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(54) **METHODS FOR MEASURING REAL TIME KINASE ACTIVITY**

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(57) **ABSTRACT**

The present invention relates to methods for detecting and/or measuring the activity of a specific kinase, with the methods comprising contacting one or more kinases with a binding agent to isolate a specific kinase of interest. The isolated kinase is then contacted with a kinase activity sensor, where the kinase activity sensor is comprised of a kinase recognition motif that is capable of being recognized by the isolated kinase, and at least one phosphorylation site. The isolated kinase phosphorylates the amino acid target of the kinase activity sensor and levels of the phosphorylated target amino acid can then be quantified.

FIG. 1

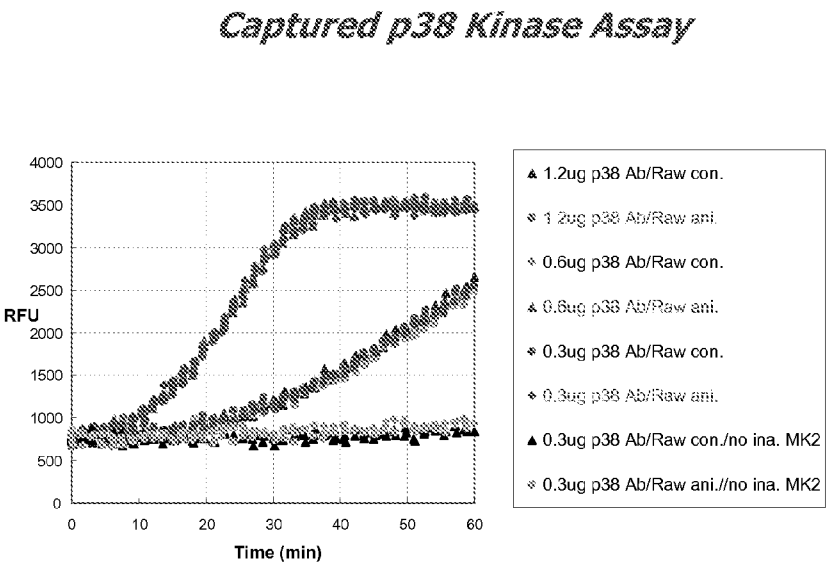

Captured p38 Kinase Assay
RFU
Time (min)
1.2ug p38 Ab/Raw con.
1.2ug p38 Ab/Raw ani.
0.6ug p38 Ab/Raw con.
0.6ug p38 Ab/Raw ani.
0.3ug p38 Ab/Raw con.
0.3ug p38 Ab/Raw ani.
0.3ug p38 Ab/Raw con./no ina. MK2
0.3ug p38 Ab/Raw ani.//no ina. MK2

FIG. 2

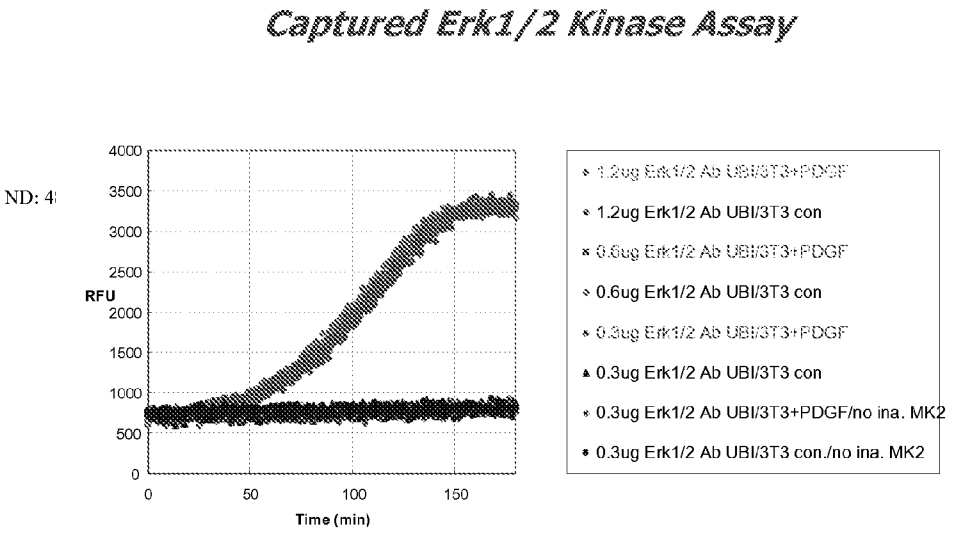

Captured Erk1/2 Kinase Assay
RFU
Time (min)
1.2ug Erk1/2 Ab UBI/3T3+PDGF
1.2ug Erk1/2 Ab UBI/3T3 con
0.6ug Erk1/2 Ab UBI/3T3+PDGF
0.6ug Erk1/2 Ab UBI/3T3 con
0.3ug Erk1/2 Ab UBI/3T3+PDGF
0.3ug Erk1/2 Ab UBI/3T3 con
0.3ug Erk1/2 Ab UBI/3T3+PDGF/no ina. MK2
0.3ug Erk1/2 Ab UBI/3T3 con./no ina. MK2

FIG. 3

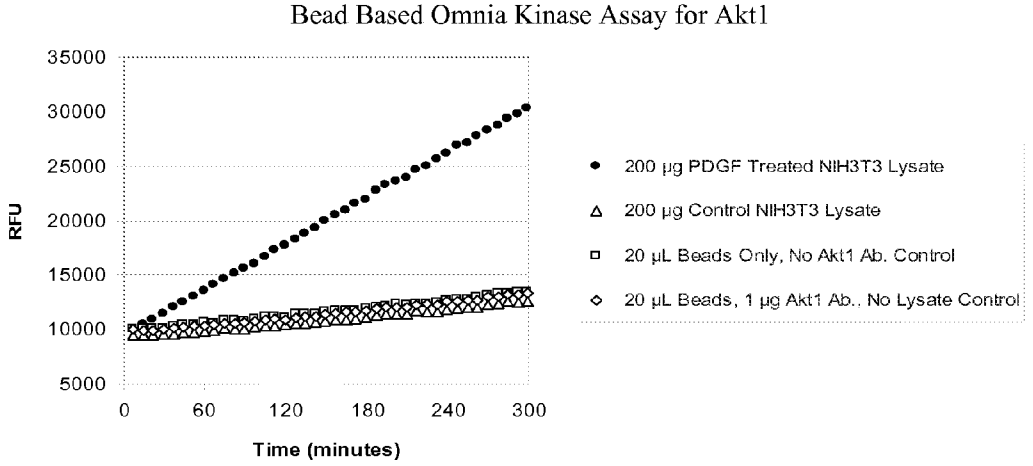

Bead Based Omnia Kinase Assay for Akt1
35000
30000
25000
20000
15000
10000
5000
RFU
0
60
120
180
240
300
Time (minutes)
200 µg PDGF Treated NIH3T3 Lysate
200 µg Control NIH3T3 Lysate
20 µL Beads Only, No Akt1 Ab. Control
20 µL Beads, 1 µg Akt1 Ab.. No Lysate Control

FIG. 4

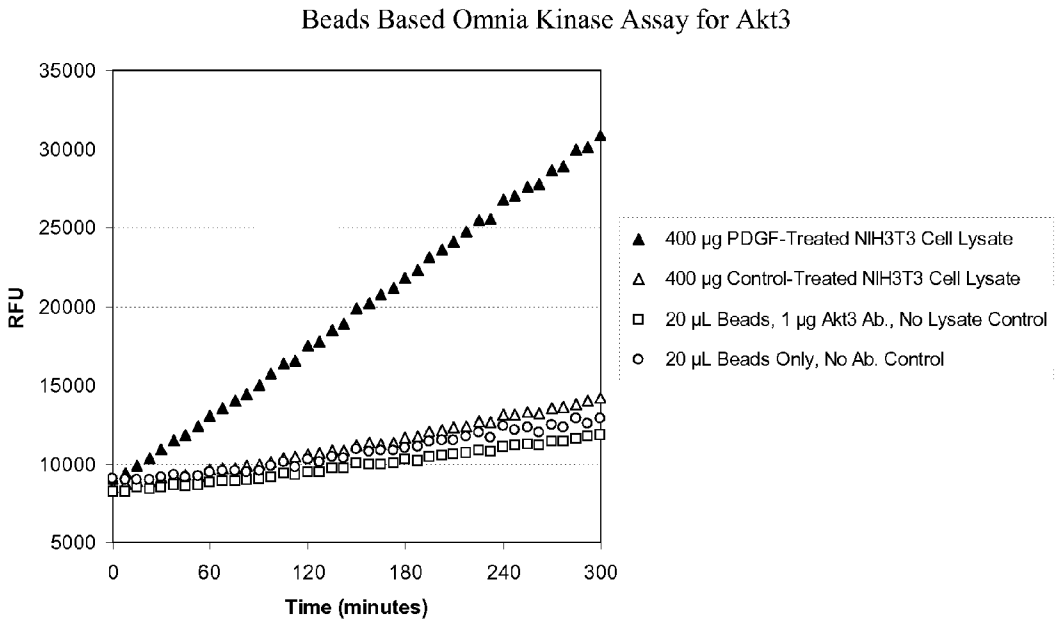

Beads Based Omnia Kinase Assay for Akt3
35000
30000
25000
20000
15000
10000
5000
RFU
0
60
120
180
240
300
Time (minutes)
400 µg PDGF-Treated NIH3T3 Cell Lysate
400 µg Control-Treated NIH3T3 Cell Lysate
20 µL Beads, 1 µg Akt3 Ab., No Lysate Control
20 µL Beads Only, No Ab. Control

FIG. 5

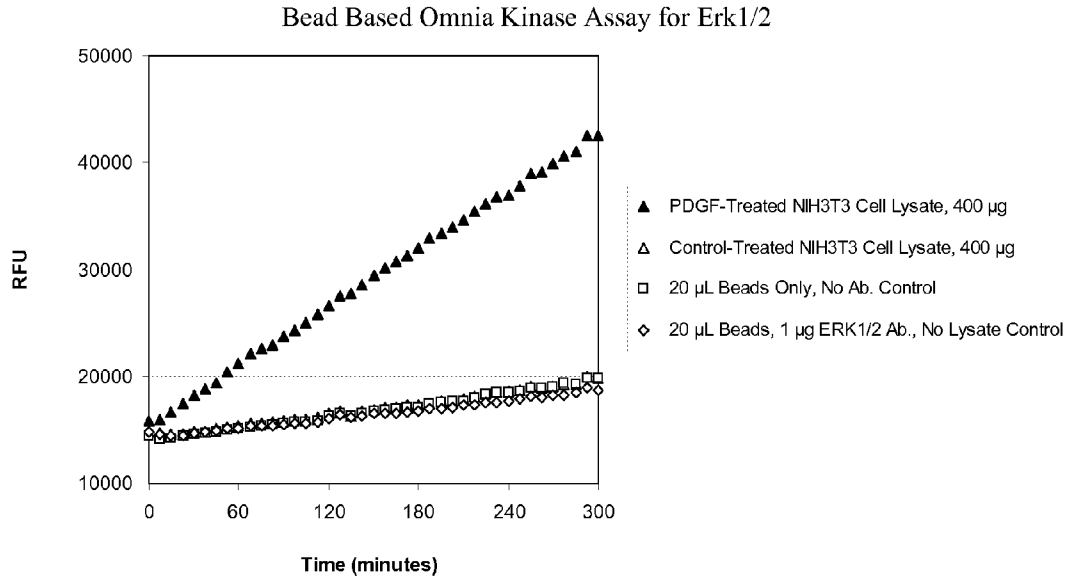

Bead Based Omnia Kinase Assay for Erk1/2
RFU
50000
40000
30000
20000
10000
0
60
120
180
240
300
Time (minutes)
PDGF-Treated NIH3T3 Cell Lysate, 400 µg
Control-Treated NIH3T3 Cell Lysate, 400 µg
20 µL Beads Only, No Ab. Control
20 µL Beads, 1 µg ERK1/2 Ab., No Lysate Control

FIG. 6

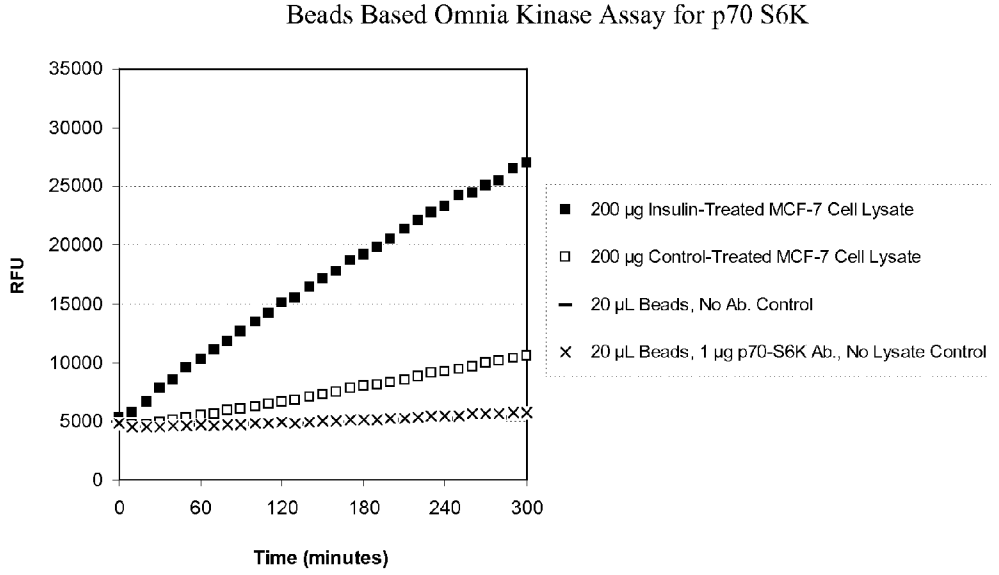

Beads Based Omnia Kinase Assay for p70 S6K
RFU
35000
30000
25000
20000
15000
10000
5000
0
0
60
120
180
240
300
Time (minutes)
200 µg Insulin-Treated MCF-7 Cell Lysate
200 µg Control-Treated MCF-7 Cell Lysate
20 µL Beads, No Ab. Control
20 µL Beads, 1 µg p70-S6K Ab., No Lysate Control

FIG. 7

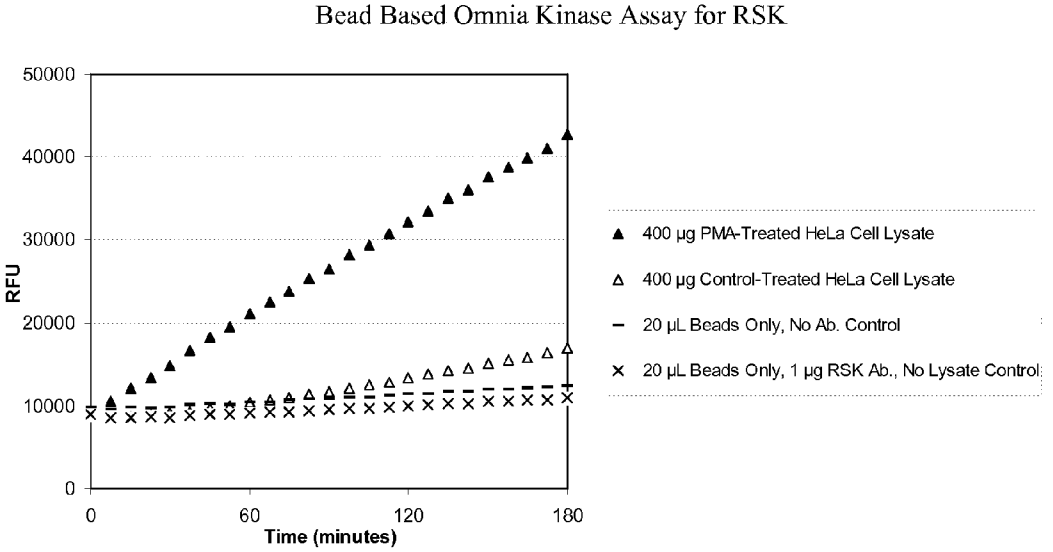

Bead Based Omnia Kinase Assay for RSK
50000
40000
30000
20000
10000
0
RFU
0
60
120
180
Time (minutes)
400 µg PMA-Treated HeLa Cell Lysate
400 µg Control-Treated HeLa Cell Lysate
20 µL Beads Only, No Ab. Control
20 µL Beads Only, 1 µg RSK Ab., No Lysate Control

FIG. 8

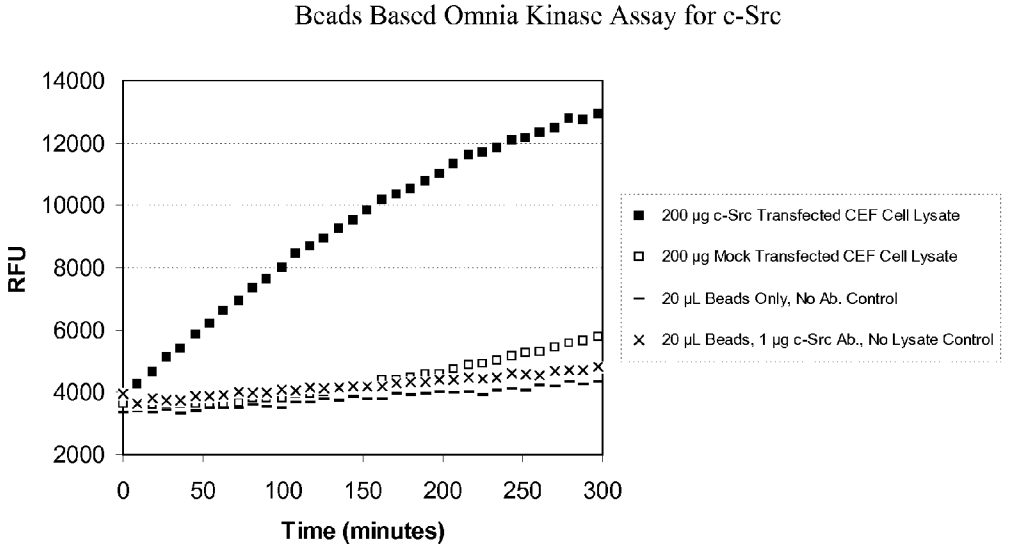

Beads Based Omnia Kinase Assay for c-Src
14000
12000
10000
8000
6000
4000
2000
RFU
0
50
100
150
200
250
300
Time (minutes)
200 µg c-Src Transfected CEF Cell Lysate
200 µg Mock Transfected CEF Cell Lysate
20 µL Beads Only, No Ab. Control
20 µL Beads, 1 µg c-Src Ab., No Lysate Control

FIG. 9

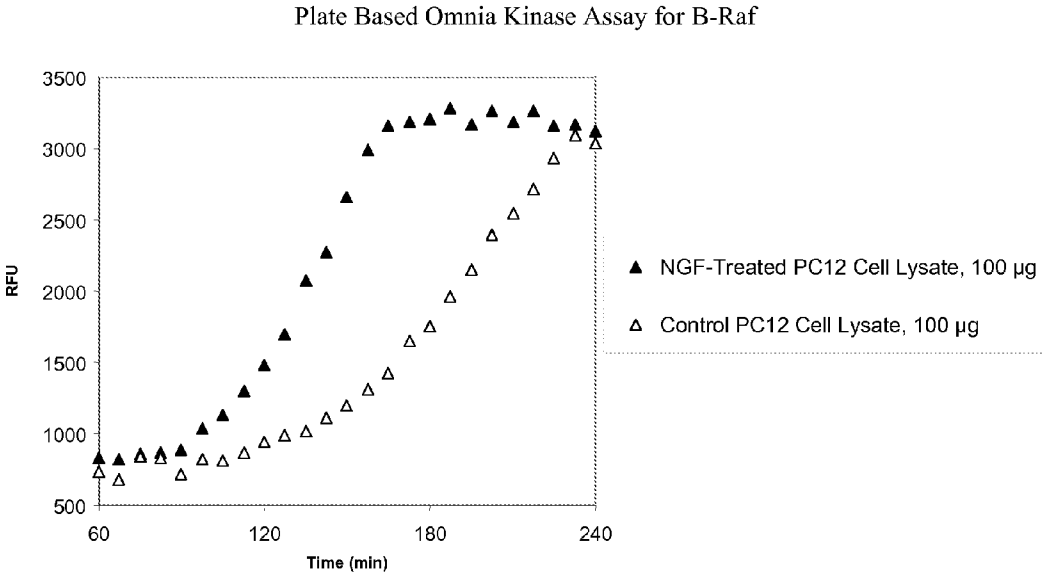


Plate Based Omnia Kinase Assay for B-Raf
RFU
Time (min)
NGF-Treated PC12 Cell Lysate, 100 µg
Control PC12 Cell Lysate, 100 µg

FIG. 10

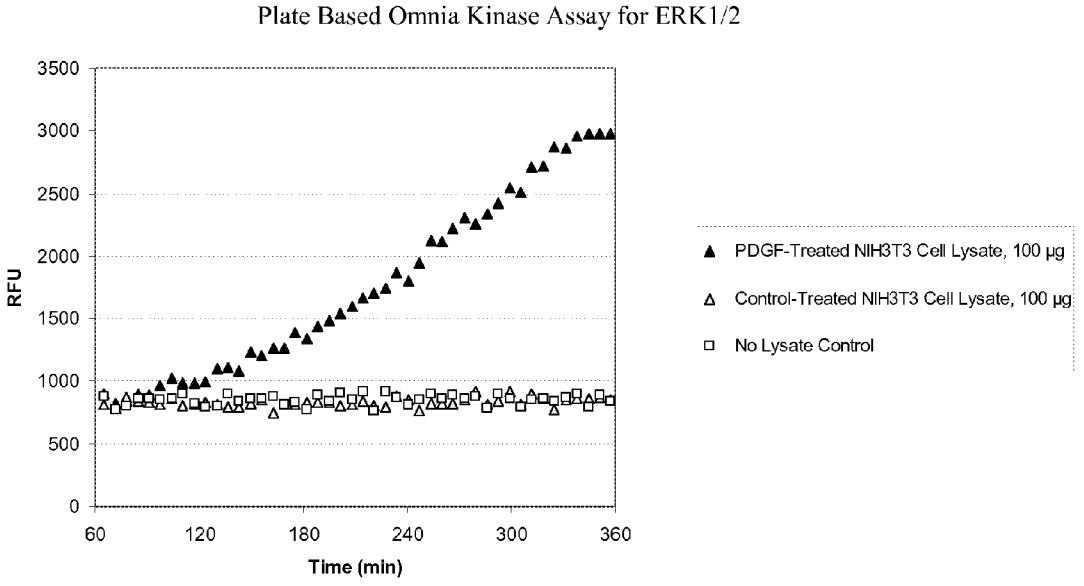


Plate Based Omnia Kinase Assay for ERK1/2
RFU
Time (min)
PDGF-Treated NIH3T3 Cell Lysate, 100 µg
Control-Treated NIH3T3 Cell Lysate, 100 µg
No Lysate Control

FIG. 11

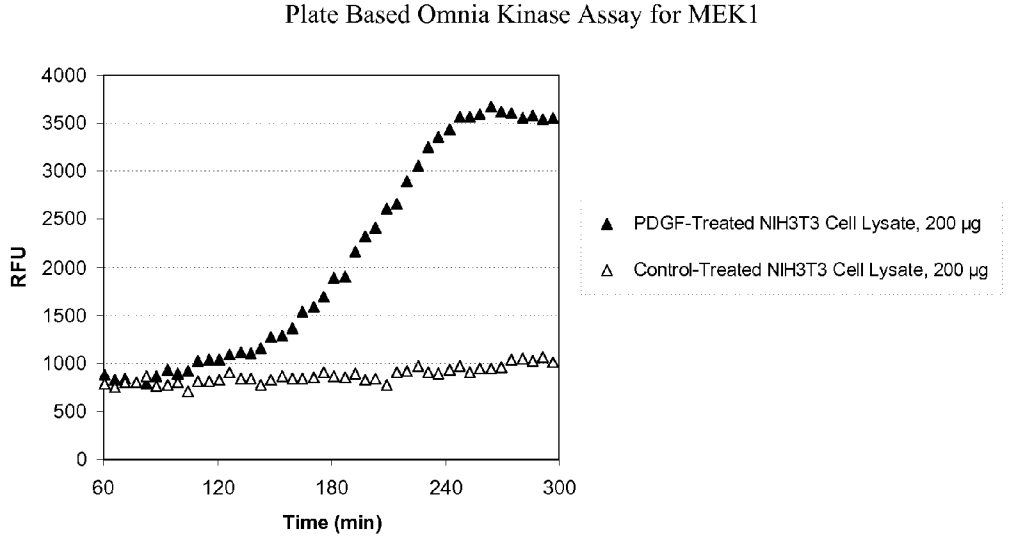


Plate Based Omnia Kinase Assay for MEK1
RFU
4000
3500
3000
2500
2000
1500
1000
500
0
60
120
180
240
300
Time (min)
PDGF-Treated NIH3T3 Cell Lysate, 200 µg
Control-Treated NIH3T3 Cell Lysate, 200 µg

FIG. 12

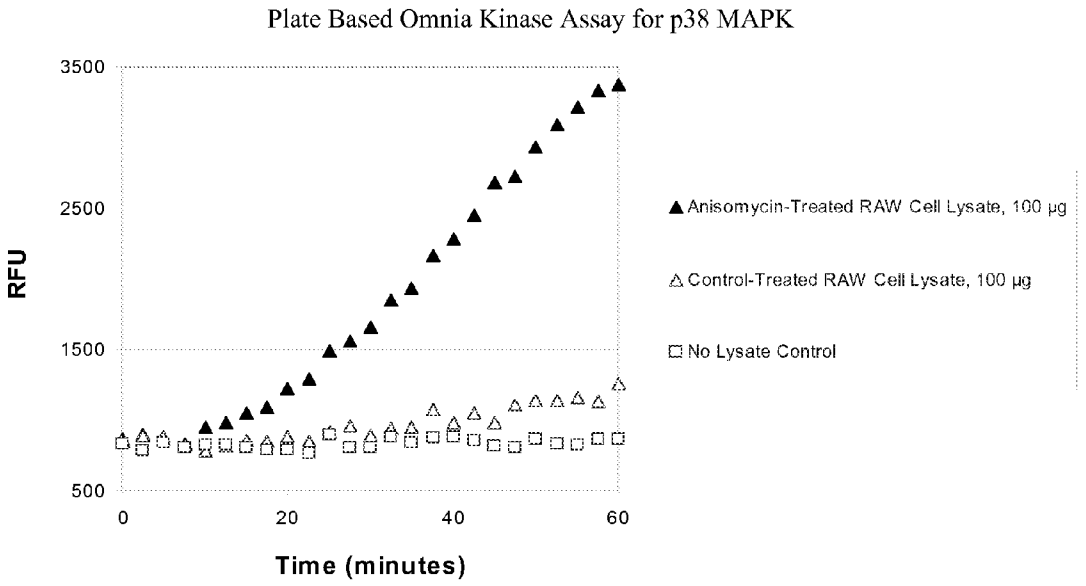


Plate Based Omnia Kinase Assay for p38 MAPK
RFU
3500
2500
1500
500
0
20
40
60
Time (minutes)
Anisomycin-Treated RAW Cell Lysate, 100 µg
Control-Treated RAW Cell Lysate, 100 µg
No Lysate Control

FIG. 13A

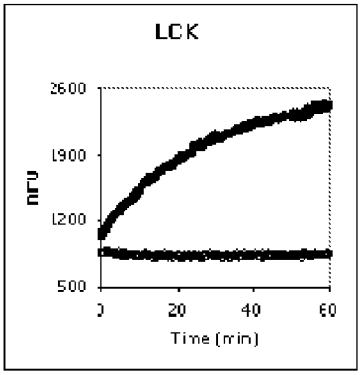


FIG. 13B

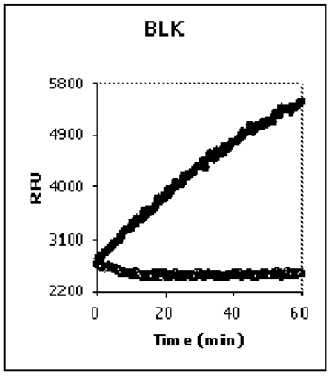


FIG. 13C

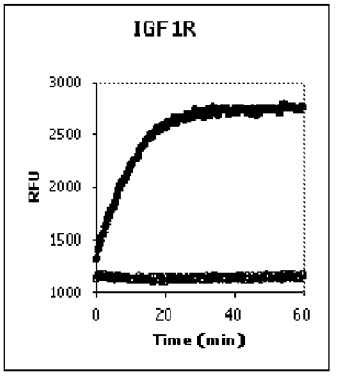


FIG. 13D

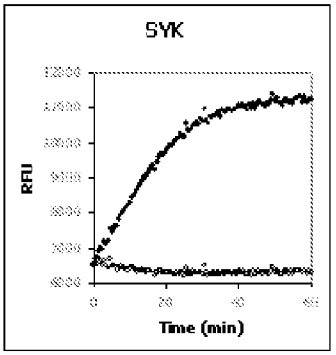


FIG. 13E

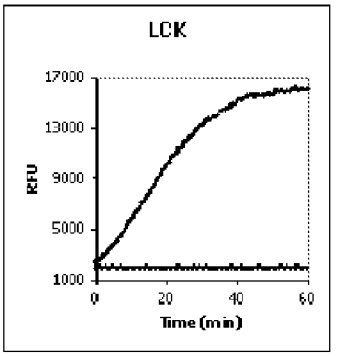


FIG. 13F

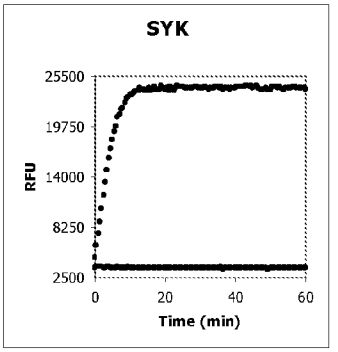


FIG. 13G

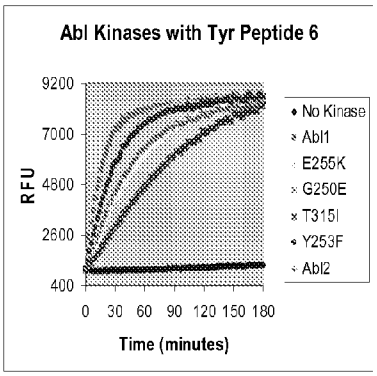


FIG. 13H

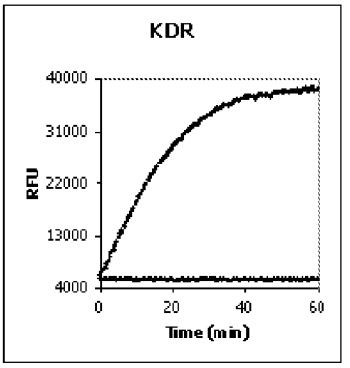
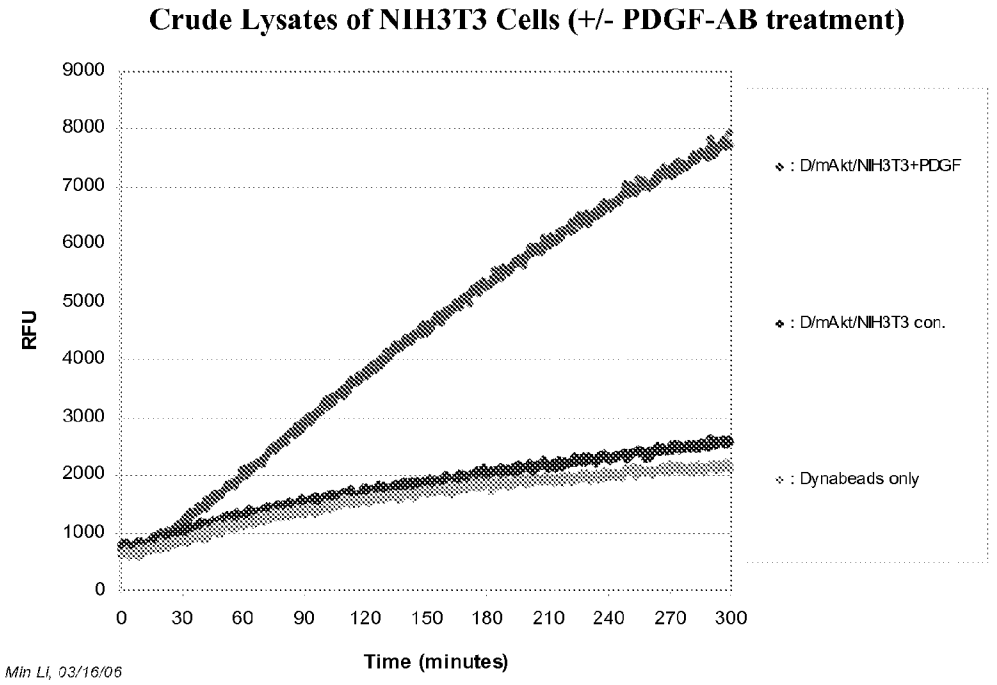


FIG. 14

Cell-based (Magnetic Bead Capture) Kinase Assay



METHODS FOR MEASURING REAL TIME KINASE ACTIVITY

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Application No. 60/759,919, filed Jan. 18, 2006 and U.S. Provisional Application No. 60/819,432, filed Jul. 7, 2006, the contents of which are incorporated by reference as if set forth fully herein.

FIELD OF THE INVENTION

[0002] The present invention relates to methods for detecting and/or measuring the activity of a specific kinase of interest.

BACKGROUND OF THE INVENTION

[0003] Protein kinases are believed to play a role in the formation of many different diseases. Therefore, new drug candidates and treatment protocols are being identified using methods for understanding and identifying protein kinase activity and molecules that affect that activity.

[0004] A protein kinase catalyzes the transfer of a phosphate group from adenosine triphosphate (ATP) to a serine, threonine or tyrosine residue in a peptide or protein sequence. Protein kinases are involved in all aspects of regulation within cells. The traditional method for assaying kinase activity is discontinuous and requires ^{32}P-labelled ATP, which requires special handling. Many companies market specialized fluorescence kinase assay systems, all of which are discontinuous, requiring sampling of the reaction mixture followed by additional handling steps to label the product of the reaction with a fluorescent moiety (e.g., Promega, Panvera, Calbiochem, Cell Signaling Technology, Molecular Devices, DiscoveRx, Upstate, PerkinElmer). A continuous fluorescence assay that can be performed in real time is of great utility. Currently, few examples of sensors capable of such assays exist.

[0005] U.S. Pat. No. 6,906,104, which is hereby incorporated by reference, describes sulfonamide substituted quinoline compounds, including "SOX", for detection of kinases free in solution. An article by Lawrence et al. describes real time visualization of protein kinase activity in living cells (Journal of Biological Chemistry Vol. 277, No. 13, Issue of March 29, pp. 11527-11532, 2002).

[0006] One way to measure kinases activity in real time is to capture the kinase using the kinase recognition motif, or amino acid sequence that a particular kinase recognizes and phosphorylates. Once captured, ATP can be added to the assay and a signaling moiety can then generate or alter a signal upon a phosphorylation event. In general, each kinase may be specific for a particular kinase recognition motif, however, there are some sequence similarities in the amino acid sequences of the kinase recognition motif surrounding the target amino acid among kinases. Thus, using the kinase recognition motif to capture a particular kinase may result in capturing a population of different kinases that all recognize the same or similar kinase recognition motif. The results of these assays have a propensity to generate false positive results when trying to identify and/or measure the activity of a specific kinase. What is needed, therefore, is a method of improving the specificity of the kinase activity assays to lower the incidences of false positives, thus adding value to assay results that assess kinase activity. Currently, there are no methods in the literature that overcome this problem caused by the promiscuity of kinase recognition motifs in capturing specific kinases.

SUMMARY OF THE INVENTION

[0007] The present invention relates to methods for detecting and/or measuring the activity of a specific kinase, with the methods comprising contacting one or more kinases with a binding agent to isolate a specific kinase of interest. The isolated kinase is then contacted with a kinase activity sensor, where the kinase activity sensor is comprised of a kinase recognition motif that is capable of being recognized by the isolated kinase, and at least one phosphorylation site. The isolated kinase, if present, phosphorylates the amino acid target of the kinase activity sensor and levels of the phosphorylated amino acid target can then be quantified.

[0008] One aspect of the present invention provides a method of measuring the activity of a specific kinase, said method comprising:

[0009] providing a solid or semi-solid support comprising an immobilized binding agent;

[0010] contacting the immobilized binding agent with a sample comprising the specific kinase to form an immobilized specific kinase;

[0011] contacting the immobilized specific kinase with a kinase activity sensor to form a contacted specific kinase, wherein the kinase activity sensor comprises at least one peptide capable of being phosphorylated and a chelator;

[0012] incubating the contacted specific kinase for a sufficient amount of time for the kinase to phosphorylate the peptide in the presence of a phosphate source and a metal ion, wherein the kinase activity sensor forms a ternary complex with the metal ion and phosphorylated peptide to generate a detectable signal; and

[0013] detecting the signal whereby the activity of the specific kinase is measured.

[0014] In a more particular embodiment, the binding agent comprises an antibody or a functional fragment thereof.

[0015] In another embodiment, the support is glass, plastic, metal, polymeric particle, polymeric gel or a polymeric membrane.

[0016] In another embodiment, the kinase activity sensor has an increased fluorescence signal when complexed with the metal ion. In another embodiment, the kinase activity sensor further comprises a signaling moiety that is separate from the chelator. More particular still, the signaling moiety comprises a coumarin, cyanine, benzofuran, a quinoline, a quinazolinone, an indole, a benzazole, a borapolyazaindacene or a xanthene.

[0017] In another embodiment, the chelator comprises a signaling moiety. More particular still, the signaling moiety comprises a coumarin, cyanine, benzofuran, a quinoline, a quinazolinone, an indole, a benzazole, a borapolyazaindacene or a xanthene. In another embodiment, the chelator

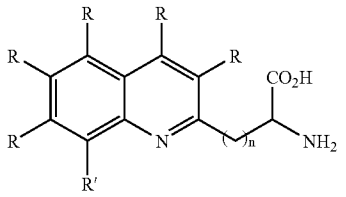
comprises a quinoline or a derivative thereof, phenanthrolines or derivatives thereof, BAPTA, IDA, DTPA and derivatives thereof.

[0018] In another embodiment, the metal ion is Ca^{2+}, Zn^{2+}, Mg^{2+}, Ga^{3+}, Tb^{3+}, La^{3+}, Pb^{2+}, Hg^{2+}, Cd^{2+}, Cu^{2+}, Ni^{2+}, Co^{2+}, Fe^{2+}, Mn^{2+}, Ba^{2+}, or Sr^{2+}.

[0019] In another embodiment, the peptide comprises an amino acid that when phosphorylated complexes magnesium.

[0020] In another embodiment the method further comprises contacting the immobilized specific kinase with ATP.

[0021] In another more particular embodiment, the kinase activity sensor comprises the formula:



[0022] where at least one R group is —SO_2X, where X is —OR" or —NR"R'";

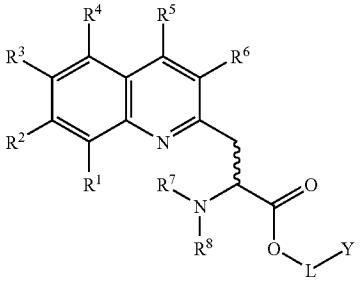
[0023] R' is hydroxy, amino, or thiol;

[0024] R" is C_{1-6} alkyl;

[0025] R'" is hydrogen or alkyl; and

[0026] n is 1, 2 or 3.

[0027] Alternatively, the kinase activity sensor has the formula:



[0028] wherein R^1, is H, alkyl, substituted alkyl, alkoxy, substituted alkoxy, acyl, acylamino, acyloxy, amino, substituted amino, aminocarbonyl, aminothiocarbonyl, aminocarbonylamino, aminothiocarbonylamino, aminocarbonyloxy, aminosulfonyl, aminosulfonyloxy, aminosulfonylamino, amidino, carboxyl, carboxyl ester, (carboxyl ester)amino, (carboxyl ester)oxy, cyano, halo, hydroxy, nitro, SO_3H, sulfonyl, substituted sulfonyl, sulfonyloxy, thioacyl, thiol, alkylthio, substituted alkylthio, aryl, substituted aryl, heteroaryl, substituted heteroaryl, cycloalkyl, substituted cycloalkyl, heterocyclyl, or substituted heterocyclyl;

[0029] R^2 is a fluorophore, H, alkyl, substituted alkyl, alkoxy, substituted alkoxy, acyl, acylamino, acyloxy, amino, substituted amino, aminocarbonyl, aminothiocarbonyl, aminocarbonylamino, aminothiocarbonylamino, aminocarbonyloxy, aminosulfonyl, aminosulfonyloxy, aminosulfonylamino, amidino, carboxyl, carboxyl ester, (carboxyl ester)amino, (carboxyl ester)oxy, cyano, halo, hydroxy, nitro, SO_3H, sulfonyl, substituted sulfonyl, sulfonyloxy, thioacyl, thiol, alkylthio, substituted alkylthio, aryl, substituted aryl, heteroaryl, substituted heteroaryl, cycloalkyl, substituted cycloalkyl, heterocyclyl, or substituted heterocyclyl;

[0030] R^3 is a fluorophore, H, alkyl, substituted alkyl, alkoxy, substituted alkoxy, acyl, acylamino, acyloxy, amino, substituted amino, aminocarbonyl, aminothiocarbonyl, aminocarbonylamino, aminothiocarbonylamino, aminocarbonyloxy, aminosulfonyl, aminosulfonyloxy, aminosulfonylamino, amidino, carboxyl, carboxyl ester, (carboxyl ester)amino, (carboxyl ester)oxy, cyano, halo, hydroxy, nitro, SO_3H, sulfonyl, substituted sulfonyl, sulfonyloxy, thioacyl, thiol, alkylthio, substituted alkylthio, aryl, substituted aryl, heteroaryl, substituted heteroaryl, cycloalkyl, substituted cycloalkyl, heterocyclyl, or substituted heterocyclyl;

[0031] R^4 is a fluorophore, H, alkyl, substituted alkyl, alkoxy, substituted alkoxy, acyl, acylamino, acyloxy, amino, substituted amino, aminocarbonyl, aminothiocarbonyl, aminocarbonylamino, aminothiocarbonylamino, aminocarbonyloxy, aminosulfonyl, aminosulfonyloxy, aminosulfonylamino, amidino, carboxyl, carboxyl ester, (carboxyl ester)amino, (carboxyl ester)oxy, cyano, halo, hydroxy, nitro, SO_3H, sulfonyl, substituted sulfonyl, sulfonyloxy, thioacyl, thiol, alkylthio, substituted alkylthio, aryl, substituted aryl, heteroaryl, substituted heteroaryl, cycloalkyl, substituted cycloalkyl, heterocyclyl, or substituted heterocyclyl;

[0032] R^5 is a fluorophore, H, alkyl, substituted alkyl, alkoxy, substituted alkoxy, acyl, acylamino, acyloxy, amino, substituted amino, aminocarbonyl, aminothiocarbonyl, aminocarbonylamino, aminothiocarbonylamino, aminocarbonyloxy, aminosulfonyl, aminosulfonyloxy, aminosulfonylamino, amidino, carboxyl, carboxyl ester, (carboxyl ester)amino, (carboxyl ester)oxy, cyano, halo, hydroxy, nitro, SO_3H, sulfonyl, substituted sulfonyl, sulfonyloxy, thioacyl, thiol, alkylthio, substituted alkylthio, aryl, substituted aryl, heteroaryl, substituted heteroaryl, cycloalkyl, substituted cycloalkyl, heterocyclyl, or substituted heterocyclyl; and

[0033] R^6 is a fluorophore, H, alkyl, substituted alkyl, alkoxy, substituted alkoxy, acyl, acylamino, acyloxy, amino, substituted amino, aminocarbonyl, aminothiocarbonyl, aminocarbonylamino, aminothiocarbonylamino, aminocarbonyloxy, aminosulfonyl, aminosulfonyloxy, aminosulfonylamino, amidino, carboxyl, carboxyl ester, (carboxyl ester)amino, (carboxyl ester)oxy, cyano, halo, hydroxy, nitro, SO_3H, sulfonyl, substituted sulfonyl, sulfonyloxy, thioacyl, thiol, alkylthio, substituted alkylthio, aryl, substituted aryl, heteroaryl, substituted heteroaryl, cycloalkyl, substituted cycloalkyl, heterocyclyl, or substituted heterocyclyl;

[0034] R^7 is H, alkyl, substituted alkyl, carbonyl, or a protecting group; and

[0035] R^3 is H, alkyl, substituted alkyl, carbonyl, or a protecting group;

[0036] L is a linker; and

[0037] Y is a peptide;

[0038] or a tautomer, stereoisomer, or salt thereof.

[0039] More particularly, at least one of R^2, R^3, R^4, R^5 and R^6 is a fluorophore.

[0040] In another embodiment, the peptide is at least one of SEQ ID NO. 1 through SEQ ID NO. 133.

[0041] Another aspect of the invention provides a composition comprising at least one of SEQ ID NO. 1 through SEQ ID NO. 133.

[0042] Another aspect of the invention provides a composition comprising:

[0043] (a) a kinase immobilized by a binding agent; and

[0044] (b) a kinase activity sensor comprising at least one peptide capable of being phosphorylated and a chelator.

[0045] Another aspect of the invention provides a kit for detecting the activity of a specific kinase, comprising a binding agent that binds to the specific kinase and a kinase activity sensor, wherein said kinase activity sensor comprises at least one peptide capable of being phosphorylated and a chelator. More particularly, the kit further comprises ATP and a metal ion that has affinity for both the chelator and the phosphorylated peptide.

[0046] Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

[0047] FIG. **1** depicts the kinase activity of p38 kinase as assessed using the methods of the present invention.

[0048] FIG. **2** depicts the kinase activity of Erk1/2 kinase as assessed using the methods of the present invention.

[0049] FIG. **3** depicts Akt1 Activity in Crude Lysates from PDGF-Treated or Control NIH3T3 Cells.

[0050] FIG. **4** depicts Akt3 Activity in Crude Lysates from PDGF-Treated or Control NIH3T3 Cells.

[0051] FIG. **5** depicts ERK1/2 Activity in Crude Lysates from PDGF-Treated or Control NIH3T3 Cells

[0052] FIG. **6** depicts p70-S6K Activity in Crude Lysates from PDGF-Treated or Control MCF-7 Cells

[0053] FIG. **7** depicts RSK Activity in Crude Lysates from PMA-Treated or Control HeLa Cells

[0054] FIG. **8** depicts c-Src Activity in Crude Lysates from c-Src-Transfected CEF Cells.

[0055] FIG. **9** depicts B-Raf Activity in Crude Lysates from NGF Treated or Control PC12 Cells.

[0056] FIG. **10** depicts ERK1/2 Activity in Crude Lysates from PDGF-Treated or Control NIH3T3 Cells.

[0057] FIG. **11** depicts MEK1 Activity in Crude Lysates from PDGF-Treated or Control NIH3T3 Cells.

[0058] FIG. **12** depicts p38 MAPK Activity in Crude Lysates from Anisomycin Treated or Control RAW Cells.

[0059] FIG. **13** depicts Omnia Tyrosine Kinase Recombinant Assays Signal to Noise Ratios for various kinase targets, wherein the kinase recognition motif for each of the figures are as follows: FIG. **13**A corresponds to SEQ ID NO. 42 (~2.8 fold); FIG. **13**B corresponds to SEQ ID NO. 43 (~2.2 fold); FIG. **13**C corresponds to SEQ ID NO. 45 (~2.4 fold); FIG. **13**D corresponds to SEQ ID NO. 44 (~1.8 fold); FIG. **13**E corresponds to SEQ ID NO. 46 (~8.3 fold); FIG. **13**F corresponds to SEQ ID NO. 48 (~6.8 fold); FIG. **13**G corresponds to SEQ ID NO. 47 (~7.9 fold); and FIG. **13**H corresponds to SEQ ID NO. 49 (~6.9 fold).

[0060] FIG. **14** depicts a Cell-based (Magnetic Bead Capture) Kinase Assay for Akt/PKB kinase. Dynabeads M-280 w/Sheep anti-Mouse IgG were used along with Akt Mouse Monoclonal Antibody (Invitrogen). 2 ug anti-Akt mAb to 400 ug lysate were added and incubated at 4° C. overnight. 20 ul Dynabeads were added to each sample at 4° C. for 2 hrs and then beads were washed 3×. 20 uM Aktide were added with 1 mM ATP and results were read in M5 for 5 hrs. The isolation and kinase activity sensor increased selectivity and adjustable signal.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

[0061] Before describing the present invention in detail, it is to be understood that this invention is not limited to specific compositions or process steps, as such may vary. It must be noted that, as used in this specification and the appended claims, the singular form "a", "an" and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a peptide" includes a plurality of peptides and reference to "an enzyme" includes a plurality of enzymes and the like.

[0062] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention is related. The following terms are defined for purposes of the invention as described herein.

[0063] "Alkyl" refers to monovalent saturated aliphatic hydrocarbyl groups having from 1 to 10 carbon atoms and preferably 1 to 6 carbon atoms. This term includes, by way of example, linear and branched hydrocarbyl groups such as methyl (CH_3—), ethyl (CH_3CH_2—), n-propyl ($CH_3CH_2CH_2$—), isopropyl ($(CH_3)_2CH$—), n-butyl ($CH_3CH_2CH_2CH_2$—), isobutyl ($(CH_3)_2CHCH_2$—), sec-butyl ($(CH_3)(CH_3CH_2)CH$—), t-butyl ($(CH_3)_3C$—), n-pentyl ($CH_3CH_2CH_2CH_2CH_2$—), and neopentyl ($(CH_3)_3CCH_2$—).

[0064] "Substituted alkyl" refers to an alkyl group having from 1 to 5, preferably 1 to 3, or more preferably 1 to 2 substituents selected from the group consisting of alkoxy,

substituted alkoxy, acyl, acylamino, acyloxy, amino, substituted amino, aminocarbonyl, aminothiocarbonyl, aminocarbonylamino, aminothiocarbonylamino, aminocarbonyloxy, aminosulfonyl, aminosulfonyloxy, aminosulfonylamino, amidino, aryl, substituted aryl, aryloxy, substituted aryloxy, arylthio, substituted arylthio, carboxyl, carboxyl ester, (carboxyl ester)amino, (carboxyl ester)oxy, cyano, cycloalkyl, substituted cycloalkyl, cycloalkyloxy, substituted cycloalkyloxy, cycloalkylthio, substituted cycloalkylthio, cycloalkenyl, substituted cycloalkenyl, cycloalkenyloxy, substituted cycloalkenyloxy, cycloalkenylthio, substituted cycloalkenylthio, guanidino, substituted guanidino, halo, hydroxy, heteroaryl, substituted heteroaryl, heteroaryloxy, substituted heteroaryloxy, heteroarylthio, substituted heteroarylthio, heterocyclic, substituted heterocyclic, heterocyclyloxy, substituted heterocyclyloxy, heterocyclylthio, substituted heterocyclylthio, nitro, SO_3H, substituted sulfonyl, sulfonyloxy, thioacyl, thiol, alkylthio, and substituted alkylthio, wherein said substituents are defined herein.

[0065] "Alkoxy" refers to the group —O-alkyl wherein alkyl is defined herein. Alkoxy includes, by way of example, methoxy, ethoxy, n-propoxy, isopropoxy, n-butoxy, t-butoxy, sec-butoxy, and n-pentoxy.

[0066] "Substituted alkoxy" refers to the group —O-(substituted alkyl) wherein substituted alkyl is defined herein.

[0067] "Acyl" refers to the groups H—C(O)—, alkyl-C(O)—, substituted alkyl-C(O)—, alkenyl-C(O)—, substituted alkenyl-C(O)—, alkynyl-C(O)—, substituted alkynyl-C(O)—, cycloalkyl-C(O)—, substituted cycloalkyl-C(O)—, cycloalkenyl-C(O)—, substituted cycloalkenyl-C(O)—, aryl-C(O)—, substituted aryl-C(O)—, heteroaryl-C(O)—, substituted heteroaryl-C(O)—, heterocyclic-C(O)—, and substituted heterocyclic-C(O)—, wherein alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, cycloalkyl, substituted cycloalkyl, cycloalkenyl, substituted cycloalkenyl, aryl, substituted aryl, heteroaryl, substituted heteroaryl, heterocyclic and substituted heterocyclic are as defined herein. Acyl includes the "acetyl" group $CH_3C(O)$—.

[0068] "Acylamino" refers to the groups —NRC(O)alkyl, —NRC(O)substituted alkyl, —NRC(O)cycloalkyl, —NRC(O)substituted cycloalkyl, —NRC(O)cycloalkenyl, —NRC(O)substituted cycloalkenyl, —NRC(O)alkenyl, —NRC(O)substituted alkenyl, —NRC(O)alkynyl, —NRC(O)substituted alkynyl, —NRC(O)aryl, —NRC(O)substituted aryl, —NRC(O)heteroaryl, —NRC(O)substituted heteroaryl, —NRC(O)heterocyclic, and —NRC(O)substituted heterocyclic wherein R is hydrogen or alkyl and wherein alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, cycloalkyl, substituted cycloalkyl, cycloalkenyl, substituted cycloalkenyl, aryl, substituted aryl, heteroaryl, substituted heteroaryl, heterocyclic and substituted heterocyclic are as defined herein.

[0069] "Acyloxy" refers to the groups alkyl-C(O)O—, substituted alkyl-C(O)O—, alkenyl-C(O)O—, substituted alkenyl-C(O)O—, alkynyl-C(O)O—, substituted alkynyl-C(O)O—, aryl-C(O)O—, substituted aryl-C(O)O—, cycloalkyl-C(O)O—, substituted cycloalkyl-C(O)O—, cycloalkenyl-C(O)O—, substituted cycloalkenyl-C(O)O—, heteroaryl-C(O)O—, substituted heteroaryl-C(O)O—, heterocyclic-C(O)O—, and substituted heterocyclic-C(O)O— wherein alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, cycloalkyl, substituted cycloalkyl, cycloalkenyl, substituted cycloalkenyl, aryl, substituted aryl, heteroaryl, substituted heteroaryl, heterocyclic, and substituted heterocyclic are as defined herein.

[0070] "Amino" refers to the group —NH_2.

[0071] "Substituted amino" refers to the group —NR'R" where R' and R" are independently selected from the group consisting of hydrogen, alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, aryl, substituted aryl, cycloalkyl, substituted cycloalkyl, cycloalkenyl, substituted cycloalkenyl, heteroaryl, substituted heteroaryl, heterocyclic, substituted heterocyclic, —SO_2-alkyl, —SO_2-substituted alkyl, —SO_2-alkenyl, —SO_2-substituted alkenyl, —SO_2-cycloalkyl, —SO_2-substituted cycloalkyl, —SO_2-cycloalkenyl, —SO_2-substituted cycloalkenyl, —SO_2-aryl, —SO_2-substituted aryl, —SO_2-heteroaryl, —SO_2-substituted heteroaryl, —SO_2-heterocyclic, and —SO_2-substituted heterocyclic and wherein R' and R" are optionally joined, together with the nitrogen bound thereto to form a heterocyclic or substituted heterocyclic group, provided that R' and R" are both not hydrogen, and wherein alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, cycloalkyl, substituted cycloalkyl, cycloalkenyl, substituted cycloalkenyl, aryl, substituted aryl, heteroaryl, substituted heteroaryl, heterocyclic, and substituted heterocyclic are as defined herein. When R' is hydrogen and R" is alkyl, the substituted amino group is sometimes referred to herein as alkylamino. When R' and R" are alkyl, the substituted amino group is sometimes referred to herein as dialkylamino. When referring to a monosubstituted amino, it is meant that either R' or R" is hydrogen but not both. When referring to a disubstituted amino, it is meant that neither R' nor R" are hydrogen.

[0072] "Aminocarbonyl" refers to the group —C(O)N$R^{10}R^{11}$ where R^{10} and R^{11} are independently selected from the group consisting of hydrogen, alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, aryl, substituted aryl, cycloalkyl, substituted cycloalkyl, cycloalkenyl, substituted cycloalkenyl, heteroaryl, substituted heteroaryl, heterocyclic, and substituted heterocyclic and where R^{10} and R^{11} are optionally joined together with the nitrogen bound thereto to form a heterocyclic or substituted heterocyclic group, and wherein alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, cycloalkyl, substituted cycloalkyl, cycloalkenyl, substituted cycloalkenyl, aryl, substituted aryl, heteroaryl, substituted heteroaryl, heterocyclic and substituted heterocyclic are as defined herein.

[0073] "Aminothiocarbonyl" refers to the group —C(S)N$R^{10}R^{11}$ where R^{10} and R^{11} are independently selected from the group consisting of hydrogen, alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, aryl, substituted aryl, cycloalkyl, substituted cycloalkyl, cycloalkenyl, substituted cycloalkenyl, heteroaryl, substituted heteroaryl, heterocyclic, and substituted heterocyclic and where R^{10} and R^{11} are optionally joined together with the nitrogen bound thereto to form a heterocyclic or substituted heterocyclic group, and wherein alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, cycloalkyl, substituted cycloalkyl, cycloalkenyl, substituted cycloalkenyl, aryl, substituted aryl,

heteroaryl, substituted heteroaryl, heterocyclic and substituted heterocyclic are as defined herein.

[0074] "Aminocarbonylamino" refers to the group —NRC(O)NR^{10}R^{11} where R is hydrogen or alkyl and R^{10} and R^{11} are independently selected from the group consisting of hydrogen, alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, aryl, substituted aryl, cycloalkyl, substituted cycloalkyl, cycloalkenyl, substituted cycloalkenyl, heteroaryl, substituted heteroaryl, heterocyclic, and substituted heterocyclic and where R^{10} and R^{11} are optionally joined together with the nitrogen bound thereto to form a heterocyclic or substituted heterocyclic group, and wherein alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, cycloalkyl, substituted cycloalkyl, cycloalkenyl, substituted cycloalkenyl, aryl, substituted aryl, heteroaryl, substituted heteroaryl, heterocyclic and substituted heterocyclic are as defined herein.

[0075] "Aminothiocarbonylamino" refers to the group —NRC(S)NR^{10}R^{11} where R is hydrogen or alkyl and R^{10} and R^{11} are independently selected from the group consisting of hydrogen, alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, aryl, substituted aryl, cycloalkyl, substituted cycloalkyl, cycloalkenyl, substituted cycloalkenyl, heteroaryl, substituted heteroaryl, heterocyclic, and substituted heterocyclic and where R^{10} and R^{11} are optionally joined together with the nitrogen bound thereto to form a heterocyclic or substituted heterocyclic group, and wherein alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, cycloalkyl, substituted cycloalkyl, cycloalkenyl, substituted cycloalkenyl, aryl, substituted aryl, heteroaryl, substituted heteroaryl, heterocyclic and substituted heterocyclic are as defined herein.

[0076] "Aminocarbonyloxy" refers to the group —O—C(O)NR^{10}R^{11} where R^{10} and R^{11} are independently selected from the group consisting of hydrogen, alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, aryl, substituted aryl, cycloalkyl, substituted cycloalkyl, cycloalkenyl, substituted cycloalkenyl, heteroaryl, substituted heteroaryl, heterocyclic, and substituted heterocyclic and where R^{10} and R^{11} are optionally joined together with the nitrogen bound thereto to form a heterocyclic or substituted heterocyclic group, and wherein alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, cycloalkyl, substituted cycloalkyl, cycloalkenyl, substituted cycloalkenyl, aryl, substituted aryl, heteroaryl, substituted heteroaryl, heterocyclic and substituted heterocyclic are as defined herein.

[0077] "Aminosulfonyl" refers to the group —SO$_2$NR^{10}R^{11} where R^{10} and R^{11} are independently selected from the group consisting of hydrogen, alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, aryl, substituted aryl, cycloalkyl, substituted cycloalkyl, cycloalkenyl, substituted cycloalkenyl, heteroaryl, substituted heteroaryl, heterocyclic, and substituted heterocyclic and where R^{10} and R^{11} are optionally joined together with the nitrogen bound thereto to form a heterocyclic or substituted heterocyclic group, and wherein alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, cycloalkyl, substituted cycloalkyl, cycloalkenyl, substituted cycloalkenyl, aryl, substituted aryl, heteroaryl, substituted heteroaryl, heterocyclic and substituted heterocyclic are as defined herein.

[0078] "Aminosulfonyloxy" refers to the group —O—SO$_2$NR^{10}R^{11} where R^{10} and R^{11} are independently selected from the group consisting of hydrogen, alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, aryl, substituted aryl, cycloalkyl, substituted cycloalkyl, cycloalkenyl, substituted cycloalkenyl, heteroaryl, substituted heteroaryl, heterocyclic, and substituted heterocyclic and where R^{10} and R^{11} are optionally joined together with the nitrogen bound thereto to form a heterocyclic or substituted heterocyclic group, and wherein alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, cycloalkyl, substituted cycloalkyl, cycloalkenyl, substituted cycloalkenyl, aryl, substituted aryl, heteroaryl, substituted heteroaryl, heterocyclic and substituted heterocyclic are as defined herein.

[0079] "Aminosulfonylamino" refers to the group —NR—SO$_2$NR^{10}R^{11} where R is hydrogen or alkyl and R^{10} and R^{11} are independently selected from the group consisting of hydrogen, alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, aryl, substituted aryl, cycloalkyl, substituted cycloalkyl, cycloalkenyl, substituted cycloalkenyl, heteroaryl, substituted heteroaryl, heterocyclic, and substituted heterocyclic and where R^{10} and R^{11} are optionally joined together with the nitrogen bound thereto to form a heterocyclic or substituted heterocyclic group, and wherein alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, cycloalkyl, substituted cycloalkyl, cycloalkenyl, substituted cycloalkenyl, aryl, substituted aryl, heteroaryl, substituted heteroaryl, heterocyclic and substituted heterocyclic are as defined herein.

[0080] "Amidino" refers to the group —C(=NR^{12})R^{10}R^{11} where R^{10}, R^{11}, and R^{12} are independently selected from the group consisting of hydrogen, alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, aryl, substituted aryl, cycloalkyl, substituted cycloalkyl, cycloalkenyl, substituted cycloalkenyl, heteroaryl, substituted heteroaryl, heterocyclic, and substituted heterocyclic and where R^{10} and R^{11} are optionally joined together with the nitrogen bound thereto to form a heterocyclic or substituted heterocyclic group, and wherein alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, cycloalkyl, substituted cycloalkyl, cycloalkenyl, substituted cycloalkenyl, aryl, substituted aryl, heteroaryl, substituted heteroaryl, heterocyclic and substituted heterocyclic are as defined herein.

[0081] "Aryl" or "Ar" refers to a monovalent aromatic carbocyclic group of from 6 to 14 carbon atoms having a single ring (e.g., phenyl) or multiple condensed rings (e.g., naphthyl or anthryl) which condensed rings may or may not be aromatic (e.g., 2-benzoxazolinone, 2H-1,4-benzoxazin-3(4H)-one-7-yl, and the like) provided that the point of attachment is at an aromatic carbon atom. Preferred aryl groups include phenyl and naphthyl.

[0082] "Substituted aryl" refers to aryl groups which are substituted with 1 to 5, preferably 1 to 3, or more preferably 1 to 2 substituents selected from the group consisting of alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, alkoxy, substituted alkoxy, acyl, acylamino, acyloxy, amino, substituted amino, aminocarbonyl, aminothiocarbonyl, aminocarbonylamino, aminothiocarbonylamino, aminocarbonyloxy, aminosulfonyl, aminosulfonyloxy, aminosulfonylamino, amidino, aryl, substituted aryl,

aryloxy, substituted aryloxy, arylthio, substituted arylthio, carboxyl, carboxyl ester, (carboxyl ester)amino, (carboxyl ester)oxy, cyano, cycloalkyl, substituted cycloalkyl, cycloalkyloxy, substituted cycloalkyloxy, cycloalkylthio, substituted cycloalkylthio, cycloalkenyl, substituted cycloalkenyl, cycloalkenyloxy, substituted cycloalkenyloxy, cycloalkenylthio, substituted cycloalkenylthio, guanidino, substituted guanidino, halo, hydroxy, heteroaryl, substituted heteroaryl, heteroaryloxy, substituted heteroaryloxy, heteroarylthio, substituted heteroarylthio, heterocyclic, substituted heterocyclic, heterocyclyloxy, substituted heterocyclyloxy, heterocyclylthio, substituted heterocyclylthio, nitro, SO_3H, substituted sulfonyl, sulfonyloxy, thioacyl, thiol, alkylthio, and substituted alkylthio, wherein said substituents are defined herein.

[0083] "Aryloxy" refers to the group —O-aryl, where aryl is as defined herein, that includes, by way of example, phenoxy and naphthoxy.

[0084] "Substituted aryloxy" refers to the group —O-(substituted aryl) where substituted aryl is as defined herein.

[0085] "Arylthio" refers to the group —S-aryl, where aryl is as defined herein.

[0086] "Substituted arylthio" refers to the group —S-(substituted aryl), where substituted aryl is as defined herein.

[0087] "Alkenyl" refers to alkenyl groups having from 2 to 6 carbon atoms and preferably 2 to 4 carbon atoms and having at least 1 and preferably from 1 to 2 sites of alkenyl unsaturation. Such groups are exemplified, for example, by vinyl, allyl, and but-3-en-1-yl.

[0088] "Substituted alkenyl" refers to alkenyl groups having from 1 to 3 substituents, and preferably 1 to 2 substituents, selected from the group consisting of alkoxy, substituted alkoxy, acyl, acylamino, acyloxy, amino, substituted amino, aminocarbonyl, aminothiocarbonyl, aminocarbonylamino, aminothiocarbonylamino, aminocarbonyloxy, aminosulfonyl, aminosulfonyloxy, aminosulfonylamino, amidino, aryl, substituted aryl, aryloxy, substituted aryloxy, arylthio, substituted arylthio, carboxyl, carboxyl ester, (carboxyl ester)amino, (carboxyl ester)oxy, cyano, cycloalkyl, substituted cycloalkyl, cycloalkyloxy, substituted cycloalkyloxy, cycloalkylthio, substituted cycloalkylthio, cycloalkenyl, substituted cycloalkenyl, cycloalkenyloxy, substituted cycloalkenyloxy, cycloalkenylthio, substituted cycloalkenylthio, guanidino, substituted guanidino, halo, hydroxy, heteroaryl, substituted heteroaryl, heteroaryloxy, substituted heteroaryloxy, heteroarylthio, substituted heteroarylthio, heterocyclic, substituted heterocyclic, heterocyclyloxy, substituted heterocyclyloxy, heterocyclylthio, substituted heterocyclylthio, nitro, SO_3H, substituted sulfonyl, sulfonyloxy, thioacyl, thiol, alkylthio, and substituted alkylthio, wherein said substituents are defined herein and with the proviso that any hydroxy substitution is not attached to a vinyl (unsaturated) carbon atom.

[0089] "Alkynyl" refers to alkynyl groups having from 2 to 6 carbon atoms and preferably 2 to 3 carbon atoms and having at least 1 and preferably from 1 to 2 sites of alkynyl unsaturation.

[0090] "Substituted alkynyl" refers to alkynyl groups having from 1 to 3 substituents, and preferably 1 to 2 substituents, selected from the group consisting of alkoxy, substituted alkoxy, acyl, acylamino, acyloxy, amino, substituted amino, aminocarbonyl, aminothiocarbonyl, aminocarbonylamino, aminothiocarbonylamino, aminocarbonyloxy, aminosulfonyl, aminosulfonyloxy, aminosulfonylamino, amidino, aryl, substituted aryl, aryloxy, substituted aryloxy, arylthio, substituted arylthio, carboxyl, carboxyl ester, (carboxyl ester)amino, (carboxyl ester)oxy, cyano, cycloalkyl, substituted cycloalkyl, cycloalkyloxy, substituted cycloalkyloxy, cycloalkylthio, substituted cycloalkylthio, cycloalkenyl, substituted cycloalkenyl, cycloalkenyloxy, substituted cycloalkenyloxy, cycloalkenylthio, substituted cycloalkenylthio, guanidino, substituted guanidino, halo, hydroxy, heteroaryl, substituted heteroaryl, heteroaryloxy, substituted heteroaryloxy, heteroarylthio, substituted heteroarylthio, heterocyclic, substituted heterocyclic, heterocyclyloxy, substituted heterocyclyloxy, heterocyclylthio, substituted heterocyclylthio, nitro, SO_3H, substituted sulfonyl, sulfonyloxy, thioacyl, thiol, alkylthio, and substituted alkylthio, wherein said substituents are defined herein and with the proviso that any hydroxy substitution is not attached to an acetylenic carbon atom.

[0091] "Carbonyl" refers to the divalent group —C(O)— which is equivalent to —C(═O)—.

[0092] "Carboxyl" or "carboxy" refers to —COOH or salts thereof.

[0093] "Carboxyl ester" or "carboxy ester" refers to the groups —C(O)O-alkyl, —C(O)O-substituted alkyl, —C(O)O-alkenyl, —C(O)O-substituted alkenyl, —C(O)O-alkynyl, —C(O)O-substituted alkynyl, —C(O)O-aryl, —C(O)O-substituted aryl, —C(O)O-cycloalkyl, —C(O)O-substituted cycloalkyl, —C(O)O-cycloalkenyl, —C(O)O-substituted cycloalkenyl, —C(O)O-heteroaryl, —C(O)O-substituted heteroaryl, —C(O)O-heterocyclic, and —C(O)O-substituted heterocyclic wherein alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, cycloalkyl, substituted cycloalkyl, cycloalkenyl, substituted cycloalkenyl, aryl, substituted aryl, heteroaryl, substituted heteroaryl, heterocyclic, and substituted heterocyclic are as defined herein.

[0094] "(Carboxyl ester)amino" refers to the group —NR—C(O)O-alkyl, substituted —NR—C(O)O-alkyl, —NR—C(O)O-alkenyl, —NR—C(O)O-substituted alkenyl, —NR—C(O)O-alkynyl, —NR—C(O)O-substituted alkynyl, —NR—C(O)O-aryl, —NR—C(O)O-substituted aryl, —NR—C(O)O-cycloalkyl, —NR—C(O)O-substituted cycloalkyl, —NR—C(O)O-cycloalkenyl, —NR—C(O)O-substituted cycloalkenyl, —NR—C(O)O-heteroaryl, —NR—C(O)O-substituted heteroaryl, —NR—C(O)O-heterocyclic, and —NR—C(O)O-substituted heterocyclic wherein R is alkyl or hydrogen, and wherein alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, cycloalkyl, substituted cycloalkyl, cycloalkenyl, substituted cycloalkenyl, aryl, substituted aryl, heteroaryl, substituted heteroaryl, heterocyclic, and substituted heterocyclic are as defined herein.

[0095] "(Carboxyl ester)oxy" refers to the group —O—C(O)O-alkyl, substituted —O—C(O)O-alkyl, —O—C(O)O-alkenyl, —O—C(O)O-substituted alkenyl, —O—C(O)O-alkynyl, —O—C(O)O-substituted alkynyl, —O—C(O)O-aryl, —O—C(O)O-substituted aryl, —O—C(O)O-cycloalkyl, —O—C(O)O-substituted cycloalkyl, —O—C(O)O-cycloalkenyl, —O—C(O)O-sub-

stituted cycloalkenyl, —O—C(O)O-heteroaryl, —O—C(O)O-substituted heteroaryl, —O—C(O)O-heterocyclic, and —O—C(O)O-substituted heterocyclic wherein alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, cycloalkyl, substituted cycloalkyl, cycloalkenyl, substituted cycloalkenyl, aryl, substituted aryl, heteroaryl, substituted heteroaryl, heterocyclic, and substituted heterocyclic are as defined herein.

[0096] "Cyano" refers to the group —CN.

[0097] "Cycloalkyl" refers to cyclic alkyl groups of from 3 to 10 carbon atoms having single or multiple cyclic rings including fused, bridged, and spiro ring systems. Examples of suitable cycloalkyl groups include, for instance, adamantyl, cyclopropyl, cyclobutyl, cyclopentyl, and cyclooctyl.

[0098] "Cycloalkenyl" refers to non-aromatic cyclic alkyl groups of from 3 to 10 carbon atoms having single or multiple cyclic rings and having at least one >C═C< ring unsaturation and preferably from 1 to 2 sites of >C═C< ring unsaturation.

[0099] "Substituted cycloalkyl" and "substituted cycloalkenyl" refers to a cycloalkyl or cycloalkenyl group having from 1 to 5 or preferably 1 to 3 substituents selected from the group consisting of oxo, thione, alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, alkoxy, substituted alkoxy, acyl, acylamino, acyloxy, amino, substituted amino, aminocarbonyl, aminothiocarbonyl, aminocarbonylamino, aminothiocarbonylamino, aminocarbonyloxy, aminosulfonyl, aminosulfonyloxy, aminosulfonylamino, amidino, aryl, substituted aryl, aryloxy, substituted aryloxy, arylthio, substituted arylthio, carboxyl, carboxyl ester, (carboxyl ester)amino, (carboxyl ester)oxy, cyano, cycloalkyl, substituted cycloalkyl, cycloalkyloxy, substituted cycloalkyloxy, cycloalkylthio, substituted cycloalkylthio, cycloalkenyl, substituted cycloalkenyl, cycloalkenyloxy, substituted cycloalkenyloxy, cycloalkenylthio, substituted cycloalkenylthio, guanidino, substituted guanidino, halo, hydroxy, heteroaryl, substituted heteroaryl, heteroaryloxy, substituted heteroaryloxy, heteroarylthio, substituted heteroarylthio, heterocyclic, substituted heterocyclic, heterocyclyloxy, substituted heterocyclyloxy, heterocyclylthio, substituted heterocyclylthio, nitro, SO_3H, substituted sulfonyl, sulfonyloxy, thioacyl, thiol, alkylthio, and substituted alkylthio, wherein said substituents are defined herein.

[0100] "Cycloalkyloxy" refers to —O-cycloalkyl.

[0101] "Substituted cycloalkyloxy refers to —O-(substituted cycloalkyl).

[0102] "Cycloalkylthio" refers to —S-cycloalkyl.

[0103] "Substituted cycloalkylthio" refers to —S-(substituted cycloalkyl).

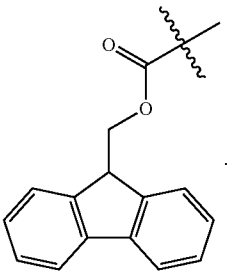
[0104] "Cycloalkenyloxy" refers to —O-cycloalkenyl.

[0105] "Substituted cycloalkenyloxy refers to —O-(substituted cycloalkenyl).

[0106] "Cycloalkenylthio" refers to —S-cycloalkenyl.

[0107] "Substituted cycloalkenylthio" refers to —S-(substituted cycloalkenyl).

[0108] "FMOC" refers to a compound having the general formula (or derivatives thereof):



[0109] "Guanidino" refers to the group —NHC(═NH)NH_2.

[0110] "Substituted guanidino" refers to —$NR_{13}C(═NR^{13})N(R^{13})_2$ where each R^{13} is independently selected from the group consisting of hydrogen, alkyl, substituted alkyl, aryl, substituted aryl, heteroaryl, substituted heteroaryl, heterocyclic, and substituted heterocyclic and two R^{13} groups attached to a common guanidino nitrogen atom are optionally joined together with the nitrogen bound thereto to form a heterocyclic or substituted heterocyclic group, provided that at least one R^{13} is not hydrogen, and wherein said substituents are as defined herein.

[0111] "Halo" or "halogen" refers to fluoro, chloro, bromo and iodo.

[0112] "Hydroxy" or "hydroxyl" refers to the group —OH.

[0113] "Heteroaryl" refers to an aromatic group of from 1 to 10 carbon atoms and 1 to 4 heteroatoms selected from the group consisting of oxygen, nitrogen and sulfur within the ring. Such heteroaryl groups can have a single ring (e.g., pyridinyl or furyl) or multiple condensed rings (e.g., indolizinyl or benzothienyl) wherein the condensed rings may or may not be aromatic and/or contain a heteroatom provided that the point of attachment is through an atom of the aromatic heteroaryl group. In one embodiment, the nitrogen and/or the sulfur ring atom(s) of the heteroaryl group are optionally oxidized to provide for the N-oxide (N→O), sulfinyl, or sulfonyl moieties. Preferred heteroaryls include pyridinyl, pyrrolyl, indolyl, thiophenyl, and furanyl.

[0114] "Substituted heteroaryl" refers to heteroaryl groups that are substituted with from 1 to 5, preferably 1 to 3, or more preferably 1 to 2 substituents selected from the group consisting of the same group of substituents defined for substituted aryl.

[0115] "Heteroaryloxy" refers to —O-heteroaryl.

[0116] "Substituted heteroaryloxy refers to the group —O-(substituted heteroaryl).

[0117] "Heteroarylthio" refers to the group —S-heteroaryl.

[0118] "Substituted heteroarylthio" refers to the group —S-(substituted heteroaryl).

[0119] "Heterocycle" or "heterocyclic" or "heterocycloalkyl" or "heterocyclyl" refers to a saturated or unsaturated group having a single ring or multiple condensed rings, including fused bridged and spiro ring systems, from 1 to 10

carbon atoms and from 1 to 4 hetero atoms selected from the group consisting of nitrogen, sulfur or oxygen within the ring wherein, in fused ring systems, one or more the rings can be cycloalkyl, aryl or heteroaryl provided that the point of attachment is through the non-aromatic ring. In one embodiment, the nitrogen and/or sulfur atom(s) of the heterocyclic group are optionally oxidized to provide for the N-oxide, sulfinyl, sulfonyl moieties.

[0120] "Substituted heterocyclic" or "substituted heterocycloalkyl" or "substituted heterocyclyl" refers to heterocyclyl groups that are substituted with from 1 to 5 or preferably 1 to 3 of the same substituents as defined for substituted cycloalkyl.

[0121] "Heterocyclyloxy" refers to the group —O-heterocyclyl.

[0122] "Substituted heterocyclyloxy refers to the group —O-(substituted heterocyclyl).

[0123] "Heterocyclylthio" refers to the group —S-heterocyclyl.

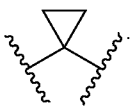
[0124] "Substituted heterocyclylthio" refers to the group —S-(substituted heterocyclyl).

[0125] Examples of heterocycle and heteroaryls include, but are not limited to, azetidine, pyrrole, imidazole, pyrazole, pyridine, pyrazine, pyrimidine, pyridazine, indolizine, isoindole, indole, dihydroindole, indazole, purine, quinolizine, isoquinoline, quinoline, phthalazine, naphthylpyridine, quinoxaline, quinazoline, cinnoline, pteridine, carbazole, carboline, phenanthridine, acridine, phenanthroline, isothiazole, phenazine, isoxazole, phenoxazine, phenothiazine, imidazolidine, imidazoline, piperidine, piperazine, indoline, phthalimide, 1,2,3,4-tetrahydroisoquinoline, 4,5,6,7-tetrahydrobenzo[b]thiophene, thiazole, thiazolidine, thiophene, benzo[b]thiophene, morpholinyl, thiomorpholinyl (also referred to as thiamorpholinyl), 1,1-dioxothiomorpholinyl, piperidinyl, pyrrolidine, and tetrahydrofuranyl.

[0126] "Nitro" refers to the group —NO_2—.

[0127] "Oxo" refers to the atom (═O) or (—O^-).

[0128] "Spirocyclyl" refers to divalent saturated cyclic group from 3 to 10 carbon atoms having a cycloalkyl or heterocyclyl ring with a spiro union (the union formed by a single atom which is the only common member of the rings) as exemplified by the following structure:



[0129] "Sulfonyl" refers to the divalent group —$S(O)_2$—.

[0130] "Substituted sulfonyl" refers to the group —SO_2-alkyl, —SO_2-substituted alkyl, —SO_2-alkenyl, —SO_2-substituted alkenyl, —SO_2-cycloalkyl, —SO_2-substituted cylcoalkyl, —SO_2-cycloalkenyl, —SO_2-substituted cycloalkenyl, —SO_2-aryl, —SO_2-substituted aryl, —SO_2-heteroaryl, —SO_2-substituted heteroaryl, —SO_2-heterocyclic, —SO_2-substituted heterocyclic, wherein alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, cycloalkyl, substituted cycloalkyl, cycloalkenyl, substituted cycloalkenyl, aryl, substituted aryl, heteroaryl, substituted heteroaryl, heterocyclic and substituted heterocyclic are as defined herein. Substituted sulfonyl includes groups such as methyl-SO_2—, phenyl-SO_2—, and 4-methylphenyl-SO_2—.

[0131] "Sulfonyloxy" refers to the group —OSO_2-alkyl, —OSO_2-substituted alkyl, —OSO_2-alkenyl, —OSO_2-substituted alkenyl, —OSO_2-cycloalkyl, —OSO_2-substituted cylcoalkyl, —OSO_2-cycloalkenyl, —OSO_2-substituted cycloalkenyl, —OSO_2-aryl, —OSO_2-substituted aryl, —OSO_2-heteroaryl, —OSO_2-substituted heteroaryl, —OSO_2-heterocyclic, —OSO_2-substituted heterocyclic, wherein alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, cycloalkyl, substituted cycloalkyl, cycloalkenyl, substituted cycloalkenyl, aryl, substituted aryl, heteroaryl, substituted heteroaryl, heterocyclic and substituted heterocyclic are as defined herein.

[0132] "Thioacyl" refers to the groups H-C(S)—, alkyl-C(S)—, substituted alkyl-C(S)—, alkenyl-C(S)—, substituted alkenyl-C(S)—, alkynyl-C(S)—, substituted alkynyl-C(S)—, cycloalkyl-C(S)—, substituted cycloalkyl-C(S)—, cycloalkenyl-C(S)—, substituted cycloalkenyl-C(S)—, aryl-C(S)—, substituted aryl-C(S)—, heteroaryl-C(S)—, substituted heteroaryl-C(S)—, heterocyclic-C(S)—, and substituted heterocyclic-C(S)—, wherein alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, cycloalkyl, substituted cycloalkyl, cycloalkenyl, substituted cycloalkenyl, aryl, substituted aryl, heteroaryl, substituted heteroaryl, heterocyclic and substituted heterocyclic are as defined herein.

[0133] "Thiol" refers to the group —SH.

[0134] "Thiocarbonyl" refers to the divalent group —C(S)— which is equivalent to —C(═S)—.

[0135] "Thione" refers to the atom (═S).

[0136] "Alkylthio" refers to the group —S-alkyl wherein alkyl is as defined herein.

[0137] "Substituted alkylthio" refers to the group —S-(substituted alkyl) wherein substituted alkyl is as defined herein.

[0138] The term "protected" or a "protecting group" with respect to hydroxyl groups, amine groups, and sulfhydryl groups refers to forms of these functionalities which are protected from undesirable reaction with a protecting group known to those skilled in the art such as those set forth in Protective Groups in Organic Synthesis, Greene, T. W. , Solon Wiley & Sons, New York, N.Y., (1st Edition, 1981) which can be added or removed using the procedures set forth therein. Examples of protected hydroxyl groups include, but are not limited to, silyl ethers such as those obtained by reaction of a hydroxyl group with a reagent such as, but not limited to, t-butyldimethyl-chlorosilane, trimethylchlorosilane, triisopropylchlorosilane, triethylchlorosilane; substituted methyl and ethyl ethers such as, but not limited to methoxymethyl ether, methythiomethyl ether, benzyloxymethyl ether, t-butoxymethyl ether, 2-methoxyethoxymethyl ether, tetrahydropyranyl ethers, 1-ethoxyethyl ether, allyl ether, benzyl ether; esters such as, but not limited to, benzoylformate, formate, acetate, trichloroacetate, and trifluoracetate. Examples of protected amine groups include,

but are not limited to, benzyl or dibenzyl, amides such as, fonnamide, acetamide, trifluoroacetamide, and benzamide; imides, such as phthalimide, BOC (tBoc), FMOC, and dithiosuccinimide; and others. In some embodiments, a protecting group for amines is an FMOC group. Examples of protected sulfhydryl groups include, but are not limited to, thioethers such as S-benzyl thioether, and S-4-picolyl thioether; substituted S-methyl derivatives such as hemithio, dithio and aminothio acetals; and others.

[0139] "Stereoisomer" or "stereoisomers" refer to compounds that differ in the chirality of one or more stereocenters. Stereoisomers include enantiomers and diastereomers.

[0140] "Tautomer" refer to alternate forms of a compound that differ in the position of a proton, such as enol-keto and imine-enamine tautomers, or the tautomeric forms of heteroaryl groups containing a ring atom attached to both a ring —NH— moiety and a ring =N— moeity such as pyrazoles, imidazoles, benzimidazoles, triazoles, and tetrazoles.

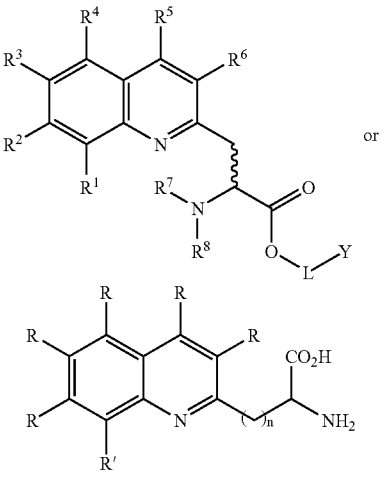
[0141] The term "affinity" as used herein refers to the strength of the binding interaction of two molecules, such as a metal-chelating compound and a metal ion.

[0142] The term "detectable response" as used herein refers to an occurrence of, or a change in, a signal that is directly or indirectly detectable either by observation or by instrumentation. Typically, the detectable response is an optical response resulting in a change in the wavelength distribution patterns or intensity of absorbance or fluorescence or a change in light scatter, fluorescence lifetime, fluorescence polarization, or a combination of these parameters. Alternatively, the detectable response is an occurrence of a signal wherein the dye is inherently fluorescent and does not produce a change in signal upon binding to a metal ion or phosphorylated target molecule. Alternatively, the detectable response is the result of a signal, such as color, fluorescence, radioactivity or another physical property of the detectable label becoming spatially localized in a subset of a sample such as in a gel, on a blot, or an array, in a well of a micoplate, in a microfluidic chamber, or on a microparticle as the result of formation of a ternary complex of the invention that comprises a phosphorylated target molecule.

[0143] The term "enzyme" as used herein refers to a protein molecule produced by living organisms, or through chemical modification of a natural protein molecule, that catalyzes chemical reaction of other substances without itself being destroyed or altered upon completion of the reactions. Examples of other substances, include, but are not limited to chemiluminescent, chromogenic and fluorogenic substances or protein-based substrates.

[0144] A "kinase" is an enzyme capable of phosphorylating a substrate. Due to the extreme versatility of the methods and components described herein, the present invention finds use in the detection of activity from all kinases. Preferred kinases for use in the present invention include but are not limited to: LCK, IRK (=INSR=Insulin receptor), IGF-1 receptor, SYK, ZAP-70, IRAK1, BLK, BMX, BTK, FRK, FGR, FYN, HCK, ITK, LYN, TEC, TXK, YES, ABL, SRC, EGF-R (=ErbB-1), ErbB-2 (=NEU=HER.sup.2), ErbB-4, FAK, FGF1R (=FGR-1), FGF2R (=FGR-2), IKK-1 (=IKK-ALPHA=CHUK), IKK-2 (=IKK-BETA), MET (=c-MET), NIK, PDGF receptor ALPHA, PDGF receptor BETA, TIE1, TIE2 (=TEK), VEGFR1 (=FLT-1), VEGFR2 (=KDR), FLT-3, FLT-4, KIT, CSK, JAK1, JAK2, JAK3, TYK2, RIP, RIP-2, LOK, TAKI, RET, ALK, MLK3, COT, TRKA, PYK2, Activin-like Kinases (Alk1-7), EPHA(1-8), EPHB(1-6), RON, GSK3(A and B), Ilk, PDK1, SGK, Fcs, Fer, MatK, Ark(1-3), Plk(1-3), LimK(1 and 2), RhoK, Pak (1-3), Raf(A, B, and C), PknB, CDK(1-10), Chk(1 and 2), CamK(I-IV), CamKK, CK1, CK2, PKR, Jnk(1-3), EPHB4, UL13, ORF47, ATM, PKA (.alpha., .beta. and .gamma.), P38(.alpha., .beta., and .gamma.), Erk(1-3), PKB (including all PKB subtypes) (=AKT-1, AKT-2, AKT-3), and PKC (including all PKC subtypes) and all subtypes of these kinases. Preferred kinases of the present invention are tyrosine and/or serine/threonine kinases.

[0145] The term "kinase activity sensor" refers to a compound or composition comprising at least one peptide substrate capable of being phosphorylated, a chelator and a detection moiety, wherein the detection moiety is the chelator per se or includes an additional detection moiety, such as a fluorophore. A preferred kinase activity sensor of the present invention has the formula:



[0146] wherein the variables are described herein.

[0147] The term "fluorophore" as used herein refers to a composition that is inherently fluorescent or demonstrates a change in fluorescence upon binding to a biological compound or metal ion, i.e., fluorogenic. Fluorophores may contain substitutents that alter the solubility, spectral properties or physical properties of the fluorophore. Numerous fluorophores are known to those skilled in the art and include, but are not limited to coumarin, cyanine, benzofuran, a quinoline, a quinazolinone, an indole, a benzazole, a borapolyazaindacene and xanthenes including fluoroscein, rhodamine and rhodol as well as semiconductor nanocrystals and other fluorophores described in RICHARD P. HAUGLAND, MOLECULAR PROBES HANDBOOK OF FLUORESCENT PROBES AND RESEARCH CHEMICALS (10th edition, 2005).

[0148] The term "linker" refers to a divalent moiety capable of linking two particles, either as a single covalent bond or a series of stable covalent bonds incorporating 1-30 nonhydrogen atoms selected from the group consisting of C,

N, O, S and P that covalently attach the fluorophore, quinoline group and amino acids to form the present kinase activity sensor. Exemplary linking members include a moiety that includes —C(O)NH—, —C(O)O—, —NH—, —S—, —O—, and the like. Exemplary linkers include covalent bonds, amino acids, succinimidyl derivatives, methines, and alkenyl groups, alkyl and substituted alkyl groups, ethyleyene, proypylene, polyethylene, and polypropylene glycols, esters, ethers, amides, carbamates, and carbonyl containing moieties, diones, squarate, adipic acid as well as other groups, such as those described in *Chemistry of Protein Conjugation and Cross-Linking* by Susan Wong.

[0149] A "cleavable linker" is a linker that has one or more cleavable groups that may be broken by the result of a reaction or condition. The term "cleavable group" refers to a moiety that allows for release of a portion, e.g., a reporter molecule, carrier molecule or solid support, of a conjugate from the remainder of the conjugate by cleaving a bond linking the released moiety to the remainder of the conjugate. Such cleavage is either chemical in nature, or enzymatically mediated. Exemplary enzymatically cleavable groups include natural amino acids or peptide sequences that end with a natural amino acid.

[0150] The present invention relates to methods for detecting and/or measuring the activity of a specific kinase. "Specific kinase" is used to indicate a known kinase of interest, where at least some information about the activity and/or structure is known to the practitioner prior to implementing the methods of the present invention.

[0151] The methods comprise contacting a sample comprising one or more kinases with a binding agent to isolate the specific kinase of interest. The one or more kinases may be present in any environment including but not limited to, a cell, a tissue, a body fluid, a buffer solution or another synthetic composition or fluid. Where more than one type of kinase is present, the collection of kinases represents a plurality or population of kinases. Not every member of the population of kinases need be a specific kinase, as understood herein. Rather, the sample, which could comprise a population of different kinases, should comprise or may be suspected to comprise the specific kinase being assayed.

[0152] The specific kinase must then be exposed to or contacted with a binding agent capable of specifically binding the specific kinase. As used herein, the term binding agent is used to mean a composition that binds specifically to the known biomarker. Examples of binding agents include, but are not limited to, receptors, antibodies and functional fragments thereof. As used herein, the term "antibody" is used to mean immunoglobulin molecules and functional fragments thereof, regardless of the source or method of producing the fragment. As used herein, a "functional fragment" of an immunoglobulin is a portion of the immunoglobulin molecule that specifically binds to a binding target. Thus, the term "antibody" as used herein encompasses whole antibodies, such as antibodies with isotypes that include but are not limited to IgG, IgM, IgA, IgD, IgE and IgY. Whole antibodies may be monoclonal or polyclonal, and they may be humanized or chimeric. The term "monoclonal antibody" as used herein is not limited to antibodies produced through hybridoma technology. Rather the term "monoclonal antibody" refers to an antibody that is derived from a single clone, including any eukaryotic, prokaryotic, or phage clone, and not the method by which it is produced. The term "antibody" also encompasses functional fragments of immunoglobulins, including but not limited to Fab fragments, Fab' fragments, $F(ab')_2$ fragments and Fd fragments. "Antibody" also encompasses fragments of immunoglobulins that comprise at least a portion of a V_L and/or V_{II} domain, such as single chain antibodies, a single-chain Fv (scFv), disulfide-linked Fvs and the like.

[0153] The antibodies used in the present invention may be monospecific, bispecific, trispecific or of even greater multispecificity. In addition the antibodies may be monovalent, bivalent, trivalent or of even greater multivalency. Furthermore, the antibodies of the invention may be from any animal origin including, but not limited to, birds and mammals. In specific embodiments, the antibodies are human, murine, rat, sheep, rabbit, goat, guinea pig, horse, or chicken. As used herein, "human" antibodies include antibodies having the amino acid sequence of a human immunoglobulin and include antibodies isolated from human immunoglobulin libraries or from animals transgenic for one or more human immunoglobulin and that do not express endogenous immunoglobulins, as described in U.S. Pat. No. 5,939,598, which is herein incorporated by reference.

[0154] The antibodies used in the present invention may be described or specified in terms of the epitope(s) or portion(s) of a polypeptide to which they recognize or specifically bind. Or the antibodies may be described based upon their ability to bind to specific conformations of the antigen. In one embodiment, a single antibody used in the methods of the present invention is specific towards an epitope presented on the specific kinase of interest.

[0155] The specificity of the antibodies used in present invention may also be described or specified in terms their binding affinity towards the antigen (epitope) or of by their cross-reactivity. Specific examples of binding affinities encompassed in the present invention include but are not limited to those with a dissociation constant (Kd) less than 5×10^{-2} M, 10^{-2} M, 5×10^{-3} M, 10^{-3} M, 5×10^{-4} M, 10^{-4} M, 5×10^{-5} M, 10^{-5} M, 5×10^{-6} M, 10^{-6} M, 5×10^{-7} M, 10^{-7} M, 5×10^{-8} M, 10^{-8}M, 5×10^{-9} M, 10^{-9} M, 5×10^{-10} M, 10^{-10} M,5×10^{-11} M, 10^{-11} M, 5×10^{-12} M, 10^{-12} M, 5×10^{-13} M, 10^{-13} M, 5×10^{-14} M, 10^{-14} M, 5×10^{-15} M, or 10^{-15} M.

[0156] The antibodies used in the invention also include derivatives that are modified, for example, by covalent attachment of any type of molecule to the antibody such that covalent attachment does not prevent the antibody from generating an anti-idiotypic response. Examples of modifications to antibodies include but are not limited to, glycosylation, acetylation, pegylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to a cellular ligand or other composition, such as a signaling moiety, a label etc. In addition, the antibodies may be linked or attached to solid substrates, such as, but not limited to, beads, particles, glass surfaces, plastic surfaces, ceramic surfaces, metal surfaces. Methods of attaching an antibody to a surface are described in Coligan, J E et al, Current Protocols In Immunology, Wiley Intersciences, (1993) and Harlow et al., Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, 2nd ed. (1988), which are incorporated by reference. Any of numerous chemical modifications may be carried out by known techniques, including, but not limited to, specific

chemical cleavage, acetylation, formylation, metabolic synthesis of tunicamycin and the like. Additionally, the derivative may contain one or more non-classical amino acids.

[0157] The antibodies used in the present invention may be generated by any suitable method known in the art. Polyclonal antibodies can be produced by various procedures well known in the art. For example, a kinase or an epitope on the kinase can be administered to various host animals including, but not limited to, rabbits, mice, rats, to induce the production of sera containing polyclonal antibodies specific for the antigen. Various adjuvants may be used to increase the immunological response, depending on the host species, and include but are not limited to, Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and *corynebacterium parvum*. Such adjuvants are also well known in the art. In addition, portions of the peptide chain making up the kinase may be attached to multiarm peptides known as multiple antigentic peptides (MAPs). The MAP can then be administered to an animal to generate an immune response that is highly tailored to the epitope presented on the arm or arms of MAP. Methods of generating antibodies using MAP technology are well-known in the art. Methods of generating antibodies using MAP technology is described in Tam, J P, *Proc. Natl. Acad. Sci.* USA 85: 5409, (1988) and Olive, C. et al., *Mini Rev. Med. Chem.* 1:429, (2001), which are incorporated by reference.

[0158] Monoclonal antibodies can be prepared using a wide variety of techniques known in the art including the use of hybridoma, recombinant, and phage display technologies, or a combination thereof. For example, monoclonal antibodies can be produced using hybridoma techniques including those known in the art and taught, for example, in Harlow et al., Antibodies: A Laboratory Manual, (Cold Spring Harbor Laboratory Press, 2nd ed. 1988); Hammerling, et al., in: Monoclonal Antibodies and T-Cell Hybridomas 563-681 (Elsevier, N.Y., 1981) (both of which are incorporated by reference).

[0159] Methods for producing and screening for specific antibodies using hybridoma technology are routine and well known in the art such as, but not limited to, immunizing a mouse. Once an immune response is detected, the mouse spleen is harvested and splenocytes isolated. The splenocytes are then fused by well known techniques to any suitable myeloma cells, for example cells from cell line SP20 available from the ATCC. Hybridomas are selected and cloned by limited dilution. The hybridoma clones can then be assayed by methods known in the art for cells that secrete antibodies capable of binding a biomarker of the present invention. Ascites fluid, which generally contains high levels of antibodies, can be generated by immunizing mice with positive hybridoma clones.

[0160] The antibodies used in the present invention can also be generated using various phage display methods known in the art. In phage display methods, functional antibody domains are displayed on the surface of phage particles which carry the polynucleotide sequences encoding them. In a particular embodiment, such phage can be utilized to display antigen binding domains expressed from a repertoire or combinatorial antibody library. Phage expressing an antigen binding domain that binds the antigen of interest can be selected or identified with the antigen of interest, such as using a labeled antigen or antigen bound or captured to a solid surface or bead. The phage used in these methods are typically filamentous phage including, but not limited to, fd and M13 binding domains expressed from phage with Fab, Fv or disulfide stabilized Fv antibody domains recombinantly fused to either the phage gene III or gene VIII protein. Examples of phage display methods that can be used to make the antibodies of the present invention include those disclosed in Brinkman et al., *J. Immunol. Methods* 182:41-50 (1995); Ames et al., *J. Immunol Methods* 184:177-186 (1995); Kettleborough et al., *Eur. J. Immunol.* 24:952-958 (1994); Persic et al., *Gene* 187 9-18 (1997); Burton et al., *Advances in Immunology* 57:191-280 (1994); PCT application No. PCT/GB91/01134; PCT publications WO 90/02809; WO 91/10737; WO 92/01047; WO 92/18619; WO 93/11236; WO 95/15982; WO 95/20401; and U.S. Pat. Nos. 5,698,426; 5,223,409; 5,403,484; 5,580,717; 5,427,908; 5,750,753; 5,821,047; 5,571,698; 5,427,908; 5,516,637; 5,780,225; 5,658,727; 5,733,743 and 5,969,108, all of which are incorporated by reference.

[0161] Antibody fragments which recognize specific epitopes may be generated by known techniques. For example, Fab and $F(ab')_2$ fragments of the invention may be produced by proteolytic cleavage of immunoglobulin molecules, using enzymes such as papain (to produce Fab fragments) or pepsin (to produce $F(ab')_2$ fragments). $F(ab')_2$ fragments contain the variable region, the light chain constant region and the CH1 domain of the heavy chain.

[0162] Other methods, such as recombinant techniques, may be used to produce Fab, Fab' and $F(ab')_2$ fragments and are disclosed in PCT publication WO 92/22324; Mullinax et al., BioTechniques 12(6):864-869 (1992); and Sawai et al., AJRI 34:26-34 (1995); and Better et al., Science 240:1041-1043 (1988), which are herein incorporated by reference. After phage selection, for example, the antibody coding regions from the phage can be isolated and used to generate whole antibodies, including human antibodies, or any other desired antigen binding fragment, and expressed in any desired host, including mammalian cells, insect cells, plant cells, yeast, and bacteria.

[0163] Examples of techniques which can be used to produce other types of fragments, such as scFvs and include those described in U.S. Pat. Nos. 4,946,778 and 5,258,498; Huston et al, *Methods in Enzymology* 203:46-88 (1991); Shu et al., *Proc. Nat'l Acad. Sci. (USA)* 90:7995-7999 (1993); and Skerra et al., *Science* 240:1038-1040 (1988), all of which are incorporated by reference. For some uses, including in vivo use of antibodies in humans and in vitro detection assays, it may be preferable to use chimeric, humanized, or human antibodies. A chimeric antibody is a molecule in which different portions of the antibody are derived from different animal species, such as antibodies having a variable region derived from a murine monoclonal antibody and a human immunoglobulin constant region. Methods for producing chimeric antibodies are known in the art. See e.g., Morrison, *Science* 229:1202 (1985); Oi et al., *BioTechniques* 4:214 (1986); Gillies et al., *J. Immunol. Methods* 125:191-202(1989); U.S. Pat. Nos. 5,807,715; 4,816,567; and 4,816,397, all of which are herein incorporated by reference.

Humanized antibodies are antibody molecules from non-human species antibody that binds the desired antigen having one or more complementarity determining regions (CDRs) from the non-human species and framework regions from a human immunoglobulin molecule. Often, framework residues in the human framework regions will be substituted with the corresponding residue from the CDR donor antibody to alter, preferably improve, antigen binding. These framework substitutions are identified by methods well known in the art, e.g., by modeling of the interactions of the CDR and framework residues to identify framework residues important for antigen binding and sequence comparison to identify unusual framework residues at particular positions. (See U.S. Pat. No. 5,585,089; Riechmann et al., *Nature* 332:323 (1988), both of which are herein incorporated by reference. Antibodies can be humanized using a variety of techniques known in the art including, for example, CDR-grafting (EP 239,400; PCT publication WO 91/09967; U.S. Pat. Nos. 5,225,539; 5,530,101; and 5,585,089), veneering or resurfacing (EP 592,106; EP 519,596; Padlan, *Molecular Immunology* 28(4/5):489-498 (1991); Studnicka et al., *Protein Engineering* 7(6):805-814 (1994); Roguska. et al., *Proc. Nat'l. Acad. Sci.* 91:969-913 (1994)), and chain shuffling (U.S. Pat. No. 5,565,332), all of which are hereby incorporated by reference.

[0164] Completely human antibodies may be particularly desirable for therapeutic treatment or diagnosis of human patients. Human antibodies can be made by a variety of methods known in the art including phage display methods described above using antibody libraries derived from human immunoglobulin sequences. See also. U.S. Pat. Nos. 4,444,887 and 4,716,111; and PCT publications WO 98/46645, WO 98/50433, WO 98/24893, WO 98/16654, WO 96/34096, WO 96/33735, and WO 91/10741; each of which is incorporated by reference.

[0165] Human antibodies can also be produced using transgenic mice which are incapable of expressing functional endogenous immunoglobulins, but which can express human immunoglobulin genes. For example, the human heavy and light chain immunoglobulin gene complexes may be introduced randomly or by homologous recombination into mouse embryonic stem cells. Alternatively, the human variable region, constant region, and diversity region may be introduced into mouse embryonic stem cells in addition to the human heavy and light chain genes. The mouse heavy and light chain immunoglobulin genes may be rendered non-functional separately or simultaneously with the introduction of human immunoglobulin loci by homologous recombination. In particular, homozygous deletion of the JH region prevents endogenous antibody production. The modified embryonic stem cells are expanded and microinjected into blastocysts to produce chimeric mice. The chimeric mice are then bred to produce homozygous offspring which express human antibodies. The transgenic mice are immunized in the normal fashion with a selected antigen. Monoclonal antibodies directed against the antigen can be obtained from the immunized, transgenic mice using conventional hybridoma technology. The human immunoglobulin transgenes harbored by the transgenic mice rearrange during B cell differentiation, and subsequently undergo class switching and somatic mutation. Thus, using such a technique, it is possible to produce therapeutically useful IgG, IgA, IgM and IgE antibodies. For an overview of this technology for producing human antibodies, see Lonberg and Huszar, *Int. Rev. Immunol* 13:65-93 (1995), which is hereby incorporated by reference. For a detailed discussion of this technology for producing human antibodies and human monoclonal antibodies and protocols for producing such antibodies, see, e.g., PCT publications WO 98/24893; WO 92/01047; WO 96/34096; WO 96/33735; European Patent No. 0 598 877; U.S. Pat. Nos. 5,413,923; 5,625,126; 5,633,425; 5,569,825; 5,661,016; 5,545,806; 5,814,318; 5,885,793; 5,916,771; and 5,939,598, which are incorporated by reference.

[0166] Still another approach for generating human antibodies utilizes a technique referred to as guided selection. In guided selection, a selected non-human monoclonal antibody, e.g., a mouse antibody, is used to guide the selection of a completely human antibody recognizing the same epitope. (Jespers et al., *Biotechnology* 12:899-903 (1988), herein incorporated by reference).

[0167] The isolated kinase is then contacted with a kinase activity sensor. As used herein, a kinase activity sensor is a composition comprising a kinase recognition motif with at least one phosphorylation site. The phosphorylation site may or may not be present within the kinase recognition motif of the kinase activity sensor. In one embodiment, the phosphorylation site of is an amino acids reside that is capable being phosphorylated. Thus, in aspect the kinase activity sensor comprises a peptide that is capable of being phosphroylated. Examples of amino acids capable of being phosphorylated include, but are not limited to, naturally occurring hydroxyl-containing amino acid residues, such as serine, threonine and tyrosine, and non-naturally occuring hydroxyl-containing amino acid residues.

Kinase Recognition Motif:

[0168] Any kinase recognition motif can be used in accordance with the present invention. While still functional, kinase recognition motifs with acidic residues may be less desirable because of the acidic conditions that these residues may create during the assay. Examples of recognition motifs that can be used in the kinase activity sensors include but are not limited to, the motifs shown in Table I:

TABLE I

SEQ ID NO.	Kinase	Kinase Recognition Motif
1	Protein Kinase C (PKC)	TFRRR
2	Protein Kinase C (PKC)	SFRRR
3	Cyclic-AMP Dependent Kinase (PKA)	LRRASL
4	Cyclic-AMP Dependent Kinase (PKA)	LRRATL
5	Abelson Kinase (Abl)	IYAAPF

[0169] Other isolated kinase recognition motifs include but are not limited to, the recognition motifs in Table II, Table III and Table IV.

[0170]

TABLE II

SEQ ID NO.	Kinase Name	Kinase Recognition Motif
6	Akt1-3, CHK1, DAPK3, MLK2, MSK1/2, p70S6K, PAK1/2/4, PKA, PKG, RSK1-3, SGK1-3	ARKRERAYSF(dP) and ARKRERAY(pS)F(dP)
7	AKT2, AuroraB, MLK2, MSK1, PAK1/2/4, PASK, PKA, PKG, PRKX, RSK2, SGK1-3	LRRASL(dP) and LRRA(pS)L(dP)
8	CHK1/2, CHK, MK2/3/5, PAK1, PHKG2, PKD	AHLQRQLSI(dP) and AHLQRQL(pS)I(dP)
9	Akt1-3, AuroaB, MSK1/2, PKA, PRKX, Rock1/2, RSK1-3, SGK1-3, MLK2, PAK1-4/7	GRPRTSSF(dP) and GRPRTS(pS)F(dP)
10	Akt1/2, MLK2, MSK1/2, p70S6K, PAK1-4/7, PKA, PKG, PRKX, RSK1-3, SGK1-3	GRTGRRNSI(dP) and GRTGRRN(pS)I(dP)
11	Akt1-3, MLK2, MSK1, PAK1/2, PASK, PKG, SGK1-3	KKRNRTLSV(dP) and KKRNRTL(pS)V(dP)
12	CSF1R, FLT3, c-Met, PDGFRa/b; Ab1, JAK2/3, Lck	PGIYAAPFAKKK and PGI(pY)AAPFAKKK
13	FYN, Src	PGIYGELEA and PGI(pY)GELEA
14	EPHA4, EPHB2, VEGFR2; FAK, Syk	ELEDDYED(dP) and ELEDD(pY)ED(dP)
15	ALK, FLT3, IGF1R, IR, TrkA; FER	PGAYGWLDF and PGA(pY)GWLDF
16	CDK1, 2, 5	PGTPKKAKKL and PG(pT)PKKAKKL
17	ERK1 & ERK2; p38 gamma	PRTPGGRR and PR(pT)PGGRR
18	p38 beta, gamma, ERK1	PGTPSGEAPNQALLR and PG(pT)PSGEAPNQALLR
8	ERK1 & ERK2	AHLQRQLSd(dP)and AHLQRQL(pS)I(dP)
8	p38 alpha (vs. beta, gamma, delta)	AHLQRQLSI(dP) and AHLQRQL(pS)I(dP)
19	PDK1	ARKRERAYSF(dP) and ARKRERAY(pS)F(dP)
8	MEK1	AHLQRQLSI(dP) and AHLQRQL(pS)I(dP)
8	MEK2	AHLQRQLSI(dP) and AHLQRQL(pS)I(dP)
8	c-Raf	AHLQRQLSI(dP) and AHLQRQL(pS)I(dP)
8	b-Raf	AHLQRQLSI(dP) and AHLQRQL(pS)I(dP)
8	MEKK1	AHLQRQLSI(dP) and AHLQRQL(pS)I(dP)

TABLE III

SEQ ID NO.	Sequence
20	Ac-ARKRERAYSFdPSxG-NH2
21	Ac-LRRASLdPSxG-NH2
22	Ac-AHLQRQLSIdPSxG-NH2
23	Ac-GRPRTSSFdPSxG-NH2
24	Ac-GRTGRRNSIdPSxG-NH2
25	Ac-KKRNRTLSVdPSxG-NH2
26	Ac-SxPKTPKKAKKL-NH2
27	Ac-SxPGSFRRR-NH2
28	Ac-SxPRTPGGRR-NH2
29	Ac-SxPGTPSGEAPNQALLR-NH2
30	Ac-ALKLSRYPSFdPSxG-NH2
31	Ac-GDQDYLSLdPSxG-NH2
32	Ac-SxPGSRRPpSYR-NH2
33	Ac-RRRQFSLdPSxG-NH2
34	Ac-ERMRPRKRQGSVdPSxG-NH2
35	Ac-ALRRFSLdPSxG-NH2
36	Ac-SxPLSPGPF-NH2
37	Ac-KRRRLASLdPSxG-NH2
38	Ac-DRHDSGLDSMdPSxG-NH2
39	Ac-DRHDpSGLDSMdPSxG-NH2
40	Ac-HAAIGDDDDAYSIdPSxG-NH2
41	Ac-SxPGSDDDDD-NH2
42	Ac-SxPGIYAAPFAKKK-NH2
43	Ac-SxPGIYGELEA-NH2
44	Ac-ELEDDYEDdPSxG-NH2
45	Ac-SxPGAYGWLDF-NH2
46	Ac-EAEAIYAAdPSxG-NH2
47	Ac-KKGEAIYAAdPSxG-NH2
48	Ac-EEEEYIQdPSxG-NH2
49	Ac-ESSDDYVNdPSxG-NH2

[0171]

TABLE IV

SEQ ID NO.	Sequence
50	Ac-SxPGLSPGPF-NH2
51	Ac-SxPGTTPITTTYF-NH2
52	Ac-AEEEIYGEdPSxG-NH2
53	Ac-SxPGEIYGELEA-NH2
54	Ac-RFARKGSLdPSxG-NH2
55	Ac-SxPGLTPSGEAPN-NH2
56	Ac-SxPGASFRGHMAR-NH2
57	Ac-RARRRLSFdPSxG-NH2
58	Ac-SxPGSPGPF-NH2
59	Ac-SxPGTPITTYFFFK-NH2
60	Ac-SxPGSFRGHMAR-NH2
61	Ac-SxPGSPGRRRK-NH2
62	Ac-SxPGTPKKAKKL-NH2
63	Ac-SxPGTPGGRR-NH2
64	Ac-EEEYEEdPSxG-NH2
65	Ac-SxPGEYEEEE-NH2
66	Ac-SxPGIYETDYYRKG-NH2
67	Ac-SxPGDIYETDFFRKG-NH2
68	Ac-SxPDIYETDFFRKG-NH2
69	Ac-LMTGDTYTAdPSxG-NH2
70	Ac-VSETDDYAEdPSxG-NH2
71	Ac-SxPGDYAEIIDEED-NH2
72	Ac-SxPGTSDFQKLKRKY-COOH
73	Ac-KKALRRQETVdPSxG-NH2
74	Ac-EVIEASFdPSxG-NH2
75	Ac-SxPGSFAEQEA-NH2
76	Ac-SxPGSPLRGPPK-NH2
77	Ac-SxPGSVPPpSPD-NH2
78	Ac-SxPGSRTPpSLPTPPTREPK-NH2
79	Ac-SxPGSPHQpSEDEEE-NH2
80	Ac-SxPGSPSLpSRHSSPH-NH2
81	Ac-SxPGSLVGpTPYWMAPE-NH2
82	Ac-KKRFSFKKSFdPSxG-NH2
83	Ac-SxPGIYAAPGD-NH2
84	Ac-SxPGIYGVIE-NH2
85	Ac-SxPGIYFELVAK-NH2

TABLE IV-continued

SEQ ID NO.	Sequence
86	Ac-SxPGDYVNVPESGEK-NH2
87	Ac-ALKRASLdPSxG-NH2
88	Ac-AKRRRLSSLdPSxG-NH2
89	Ac-EKNGKKARKSLdPSxG-NH2
90	Ac-VPKQKRKSVdPSxG-NH2
91	Ac-AMARAASAdPSxG-NH2
92	Ac-APSSRRTTLdPSxG-NH2
93	Ac-DSDVHVNATYVNdPSxG-NH2
94	Ac-EEEIYFEdPSxG-NH2
95	Ac-EYCPDPLYEVdPSxG-NH2
96	Ac-KKKKEEIYFFdPSxG-NH2
97	Ac-SxPGDYRATFPEDQFP-NH2
98	Ac-SxPGEYVNIEFG-NH2
99	Ac-SxPGHYVHVNATYVNVK-NH2
100	Ac-SxPGLYEVMLKCWHPK-NH2
101	Ac-TEMVSNESVDYRAdPSxG-NH2
102	Ac-ARRRGVTTKTFdPSxG-NH2
103	Ac-GVTTKTFdPSxG-NH2
104	Ac-SxPKTFCGTPEYLAPEVRR-NH2
105	Ac-SxPLSVSSLPGL-NH2
106	Ac-SxPDSGLDpSMKDE-NH2
107	Ac-SxPDSGLDSMKDE-NH2
108	Ac-SxPGSGLDpSMKDE-NH2
109	Ac-SxPGSGLDSMKDE-NH2
110	Ac-RDKYKTLdPSxG-NH2
111	Ac-SxPKTLRQIRQ-NH2
112	Ac-SxPRTGRGRRGIYR-NH2
113	Ac-SxPSSMVARTQTVR-NH2
114	Ac-SxPFSLRRKAK-NH2
115	Ac-PLLRDASTdPSxG-NH2
116	Ac-SxPASTRDRHA-NH2
117	Ac-KKRNRRLSVdPSxG-NH2
118	Ac-PLSRTLSVdPSxG-NH2
119	Ac-SxPASFAEQEAK-NH2
120	Ac-LKKLRRRLSDdPSxG-NH2
121	Ac-TRPRKRQGSFdPSxG-NH2

TABLE IV-continued

SEQ ID NO.	Sequence
122	Ac-RRRDDDSDdPSxG-NH2
123	Ac-RRKDLHDDEEDEAMSIdPSxG-NH2
124	Ac-RRRADDSDdPSxG-NH2
125	Ac-SxPDSDDDDD-NH2
126	Ac-SxPLSQEAFADLWKK-NH2
127	Ac-RKKFGESEKTKdPSxG-NH2
128	Ac-SxPKSWAIPNRARK-NH2
129	Ac-KKKALSRQFSVdPSxG-NH2
130	Ac-KLNRVFSVdPSxG-NH2
131	Ac-Biotin-LC-SxPKTPKKAKKL-NH2
132	Ac-KRRRALS(pS)VASLdPSxG-NH2
133	Ac-KVEKIGEGTYGVdPSxG-NH2

[0172] Reference to "Sx" indicates SOX (a kinase activity sensor described herein), "dP" indicates D-proline, "Ac" indicates acyl, —NH2 indicates amino.

[0173] A preferred embodiment of the present invention provides a composition comprising at least one of SEQ ID NO. 1-133. More particularly, the invention provides a composition comprising or consisting of SEQ ID NO. 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, or SEQ ID NO. 49. More particular still, at least one of SEQ ID NO. 1-133 is used as a kinase recognition motif described herein.

[0174] If present, the isolated kinase, will then phosphorylate the amino acid target of the kinase activity sensor. The inventors have discovered that kinases isolated and immobilized with binding agents are still active enzymes. Thus, the active specific kinase is available to phosphorylate the target amino acid residue or residues. In one embodiment, adenosine tri-phosphate (ATP) and a metal ion that has affinity for the chelator of the kinase activity sensor and the phosphorylated amino acid are added to the assay either, before, during or after, the isolated kinase is contacted with the kinase activity sensor. In a particular aspect the metal ion is magnesium, however any metal ion as described above is part of the invention.

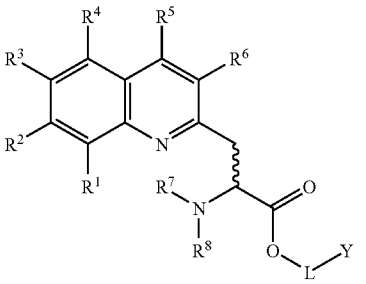
Kinase Activity Sensors:

[0175] Levels of phosphorylation activity on the phosphorylation site can then be quantified using various techniques. In one embodiment, the kinase activity sensor further comprises a signaling moiety that is capable of generating or altering a signal in response to a phosphorylation event on the target phosphorylation site. In a more specific embodiment, the signaling moiety is capable of generating a fluorescent signal. Examples of a signaling moiety include but are not limited to Fluorescent Magnesium Indicators (Molecular Probes, Eugene, Oreg., USA) and metal-binding amino acids that fluoresce upon a chelation event. These amino acids are described in U.S. Pat. No. 6,906,194, which is hereby incorporated by reference.

[0176] As used herein, the term metal chelator is used as it is in the art. Namely, a metal chelator is a compound that can form two or more coordination bonds with metal ion. The term "coordination bond" is also well known in the art and is used to indicate a coordinate covalent bond between the metal ion and the chelator.

[0177] The present metal-chelating moieties are moieties that simultaneously bind metal ions and have affinity for exposed phosphate groups on serine, threonine, or tyrosine residues of the kinase substrate, wherein a ternary complex is formed between the metal-chelating moiety, the metal ion and the phosphorylated serine, threonine, or tyrosine residues. Metal ions that have been found to bind phosphate groups include Ca^{2+}, Zn^{2+}, Mg^{2+}, Ga^{3+}, Tb^{3+}, La^{3+}, Pb^{2+}, Hg^{2+}, $Cd^{2|}$, $Cu^{2|}$, $Ni^{2|}$, $Co^{2|}$, $Fe^{2|}$, $Mn^{2|}$, $Ba^{2|}$, and $Sr^{2|}$. A preferred metal ion for chelation by the kinase activity sensor of the present invention is magnesium. Thus, the metal-chelating moieties must 1) bind metal ions that have affinity for phosphate groups, 2) not interfere with the binding of the metal ion for the phosphate groups and 3) maintain a stable ternary complex. Metal-chelating moieties that fit these three criteria include quinoline or a derivative thereof, phenanthrolines or derivatives thereof, BAPTA, IDA, DTPA and derivatives thereof.

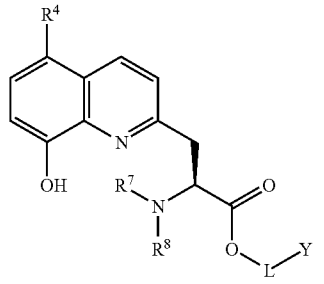
[0178] In one embodiment the present kinase activity sensor is represented by the following formula:



[0179] wherein R^1, R^2, R^3, R^4, R^5 and R^6 are independently a fluorophore, H, alkyl, substituted alkyl, alkoxy, substituted alkoxy, acyl, acylamino, acyloxy, amino, substituted amino, aminocarbonyl, aminothiocarbonyl, aminocarbonylamino, aminothiocarbonylamino, aminocarbonyloxy, aminosulfonyl, aminosulfonyloxy, aminosulfonylamino, amidino, carboxyl, carboxyl ester, (carboxyl ester)amino, (carboxyl ester)oxy, cyano, halo, hydroxy, nitro, SO_3H, sulfonyl, substituted sulfonyl, sulfonyloxy, thioacyl, thiol, alkylthio, substituted alkylthio, aryl, substituted aryl, heteroaryl, substituted heteroaryl, cycloalkyl, substituted cycloalkyl, heterocyclyl, or substituted heterocyclyl;

[0180] R^7 and R^8 are independently H, alkyl, substituted alkyl, carbonyl, or FMOC; and L is a linker; and Y is a peptide or H; or a tautomer, stereoisomer, or salt thereof.

[0181] In a preferred embodiment the kinase activity sensor has the formula:

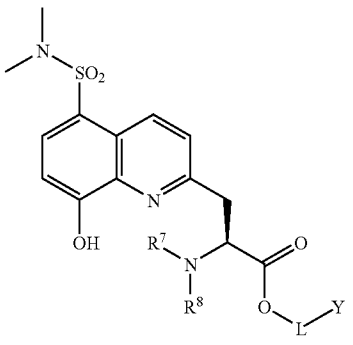


wherein,

[0182] R^4 is a sulfonamide group or a fluorophore

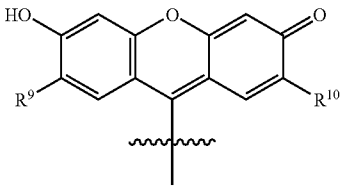
[0183] R^7 and R^8 are independently H, alkyl, substituted alkyl, carbonyl, or FMOC; and L is a linker; and Y is a peptide or H; or a tautomer, stereoisomer, or salt thereof.

[0184] In another preferred embodiment the kinase activity sensor has the formula:



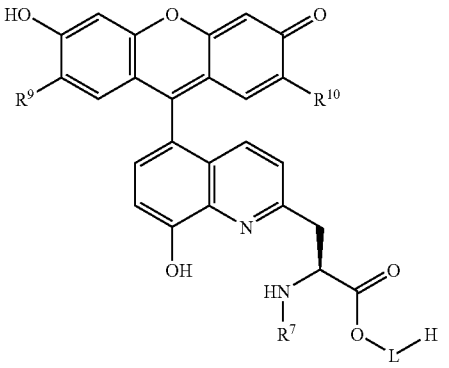
[0185] R^7 and R^8 are independently H, alkyl, substituted alkyl, carbonyl, or FMOC; and L is a linker; and Y is a peptide; or a tautomer, stereoisomer, or salt thereof.

[0186] In another preferred embodiment, the fluorophore on the kinase activity sensor has the formula:



wherein R^9 and R^{10} are each independently a halogen, —SO_3H, substituted sulfonyl, or H.

[0187] In another preferred embodiment, the kinase activity sensor has the formula



[0188] wherein R^7 is H, or a protecting group;

[0189] R^9 is a halogen, —SO_3H, substituted sulfonyl, or H;

[0190] R^{10} is a halogen, —SO_3H, substituted sulfonyl, or H;

[0191] L is a linker; and

[0192] Y is a peptide or H;

[0193] or a tautomer, stereoisomer, or salt thereof.

[0194] In a more particular embodiment of any of the foregoing, R^4 is a fluorophore. In another embodiment, the fluorophore is dansyl, xanthene, cyanine, borapolyazaindacene, pyrene, naphthalene, coumarin, oxazine, or derivatives thereof. In another embodiment the fluorophore is a coumarin, a xanthene or a derivative thereof. In another embodiment, R^1 is hydroxy. In another embodiment, R^2, R^3, R^5 and R^6 are all H. In another embodiment, R^7 is H or FMOC. In another embodiment, R^8 is H. In another embodiment, Y is a peptide. In another embodiment, the peptide comprises a kinase recognition site and at least one amino acid residue selected from serine, threonine, or tyrosine that is subject to phosphorylation by a kinase. In another embodiment, the position indicated with a ~~~ bond is in the S configuration; alternatively it is in the R-configuration; alternatively the kinase activity sensor is a racemate. In another embodiment, the salt of the kinase activity sensor is magnesium. In another embodiment, L is a covalent bond. In another embodiment, Y is H. In another embodiment, R^9 and R^{10} are both fluorine.

[0195] The present fluorophores can be any fluorophore known in the art that when conjugated to a chelating moiety are fluorogenic or essentially non-fluorescence. A fluorophore of the present invention is any chemical moiety that exhibits an absorption maximum beyond 280 nm, that when part of a kinase activity sensor compound retains its unique spectral properties to provide a detectable signal. In one embodiment the present fluorphores are a separate moiety from the chelating moiety of the kinase activity sensor. In another embodiment the chelator is also the fluorophore.

[0196] Examples of fluorophores that can be used in the present invention include, but are not limited to; a pyrene, an

anthracene, a naphthalene, an acridine, a stilbene, an indole or benzindole, an oxazole or benzoxazole, a thiazole or benzothiazole, a 4-amino-7-nitrobenz-2-oxa-1,3-diazole (NBD), a carbocyanine (including any corresponding compounds in U.S. Ser. Nos. 09/557,275; 09/968,401 and 09/969,853 and U.S. Pat. Nos. 6,403,807; 6,348,599; 5,486,616; 5,268,486; 5,569,587; 5,569,766; 5,627,027 and 6,048,982), a carbostyryl, a porphyrin, a salicylate, an anthranilate, an azulene, a perylene, a pyridine, a quinoline, a borapolyazaindacene (including any corresponding compounds disclosed in U.S. Pat. Nos. 4,774,339; 5,187,288; 5,248,782; 5,274,113; and 5,433,896), a xanthene (including any corresponding compounds disclosed in U.S. Pat. No. 6,162,931; 6,130,101; 6,229,055; 6,339,392; 5,451,343 and U.S. Ser. No. 09/922,333), an oxazine or a benzoxazine, a carbazine (including any corresponding compounds disclosed in U.S. Pat. No. 4,810,636), a phenalenone, a coumarin (including an corresponding compounds disclosed in U.S. Pat. Nos. 5,696,157; 5,459,276; 5,501,980 and 5,830,912), a benzofuran (including an corresponding compounds disclosed in U.S. Pat. Nos. 4,603,209 and 4,849,362) and benzphenalenone (including any corresponding compounds disclosed in U.S. Pat. No. 4,812,409) and derivatives thereof. As used herein, oxazines include resorufins (including any corresponding compounds disclosed in U.S. Pat. No. 5,242,805), aminooxazinones, diaminooxazines, and their benzo-substituted analogs.

[0197] In particular embodiments, the fluorophore of the present invention is selected from the group consisting of acridine, anthracene, benzofuran, indole, dansyl, cyanine, borapolyazaindacene, pyrene, naphthalene, coumarin, oxazine, boron dipyromethene difluoride, and xanthenes, including but not limited to fluorescein, rhodamine, and rhodol, and derivatives thereof Additional fluorophores that may be used in the present invention are listed in Richard P. Haugland, Molecular Probes Handbook of Fluorescent Probes and Research Chemicals (9^{th} Ed.),. In a more particular embodiment, the xanthenes are fluorescein, or rhodamine. The fluorophores may be substituted to adjust solubility, spectral or other physical properties.

[0198] Where the fluorophore is a xanthene, the fluorophore may, but need not be, a fluorescein, a rhodol (including any corresponding compounds disclosed in U.S. Pat. Nos. 5,227,487 and 5,442,045), a rosamine or a rhodamine (including any corresponding compounds in U.S. Pat. Nos. 5,798,276; 5,846,737; 5,847,162; 6,017,712; 6,025,505; 6,080,852; 6,716,979; 6,562,632). As used herein, fluorescein includes benzo- or dibenzofluoresceins, seminaphthofluoresceins, or naphthofluoresceins. Similarly, as used herein rhodol includes seminaphthorhodafluors (including any corresponding compounds disclosed in U.S. Pat. No. 4,945,171). Fluorinated xanthene fluorophores have been described previously as possessing particularly useful fluorescence properties (Int. Publ. No. WO 97/39064 and U.S. Pat. No. 6,162,931) including those sold under the tradename OREGON GREEN.

[0199] In particular the Oregon Green precursor will allow for strategic placement of a very bright and highly absorbing pH-insensitive fluorescein derivative in peptide kinase substrates. The fluorescence will initially be quenched via PET by the 8-hydroxyquinoline moiety, but chelation induced fluorescence increase, mediated by magnesium(II) ion will be observed upon phosphorylation of the appropriate Ser/Thr/Tyr residue that is separated from the dye by a beta-turn dipeptide sequence. The fluorescence (490 nm excitation/520 nm emission) increase will be similar in mechanism and magnitude to that observed upon using Fluo-4 for calcium measurements. Key synthetic steps will be regioselective formylation of the 8-hyroxyquinoline amino acid, followed by condensation of 4-fluororesorcinol and dehydrogenative oxidation. Key to the success of the invention is appropriate construction of the fluorophore-amino acid moiety, and placement of the fluorophore-amino acid moiety in three dimensional geometry such that the chelating moiety and phosphate moiety can be brought close together by an intervening metal ion. This geometric optimization is visualized by semi-empirical molecular modeling energy minimization of the PKCa peptide substrate Ac-Oregon Green alanine-Pro-Gly-Ser-Phe-Arg-Arg-Arg-NH_2. The fluorophore-substituted amino acid is placed two amino acid residues away from the serine to be phosphorylated so that the metal chelating portion of the Oregon Green alanine derivative can be oriented toward the phosphoserine moiety. The hydroxyquinoline-fluorophore moiety is constructed so that the fluorescence is quenched in the absence of metal ion chelation, and the hydroxyquinoline-amino acid connection point is also chosen for optimal geometry.

[0200] Typically the fluorophore will contain one or more aromatic or heteroaromatic rings, that are optionally substituted one or more times by a variety of substituents, including without limitation, halogen, nitro, sulfo, cyano, alkyl, perfluoroalkyl, alkoxy, alkenyl, alkynyl, cycloalkyl, arylalkyl, acyl, aryl or heteroaryl ring system, benzo, or other substituents typically present on chromophores or fluorophores known in the art.

[0201] In an exemplary embodiment, the fluorophores are independently substituted by substituents selected from the group consisting of hydrogen, halogen, amino, substituted amino, alkyl, substituted alkyl, aryl, substituted aryl, heteroaryl, substituted heteroaryl, alkoxy, sulfo, reactive group and carrier molecule. In another embodiment, the xanthene fluorophores of this invention comprise both compounds substituted and unsubstituted on the carbon atom of the central ring of the xanthene by substituents typically found in the xanthene-based fluorophores such as phenyl and substituted-phenyl moieties. In still another embodiment, the fluorophores used in the amino acids and compositions of the present invention are rhodamine, fluorescein, dansyl, naphthalene and derivatives thereof. The choice of the fluorophore attached to the chelating moiety will determine the absorption and fluorescence emission properties of the amino acids and compositions of the present invention as well as its live cell properties.

[0202] Selected sulfonated fluorophores also exhibit advantageous properties, and include sulfonated pyrenes,

coumarins, carbocyanines, and xanthenes (as described in U.S. Pat. Nos. 5,132,432; 5,696,157; 5,268,486; 6,130,101). Sulfonatedpyrenes and coumarins are typically excited at wavelengths below about 450 nm (U.S. Pat. Nos. 5,132,432 and 5,696,157).

[0203] In one embodiment, the label is a fluorophore selected from the group consisting of fluorescein, coumarins, rhodamines, 5-TMRIA (tetramethylrhodamine-5-iodoacetamide), (9-(2(or 4)-(N-(2-maleimdylethyl)-sulfonamidyl)-4 (or 2)-sulfophenyl)-2,3,6,7,12,13,16,17-octahydro-(1H,5H,11H,15H-xantheno(2,3,4-ij:5,6,7-i'j')diquinolizin-18-ium salt) (Texas Red®), 2-(5-(1-(6-(N-(2-maleimdylethyl)-amino)-6-oxohexyl)-1,3-dihydro-3,3-dimethyl-5-sulfo-2H-indol-2-ylidene)-1,3-propyldienyl)-1-ethyl-3,3-dimethyl-5-sulfo-3H-indolium salt (Cy™3), N,N'-dimethyl-N-(iodoacetyl)-N'-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)ethylenediamine (IANBD amide), 6-acryloyl-2-dimethylaminonaphthalene (acrylodan), pyrene, 6-amino-2,3-dihydro-2-(2-((iodoacetyl)amino)ethyl)-1,3-dioxo-1H-benz(de)isoquinoline-5,8-disulfonic acid salt (lucifer yellow), 2-(5-(1-(6-(N-(2-maleimdylethyl)-amino)-6-oxohexyl)-1,3-dihydro-3,3-dimethyl-5-sulfo-2H-indol-2-ylidene)-1,3-pentadienyl)-1-ethyl-3,3-dimethyl-5-sulfo-3H-indolium salt (Cy™5), 4-(5-(4-dimethylaminophenyl)oxazol-2-yl)phenyl-N-(2-bromoacetamidoethyl)sulfonamide (Dapoxyl® (2-bromoacetamidoethyl)sulfonamide)), (N-(4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacene-2-yl)iodoacetamide (BODIPY® 507/545 IA), N-(4,4-difluoro-5,7-diphenyl-4-bora-3a,4a-diaza-s-indacene-3-propionyl)-N'-iodoacetylethylenediamine (BODIPY 530/550 IA), 5-((((2-iodoacetyl)amino)ethyl)amino)naphthalene-1-sulfonic acid (1,5-IAEDANS), and carboxy-X-rhodamine, 5/6-iodoacetamide (XRIA 5,6). Another example of a label is BODIPY-FL-hydrazide.

[0204] In an exemplary embodiment, the fluorophore has a Stokes shift larger than about 50 nm. In particularly useful embodiments, the fluorophore has a Stokes shift larger than about 100 nm or larger than about 150 nm. In still more embodiments, the present compounds have a Stokes shift larger than about 200 nm or larger than about 250 nm. The term "Stokes shift" as used herein refers to the difference in wavelength between absorbed and emitted energy. Specifically, the Stokes shift is the difference (usually in frequency units) between the spectral positions and the band maxima (or band origin) of the absorption and luminescence arising from the same electronic transitions.

[0205] To facilitate correct kinase substrate recognition motifs, S/T or Y peptide substrate sampler plates with 14 substrates (1 substrate per well and 6 wells per substrate) dried onto the surface of a 96-well plate are provided. Each substrate is resuspended in reaction buffer and the kinase(s) of interest are added to each well, followed by monitoring the increase in fluorescence in real time.

[0206] Another embodiment of the present invention provides a method of detecting kinase activity comprising

[0207] measuring the fluorescence of a kinase activity sensor comprising a metal chelator, one or more amino acids and a fluorophore, wherein the chelator comprises a quinoline group and both the fluorophore and amino acid are conjugated to the quinoline group and, and wherein the amino acids comprise a kinase recognition site and a phosphorylation site;

[0208] providing a solid or semi-solid support comprising an immobilized binding agent;

[0209] contacting the immobilized binding agent with a sample comprising the specific kinase to form an immobilized specific kinase;

[0210] contacting the immobilized specific kinase with a kinase activity sensor to form a contacted specific kinase, wherein the kinase activity sensor comprises at least one peptide capable of being phosphorylated and a chelator;

[0211] incubating the contacted specific kinase for a sufficient amount of time for the kinase to phosphorylate the peptide in the presence of a phosphate source and a metal ion, wherein the kinase activity sensor forms a ternary complex with the metal ion and phosphorylated peptide to generate a detectable signal; and

[0212] detecting the signal whereby the activity of the specific kinase is measured

[0213] determining the difference in fluorescence of the kinase activity sensor between the non-contacted and contacted states;

[0214] wherein a difference in fluorescence indicates the presence kinase activity.

[0215] In another more particular embodiment, the measured fluorescence is selected from the group consisting of intensity, excitation or emission wavelength, distribution of fluorescence, fluorescence lifetime, fluorescence polarization, or a combination thereof.

[0216] Another embodiment of the present invention provides a method of detecting kinase activity, wherein the method comprises:

[0217] contacting one or more kinases with a binding agent to isolate said specific kinase, thereby forming an isolated kinase;

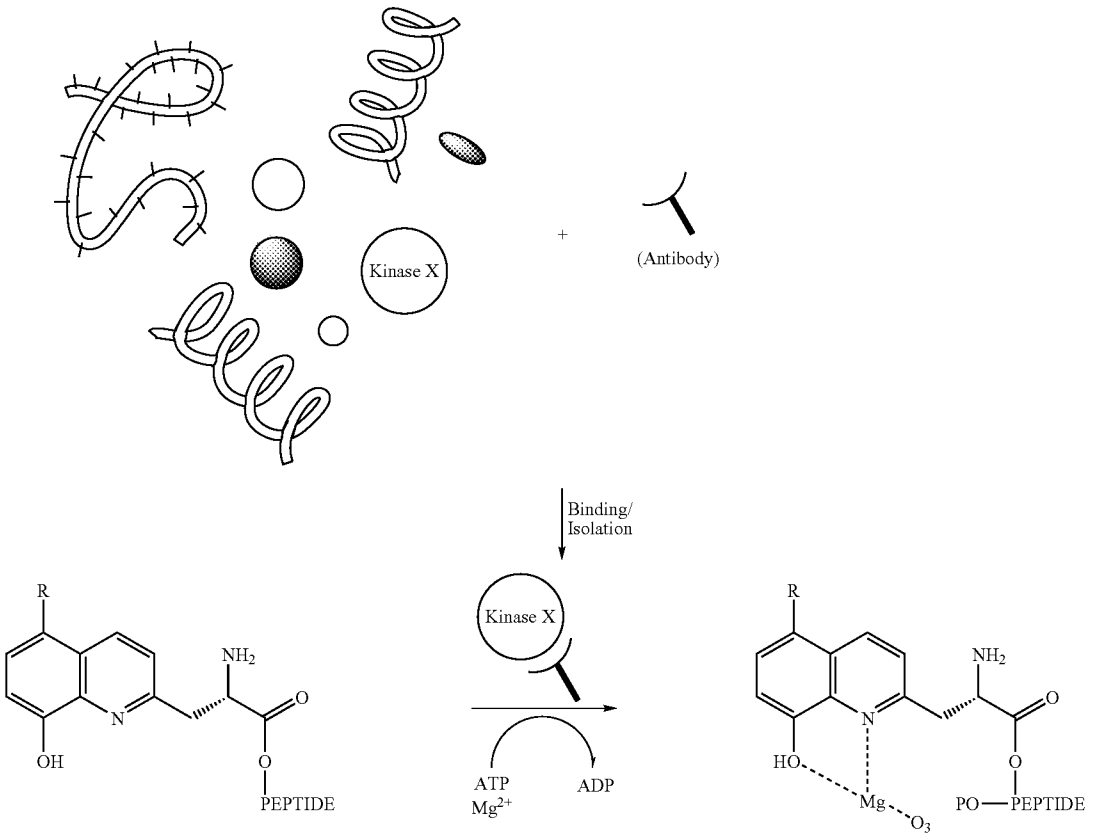
[0218] contacting said isolated kinase with a kinase activity sensor, wherein said kinase activity sensor comprises a kinase recognition motif that is capable of being recognized by said isolated kinase, and at least one phosphorylation site; and

[0219] illuminating the incubated sample with an appropriate wavelength to form an illuminated sample;

[0220] observing the illuminated sample wherein the kinase activity is detected by the presences of an observable fluorescent signal.

[0221] Scheme 1 provides an illustration of one embodiment of the present invention employing a kinase activity sensor:

Scheme 1:



[0222] The magnesium complex modifies the emission and/or excitation spectrum of the kinase activity sensor, such that phosphorylation of the peptide can be detected and/or quantified.

[0223] The present kinase activity sensors comprise one or more amino acids. In one embodiment, the amino acids form a peptide that comprise at least one kinase recognition motif, wherein the kinase recognition motifs comprise at least one phosphorylatable amino acid residue.

[0224] Any kinase recognition motif can be used in accordance with the present invention. Recognition sequences with acidic residues may show a lesser magnitude of fluorescence increase upon phosphorylation than comparable sequences, as the affinity of the unphosphorylated peptide for magnesium tends to increase under acidic conditions. Phosphorylation sites within the kinase recognition motif accordance with the present invention include, but are not limited to, amino acids comprising hydroxyl groups. Examples include, but are not limited to, naturally occurring hydroxyl-containing amino acid residues, such as serine, threonine and tyrosine, as well as non-naturally occurring hydroxyl-containing amino acid residues. Preferred recognition motifs are listed in Tables I, II, and III.

[0225] Other methods of detecting levels of phosphorylation activity include but are not limited to the use of phosphorylation site specific antibodies that can recognize and bind to phosphorylated amino acid residues. Examples of antibodies that recognize phosphorylated amino acid residues include those antibodies described in United Stated Patent Application Publication No. 2003/0162330, Mandel, J., Phosphorylation State-Specific Antibodies, *Amer. J. Pathol.*, 163:1687 (2003) and Glenney, J R et al., Monoclonal antibodies to phosphotyrosin. *J. Immunol. Methods.* 109:277 (1988), which are hereby incorporated by reference.

[0226] The methods of the present invention are useful for drug discovery settings. In one particular embodiment, the methods of the present invention comprise exposing a cell or tissue to compound, such as drug or drug candidate, prior to isolating the specific kinase of interest. In this regard, the collection of kinases is present in the cells or tissue and the drug or drug candidate is administered to the cells or tissue. Once exposed, the cells tissue can be lysed and the specific kinase of interest can then be isolated and its activity quantified using the methods of the present invention.

Solid or Semi-Solid Supports:

[0227] In an exemplary embodiment, the compounds or binding agents (e.g. antibodies) of the invention are bonded to a solid or semi-solid support. A support suitable for use in

the present invention is typically substantially insoluble in liquid phases. Solid or semi-solid supports of the current invention are not limited to a specific type of support. Rather, a large number of supports are available and are known to one of ordinary skill in the art. Thus, useful solid supports include semi-solids, such as aerogels and hydrogels, resins, beads, biochips (including thin film coated biochips), multi-well plates (also referred to as microtitre plates), membranes, conducting and nonconducting metals and magnetic supports. More specific examples of useful solid supports include silica gels, polymeric membranes, particles, derivatized plastic films, glass beads, cotton, plastic beads, alumina gels, polysaccharides such as Sepharose, poly(acrylate), polystyrene, poly(acrylamide), polyol, agarose, agar, cellulose, dextran, starch, FICOLL, heparin, glycogen, amylopectin, mannan, inulin, nitrocellulose, diazocellulose, polyvinylchloride, polypropylene, polyethylene (including poly(ethylene glycol)), nylon, latex bead, magnetic bead, paramagnetic bead, superparamagnetic bead (Dynabeads (e.g. M-280)), starch and the like. Preferred supports include agarose and magnetic beads.

[0228] In some embodiments, the solid or semi-solid support may include a support reactive functional group, including, but not limited to, hydroxyl, carboxyl, amino, thiol, aldehyde, halogen, nitro, cyano, amido, urea, carbonate, carbamate, isocyanate, sulfone, sulfonate, sulfonamide, sulfoxide, etc., for attaching the compounds of the invention. In a preferred embodiment, the solid supports contain a nucleophile (x), such as amino, thiol, or hydroxyl.

[0229] A suitable solid or semi-solid support can be selected on the basis of desired end use and suitability for various synthetic protocols. For example, where amide bond formation is desirable to attach the compounds of the invention to the solid support, resins generally useful in peptide synthesis may be employed, such as polystyrene (e.g., PAM-resin obtained from Bachem Inc., Peninsula Laboratories, etc.), POLYHIPE™ resin (obtained from Aminotech, Canada), polyamide resin (obtained from Peninsula Laboratories), polystyrene resin grafted with polyethylene glycol (TentaGel™, Rapp Polymere, Tubingen, Germany), polydimethyl-acrylamide resin (available from Milligen/Biosearch, California), or PEGA beads (obtained from Polymer Laboratories).

Kits:

[0230] Additional embodiments of the present invention include kits comprising the compositions described herein for use in detection of kinase activity. In addition, the kits include instructions on how to best detect the kinase of interest.

[0231] Another aspect of the invention provides a kit for detecting the activity of a specific kinase, comprising a binding agent that binds to the specific kinase and a kinase activity sensor, wherein said kinase activity sensor comprises at least one peptide capable of being phosphorylated and a chelator. More particularly, the kit further comprises ATP and a metal ion that has affinity for both the chelator and the phosphorylated peptide.

[0232] To facilitate correct kinase substrate recognition motifs, S/T or Y peptide substrate sampler plates with 14 substrates (1 substrate per well and 6 wells per substrate) dried onto the surface of a 96-well plate are provided. Each substrate is resuspended in reaction buffer and the kinase(s) of interest are added to each well, followed by monitoring the increase in fluorescence in real time.

[0233] A preferred kit of the invention includes 14 Sox-based peptide substrates that can be used to measure the activity of a variety of Ser/Thr kinases. The peptides are arranged in sets of 6 replicates with a bottom row of 12 replicates of a control peptide. These substrates correspond to peptides listed in SEQ ID NOs 20-33.

[0234] Another preferred kit includes 14 Sox-based peptide substrates (kinase activity sensors) that can be used to measure the activity of a variety of Ser/Thr kinases. The peptides are arranged in sets of 6 replicates with a bottom row of 12 replicates of a control peptide. These substrates correspond to peptides listed in SEQ ID NOs 4 & 34-36. Also, optionally included in this kit are additional kinase activity sensors for detection of Ser/Thr kinases.

[0235] Another preferred kit includes includes 14 different kinase activity sensors that can be used to measure the activity of a variety of Tyr kinases. The peptides are arranged in sets of 6 replicates with a bottom row of 12 replicates of a control peptide. These substrates correspond to peptides listed in SEQ ID NOs 42-49. Also, optionally included in the kit are additional kinase activity sensors for the detection of tyrosine kinases.

[0236] Various ancillary materials will frequently be employed in an assay or kit in accordance with the present invention. In an exemplary embodiment, buffers and/or stabilizers are present in the kit components. Further components include gels, beads, supports, reagents and the like. In another exemplary embodiment, the kits comprise indicator solutions or indicator "dipsticks", blotters, culture media, cuvettes, and the like. In yet another exemplary embodiment, the kits comprise indicator cartridges (where a kit component is bound to a solid support) for use in an automated detector. In another exemplary embodiment, the kit further comprises addititives, wherein said additives are selected from phosphorylated and non-phosphorylated polypeptides, calcium-binding and non-calcium binding polypeptides, sulfonated and non-sulfonated polypeptides, and sialylated and non-sialylated polypeptides. In another exemplary embodiment, the kit further comprises a member selected from a fixing solution, a detection reagent, a standard, a wash solution, and combinations thereof.

Illumination:

[0237] The sample or medium in which the complex of the phosphorylated amino acid, metal ion and chelator of the kinase activity sensor of the present invention is present is illuminated with a wavelength of light selected to give a detectable optical response, and observed with a means for detecting the optical response. Equipment that is useful for illuminating the present compounds and compositions of the invention includes, but is not limited to, hand-held ultraviolet lamps, mercury arc lamps, xenon lamps, lasers and laser diodes. These illumination sources are optically integrated into laser scanners, fluorescence microplate readers or standard or microfluorometers.

[0238] The the kinase activity sensor of the invention may, at any time after or during an assay, be illuminated with a wavelength of light that results in a detectable optical response, and observed with a means for detecting the

optical response. Selected equipment that is useful for illuminating the kinase activity sensor of the invention includes, but is not limited to, hand-held ultraviolet lamps, mercury arc lamps, xenon lamps, argon lasers, laser diodes, and YAG lasers. These illumination sources are optionally integrated into laser scanners, fluorescence microplate readers, standard or mini fluorometers, or chromatographic detectors. This fluorescence emission is optionally detected by visual inspection, or by use of any of the following devices: CCD cameras, video cameras, photographic film, laser scanning devices, fluorometers, photodiodes, quantum counters, epifluorescence microscopes, scanning microscopes, flow cytometers, fluorescence microplate readers, or by means for amplifying the signal such as photomultiplier tubes.

[0239] A detailed description of the invention having been provided above, the following examples are given for the purpose of illustrating the invention and shall not be construed as being a limitation on the scope of the invention or claims.

EXAMPLES

Example 1

Quantification of p38 Kinase Activity

[0240] A mouse monoclonal antibody specific for p38 kinase was attached to the wells of a 96-well plate. The concentrations of p38 antibody in carbonate buffer were 3 μg/ml, 6 μg/ml and 12 μg/ml and 100 μl of each concentration was used per well.

[0241] Murine macrophage cells (RAW264.7) were exposed to anisomycine and incubated for about 2 hours in culture. Control cells were untreated. After incubation, cell culture was removed from the cells and the cells were washed. Cells were then lysed using commercially available cell lysis reagents, and the cell lysate was added to the wells of the coated plate.

[0242] After the binding agent was allowed to capture the specific kinase of interest (p38), the kinase activity sensor was added to the wells (50 ng) along with 1 mM ATP. In this instance, the kinase recognition motif of the activity sensor comprised the amino acid sequence: AHLQRLSI(dP) (SEQ ID NO. 134), where the serine was the phosphorylation target site. The kinase activity sensor further comprised the metal binding amino acid that is described in U.S. Pat. No. 6,906,194 and referred to as SOX. The SOX amino acid fluoresces upon chelation of magnesium. FIG. **1** depicts levels phosphorylation activity that can be solely attributed to p38.

Example 2

Quantification of Erk1/2 Kinase Activity

[0243] A rabbit monoclonal antibody specific for Erk1/2 kinase was attached to the wells of a 96-well plate. The concentrations of Erk1/2 antibody in carbonate buffer were 3 μg/ml, 6 μg/ml and 12 μg/ml and 100 μl of each concentration was used per well.

[0244] Murine embryonic fibroblast cells (3T3) were exposed to platelet derived growth factor (PDGF) and incubated for about 2 hours in culture. Control cells were untreated. After incubation, cell culture was removed from the cells and the cells were washed. Cells were then lysed using commercially available cell lysis reagents, and the cell lysate was added to the wells of the coated plate.

[0245] After the binding agent was allowed to capture the specific kinase of interest (Erk1/2), the kinase activity sensor was added to the wells (50 ng) along with 1 mM ATP. In this instance, the kinase recognition motif of the activity sensor comprised the amino acid sequence: AHLQRLSI(dP) (SEQ ID NO. 134), where the serine was the phosphorylation target site. The kinase activity sensor further comprised the metal binding amino acid that is described in U.S. Pat. No. 6,906,194 and referred to as SOX. The SOX amino acid fluoresces upon chelation of magnesium. FIG. **2** depicts levels phosphorylation activity that can be solely attributed to Erk1/2 kinase.

Example 3

Measurement of Akt1 Activity in Crude Lysates from PDGF-Treated or Control NIH3T3 Cells

[0246] NIH3T3 cells were seeded in 100 mm dishes and grown in DMEM plus 10% fetal bovine serum until 90% confluent. The cells were then incubated overnight in serum-free medium to induce quiescence, followed by treatment with PDGF A/B (50 ng/mL, 10 min) or control media. Cell lysates were prepared and Akt1 activity was assayed as described below in the assay procedure.

[0247] The PDGF-treated sample showed a reaction rate of 1.13 RFU/sec, whereas the control treated sample had a markedly reduced rate of 0.12 RFU/sec, resulting in a dramatic signal-to-noise ratio of 9. Other assay control groups (bead only group or bead and antibody only group) also had reaction rates less than 0.12 RFU/sec. Activity was assayed as described in the Assay Procedure section below using SEQ ID NO. 25. Results are shown in FIG. **3**.

Assay Procedure:

Reagents:

[0248] Wash Buffer (prepare 1× stock): Dilute an appropriate amount of the 10× Wash Buffer Concentrate 10-fold with ultrapure water (e.g., 5 mL of 10× Wash Buffer+45 mL of ultrapure water).

[0249] Omnia™ Kinase Reaction Buffer (prepare 1× stock): Dilute an appropriate amount of the 10× Omnia™ Kinase Reaction Buffer 10-fold with ultrapure water and add DTT (provided) to a final concentration of 0.2 mM (e.g., 500 μL of 10× Omnia™ Kinase Reaction Buffer+10 μL of 100 mM DTT solution+4490 μL ultrapure water).

[0250] We have found that a useful reaction buffer for phosphorylation Ser/Thr kinases comprises: 20 mM Tris, pH 7.5; 15 mM $MgCl_2$; 1 mM EGTA; 5 mM beta-glycerophosphate; 5% glycerol; 1 mM ATP (added from separate stock); 0.2 mM DTT (added from separate stock; 1.5 mM $CaCl_2$ (PKC only; added from separate stock); 2.5 ug/mL phosphatidylserine (PKC only; added from separate stock); and 0.5 ug/mL diacylglycerol (PKC only; added from separate stock).

[0251] Alterantively, we have found that a useful reaction buffer for phosphorylation of Tyr kinases comprises: 20 mM Tris, pH 7.5; 5 mM $MgCl_2$; 1 mM EGTA; 5 mM beta-

glycerophosphate; 5% glycerol; 1 mM ATP (added from separate stock); and 0.2 mM DTT (added from separate stock).

[0252] Kinase Activity Sensor (prepare 100 μM stock): Dilute an appropriate amount of the peptide substrate solution (1 mM) 10-fold with 1× Omnia™ Kinase Reaction Buffer (e.g., 10 μL of 1 mM peptide+90 μL of 1× Omnia™ Kinase Reaction Buffer).

[0253] ATP Solution (prepare 2 mM stock): Dilute an appropriate amount of 100 mM ATP solution 50-fold with 1× Omnia™ Kinase Reaction Buffer (e.g., 10 μL of 100 mM ATP+490 μL of 1× Omnia™ Kinase Reaction Buffer).

[0254] Cell lysates: Dilute the lysate to 0.5 to 1 mg/mL total protein with Omnia™ Cell Lysis Buffer. The amount of cell lysate protein used in the assay varies depending on the quantity and activity of kinase enzyme in the individual cell line.

[0255] We have found that a useful buffer for preserving the kinase activity of kinases from crude cell or tissue lysates, comprises: 50 mM Tris, pH 7.5; 150 mM NaCl; 2 mM EGTA; 30 mM NaF; 10 mM $Na_4P_2O_7$; 100 mM Na_3VO_4; 1% Triton X-100; 50 mM b-glycerophosphate; 1 mM DTT; Sigma Protease Inhibitor cocktail (Cat. # P8340) and Sigma Phosphatase Inhibitor cocktail 1 (Cat. # P2850).

[0256] Kinase Mix (prepare 1× stock): Dilute an appropriate amount of the Kinase solution with 1× Omnia™ Kinase Reaction Buffer (e.g., 10 μL of Kinase Mix+290 μL of 1× Kinase Reaction Buffer).

[0257] Kinase control: Recombinant kinase enzyme can be used as a positive control to quantify the activity of kinase in the cell lysates.

Procedure:

[0258] 100 μL of cell lysate containing 25-100 μg of total protein prepared in Omnia™ Cell Lysis Buffer is added to each sample well in the antibody coated plate strips. The plates are sealed with Adhesive Plate Cover Strips and incubated at room temperature for 2 hours. Cell lysate is removed and discarded and wells and plates are washed multiple times with 200 μL of Wash Buffer. Wash buffer is aspirated or decanted and any residual liquid is removed between washes by tapping the plate firmly on absorbent paper.

[0259] 30 μL of the kinase mix is added to 20 μL of the kinase activity sensor solution (100 μM) and 50 μL of ATP (2 mM). The final concentration of kinase activity sensor in the reaction mixture is 20 μM and the final concentration of ATP is 1 mM. The final volume of the reaction is 100 μL.

[0260] The solution is incubated at 30° C. for 1 hour. The plate is transferred to a fluorescence plate reader (such as SpectraMax M5® by Molecular Devices or comparable instrument). The fluorescence values are read from each well every 30 seconds at an excitation wavelength of 360 nm and an emission wavelength of 485 nm for up to 5 hours at 30° C. in a kinetic mode.

[0261] The Assay Procedure is also described in the IP Kinase Activity Assay Kit, Catalog # KNZ7011, Omnia™, Plate IP Kit for B-Raf, which is hereby incorporated by reference.

Example 4

Measurement of Akt3 Activity in Crude Lysates from PDGF-Treated or Control NIH3T3 Cells

[0262] NIH3T3 cells were seeded in 100 mm dishes and grown in DMEM plus 10% fetal bovine serum until 90% confluent. The cells were then incubated overnight in serum-free medium to induce quiescence, followed by treatment with PDGF A/B (50 ng/mL, 10 min) or control media. Cell lysates were prepared and Akt3 activity was assayed as described in the Assay Procedure of Example 3. The PDGF-treated sample showed a reaction rate of 1.24 RFU/sec, whereas the control treated sample had a markedly reduced rate of 0.28 RFU/sec, resulting in a dramatic signal-to-noise ratio of 4.42. Other assay control groups (bead only group or bead and antibody only group) also had reaction rates less than 0.28 RFU/sec. Activity was assayed as described in the Assay Procedure section in Example 3 using SEQ ID NO. 25. Results are shown in FIG. **4**.

Example 5

Measurement of ERK1/2 Activity in Crude Lysates from PDGF-Treated or Control NIH3T3 Cells

[0263] NIH3T3 cells were seeded in 100 mm dishes and grown in DMEM plus 10% fetal bovine serum until 90% confluent. The cells were then incubated overnight in serum-free medium to induce quiescence, followed by treatment with PDGF A/B (50 ng/mL, 10 min) or control media. Cell lysates were prepared and ERK1/2 activity was assayed as described in the Assay Procedure of Example 3. The PDGF-treated sample showed a reaction rate of 1.55 RFU/sec, whereas the control treated sample had a markedly reduced rate of 0.27 RFU/sec, resulting in a dramatic signal-to-noise ratio of 5.7. Other assay control groups (bead only group or bead and antibody only group) also had reaction rates less than 0.27 RFU/sec. Activity was assayed as described in the Assay Procedure section in Example 3 using SEQ ID NO. 41. Results are shown in FIG. **5**.

Example 6

Measurement of p70-S6K Activity in Crude Lysates from PDGF-Treated or Control MCF-7 Cells

[0264] MCF-7 cells were seeded in 100 mm dishes and grown in DMEM plus 10% fetal bovine serum until 90% confluent. The cells were then incubated overnight in serum-free medium to induce quiescence, followed by treatment with insulin (100 nM, 10 min) or control media. Cell lysates were prepared and p70-S6K activity was assayed as described in the Assay Procedure of Example 3. The insulin-treated sample showed a reaction rate of 1.13 RFU/sec, whereas the control treated sample had a markedly reduced rate of 0.12 RFU/sec, resulting in a dramatic signal-to-noise ratio of 9.4. Other assay control groups (bead only group or bead and antibody only group) also had reaction rates less than 0.12 RFU/sec. Activity was assayed as described in the Assay Procedure section using SEQ ID NO. 42.

Example 7

Measurement of RSK Activity in Crude Lysates from PMA-Treated or Control HeLa Cells

[0265] HeLa cells were seeded in 100 mm dishes and grown in DMEM plus 10% fetal bovine serum until 90%

confluent. The cells were then incubated overnight in serum-free medium to induce quiescence, followed by treatment with PMA(200 nM, 30 min) or control media. Cell lysates were prepared and RSK activity was assayed as described in the Assay Procedure of Example 3. The PMA-treated sample showed a reaction rate of 3.17 RFU/sec, whereas the control treated sample had a markedly reduced rate of 0.64 RFU/sec, resulting in a dramatic signal-to-noise ratio of 4.95. Other assay control groups (bead only group or bead and antibody only group) also had reaction rates less than 0.64 RFU/sec. Activity was assayed as described in the Assay Procedure section using SEQ ID NO. 30. Results are shown in FIG. **7**.

Example 8

Measurement of c-Src Activity in Crude Lysates from c-Src-Transfected CEF Cells

[**0266**] C-Src transfected and mock transfected chicken embryo fibroblast (CEF) cell lysates were prepared as described (Thomas, et al., (1999) J. Biol. Chem., 74:36684-36692). The c-Src transfected CEF sample showed a reaction rate of 50 RFU/sec, whereas the control treated sample had a markedly reduced rate of 0.048 RFU/sec, resulting in a dramatic signal-to-noise ratio of 10.5. Other assay control groups (bead only group or bead and antibody only group) also had reaction rates less than 0.05 RFU/sec. Activity was assayed as described in the Assay Procedure of Example 3 using SEQ ID NO. 48. Results are shown in FIG. **8**.

Example 9

Measurement of B-Raf Activity in Crude Lysates from NGF Treated or Control PC12 Cells

[**0267**] PC12 cells were seeded in 100 mm Petri dishes and grown in DMEM plus 2.5% fetal bovine serum and 15% horse serum. Cells were then treated with NGF (50 ng/mL, 15 minutes) or with control media. Cell lysates were prepared and B-Raf activity was assayed as described in Example 3. The NGF-treated sample produced fluorescence signals that reached a plateau at 150 minutes of incubation (3200 RFU), while the control group reached a plateau at 240 minutes after the reaction started. Activity was assayed as described in the Assay Procedure section using a cascade reaction with SEQ ID NO. 27, used to measure the level of activated MK2 enzyme. Results are shown in FIG. **9**.

Example 10

Measurement of ERK1/2 Activity in Crude Lysates from PDGF-Treated or Control NIH3T3 Cells

[**0268**] NIH3T3 cells were seeded in 100 mm Petri dishes and grown in DMEM plus 10% fetal bovine serum. Cells then were treated with PDGF (50 ng/mL, 15 minutes) or with control media. Cell lysates then were prepared and ERK1/2 activity was assayed as described in Example 1. The PDGF-treated sample produced a fluorescence signal of 3000 RFU that reached a plateau at 335 minutes of incubation, while the control group produced fluorescence signal of 845 RFU. Activity was assayed as described in the Example 3 using a cascade reaction with SEQ ID NO. 27 used to measure the level of activated MK2 enzyme. Results are shown in FIG. **10**.

Example 11

Measurement of MEK1 Activity in Crude Lysates from PDGF-Treated or Control NIH3T3 Cells

[**0269**] NIH3T3 cells were seeded in 100 mm Petri dishes and grown in DMEM plus 10% fetal bovine serum. Cells then were treated with PDGF (50 ng/mL, 15 minutes) or with control media. Cell lysates then were prepared and MEK1 activity was assayed as described in the Assay Procedure of Example 3. The PDGF-treated sample produced fluorescence signal of 3650 RFU that reached a plateau at 250 minutes of incubation, while the control group produced fluorescence signal of 1050 RFU. Activity was assayed as described in the Assay Procedure section using a cascade reaction with SEQ ID NO. 27 used to measure the level of activated MK2 enzyme. Results are shown in FIG. **11**.

Example 12

Measurement of p38 MAPK Activity in Crude Lysates from Anisomycin Treated or Control RAW Cells

[**0270**] RAW cells were seeded in 100 mm Petri dishes and grown in DMEM plus 10% fetal bovine serum. Cells then were treated with anisomycin (10 μg/mL, 15 minutes) or with control media. Cell lysates then were prepared and p38 MAPK activity was assayed as described in the Assay Procedures of Example 3. The anisomycin treated sample produced fluorescence signal of 3350 RFU that reached a plateau at 60 minutes of incubation, while the control group produced fluorescence signal of 1250 RFU. Activity was assayed as described in the Assay Procedure section using a cascade reaction with SEQ ID NO. 27 used to measure the level of activated MK2 enzyme. Results are shown in FIG. **12**.

[**0271**] Each of the aforementioned references are hereby incorporated by reference as if set forth fully herein.

What is claimed is:

1. A method of measuring the activity of a specific kinase, said method comprising:

providing a solid or semi-solid support comprising an immobilized binding agent;

contacting the immobilized binding agent with a sample comprising the specific kinase to form an immobilized specific kinase;

contacting the immobilized specific kinase with a kinase activity sensor to form a contacted specific kinase, wherein the kinase activity sensor comprises at least one peptide capable of being phosphorylated and a chelator;

incubating the contacted specific kinase for a sufficient amount of time for the kinase to phosphorylate the peptide in the presence of a phosphate source and a metal ion, wherein the kinase activity sensor forms a ternary complex with the metal ion and phosphorylated peptide to generate a detectable signal; and

detecting the signal whereby the activity of the specific kinase is measured.

2. The method of claim 1, wherein the binding agent comprises an antibody or a functional fragment thereof.

3. The method of claim 1, wherein the support is glass, plastic, metal, polymeric particle, polymeric gel or a polymeric membrane.

4. The method according to claim 1, wherein the kinase activity sensor has an increased fluorescence signal when complexed with the metal ion.

5. The method of claim 1, wherein the chelator comprises a signaling moiety.

6. The method according to claim 5, wherein the signaling moiety comprises a coumarin, cyanine, benzofuran, a quinoline, a quinazolinone, an indole, a benzazole, a borapolyazaindacene or a xanthene.

7. The method according to claim 1, wherein the chelator comprises a quinoline or a derivative thereof, phenanthrolines or derivatives thereof, BAPTA, IDA, DTPA and derivatives thereof.

8. The method according to claim 1, wherein the kinase activity sensor further comprises a signaling moiety that is separate from the chelator.

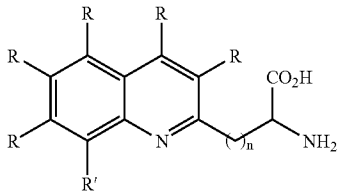
9. The method according to claim 8, wherein the signaling moiety comprises a coumarin, cyanine, benzofuran, a quinoline, a quinazolinone, an indole, a benzazole, a borapolyazaindacene or a xanthene.

10. The method according to claim 1, wherein the method further comprises contacting the immobilized specific kinase with ATP.

11. The method according to claim 1, wherein the metal ion is Ca^{2+}, Zn^{2+}, Mg^{2+}, Ga^{3+}, Tb^{3-}, La^{3+}, Pb^{2+}, Hg^{2+}, Cd^{2+}, Cu^{2+}, Ni^{2+}, Co^{2+}, Fe^{2+}, Mn^{2+}, Ba^{2+}, or Sr^{2+}.

12. The method of claim 1, wherein the peptide comprises an amino acid that when phosphorylated complexes magnesium.

13. The method according to claim 1, wherein the kinase activity sensor comprises the formula:



where at least one R group is —SO_2X, where X is —OR" or —NR"R'";

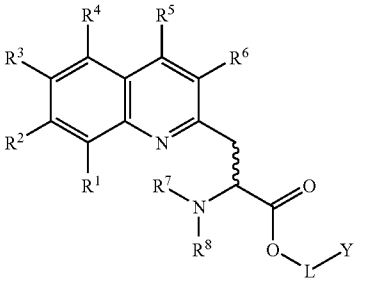
R' is hydroxy, amino, or thiol;

R" is C_{1-6} alkyl;

R'" is hydrogen or alkyl; and

n is 1, 2 or 3.

14. The method of claim 1, wherein said kinase activity sensor has the formula:



wherein R^1, is H, alkyl, substituted alkyl, alkoxy, substituted alkoxy, acyl, acylamino, acyloxy, amino, substituted amino, aminocarbonyl, aminothiocarbonyl, aminocarbonylamino, aminothiocarbonylamino, aminocarbonyloxy, aminosulfonyl, aminosulfonyloxy, aminosulfonylamino, amidino, carboxyl, carboxyl ester, (carboxyl ester)amino, (carboxyl ester)oxy, cyano, halo, hydroxy, nitro, SO_3H, sulfonyl, substituted sulfonyl, sulfonyloxy, thioacyl, thiol, alkylthio, substituted alkylthio, aryl, substituted aryl, heteroaryl, substituted heteroaryl, cycloalkyl, substituted cycloalkyl, heterocyclyl, or substituted heterocyclyl;

R^2 is a fluorophore, H, alkyl, substituted alkyl, alkoxy, substituted alkoxy, acyl, acylamino, acyloxy, amino, substituted amino, aminocarbonyl, aminothiocarbonyl, aminocarbonylamino, aminothiocarbonylamino, aminocarbonyloxy, aminosulfonyl, aminosulfonyloxy, aminosulfonylamino, amidino, carboxyl, carboxyl ester, (carboxyl ester)amino, (carboxyl ester)oxy, cyano, halo, hydroxy, nitro, SO_3H, sulfonyl, substituted sulfonyl, sulfonyloxy, thioacyl, thiol, alkylthio, substituted alkylthio, aryl, substituted aryl, heteroaryl, substituted heteroaryl, cycloalkyl, substituted cycloalkyl, heterocyclyl, or substituted heterocyclyl;

R^3 is a fluorophore, H, alkyl, substituted alkyl, alkoxy, substituted alkoxy, acyl, acylamino, acyloxy, amino, substituted amino, aminocarbonyl, aminothiocarbonyl, aminocarbonylamino, aminothiocarbonylamino, aminocarbonyloxy, aminosulfonyl, aminosulfonyloxy, aminosulfonylamino, amidino, carboxyl, carboxyl ester, (carboxyl ester)amino, (carboxyl ester)oxy, cyano, halo, hydroxy, nitro, SO_3H, sulfonyl, substituted sulfonyl, sulfonyloxy, thioacyl, thiol, alkylthio, substituted alkylthio, aryl, substituted aryl, heteroaryl, substituted heteroaryl, cycloalkyl, substituted cycloalkyl, heterocyclyl, or substituted heterocyclyl;

R^4 is a fluorophore, H, alkyl, substituted alkyl, alkoxy, substituted alkoxy, acyl, acylamino, acyloxy, amino, substituted amino, aminocarbonyl, aminothiocarbonyl, aminocarbonylamino, aminothiocarbonylamino, aminocarbonyloxy, aminosulfonyl, aminosulfonyloxy, aminosulfonylamino, amidino, carboxyl, carboxyl ester, (carboxyl ester)amino, (carboxyl ester)oxy, cyano, halo, hydroxy, nitro, SO_3H, sulfonyl, substituted sulfonyl, sulfonyloxy, thioacyl, thiol, alkylthio, substituted alkylthio, aryl, substituted aryl, heteroaryl, sub-

stituted heteroaryl, cycloalkyl, substituted cycloalkyl, heterocyclyl, or substituted heterocyclyl;

R^5 is a fluorophore, H, alkyl, substituted alkyl, alkoxy, substituted alkoxy, acyl, acylamino, acyloxy, amino, substituted amino, aminocarbonyl, aminothiocarbonyl, aminocarbonylamino, aminothiocarbonylamino, aminocarbonyloxy, aminosulfonyl, aminosulfonyloxy, aminosulfonylamino, amidino, carboxyl, carboxyl ester, (carboxyl ester)amino, (carboxyl ester)oxy, cyano, halo, hydroxy, nitro, SO_3H, sulfonyl, substituted sulfonyl, sulfonyloxy, thioacyl, thiol, alkylthio, substituted alkylthio, aryl, substituted aryl, heteroaryl, substituted heteroaryl, cycloalkyl, substituted cycloalkyl, heterocyclyl, or substituted heterocyclyl; and

R^6 is a fluorophore, H, alkyl, substituted alkyl, alkoxy, substituted alkoxy, acyl, acylamino, acyloxy, amino, substituted amino, aminocarbonyl, aminothiocarbonyl, aminocarbonylamino, aminothiocarbonylamino, aminocarbonyloxy, aminosulfonyl, aminosulfonyloxy, aminosulfonylamino, amidino, carboxyl, carboxyl ester, (carboxyl ester)amino, (carboxyl ester)oxy, cyano, halo, hydroxy, nitro, SO_3H, sulfonyl, substituted sulfonyl, sulfonyloxy, thioacyl, thiol, alkylthio, substituted alkylthio, aryl, substituted aryl, heteroaryl, substituted heteroaryl, cycloalkyl, substituted cycloalkyl, heterocyclyl, or substituted heterocyclyl;

R^7 is H, alkyl, substituted alkyl, carbonyl, or a protecting group; and

R^8 is H, alkyl, substituted alkyl, carbonyl, or a protecting group;

L is a linker; and

Y is a peptide;

or a tautomer, stereoisomer, or salt thereof.

15. The method of claim 13, wherein at least one of R^2, R^3, R^4, R^5 and R^6 is a fluorophore.

16. The method according to claim 1, wherein the peptide is at least one of SEQ ID NO. 1 through SEQ ID NO. 133.

17. A composition comprising:

(a) a kinase immobilized by a binding agent; and

(b) a kinase activity sensor comprising at least one peptide capable of being phosphorylated and a chelator.

18. A kit for detecting the activity of a specific kinase, comprising a binding agent that binds to the specific kinase and a kinase activity sensor, wherein said kinase activity sensor comprises at least one peptide capable of being phosphorylated and a chelator.

19. The kit according to claim 18, further comprising ATP and a metal ion that has affinity for both the chelator and the phosphorylated peptide.

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