SUPPORTING INFORMATION

A Time-Resolved Luminescence Biosensor Assay for Anaplastic Lymphoma Kinase (ALK) Activity

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1. Experimental Procedures

Peptide synthesis and purification. Peptides were synthesized using a Prelude Parallel peptide synthesizer (Protein Technologies, USA) on Rink-amide-MBHA resin (Peptides International, USA). The peptides were synthesized at a 50 μmol scale. The synthesis was performed using Fmoc (9- fluorenylmethoxy-carbonyl)-protected amino acids (Peptides International, USA) with coupling reagent 2-(6-chloro-1H-benzotria- zole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HCTU, AnaSpec, USA) in the presence of N-methylmorpholine (NMM, Sigma-Aldrich, USA) in dimethylformamide (DMF, VWR International). A 20% piperidine solution in DMF was used for Fmoc deprotection. Peptide cleavage and side-chain deprotection were performed simultaneously by using 10 mL mixture of trifluoroacetic acid (TFA)/water/ethane dithiol (EDT)/ triisopropylsilane (TIS) (94:2.5:2.5:1, v/v). Cleaved peptides were then precipitated and washed three times by cold diethyl ether. Precipitated peptides were re-dissolved in acetonitrile/water/TFA (50:50:0.1, v/v), flash frozen by liquid nitrogen, and lyophilized. The lyophilized peptides were verified by liquid chromatography-mass spectrometry (LC-MS, Accela/LTQ, Thermo Scientific, USA) and purified by 1200 Series preparative reverse-phase high performance liquid chromatography (HPLC, Agilent Technologies, Agilent, USA) equipped with a C18 reverse-phase column. Purified peptides were then tested again on LC-MS (Accela/LTQ, Thermo Scientific, USA).

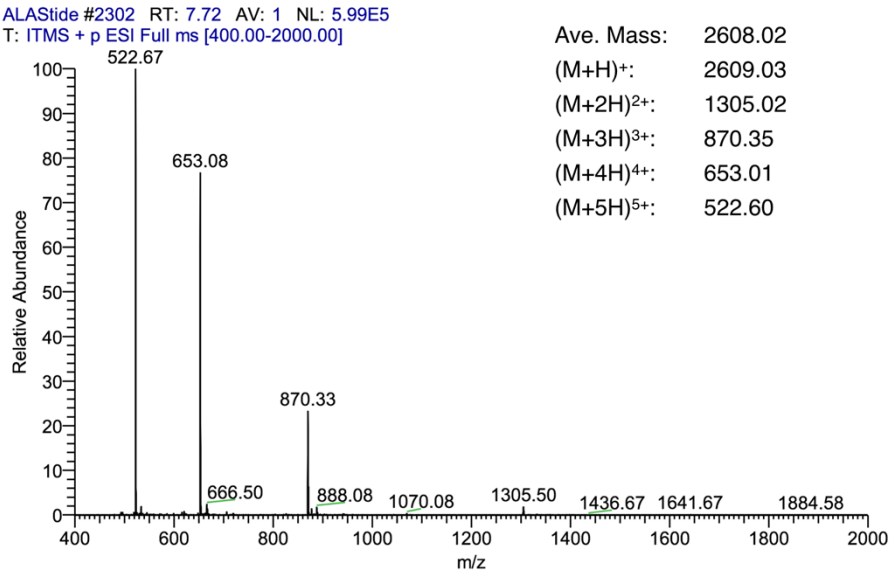

Figure S1. ESI-MS spectrum of ALAStide.

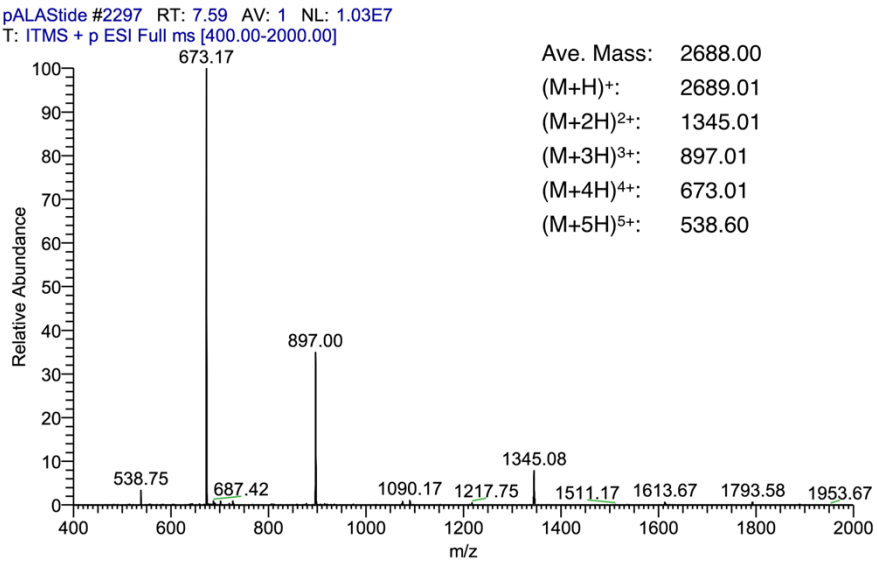

Figure S2. ESI-MS spectrum of pALAStide.

Luminescence emission measurements. Lanthanide luminescence emission spectra were measured on a Synergy4 plate reader (Biotek, USA) at room temperature in 384-well black plates (Fluortrac 200, Greiner bio-one, Germany). The excitation wavelength was 266 nm. Ten technical replicate readings were performed to collect time-resolved luminescence spectra between 450 and 650 nm with 2 nm increments with 50 μs delay time and 1 ms measurement time. All other instrumental parameters (e.g. sensitivity, a parameter of the Synergy4 that is similar to gain) were fixed for all experiments. The spectral area under the curve (AUC) was used for quantification of luminescence emission.

Tb^{3+} binding experiments. Tb^{3+} binding of ALAStide and pALAStide were measured by time-resolved Tb^{3+} luminescence emission as described above. For the Job's plot, the total combined concentration of peptide and Tb^{3+} was fixed at 20 μM while their ratio was varied (i.e., 2 μM peptide and 18 μM Tb^{3+}, 4 μM peptide and 16 μM Tb^{3+}, 18 μM peptide and 1 μM Tb^{3+}). To determine binding affinity, peptide (final concentration 2 μM) was mixed with different concentrations of Tb^{3+} ranging from 0 to 20 μM. Reference solutions that contained the varying concentrations of Tb^{3+} but without peptide were used to subtract background luminescence from experimental data. All samples were prepared in 10 mM 2-[4-(2-hydroxyethyl)-piperazin-1-yl]ethanesulfonic acid (HEPES) buffer (pH = 7.5) and 100 mM NaCl at a volume of 100 μL. The dissociation constant was calculated by nonlinear fitting in GraphPad Prism software. Detailed equations are provided below.

***In vitro* kinase assay.** Active recombinant ALK, Lyn, Abl and Csk were purchased from a commercial manufacturer (Millipore, USA). Amounts of each kinase that corresponded to equal units were incubated with the kinase reaction buffer (e.g. 40nM ALK, 100 μM adenosine triphosphate (ATP), 10 mM $MgCl_2$, and 25 mM HEPES, pH 7.5, total volume 200 μL) at 37 °C, and the reactions were initiated by adding ALAStide to a final concentration of 100 μM. At selected time points, aliquots were taken and quenched. For the Tb^{3+} luminescence assay, 20 μL of sample was quenched in 20 μL 6 M urea, followed by addition of luminescence buffer ($TbCl_3$ and NaCl) such that the final concentration of Tb^{3+} was 200 μM and that of NaCl was 100 mM (in a total volume of 50 μL). Time-resolved luminescence emission spectra were collected as described above.

For the ELISA-based chemifluorescent assay, 5 μL sample was quenched in 20 μL of 40mM Na_2EDTA. Each quenched sample was incubated for 1 h in a 96-well Neutravidin coated plate (15 pmol biotin binding capacity per well, Thermo Scientific, USA) in Tris-buffered saline (TBS) containing 5% milk protein and 0.05% Tween 20 (termed blocking buffer). Each well was then washed with the TBS buffer and incubated with mouse antiphosphotyrosine monoclonal antibody 4G10 (1:12000 dilution in blocking buffer, Millipore, USA) for 1 h. Each well was again washed, and incubated with horseradish peroxidase-conjugated goat antimouse

immunoglobulin G (IgG) secondary antibody (1:1000 dilution in blocking buffer, Abcam, USA) for 1 h. Wells were washed once again and treated with 100 μL Amplex Red (Invitrogen, USA) reaction buffer (100 μM Amplex Red reagent, 1.8 mM H_2O_2, 50mM sodium phosphate buffer, pH=7.5) for 20 min. Fluorescence was measured using an excitation wavelength of 532 nm and an emission wavelength of 590 nm.

ALK inhibition assay. Recombinant active ALK kinase domain was incubated at 37 °C with the kinase reaction buffer described above, including dimethylsulfoxide (DMSO, Sigma, USA) or various concentrations of crizotinib (Selleck Chemicals, USA), for 5 min prior to the start of the reaction. The final concentration of DMSO was limited to 0.1% v/v in each sample. The kinase reactions were initiated by adding ALAStide to a final concentration of 100 μM (in a total volume of 20 μL). Each reaction was quenched after 90 min in 20 μL 6 M urea, followed by addition of the luminescence buffer and luminescence measurement as described above. Equations for calculating high-throughput screening parameters are described below. *Substrate concentration choice:* The concentration of ALAStide was set to 100 μM in this assay for optimized luminescence spectrum recording. The +4F, +5F, +6R and +7K residues have been shown to be critical for a high K_m (340 μM) for the YFFtide on which ALAStide is based (L. A. Pinna et al., 2005, ref. 9 in manuscript). The YFFtide did not show substrate inhibition at concentrations up to 600 μM. These residues are retained in ALAStide; therefore, though the K_m of ALAStide has not yet been determined, 100 μM is very likely to be lower than the K_m of ALAStide (a requirement for optimal inhibitor screening).

Effect of reaction buffer components on luminescence signal. The signal to noise ratio of luminescence signal in kinase reaction buffer was attenuated by the presence of ATP (Figure S3).

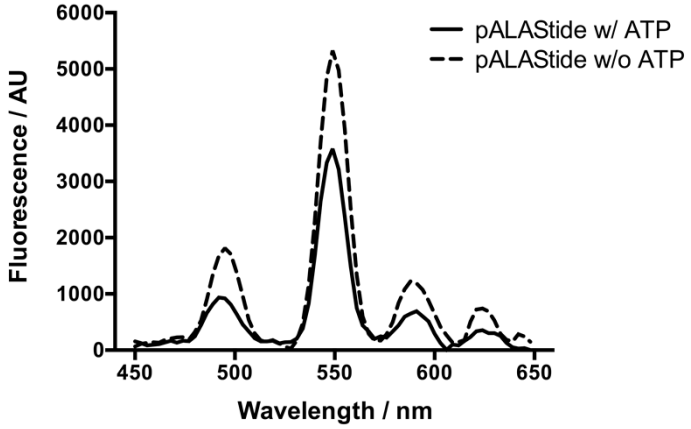

Figure S3. ATP attenuation of signal to noise ratio for luminescence signal

As previously discussed (Lipchik A.M., Parker L.L.. Anal. Chem., 2013, 85: 2582-8), most components in the reaction mixture did not interfere with luminescence signal, except ATP. The three phosphate groups of ATP can act as hard base ligand of Tb^{3+} and compete with pALAStide for Tb^{3+} binding. In this study, the introduction of ATP to the kinase reaction buffer led to approximately 30% decrease in luminescence signal from pALAStide-Tb^{3+} complex. Nevertheless, the parameters calculated from the calibration curve (for which samples were prepared in the kinase assay buffer containing ATP) still satisfy inhibitor screening requirements. A future comprehensive optimization may be able to alleviate such effect and provide further improvement of this assay.

2. Calculations.

Dissociation Constant Calculation. The data collected from Tb^{3+} binding experiments were fit to the following equation:

$$F = \frac{F_m}{2[P]_0}\{([Tb^{3+}]_0 + [P]_0 + K_d) - \sqrt{([Tb^{3+}]_0 + [P]_0 + K_d)^2 - 4[Tb^{3+}]_0[P]_0}\ \}$$

In this equation, F is the Tb^{3+} luminescence and the dependent variable. F_m is the maximum luminescence emission. $[Tb^{3+}]_0$ is the total Tb^{3+} concentration. $[P]_0$ is the total peptide concentration. K_d is the equilibrium dissociation constant.

High-Throughput Screening Parameters Calculation. The Z′ factor was calculated according to the following equation:

$$Z' = \frac{(\mu_p - \frac{3\sigma_p}{\sqrt{n}}) - (\mu_n + \frac{3\sigma_n}{\sqrt{n}})}{\mu_p - \mu_n}$$

The signal window (SW) was calculated according to the following equation:

$$SW = \frac{(\mu_p - \frac{3\sigma_p}{\sqrt{n}}) - (\mu_n + \frac{3\sigma_n}{\sqrt{n}})}{\frac{\sigma_p}{\sqrt{n}}}$$

In both equations, n is the number of replicates of measurements. μ_p and μ_n are the average AUC (area under spectrum curve) value of the positive (pALAStide or uninhibited) and negative (ALAStide or excess crizotinib treated ALK) controls, respectively. σ_p and σ_n are the standard deviation of the corresponding values.