I. Methods

Peptide synthesis. Peptides were synthesized via standard solid phase synthesis using Fmoc chemistry, as described previously.[1] To label the amino-terminus of peptide 5 with ROX for the FRET assay, the ROX fluorophore (0.45 mmol, Fluka) was coupled as described for each amino acid using HOBT (0.9 mmol, Acros), N,N'-diisopropylcarbodiimide (0.60 mmol, Acros) in DMF (5 resin volumes). The resin was then washed with DMF (10 x with 10 resin volumes), CH₂Cl₂ (3 x with 10 resin volumes), CH₂Cl₂:ether (1:1) (3 x with 10 resin volumes), and ether (3 x with 10 resin volumes) in preparation for resin cleavage or storage, as described previously.[1] All peptides are purified using a reverse-phase C18 Altima column (250 x 10mm, 5 µm particle, 300 Å) on a Varian(prep star) HPLC system, as described.[1]

Dansylation of peptides. All reactions were performed as previously described.[2] Each reaction contained either 200 units of PKA (New England Biolabs), 40 units of Abl kinase (New England Biolabs), or 40 units of CK2 (New England Biolabs). Either 37.5 µM of PKA substrate peptide 3, 15.0 µM of CK2 substrate peptide 4 (Promega), or 15.0 µM of Abl peptide substrate 5 (New England Biolabs) were also used. To create a phosphorylated peptide, 2 mM ATP was used. To create a dansylated phosphopeptide, 2 mM ATP-dansyl (Adenosine 5'-triphosphate [g]-N-(Dimethylaminonaphthalenesulfonyl)-1,5-pentanediamine from Affinity Labeling Technologies Inc) was used after evaporation of the methanol storage solvent. All reactions were performed in a 10 µL total volume with the manufacturers specified kinase buffer and were incubated at 30°C for 2 h. In the case of the reactions with CK2, better yields were obtained when a final concentration of 50 µM MgCl₂ and 2% glycerol were added to the manufacturers recommended buffer. To analyze the products of the reactions by MALDI-TOF MS, 1 µL of the crude reaction mixture was combined with 5 µL of matrix (α-cyano-4-hydroxy cinnamic acid saturated in 70% acetonitrile, 30% water and 0.1% TFA),
evaporated on a MALDI plate, and analyzed using a Bruker Ultraflex MALDI-TOF mass spectrometer.

**Quantitative mass spectrometric analysis.** Dansylated phosphopeptides (produced with ATP-dansyl) and phosphorylated peptides (produced from ATP) were generated as described above. Each peptide was incubated in a solution of 2 N acetyl chloride in [D$_2$]methanol or [D$_4$]methanol (200 µL, which produces anhydrous HCl in methanol or [D$_4$]methanol) at 24 °C for 2 h to afford methyl esterification, as described (Scheme S1). Because the conditions of esterification were acidic, the phosphoramidate bond linking the dansyl label to the peptide was cleaved, creating the phosphopeptide required for quantitative mass spectrometric analysis. For MALDI-TOF MS, the solvent was evaporated and equal quantities of the isotopically differentiated peptide samples were combined and prepared as described above (Figures S1-S3).

![Scheme S1: Schematic representation of quantitative mass analysis.](image)

**Dansylation of β-casein.** β-Casein (1.8 mM, Sigma) was combined with 80 units CK2 and 2 mM of either ATP or ATP-dansyl, diluted to 10 µL with the manufacturer’s kinase reaction buffer supplemented with a final concentration of 50mM MgCl$_2$ and 2% glycerol, and incubated for 2 h at 30 °C. The reaction was diluted with an equal volume of 50 mM NH$_4$HCO$_3$ before heat denaturing at 90 °C for 60 seconds. To afford complete proteolysis, 2.25 µg of sequencing grade trypsin (Promega) was incubated with the protein at 37 °C overnight. The solvent was removed and the peptide fragments were subjected to acidic esterification and quantitative MS analysis as described above (Figure S4).

**Enzyme coupled kinetics analysis.** Kinetics were assessed using a previously reported assay$^{[4, 5]}$ that monitors NADH absorbance at 360 nm after a series of enzyme coupled reactions initiated by production of the ADP byproduct of the kinase reaction. To test if the ATP analogues would interfere with the enzyme coupled assay, we tested the kinetics system with ADP in the presence of the desired ATP derivative. Briefly pyruvate kinase (PK), lactic dehydrogenase (LDH), phosphoenolpyruvate (PEP) and NADH were obtained from Sigma.
ADP (125 µM) and ATP derivative (250 µM for ATP-dansyl and 1mM for ATP or ATP-γS) were added to a pre-incubated solution of 30 units/mL lactic LDH, 12 units/mL PK, 1 mM PEP, 0.128 mM NADH, 0.5 mg/mL bovine serum albumin, 50 mM HEPES (pH 7.5), 150 mM NaCl, 10 mM KCl, and 10 mM MgCl₂ in a total volume of 25 µL. There was no significant change in rate from experiments containing only ADP and those with the addition of ATP analogue. It is important to note that to avoid a significant rate decrease, ATP analogue should be added after the pre-incubation period.

Kinase kinetic experiments were typically performed using the above conditions with the exclusion of ADP and pre-equilibrated with 1.29 mM of kemptide (LRRASLG). 5-250 µM ATP-dansyl was used, while 5 µM- 2 mM of ATP or ATP-γS was used. We note that a maximum of 250 µM ATP-dansyl was used to avoid significant absorbance by dansyl at 360 nm. PKA (240 units, 12.63 nM, 0.48 µg/mL) was added to commence the reaction in a 100 µL total volume. In the case with Abl, 40 units of Abl (2.73 nM, 0.31 µg/mL) were used in a total volume of 25 µL with 1.25 mM peptide 5, with all other components used at the same final concentrations. Absorbance at 360 nm was taken every minute for 20 min on a GENios Plus Fluorimeter (Tecan). $K_m$ and $V_{\text{max}}$ were calculated using a Lineweaver-Burke plot. The $k_{\text{cat}}$ value was then calculated by dividing the $V_{\text{max}}$ by the concentration of enzyme in appropriate units.

**FRET assay.** We chose ROX as the FRET donor in these experiments due to the appreciable overlap between the emission of dansyl and excitation of ROX, and the minimal overlap of the dansyl and ROX emission wavelengths. In addition, rhodamine derivatives have been coupled with dansyl previously in FRET assays. ATP or ATP-dansyl (0.6 mM), ROX 5 peptide substrate (0.1 mM) and Abl kinase (200 units) were combined, in the manufacturer kinase buffer, and incubated at 30 °C for 2 h. Typical reaction volumes were 10 µL. The reactions were diluted to 75 µL with water, transferred to a 384-well plate, and the fluorescence signal due to FRET emission at 595 nm was observed after excitation at 360 nm using a GENios Plus Fluorimeter (Tecan). The data are shown in Table S1 and Figure S7. To verify that changes in lipophilicity and viscosity have no effect on ATP-dansyl fluorescence, we tested the environmental sensitivity of dansyl by comparing the fluorescence of ATP-dansyl alone to the fluorescence of ATP-dansyl in the presence of Abl or 10% glycerol. In all cases, fluorescence intensity at 595 nm was equivalent.

For the reactions containing mammalian cell lysates, 3 X 10⁸ HeLa cells obtained from the National Cell Culture Center (www.nccc.com) were lysed in 3 mL of lysis buffer (50 mM Tris pH 8, 150 mM NaCl, 10% Glycerol, 0.5% Triton X-100) containing protease inhibitor cocktail V (1X- Calbiochem) by rotation at 4°C for 10 minutes. The cell debris was collected at 12 000 rpm and the supernatant (6 mg/mL of total protein content) was filtered through an
EconoPak 10DG desalting column (BioRad), which likely removes endogenous ATP. We note that unfiltered lysates were also compatible with the kinase-catalyzed dansylation reaction (data not shown), consistent with our previous work. The lysates were stored at 4 °C until use in subsequent phosphorylation reactions. The use of cell lysates a 384-well plate format was unfeasible because the low volume required in this format (6 µL) did not contain enough active, endogenous kinase activity for the reaction to produce signal. As an alternative, a 96-well plate format was used. Here, ATP or ATP-dansyl (1 mM), peptide (35 µM) and Abl kinase (200 units) were combined in the manufacturer kinase buffer using a typical reaction volume of 50 µL. For the FRET assays containing lysates, the reactions were performed as described except no kinase was added and lysates (32 µL) were used in the place of buffer to obtain the 50 µL total reaction volume. Reactions were also performed with unfiltered lysates to determine the activity with competitive ATP. Results were identical to those with filtered lysates.

**FRET inhibition assay.** Conditions identical to the FRET assay were used. In reactions utilizing inhibitors, a solution of inhibitor in 10% DMSO in water was incubated with the enzyme for at least ten minutes prior to addition to the remaining reaction components. A final concentration of 30 µM staurosporine (MP Biomedicals), 30 µM DRB (5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole; Sigma), or 1 mM Imatinib (STI-571; Toronto Research Chemicals) was used.

**FRET kinetics analysis.** Abl (40 units, 6.83 nM, 0.12 µg/mL) and ROX 5 peptide substrate (1.25 mM) were incubated with 5-250 µM ATP-dansyl in provided manufacturer's buffer in a 384-well plate format. Reactions were performed in a 25 µL volume. Measurements of FRET were taken every two minutes for 30 min on a GENios Plus Fluorimeter (Tecan), using an excitation wavelength of 360 nm and an emission of 595 nm. To convert fluorescence intensity into micromolar values, the following, previously published equation was used:

\[
P(t) = \frac{I(t) - f_s S_0}{f_p - f_s}
\]

where \( P(t) \) is the concentration of product, \( I(t) \) the fluorescent signal, \( f_s \) the fluorescence per µmol of starting material, \( S_0 \) the original concentration of starting material, and \( f_p \) the fluorescence per µmol of product. The fluorescence constants \( (f_s \) and \( f_p \) for the starting material and product were obtained by plotting fluorescence intensity versus concentration of compound and using the slope of the resulting line. The slopes were 3892.3 FU/µmol for ATP-dansyl \( (f_s) \) and 5880.9 FU/µmol for peptide 6 \( (f_p) \). \( K_m \) and \( V_{max} \) were calculated using a Line-weaver-Burke plot (Figure S6 and S7). The \( K_{cat} \) was then calculated by dividing the \( V_{max} \) by the concentration of enzyme in appropriate units.
References


Table S1: Fluorescence data from Figure 2B, with additional controls[^a]

<table>
<thead>
<tr>
<th>Reaction Components</th>
<th>Peptide 5</th>
<th>ROX 5</th>
<th>Peptide 5</th>
<th>ROX 5</th>
<th>ROX 5</th>
<th>ROX 5</th>
<th>ROX 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>ATP</td>
<td>ATP-dansyl</td>
<td>ATP-dansyl</td>
<td>ATP-dansyl</td>
<td>ATP-dansyl</td>
<td>ATP-dansyl</td>
<td>ATP-dansyl</td>
</tr>
<tr>
<td>Abl</td>
<td>Abl</td>
<td>Abl[^a]</td>
<td>Abl</td>
<td>Abl</td>
<td>Abl</td>
<td>Abl</td>
<td>Abl</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Column 1[^c]</td>
<td>Column 2[^c]</td>
<td>Column 3[^c]</td>
<td>Column 4[^c]</td>
<td>Column 5[^c]</td>
</tr>
<tr>
<td>Normalized Fluorescence</td>
<td>0.016 ± 0.0063</td>
<td>0.40 ± 0.10</td>
<td>0.73 ± 0.07</td>
<td>1.0 ± 0</td>
<td>1.30 ± 0.05</td>
<td>1.03 ± 0.05</td>
<td>1.01 ± 0.02</td>
</tr>
</tbody>
</table>

[^a]: Normalized fluorescence values were calculated by dividing the fluorescence intensities of each reaction by the intensity of the reaction with ROX 5 and ATP-dansyl but without Abl kinase (Column 1), with the baseline set to 1.0. The average and standard error are shown from three independent trials. bThe background signal is due to emission of ATP-dansyl; ATP-dansyl alone gave equivalent fluorescence signal. cColumn number corresponds to the column in Figure 2B.
**Table S2:** Fluorescence data from Figure 2C

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Components</th>
<th>ROX 5</th>
<th>ROX 5</th>
<th>ROX 5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ATP-dansyl</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>lysates</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Staurosporine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Column 6[^b]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Column 7[^b]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Column 8[^b]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normalized Fluorescence</td>
<td>1.0 ± 0</td>
<td>1.32 ± 0.02</td>
<td>1.06 ± 0.02</td>
<td></td>
</tr>
</tbody>
</table>

[^a]: Normalized fluorescence values were calculated by dividing the fluorescence intensities of each reaction to the intensity of the reaction with ROX 5 and ATP-dansyl but without HeLa cell lysates (Column 6), with the baseline set to 1.0. The average and standard error are shown from at least three independent trials.

[^b]: Column number corresponds to the column in Figure 2C.

**Figure S1:** Quantitative MALDI analysis of PKA substrate (3) with PKA and ATP-dansyl (1c). The peak at 1152.565 m/z corresponds to methylated phosphopeptide after phosphorylation with ATP-dansyl and acidic dansyl cleavage, while the peak at 1155.595 m/z corresponds to deuterated, methylated phosphopeptide due to ATP phosphorylation. The percent conversion in this single trial was 96%.
**Figure S2:** Quantitative MALDI analysis of CK2 peptide (4) with CK2 and ATP-dansyl (1c). The peak at 1540.567 m/z corresponds to the hepta-methylated phosphopeptide after phosphorylation with ATP-dansyl and acidic dansyl cleavage, while the peak at 1561.713 m/z corresponds to the deuterated, hepta-methylated phosphopeptide due to ATP phosphorylation. The conversion of this single experiment was 84%.

**Figure S3:** Quantitative MALDI analysis of Abl peptide (5) reaction with Abl and ATP-dansyl (1c). The peak at 1442.915 m/z corresponds to the dimethylated phosphopeptide due to phosphorylation by ATP-dansyl and acidic dansyl cleavage, while the peak at 1448.960 m/z corresponds to the phosphopeptide due to deuterated, dimethylated ATP phosphorylation. The conversion of this single reaction was 99%.
**Figure S4:** Quantitative MALDI analysis of trypsin digested β-casein after reaction with CK2 and either ATP (1a) or ATP-dansyl (1c). The peaks at 2237.770 and 2223.787 m/z correspond to heptamethylated and hexamethylated phosphopeptides, respectively, due to partial methyl esterification of the acid-cleaved, ATP-dansyl phosphorylated sample. The peaks at 2260.015 and 2241.873 m/z correspond to the deuterated hexamethylated and heptamethylated phosphopeptide, respectively, due to partial methyl esterification of the after ATP phosphorylated sample. The percentage conversion was determined by comparing the areas under both sets of peaks. The peak at 2252.811 m/z represents a peptide present in the trypsin digestion of β-casein. The percent conversion in this single experiment was 52%.
Figure S5: Lineweaver-Burke plots of ATP and derivatives with PKA and kemptide substrate (LRRASLG) obtained with the enzyme-coupled assay for kinase activity. Each plot is a single representative of at least three independent trials. The $k_{cat}$ was then calculated by dividing the $V_{max}$ by the concentration of enzyme in appropriate units.
Figure S6: Lineweaver-Burke plots of ATP and derivatives with Abl and peptide substrate (EAIY-AAPFAKKK) using data obtained with the enzyme-coupled assay for kinase activity (A and B) or the FRET-based assay (C). Each plot is a single representative of at least three independent trials. The $k_{cat}$ was then calculated by dividing the $V_{max}$ by the concentration of enzyme in appropriate units.
Figure S7: Full spectra of FRET assay without kinase (blue) and with kinase (pink) observed between 400 and 625 nm. Reactions were performed in 96-well format discussed above with measurements taken on an Aviv Automated Titrating Differential/Ratio Spectrofluorimeter ATF 105. A 25% increase in fluorescence at 600 nm was observed in the presence of kinase in this single trial.