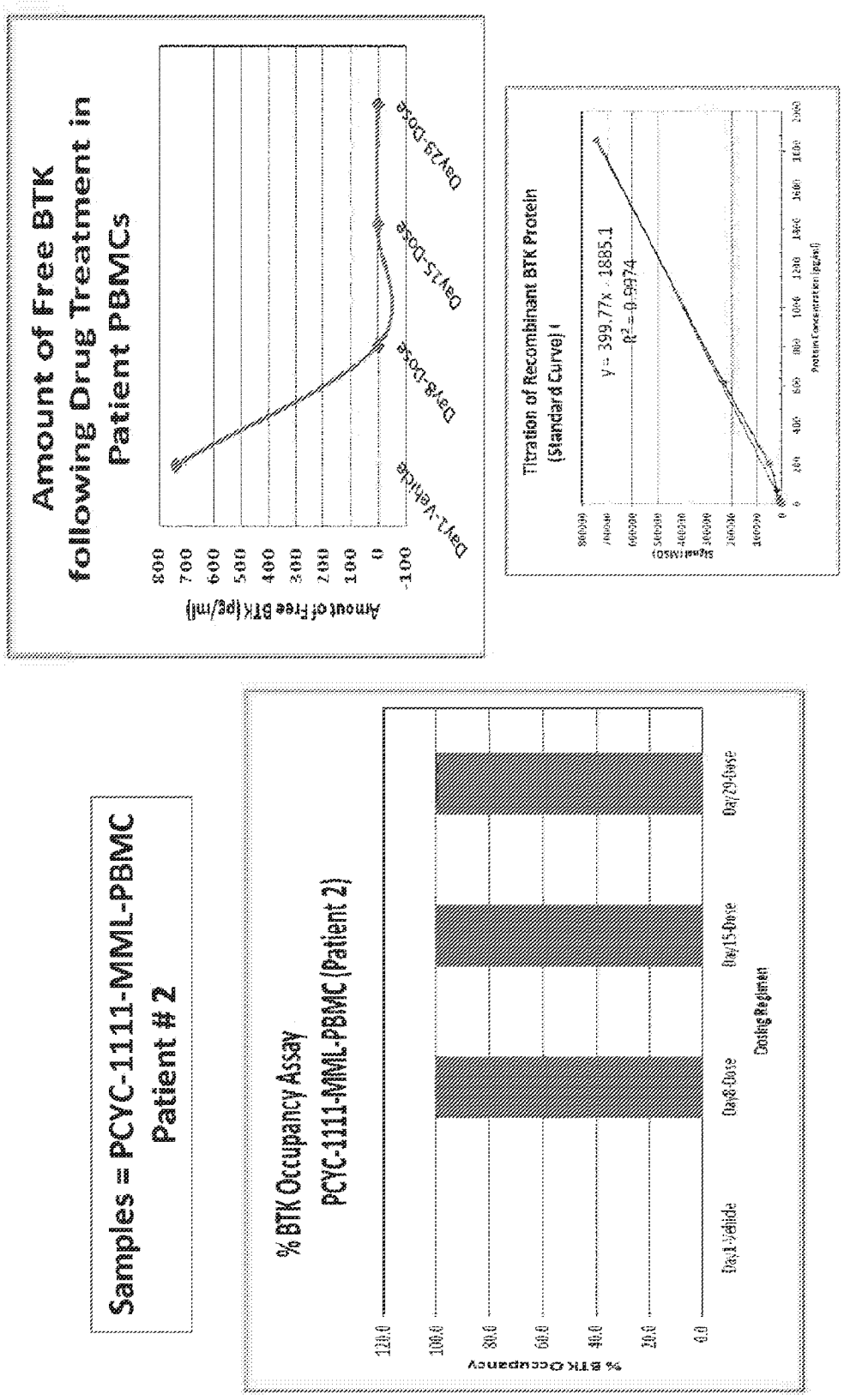

Samples = PCYC-1111-MML-PBMC
Patient # 2
% BTK Occupancy Assay
PCYC-1111-MML-PBMC (Patient 2)
% BTK Occupancy
120.0
100.0
80.0
60.0
40.0
20.0
0.0
Day1 Vehicle
Day8 Dose
Day15 Dose
Day29 Dose
Dosing Regimen
Amount of Free BTK following Drug Treatment in Patient PBMCs
Amount of Free BTK (pg/ml)
800
700
600
500
400
300
200
100
0
-100
Day1-Vehicle
Day8-Dose
Day15-Dose
Day29-Dose
Titration of Recombinant BTK Protein (Standard Curve) I
y = 399.77x - 1885.1
R² = 0.9974

FIGURE 36

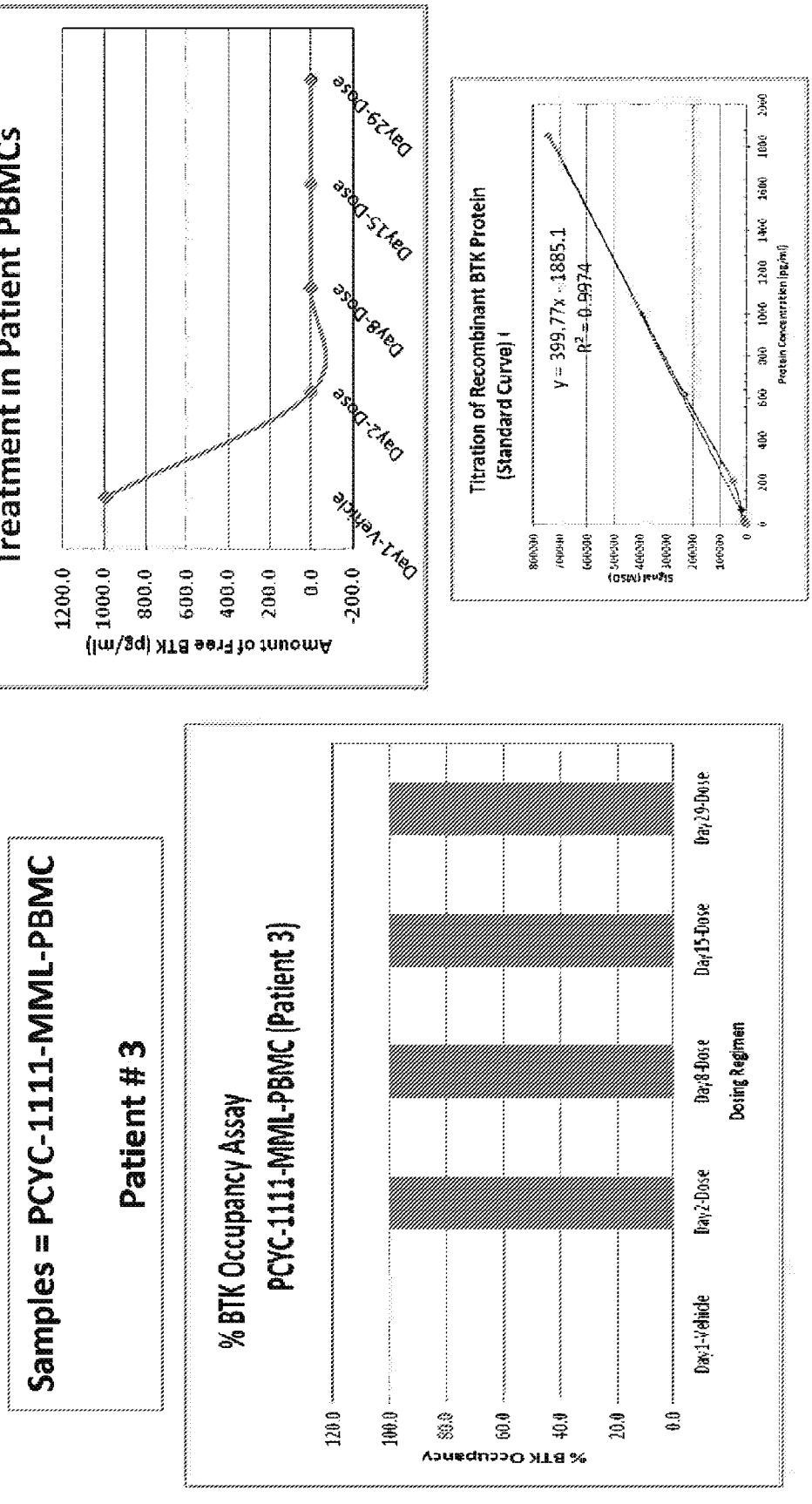

Samples = PCYC-1111-MML-PBMC
Patient # 3
% BTK Occupancy Assay
PCYC-1111-MML-PBMC (Patient 3)
% BTK Occupancy
120.0
100.0
80.0
60.0
40.0
20.0
0.0
Day1-Vehicle
Day2-Dose
Day8-Dose
Day15-Dose
Day29-Dose
Dosing Regimen
Amount of Free BTK after Drug Treatment in Patient PBMCs
Amount of Free BTK (pg/ml)
1200.0
1000.0
800.0
600.0
400.0
200.0
0.0
-200.0
Day1-Vehicle
Day2-Dose
Day8-Dose
Day15-Dose
Day29-Dose
Titration of Recombinant BTK Protein (Standard Curve) I
y = 399.77x - 1885.1
R² = 0.9974
Signal (MSD)
Protein Concentration (pg/ml)

FIGURE 37

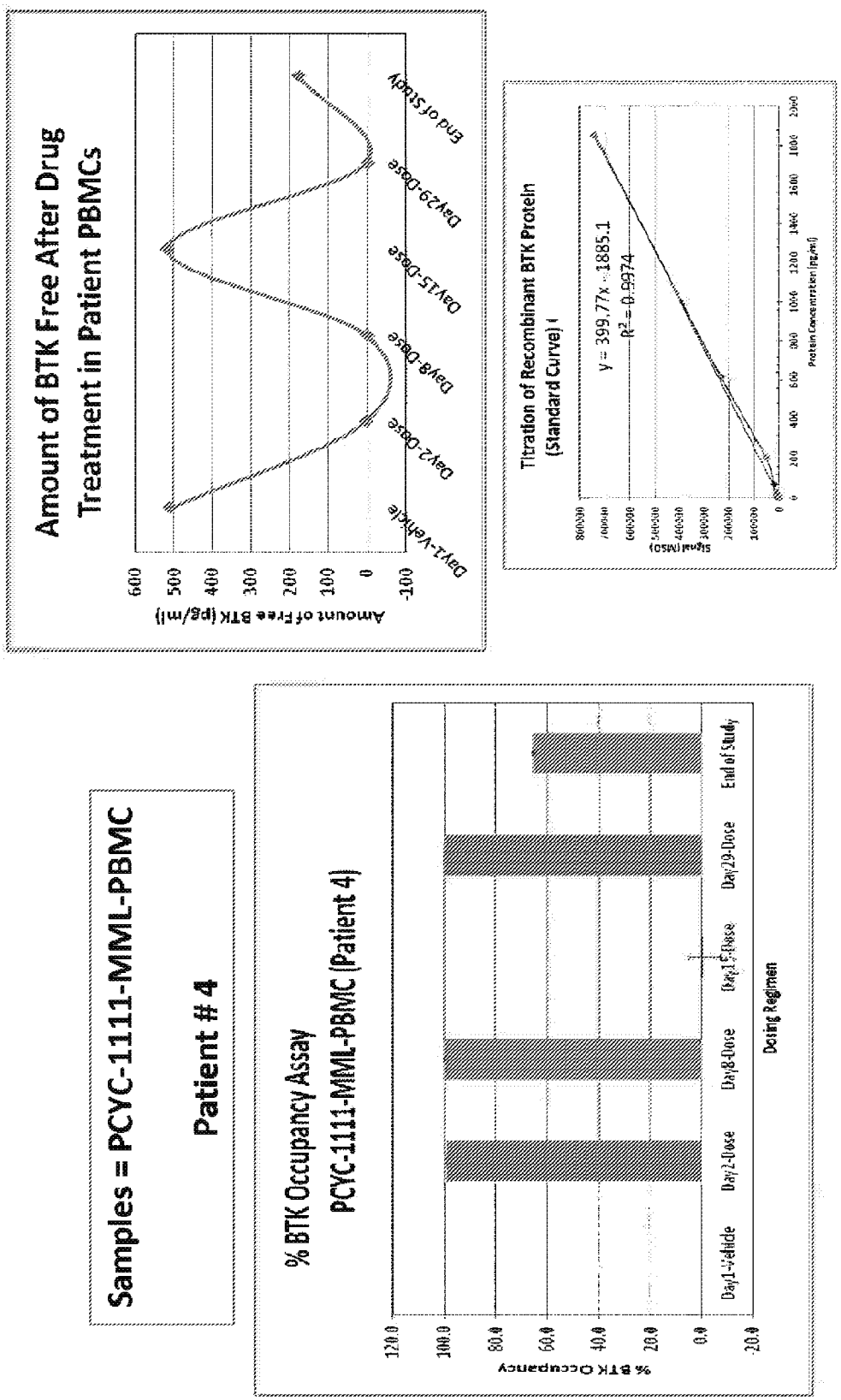
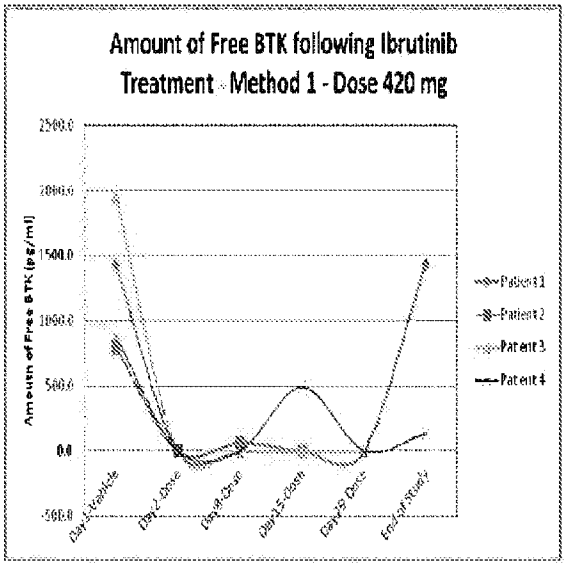
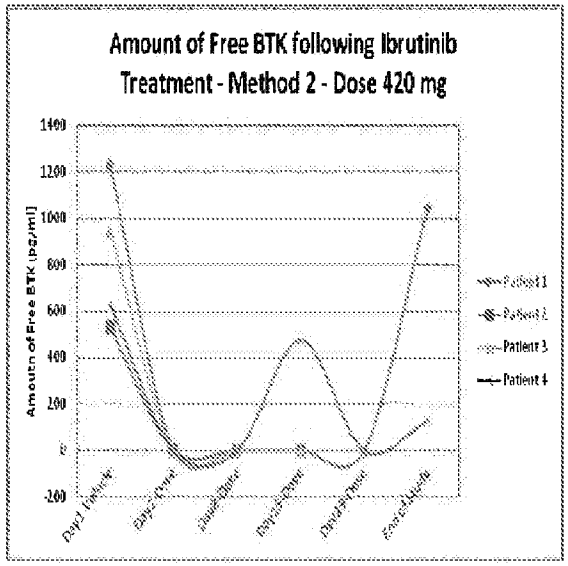

Samples = PCYC-1111-MML-PBMC
Patient # 4
% BTK Occupancy Assay
PCYC-1111-MML-PBMC (Patient 4)
% BTK Occupancy
120.0
100.0
80.0
60.0
40.0
20.0
0.0
-20.0
Day1-Vehicle
Day2-Dose
Day8-Dose
Day15-Dose
Day29-Dose
End of Study
Dosing Regimen
Amount of BTK Free After Drug
Treatment in Patient PBMCs
Amount of Free BTK (pg/ml)
600
500
400
300
200
100
0
-100
Day1-Vehicle
Day2-Dose
Day8-Dose
Day15-Dose
Day29-Dose
End of Study
Titration of Recombinant BTK Protein
(Standard Curve) I
y = 399.77x - 1885.1
R² = 0.9974
Signal (MSD)
Protein Concentration (pg/ml)

FIGURE 38

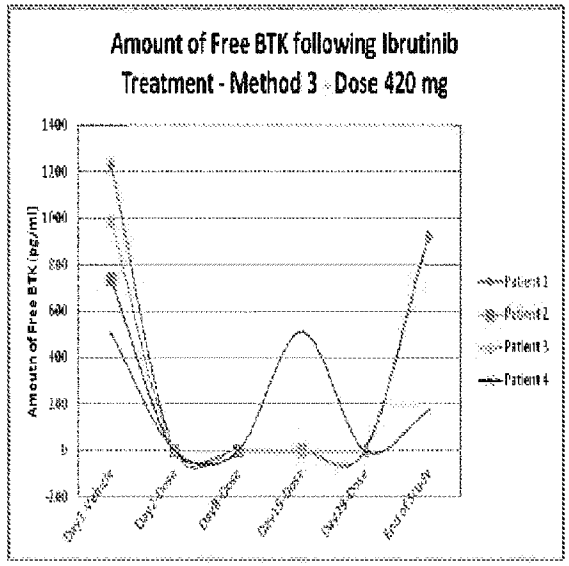
Method 1

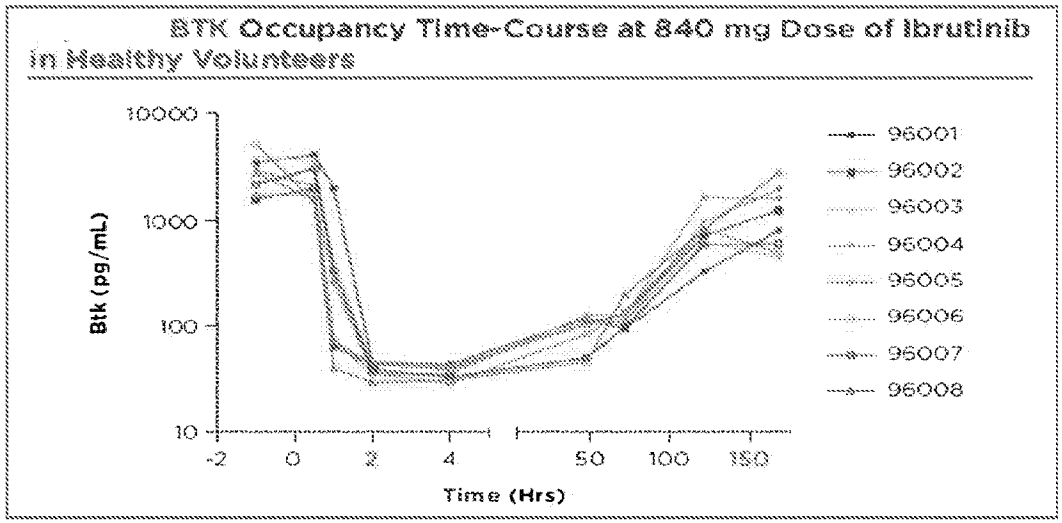


Method 2



Method 3





INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2015/066244

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12Q 1/48 (2016.01)
CPC - C12Q 1/485 (2016.02)
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8) - A61K 31/506; C07D 403/12; C12Q 1/48; G01N 33/53, 33/532 (2016.01)
CPC - A61K 31/506; C07D 403/12; C12Q 1/485; G01N 2333/912, 33/532, 33/581 (2016.02)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC 424/143.1, 1.49 (keyword delimited)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PatBase, Google Patents, Google Scholar, PubMed
Search terms used: Tec kinase BMX BTK ITK TEC TXK occupancy

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2014/059368 A1 (PHARMACYCLICS, INC) 17 April 2014 (17.04.2014) entire document	1-21
A	WO 2014/018567 A1 (PHARMACYCLICS, INC) 30 January 2014 (30.01.2014) entire document	1-21
A	US 2008/0214501 A1 (PAN et al) 04 September 2008 (04.09.2008) entire document	1-21
A	Advani et al. "Bruton tyrosine kinase inhibitor ibrutinib (PCI-32765) has significant activity in patients with relapsed/refractory B-cell malignancies," J Clin Oncol. 08 October 2012 (08.10.2012), Vol. 31, Pgs. 88-94 entire document	1-21

☐ Further documents are listed in the continuation of Box C. ☐ 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
23 February 2016	07 MAR 2016
Name and mailing address of the ISA/ Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, VA 22313-1450 Facsimile No. 571-273-8300	Authorized officer Blaine R. Copenheaver PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (January 2015)