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Application Note

Gyrasol Kinase Assays

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Gyrasol Technologies method for screening of kinases and phosphatases is a proprietary, robust and homogeneous fluorescence-based detection platform that directly measures the activity of a target enzyme. Assays tolerate high concentrations of substrate (between 100 nM to 200 µM) and ATP (1 mM) and thus allows for identification of substrate or ATP competitive inhibitors. Assays can be performed in kinetic mode for many kinases, thus greatly simplifying assay development and determination of the mode of action of inhibitors.

Advantages

- Direct detection of substrate phosphorylation
- Linear dose response
- Operates with green to red fluors (Fluorescein to TAMRA)
- Adaptable to a variety of substrates (lipid, protein)
- Continuous monitoring
- High substrate tolerance (up to 200 µM)
- High ATP tolerance (up to 1 mM)

Assay Principle

The Gyrasol Sensor is a fluorescent small molecule containing trivalent metal ions that specifically coordinate with phosphoryl groups to detect post-translational modifications. The phosphoryl groups can be associated to serine, threonine or tyrosine amino acids on peptide substrates as well as on phosphorylated lipids, thus making Gyrasol assays truly universal and generic for the detection of kinase and phosphatase activities.

Gyrasol assays involve 2 steps; the enzymatic conversion of dye-labeled substrate, and the detection of phosphorylation with the Gyrasol Sensor. The enzymatic reaction is started by incubating the substrate with the enzyme of interest in the presence of ATP. Following an incubation period, the reaction is terminated by addition of stop buffer and Sensor is added. The Sensor associates to the phosphoryl groups of the dye-labeled substrate and is brought into a proximity that allows energy transfer from the Substrate to the Sensor to occur. The fluorescence intensity of the substrate decreases proportionally to the increased percentage of phosphorylation.

When calibrator peptides are used, the precise amount of phosphorylation can be quantified.

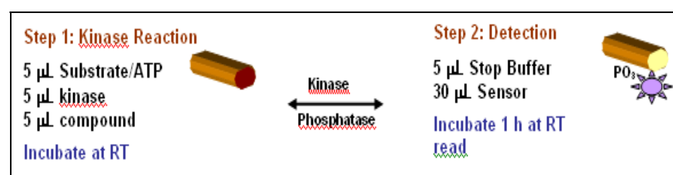

Figure 1: **Schematic of a Gyrasol assay.** In Step 1, dye-labeled substrate (block with red starburst) is phosphorylated by a kinase of interest in the presence of ATP or dephosphorylated by a phosphatase. In step 2, following metal-ion mediated association of the Sensor (purple star) to the phosphorylated substrate, fluorescence quench of the substrate is monitored.

Materials and Methods

Dye-labeled substrate and calibrator peptides were obtained from Anaspec (San Jose, CA). The lipid substrates BODIPY-TMR Phosphoinositide and TAMRA-Sphingosine were from Echelon Biosciences (Salt Lake City, UT). Dye-labeled substrate and calibrator peptides were obtained from Anaspec (San Jose, CA). The lipid Substrates BODIPY-TMR Phosphoinositide and TAMRA-Sphingosine were from Echelon Biosciences. The enzyme PKA was from Biomol (Plymouth Meeting, PA), Fyn and PI3Kα from Millipore (Temecula, CA) and Sphingosine1 from BPS Biosciences (San Diego, CA). Reactions were carried out for 1 hour at room temperature in assay buffer in black 384-well Cliniplates (Fisher Scientific, Santa Clara, CA) and terminated by addition of 5 µL stop buffer. A 2-fold volume of Gyrasol Sensor (Gyrasol Technologies, Santa Fe, NM) was added for 1 hour at room temperature and the decrease of fluorescence monitored using a BioTek Synergy 4 plate reader (Winooski, VT).

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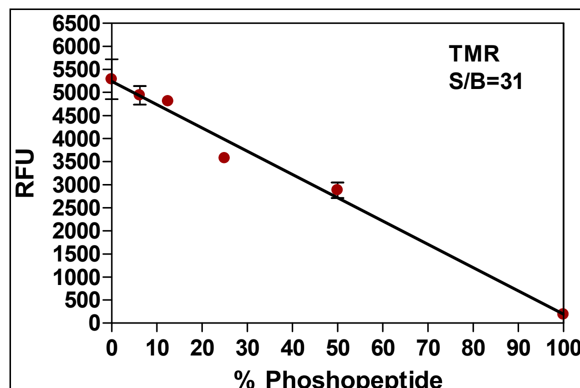
Excitation of 490 nm or 540 nm was used with monochromator setting and emission monitored at 520 nm and 590 nm for fluorescein or TMR analogs, respectively. Data was graphed using GraphPad Prism (San Diego, CA).

Linear Dose Response with a Variety of Substrate Fluors

Substrates labeled with Fluorescein, TAMRA and their analogs are suitable substrates for the Gyrasol Sensor. When mixtures of phosphorylated and non-phosphorylated peptides are combined in various ratios, a linear calibrator curve is obtained.



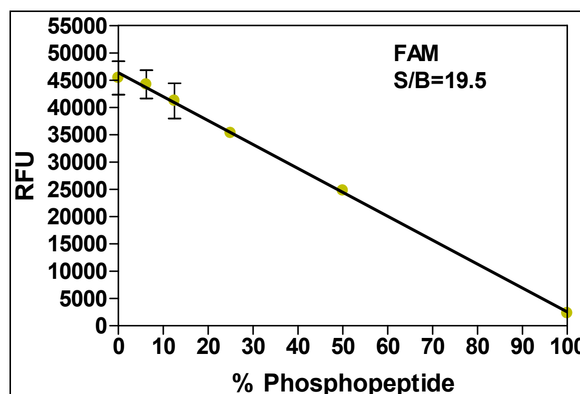
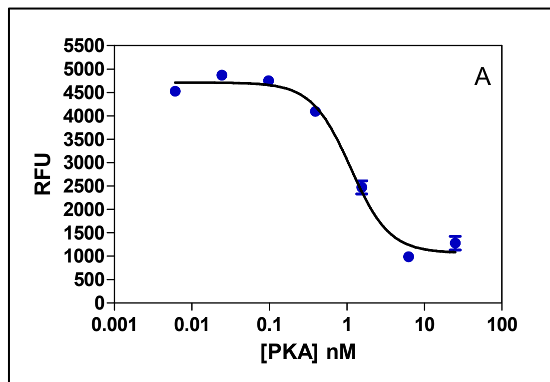
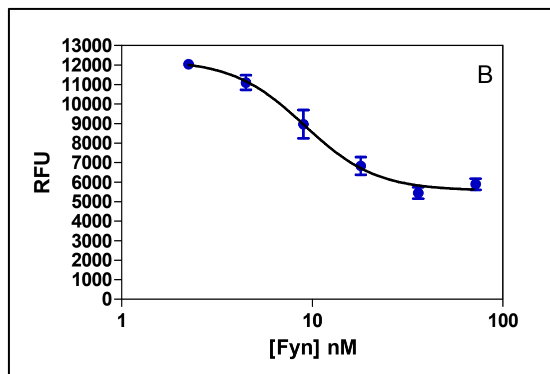


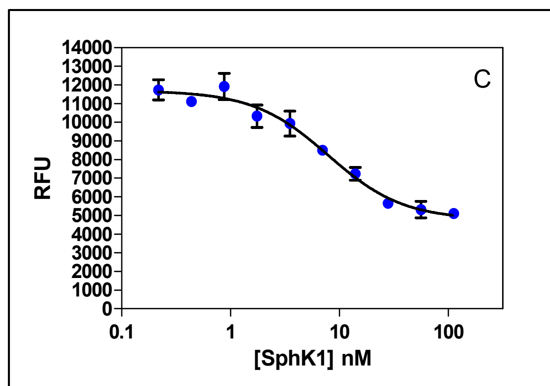
Figure 2: Phosphopeptide Calibrator Curves: TMR- or FAM-labeled, peptide substrates (10 μM) were mixed in various ratios of phosphorylated and non-phosphorylated. Following addition of Sensor, linear calibration curves with Signal to Backgrounds (S/B) of 31 and 19.5 for TMR and FAM, respectively, were obtained.

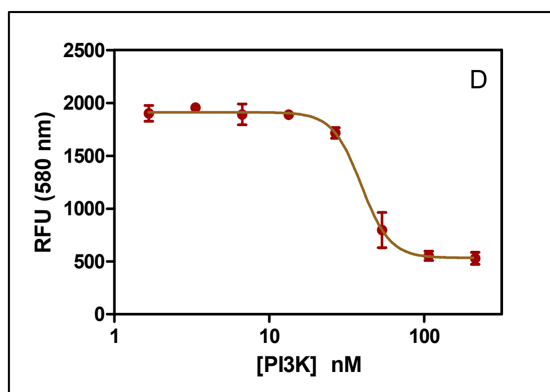
Adaptable to a Variety of Substrates

The Gyrasol Sensor associates to phosphoryl groups on substrates of various chemical natures. Peptide substrates containing phosphoserine, phosphothreonine or phosphotyrosine can be detected as well as phosphorylated lipid substrates (Figure 3). When calibration curves are included in the experimental setup, the precise amount of substrate conversion can be determined (Figure 3 E).









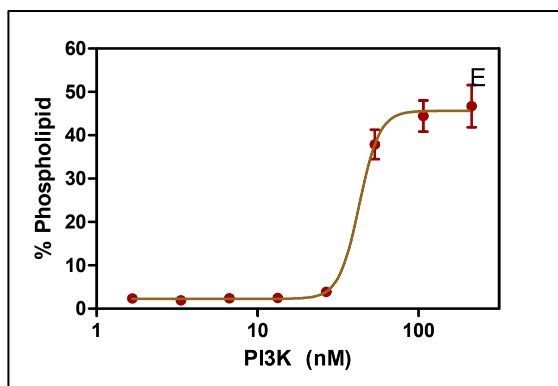


Figure 3: Enzyme Dose Response Curves: (A) PKA was serially diluted in assay buffer (10 mM TRIS, 10 mM $MgCl_2$, 0.1% BSA, pH 7.2) in the presence of 10 µM ATP and substrate (10 µM HiLyte$_{488}$-LRRASLG). (B) Reaction conditions for Fyn were 50 µM ATP and 10 µM substrate (TAMRA-KVEKIGEGTYGVVYK) in assay buffer (10 mM TRIS, 10 mM $MgCl_2$, 0.1% BSA, pH 7.2). (C) Sphingosine Kinase I was reacted with 4 µM TAMRA-Sphingosine in 10 mM TRIS, pH 7.2, 10 mM $MgCl_2$, 0.01 % Triton X-100 buffer in the presence of 50 µM ATP. (D) PI3Kα was reacted in 25 mM HEPES, pH 7.4, 50 mM $MgCl_2$, 5 mM DTT and 50 µM ATP with 1 µM BODIPY-TMR-Phosphatidylinositol and the product conversion determined (E) using back calculation with a calibration curve performed simultaneously with the enzyme reaction. Reactions proceeded for 1 hour at room temperature in wells of a 384-well plate followed by addition of 5 µL stop buffer and 30 µL Sensor, diluted 1:20 in Sensor dilution buffer. Fluorescence quench was monitored after 1 hour with an excitation wavelength of 450 or 540 nm and 490 or 590 nm emission for HiLyte$_{488}$ or BODIPY-TAMRA, respectively. Curve fit was performed using sigmoidal dose response.

High ATP Tolerance

The Gyrasol platform tolerates concentrations of ATP up to 1 mM with minimal loss of signal. This allows to establish relevant ATP K_m and further, the screening of structurally diverse libraries for non-ATP competitive inhibitors, which requires high ATP tolerance of the screening platform.

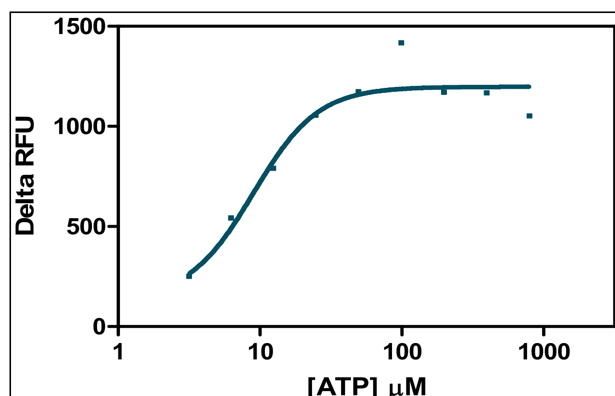
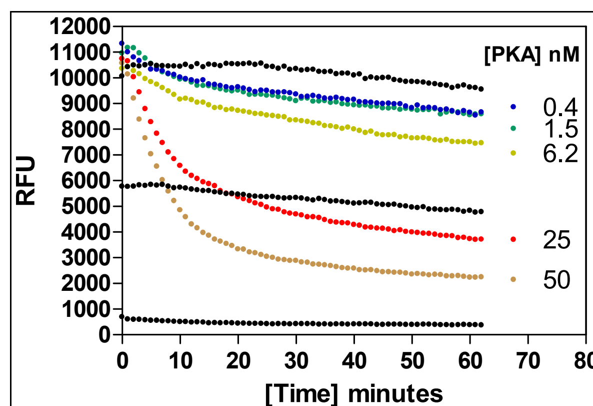


Figure 4: ATP tolerance curve for PI3Kα BODIPY-TMR Phosphatidylinositol (1 µM) was added to various concentrations of ATP in the presence or absence of 44 nM PI3Kα and the reaction stopped by addition of stop buffer. Sensor was added and the delta RFU between reactions with and without enzyme calculated.

Continuous Monitoring Mode

The monitoring of enzyme activity as it occurs in “real time” simplifies the optimization of assay parameters, the establishment of Michaelis-Menten constants for substrate and ATP well as the determination of the mode of action of inhibitors. The presence of Sensor does not interfere with PKA activity and dose responses similar to those obtained in endpoint mode were obtained. However, Sensor does interfere with the activity of some enzymes, such as Sphingosine kinase, and kinetic monitoring is not possible for every enzyme.



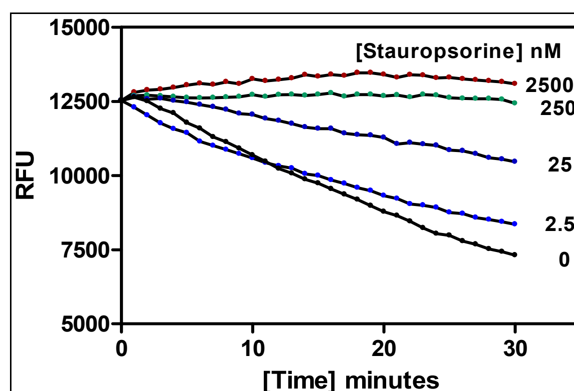


Figure 5: Continuous Monitoring. (Top) Various dilutions of PKA in assay buffer and 50 µM ATP were added to wells of a 384-well plate in the presence of 250 nM Sensor. (Bottom) Dilutions of Staurosporine were added to assay buffer containing 50 µM ATP in the presence of 250 nM Sensor. Fluorescence quench was monitored at 450 nm excitation and 490 nm emission in 1-minute intervals.

High Substrate Tolerance

In contrast to most fluorescence-based platforms, the Gyrasol platform tolerates high concentrations of substrate. Substrate concentrations can vary between 100 nM to 200 μM, which allows establishing relevant K_m and identification of substrate-competitive inhibitors in the presence of high concentrations of substrate.

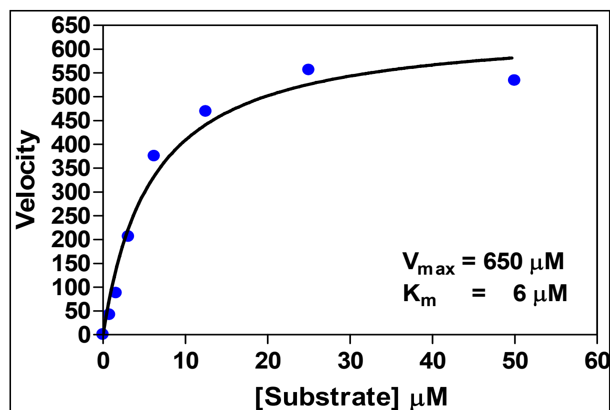


Figure 6: Michaelis-Menten Plot for Substrate PKA (12 nM) and Sensor were added to various concentrations of $Hilyte_{488}$-labeled Kemptide and substrate conversion monitored in kinetic mode using 450 nm excitation and 490 nm emission. Slopes were plotted against the concentration of substrate and V_{max} and K_m calculated using Michaelis-Menten equation in GraphPad Prism.

Conclusion

The Gyrasol assay is a robust platform suitable for diverse substrates that can be labeled with various fluors. Assay development is facilitated by readout in either endpoint or kinetic mode. The high ATP and substrate tolerance make this an attractive platform for screening of inhibitors.

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