Generic Kinase Assay

The present invention relates to a method of determining kinase or phosphatase activity comprising interacting a binding partner, a detection molecule and a substrate molecule.

The physiological modification of molecules and supramolecular assemblies plays a major role in the structure and regulation of biological systems. These modifications may include phosphorylation, cyclization, glycosylation, acylation, and/or sulfation, among others, and the modified molecules may include polypeptides, nucleic acids, and/or lipids, among others. The importance of modifications is particularly evident in the cell-signaling pathway, in which extracellular and intracellular substances related by phosphate modifications such as phosphorylation and cyclization influence the position, nature, and activity of cells.

Compounds which can interfere with phosphorylation or dephosphorylation catalysed by specific enzymes, or of specific substrates, are of interest since they may interfere with specific signaling events and thus be useful for treatment or prevention of diseases which occur through dysregulation of such pathways.

Therefore, it is of interest to develop methods of determining kinase or phosphatase activity for screening for compounds that can modulate specific signaling pathways.

EP1156329 discloses a method of determining kinase activity based on a system of *two* parts, the binding partner (BP) and the substrate molecule which is converted A ->A*. In general, the binding partner is an entity that binds the enzyme product and can itself be attached to a solid phase e.g. a bead by any kind of interaction, covalent or non-covalent. For detection, a label (fluorophore, luminophore, acceptor or quencer) is attached to A or the BP. The list of detection methods includes intensity, polarization and energy transfer measurements.

WO00/75167 discloses a method of detecting addition or removal of a substrate group to or from a substrate which is also based on a system of *two* parts, the binding partner, which is preferably a macromolecule having entrapped metal ions, and a peptide

A, wherein the conversion of phosphorylated to unphosphorylated peptide or vice versa is measured, e.g. by FRET.

One drawback of the two parts systems is that for optimal energy transfer, for a given BP and A* ratio, a certain ratio of donor and acceptor is required. Thus, for optimal FRET generation, it may be necessary to titrate BP, A and A*.

However, the optimum ratios between BP, A and A* are generally influenced by the binding capacity of BP for A* and the respective binding constants on one hand, and also by the kinetic parameters of the enzyme reaction for the target of interest on the other hand. Therefore, the fixed ratio of BP, A and A* which is necessary for FRET optimization in a two parts system leads to a compromise in either assay signal and quality or enzyme kinetics and biological meaning of the results, i.e. in the worst case it may be impossible in a two parts system to achieve the optimum for all components: enzyme reaction for optimum kinetics, BP and A* for optimum binding of converted product, acceptor – donor for optimum FRET.

One embodiment of the two parts system disclosed in EP1156329 and WO00/75167 comprises plastic beads that are doped with either luminophore or acceptor and a surface coating to bind the labeled A*. In other words IMAP™ beads with optical label in the core of the beads.

To achieve sufficient binding capacity the IMAP™ beads typically have a diameter of 100 nm or more. This is big compared to the energy transfer distance of a typical donor acceptor pair which is 3 to maximally 9 nm. As a consequence the energy transfer between a doped bead and a bound peptide is rather in efficient, there are many luminophores inside the bead which don't see a FRET partner but still contribute to assay background signal. This leads to a reduced dynamic range and decrease in sensitivity.

Therefore, it was the aim of the present invention to develop a more efficient method for determining kinase or phosphatase activity by energy transfer measurements between an energy donor and an energy acceptor.

The assay of the present disclosure consists of *three* parts: (i) a "*binding partner*" (ii) a *detection molecule* which is labeled with donor or acceptor of a FRET pair (iii) a *substrate molecule – a small molecule,peptide or protein-* which is converted by the

enzyme and thereby an amino acid (Ser, Thr, Tyr) is phosphorylated if the enzyme is a protein kinase. The same setup can be used for protein phospatases where the respective amino acid is de-phosphorylated. Thus, in a first aspect the disclosure provides a method for determining kinase or phosphatase activity comprising interacting a binding partner, a detection molecule and a substrate molecule, wherein said detection molecule is labeled with an energy donor or acceptor and said substrate molecule is labeled with an energy donor, if the detection molecule is labeled with an energy acceptor, or with an energy acceptor, if the detection molecule is labeled with an energy donor, wherein said binding partner only binds phosphorylated substrate molecule and the detection molecule, which method comprises the steps of: a) phosphorylating said substrate molecule with a kinase or dephosphorylating a phosphorylated substrate molecule with a phosphatase; and b) interacting the substrate molecule obtained in step a) with said binding partner and said detection molecule; and c) determining kinase or phosphatase activity by measuring energy transfer from the energy donor to the energy acceptor of said substrate molecule and said detector molecule bound to said binding partner. Preferably, said binding partner are metal ions which are preferably associated with a solid phase, e.g. a bead, membrane, sample holder. In a most preferred embodiment, said binding partner are metal ions on IMAP™ beads. IMAP™ beads may be obtained from Molecular Devices Corp. It has been shown that IMAP™ beads can be used as binding platform to bring two fluorophores close enough together for fluorescence resonance energy transfer (FRET) being thus a universal detection system for kinase assays and phosphatase assays.

In another preferred embodiment, the substrate molecule is a protein or a peptide.

In a second aspect, the disclosure provides for a kit for performing the method as defined above, comprising, in separate containers, beads which are doped with metal ions, more than one detection molecule comprising an energy donor or an energy acceptor, more than one substrate peptide comprising an energy acceptor or an energy donor, wherein said detection molecules and said substrate peptides are provided as sets of FRET pairs.

As used herein, "binding partner" refers to an entity that binds the enzyme product and can itself be attached to a solid phase e.g. a bead by any kind of interaction, covalent or non-covalent.

As used herein, "detection molecule" refers to a molecule which carries a label, e.g. a fluorescent label, that allows detection of the detection molecule, and which binds to the binding partner.

As used herein, "substrate molecule" refers to a small molecule, peptide or protein which is converted by the enzyme through an enzymatic reaction.

As used herein, "energy donor" refers to a fluorescent label which, upon excitation, can transfer its energy to the energy acceptor.

As used herein, "energy acceptor" refers to a molecule which can recieve energy from the energy donor.
As used herein, "kinase activity" refers to phosphorylation of a substrate by an enzyme (kinase)

As used herein, "phosphatase activity" refers to dephosphorylation of a substrate by an enzyme (phosphatase)

As used herein, "phosphorylated substrate molecule" refers to a small molecule, peptide or protein which is phosphorylated by the enzyme (kinase).

As used herein, "emission lifetime" refers to the average amount of time a fluorophore remains in the excited state following excitation.

In comparison to a fluorescence polarization readout, an implementation with TR-FRET as readout offers the following advantages:

- Increased quality of the assay signal which is expressed by the statistical parameter z', in particular for low substrate turnover.
- Increased robustness of the assay signal vs. interferences of background fluorescence due to the time gated, delayed detection.
- Increased sensitivity with respect to amount of substrate which needs to be converted (the FP readout measures the average signal of the fluorophores for all substrate molecules in the solution; the FRET sees only the fraction of the fluorophores bound to the phosphorylated substrate molecules).
- Reduced reagent consumption by a factor of more than 20 fold.

In addition, the three parts system used in the method of the present invention has the follwing advantages over the two parts system of the prior art even if the latter is also implemented by (TR)-FRET:

- The amount of beads needed for best binding of the substrate product and the amount of detection molecule for best energy transfer signal can be titrated separately.

- The energy transfer between the detection molecule and the substrate molecule which are both bound to the bead surface leads to a more efficient FRET signal.
- Furthermore, it is possible to select beads (BP), detection molecule and substrate molecule separately i.e. one can imagine an assay toolbox which contains several universal items such as beads, detection molecules, substrates and sets of FRET pairs. Selection from this toolbox yields the different assay applications. Such flexibility and universality is more difficult and practically even impossible for a two part system.

Thus, a kit for performing the method according hereinbefore and hereinafter described is also provided, comprising, in separate containers, beads, more than one detection molecule, more than one substrate molecule, wherein said detection molecules and said substrate molecule are provided as sets of FRET pairs.

Preferably, the energy donor has an emission lifetime less than 50 microsecs, more preferably less than 10 microsecs, even more preferably less than 5 microsecs. Most preferably, the energy donor has a emission lifetime of 2 to 3 microsecs.

In a preferred embodiment, a Ru-luminophore is used as energy donor. Compared to the Lanthanide (Eu, Tb, Sm) chelates and cryptates, the Ru-luminophore has several general advantages

- Broad emission spectrum with good overlap with acceptor, unlike the peaked spectrum of lanthanide dyes.
- Higher chemical stability which is of advantage for the labeling chemistry (more harsh conditions possible, also during purification) and assays (no interference from reagent, Lanthanide dyes are known to be sensitive to important ingredients of typical kinase assays such as EDTA, Mg-ions or Mn-ions).
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In another preferred embodiment, a Tb-luminophore is used as energy donor.

A particular advantage is given by the microsecond lifetime of the excited state and the resulting emission. The typical emission lifetime of the $Ru(batho)_2bpy$ – complex under assay conditions is 2-3 µs. This microsecond lifetime allows efficient background suppression against fluorescence from other assay ingredients and fluorescent compounds. However, the photon emission time is 100x faster than for the lanthanides i.e. the detector integrates 100x less of its electronic background signal.

In one embodiment of the present invention, the method is performed with a system wherein the sample moves with respect to the optical detection pathway. Preferably, said system is a labware spin system, more preferably a Tecan lab CD or the system from SpinX. In another preferred embodiment, said system is a microfluidic system or a flow through cell. In such a setting, the lanthanide emission is too slow to allow readout during the movement of the sample. In order to make use of the TR-FRET readout, the labware/disk or the sample flow, respectively, would have to go through start-stop cycles, which is practically impossible and time consuming. Alternatively one would have to modify the instrument to have a different excitation / detection path where the positions of the pathways would have to be adjusted according to the timing of the readout.

The "Fast-TR-FRET" with Ru-complexes and short microsecond lifetimes allows online readout while the sample is moving without major modification and offers a special advantage. The IMAP™ technology is a universal assay for kinases and therefore preferably used. The FP readout, however, has the disadvantages mentioned above. The lanthanide TR-FRET cannot be readily used in such a setting.

In a preferred embodiment, the energy acceptor is a fluorophore exhibiting a spectral overlap with the Ru-complexes which yields an R_0 (Förster Radius) of at least 10 Å, preferably at least 20 Å, more preferably at least 50 Å. The relation between R_0 and the spectral overlap $J(\lambda)$ is defined by the following formulae (from: Lakowicz, J.R., Principles of Fluorescence Spectroscopy, 2nd ed. Kluwer Academin/Plenum Publishers, p. 369):

$$R_0 = 0.211[\kappa^2 n^{-4} Q_D J(\lambda)]^{1/6} \quad \text{(in Å)}$$

wherein

$$J(\lambda) = \int_0^\infty F_D(\lambda)\varepsilon_A(\lambda)\lambda^4\, d\lambda = \frac{\int_0^\infty F_D(\lambda)\varepsilon_A(\lambda)\lambda^4\, d\lambda}{\int_0^\infty F_D(\lambda)\, d\lambda}$$

In a more preferred embodiment, the energy acceptor is Alexa Fluor 700, Atto 700, Dy701 or Alexa Fluor 750. In a most preferred embodiment, said energy acceptor is Alexa Fluor 700.

In a most preferred embodiment, the Ru-luminophore is $Ru(batho)_2bpy$.

Additional dye pairs for such fluorescence resonance energy transfer measurements between Ru-complexes and fluorophores which can be used in the present invention are disclosed in WO02/41001.

The dilution of the binding partner is at least 500 fold, preferably at least 1000 fold or between 400 fold or 1000 fold and 10,000 fold, wherein the dilution is between 2000 fold and 10,000 fold or between 2000 fold and 8000 fold.

In the following section a non-limiting embodiment disclosed in the examples is discussed:

As a test system phosphorylated und unphosphorylated substrate peptide was labeled with Alexa Fluor 700 as energy acceptor and phosphorylated substrate peptide was labeled with $Ru(batho)_2bpy$ as energy donor. The Alexa labeled kinase substrate peptide is phosphorylated by the enzyme. For the detection a phosphorylated $Ru(batho)_2bpy$ detection peptide and IMAP™ beads are added to the reaction mixture. Both phosphorylated substrate and detection peptides bind to the beads, come thus into close proximity so that fluorescence resonance energy transfer (FRET) can occur (Figure 1).

As FRET pair the long lifetime metal-ligand complex $Ru(batho)_2bpy$ (τ=3 µs) was used as energy donor and Alexa Fluor 700 as energy acceptor. Binding of the Ru labeled peptide to the IMAP™ beads did not influence the fluorescence polarization measurements with Alexa Fluor 700 as fluorophore. In a comparison of both readout techniques (FP and FRET) FRET was proven to be the more sensitive method. Already 10% phosphorylation gives a z' factor above 0.5. Furthermore, for FRET measurements at least ten times less IMAP™ beads are needed as compared to FP. Thus, a significant reduction of reagent consumption is achieved without the necessity of miniaturized liquid handling and readout which is typically done to save costly reagents but complicates the assay handling and thereby may lead to unstable and irreproducible processing.

Summarized, the Fast-TR FRET kinase IMAP™ assay disclosed herein is *robust, sensitive, needs less IMAP™ beads* and has *low background fluorescence* because of the time gated detection.

As known from FP experiments the signal is affected by ATP because free binding sites on the beads are occupied by ATP preventing the phosphorylated peptide to bind. In contrast to ATP, EDTA had no effect on the FRET signal. The effect of ATP could be neglected after titration of the assay components of the herein described three parts system.

In principle both fluorophores could be used as labels for the substrate. Advantages of the Ru complex as substrate label are the high chemical stability and – as compared to Alexa Fluor 700 - its availability as NHS ester and maleimide which enhances the flexibility of the labeling process.

An alternative to Alexa Fluor 700 may be Atto 700 which has comparable excitation and emission spectra and is available as NHS ester and maleimide. Due to its smaller absorption coefficient (ε=120000 $M^{-1}cm^{-1}$) R_0 is reduced by ~5 Å for the FRET pair Ru-Atto 700 compared to Ru-Alexa Fluor 700 (ε=192000 $M^{-1}cm^{-1}$, R_0=62 Å). Phosphotyrosine, other phosphorylated amino acids or other small molecules carrying a phosphate group, which are labeled with the Ru complex (or alternatively Alexa Fluor 700 or Atto 700) may be used as a "universal" detection molecule.

Figure 1: Scheme of the kinase reaction using IMAP™ beads for Fast-TR FRET readout.

Figure 2: Observed FRET signal (black) with 15 nM phosphorylated Alexa-peptide and 100 nM and 300 nM phosphorylated Ru-peptide, respectively, IMAP™ dilution: 1:800. Grey: 15 nM Alexa-peptide and phosphorylated Ru-peptide and IMAP™ beads, white: phosphorylated Ru-peptide and IMAP™ beads. The beads were incubated at RT for 30 minutes. Readout: 730 (30) nm, delay 100 ns, gate 100 ns, exposure time 4 s. The part (a) on the left shows the raw data, part (b) on the right shows the effective FRET signal.

Figure 3: Raw data of 20 nM phosphorylated Ru-peptide and 100 nM phosphorylated Alexa-peptide (IMAP™ dilution 1:4000) (white) and 15 nM phosphorylated Alexa-

peptide and 100 nM Phos-PDHK-Ru (IMAP™ dilution 1:800) (black). Readout: 730 (30) nm, delay 100 ns, gate 100 ns, exposure time 10 s (white) and 4 s (black).

Figure 4: FRET signal with different IMAP™ dilutions using 20 nM phosphorylated Alexa-peptide and 100 nM phosphorylated Ru-peptide, incubation 60 minutes, readout: 730 (30) nm, delay 100 ns, gate 100 ns, exposure time 5 s.

Figure 5: Influence of ATP on the FRET signal. 20 nM phosphorylated Alexa-peptide, 100 nM phosphorylated Ru-peptide, incubation 60 minutes at RT, readout: 730 (39) nm, delay 100 ns, gate 100 ns, exposure time 8 s.

Figure 6: Influence of EDTA on the FRET signal. 20 nM phosphorylated Alexa-peptide, 100 nM phosphorylated Ru-peptide, incubation 60 minutes at RT, readout: 730 (30) nm, delay 100 ns, gate 100 ns, exposure time 8s.

Figure 7: Simulation of a kinase assay with 20 nM (phosphorylated) Alexa-peptide and 100 nM phosphorylated Ru-peptide, IMAP™ dilution 1:400 ((a) and (b)) and 1:4000 ((c) and (d)). Incubation 60 minutes at RT. Readout: FRET: 730 (30) nm, delay 100 ns, gate 100 ns, exposure time 10 s, FP: excitation 655 (50) nm, emission 710 (40) nm.

Figure 8: Kinetic of the kinase assay. Readout: 730 (30) nm, delay 100 ns, gate 100 ns, exposure time 7 s.

Figure 9: Dose response curve for ATP, using 1 µM Alexa-peptide for the kinase reaction. Final concentrations in the readout were: 20 nM (phosphorylated) Alexa-peptide, 100 nM phosphorylated Ru-peptide, 1:4000 diluted IMAP™ beads. Readout: 730 (30) nm, delay 100 ns, gate 100 ns, exposure time 10 s.

Examples:

Example 1: Observation of FRET due to Binding to IMAP beads

Materials:

- Substrate peptide (YHGHSMSAPGVSTAC) from Biosyntan labeled with Alexa Fluor 700
- Phosphorylated substrate peptide (YHGHS(H_2PO_4)MSAPGVSTAC) from Biosyntan labeled with Alexa Fluor 700 and Ru(batho)$_2$bpy
- Kinase enzyme: PDHK2 (recombinant pyruvate dehydrogenase kinase 2, expressed according to standard methods)
- IMAP™ beads from Molecular Devices (IMAP™ Explorer Kit (IPP) Product # R8062)
- Buffer: IMAP™ Binding Buffer 1:5 diluted with dest. H_2O + 0.05% BSA

Instrumentation:

- Zeiss plate::vision reader
- Plate: Costar 384, UV-NBS
- Excitation wavelength: 355 nm
- Emission filter: 730 (30) nm, 615 (10) nm

In a first experiment the 1:800 diluted IMAP™ beads (recommended dilution for FP measurements 1:400) were added to a mixture of 15 nM (end concentration) phosphorylated or unphosphorylated substrate peptide labeled with Alexa Fluor 700 (hereafter (phosphorylated) Alexa-peptide) and 100 nM and 300 nM (end concentrations) phosphorylated substrate peptide labeled with Ru(batho)$_2$bpy (hereafter phosphorylated Ru-peptide), respectively. The reaction mixture was then diluted 50 times for the readout with the IMAP™ beads and phosphorylated Ru-peptide. Figure 2a shows the raw data of this measurement. Only the FRET signal (black) was above the background signal of the Ru complex alone (white). There was no FRET due to unspecific binding of unphosphorylated Alexa-peptide to the beads (grey). The background corrected FRET signal (Figure 2b) was increasing with increasing phosphorylated Ru-

peptide concentration indicating that with 100 nM phosphorylated Ru-peptide and 15 nM phosphorylated Alexa-peptide the IMAP™ beads were not saturated.

Example 2: Comparison of Alexa-peptide and Ru-peptide as substrate

The experiment was also done using 20 nM phosphorylated Ru-peptide and 100 nM phosphorylated Alexa-peptide with 1:4000 diluted IMAP™ beads. Figure 3 compares the raw data of the experiment using either Ru-peptide (white) or Alexa-peptide (black) as substrate. Because different measuring parameters were used for the two experiments, only the ratios of the FRET signal to the Ru background could be compared directly. Whereas the FRET signal was twice the Ru background when using the Alexa labeled substrate, this ratio was increased to six when using the Ru labeled substrate. In both experiments Alexa Fluor 700 did not contribute to the background due to the time gated detection of the FRET signal (delay 100 ns).

Example 3: Titration of IMAP™ beads

To determine optimal conditions for the readout different IMAP™ dilutions were tested with 20 nM phosphorylated Alexa-peptide and 100 nM phosphorylated Ru-peptide (Figure 4). The FRET signal was increasing by a factor of three when diluting the IMAP™ beads 1:2000 compared to a dilution of 1:400. With further dilution the signal was decreasing again reaching about the same signal as with 1:400 with 1:10'000. As stated before, with a 1:400 dilution the IMAP™ beads were not saturated. Further dilution yielded in more phosphorylated Ru-peptide per IMAP™ bead and resulted in an increase of the FRET signal. With a dilution larger than 1:2000 the number of phosphorylated Ru-peptide per IMAP™ bead was decreased again.

Example 4: Influence of ATP and EDTA on the FRET signal

FP experiments showed that addition of ATP decreases the polarization window because ATP binds to the beads and thus prevents the phosphorylated kinase substrate to bind. Therefore ATP was expected to also influence the FRET signal. The kinase reaction was typically done with 100 µM ATP and 1 µM substrate. Before adding the IMAP™ beads for the FRET readout, the reaction mixture was diluted 50 times giving an ATP end

concentration of 2 µM. Figure 5 shows the decrease of the FRET signal with increasing ATP concentration. The FRET signal decreased by a factor of two with 2 µM ATP.

Figure 6 shows that the FRET signal was not influenced by addition of EDTA which was used to stop the kinase reaction. EDTA had no effect on the binding of the phosphorylated substrate to the IMAP™ bead, nor does it affect the Ruthenium complex in any way. The tested EDTA concentrations (up to 64 µM) also had no measurable influence on the IMAP™ beads. Higher concentrations may complex the M^{3+} on the beads and thus influence the binding of the phosphorylated peptide.

Example 5: Kinase assay

To test the sensitivity of the Fast-TR FRET kinase IMAP™ assay and to compare it to the established FP IMAP™ assay a simulation of the kinase assay was done. The level of phosphorylation of 20 nM Alexa-peptide was increased from 0% to 100% by mixing unphosphorylated and phosphorylated Alexa-peptides. Two IMAP dilutions were used, namely 1:400 which is the recommended dilution for FP measurements and 1:4000 which showed still a good FRET signal with 100% phosphorylated Alexa-peptide (Figure 4). The FRET signal increased with increasing phosphorylation for both IMAP™ dilutions (Figure 7 (a) and (c)). As expected the FRET signal was higher for the 1:4000 dilution compared to the 1:400 dilution giving z' factors (open diamonds) larger than 0.5 from 10% to 100% phosphorylation. For the FP measurements, the detection window with an IMAP™ dilution of 1:400 was 200 mP and resulted in z' factors of 0.5 or better from 20% to 100% phosphorylation (Figure 7 (b)). A dilution of the IMAP™ beads of 1:4000 drastically decreased the window to 70 mP (Figure 7 (d)) and reduced the sensitivity of the FP experiment.

Example 6: Kinetic of the kinase reaction

1 µl of the PDHK2 enzyme was diluted in 11 µl kinase buffer (25 mM Hepes pH 7.4, 150 mM NaCl, 18.7 mM $MgCl_2$, 0.4 mM NH_4Ac, 0.025% Triton X-100, 1.87 mM DTT). 3 µl of a 5 µM solution of the Alexa labeled substrate-peptide in substrate buffer (25 mM Hepes pH 7.4, 150 mM NaCl, 0.01% Triton X-100) were then added to the enzyme. As a positive control the substrate solution contained 500 µM ATP whereas for the negative control no ATP was present in the reaction. This resulted in final

concentrations for the reaction of 1 µM substrate and 100 µM ATP. After incubation for 2, 3, 4, and 5 hours at 30°C the reaction was stopped by taking 1 µl of the reaction mixture and adding 24 µl of 200 nM phosphorylated Ru-peptide and 25 µl IMAP beads in IMAP binding buffer, giving a final phosphorylated Ru-peptide concentration of 100 nM. Three different dilutions of IMAP™ beads were used, 1:2000, 1:4000 and 1:8000. The beads were incubated for 60 minutes at RT. For the readout the final substrate concentration was 20 nM (phosphorylated) Alexa-peptide and with the final ATP concentration of 2 µM the FRET signal should be easily detectable (Figure 5). Figure 8 shows the kinetic of the kinase reaction for the positive and negative controls for the three IMAP dilutions (blue 1:2000, red 1:4000, green 1.6000). The observed FRET signal was sufficiently strong for all three dilutions.

Example 7: ATP dependence of the kinase reaction

The dose response curve for ATP (Figure 9) shows that the ATP used in the assay (100 µM) was well above the EC_{50} of 3.6 µM. Note that these were the concentrations in the kinase reaction, in the readout they were 50 times less. An excess of ATP prevented from finding a competitve inhibitor for the ATP binding site and assured maximal phosphorylation without inhibitor. The signal reached a maximum at 32 µM ATP. With higher ATP concentrations the signal decreased again due to free ATP in the readout mixture which bound to the IMAP™ beads and thus decreased the binding capacity of the IMAP™ beads. This problem of the binding capacity of the IMAP™ beads is well known and needs to be adjusted in the assay by proper titration of the assay reagents.

The decrease of the FRET signal due to increasing ATP concentration measured here (decrease by a factor of 1.6 and 1.9 from maximal signal (32 µM, 0.64 µM in the readout) to 128 µM (2.56 µM in the readout) and 256 µM (5.12 µM in the readout)) compared very well to the titration of ATP (Figure 5) which resulted in a decrease of a factor of 1.6 and 2.1 for these ATP concentrations.

Claims

1. A method for determining kinase or phosphatase activity comprising interacting a binding partner, a detection molecule and a substrate molecule, wherein said detection molecule is labeled with an energy donor or acceptor and said substrate molecule is labeled with an energy donor, if the detection molecule is labeled with an energy acceptor, or with an energy acceptor, if the detection molecule is labeled with an energy donor, wherein said binding partner only binds phosphorylated substrate molecule and the detection molecule, which method comprises the steps of:

a) phosphorylating said substrate molecule with a kinase or dephosphorylating a phosphorylated substrate molecule with a phosphatase; and

b) interacting the substrate molecule obtained in step a) with said binding partner and said detection molecule; and

c) determining kinase or phosphatase activity by measuring energy transfer from the energy donor to the energy acceptor of said substrate molecule and said detector molecule bound to said binding partner.

2. The method of claim 1, wherein said binding partner are metal ions on immobilized metal ion affinity-based fluorescence polarization (IMAP)^{TM} beads.

3. The method of claim 1 or 2, wherein the energy donor has an emission lifetime of less than 50 microsecs.

4. The method of claim 3, wherein said emission lifetime is less than 10 microsecs.

5. The method of claim 3, wherein said emission lifetime is less than 5 microsecs.

6. The method of claim 3, wherein said emission lifetime is 2 to 3 microsecs.

7. The method of any one of claims 1 to 6, wherein the energy donor is a Ru-luminophore.

8. The method of any one of claims 1 to 7, wherein the energy acceptor is a fluorophore exhibiting a spectral overlap with the Ru-complexes which yields an R_0 of at least 10 Å.

9. The method of claim 8, wherein said energy acceptor is Alexa Fluor 700, Atto 700, Dy701 or Alexa Fluor 750.

10. The method of claim 9, wherein said energy acceptor is Alexa Fluor 700.

11. The method of claims 7 to 10, wherein said Ru-luminophore is Ru(batho)2bpy.

12. The method of claims 1 to 11, wherein said method is performed with a system wherein the sample moves with respect to the optical detection pathway.

13. The method of claims 1 to 12, wherein said system is a labware spin system.

14. The method of claims 1 to 12, wherein said system is a microfluidic system or a flow through cell.

15. The method of any one of claims 1 to 14, wherein the dilution of the binding partner is at least 500 fold.

16. The method of claim 15, wherein the dilution is between 1000 fold and 10,000 fold.

17. A kit for performing the method according to claims 1 to 16, comprising, in separate containers, beads which are doped with metal ions, more than one detection molecule comprising an energy donor or an energy acceptor, more than one substrate peptide comprising an energy acceptor or an energy donor, wherein said detection molecules and said substrate peptides are provided as sets of FRET pairs.