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A Multiplexed Protein Kinase Assay

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We report a novel protein kinase assay designed for high-throughput detection of one or many kinases in a complex mixture. A solution-phase phosphorylation reaction is performed on 900 different peptide substrates, each covalently linked to an oligonucleotide tag. After incubation, phosphoserine, phosphothreonine, and phosphotyrosine are chemically labeled, and the substrates are hybridized to a microarray with oligonucleotides complementary to the tags to read out the phosphorylation state of each peptide. Because protein kinases act on more than one peptide sequence, each kinase can be characterized by a unique signature of phosphorylation activity on multiple substrates. Using this method, we determined signatures for 26 purified kinases and demonstrated that enzyme mixtures can be screened for activity and selectivity of inhibition.

Introduction

Signal transduction pathways that utilize phosphorylation play critical roles in a multitude of cellular functions including homeostasis, differentiation, development, and growth. Phosphorylation events that conduct and amplify signals along these cascades are mediated by specific protein kinases, which comprise one of the largest gene families in eukaryotes. Improper function or response of signal transduction pathways has been found to play an important role in a variety of diseases, including cancer, allergic responses, autoimmune diseases, and degenerative diseases. Monitoring the activity of protein kinases can provide an understanding of many fundamental biological processes, and can be beneficial in a clinical setting for the diagnosis and prognosis of various diseases. Furthermore, it can be valuable for the design or identification of therapeutic drugs that target specific kinases or signal-transduction pathways.

Currently, many technologies are available for analyzing and detecting the phosphorylation of biological molecules (for reviews, see refs. [1–3]). Most methods are uniplex—that is, one kinase is assayed against a single substrate. Alternatively, kinase substrate microarrays can be used as powerful tools for assessing complex samples, because many analyses can be carried out in parallel on a single sample.[4] Microarray phosphorylation analysis requires a method that is widely applicable to the detection of phosphoserine, phosphothreonine, and phosphotyrosine in substrates containing a wide variety of flanking amino acids. The most common detection approach involves autoradiography to detect the transfer of radiolabeled phosphate from ATP.[5,6] Alternatively, a phosphate-binding reagent can be used, for example, a fluorescently labeled anti-phosphoamino acid antibody[7] or chelate,[8,9] or unlabeled versions of these reagents (detected by surface plasmon resonance).[8,10] On the other hand, a mass spectrometry[11] readout does not require external reagents. Generality, safety, and simplicity remain important concerns about existing methods. Additionally, substrates are immobilized on a solid surface; this requires the enzyme to phosphorylate on the solid phase. The reaction is then diffusion limited and sensitive to substrate density.

Solution-phase assays are preferable, and, for compatibility with microarray analysis, substrates can be encoded for subsequent deconvolution. Such an approach has been applied to protease-activity analysis by utilizing peptide nucleic acid (PNA) encoding,[12–14] for phosphatase-activity screening with activity-based probes by expression display,[15] and we have used a similar approach to develop a protease-activity assay (I. A. Kozlov, et al., unpublished results). We introduce here a solution-phase kinase assay in which 900 peptide substrates are encoded by attachment to unique oligonucleotide sequences and presented together. In the assay, following phosphorylation, the substrate mixture is deconvolved by hybridization to a set of complementary DNA sequences affixed to a microarray. Our assay is based on Illumina's BeadArray platform, which employs silica beads derivatized with specific oligonucleotide sequences and seated in etched microwells of fiber-optic bundles.[16,17] These microarrays have been successfully applied to a variety of biological assays, such as SNP-genotyping and gene expression (reviewed in ref. [18]). The high density of these arrays allows multiple analyses from a single sample to be performed in a very small sample volume, and

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provides multiple copies of each bead type on the microarray for averaging, to reduce variability.

To detect the phosphorylation of peptide substrates, we developed a specific chemical modification of the electrophilic phosphate group. Previously, researchers have labeled phosphate groups in several steps, by either 1) modification of all electrophilic side chains followed by selective deprotection and relabeling of the phosphate[19] or 2) β-elimination of phosphoserine and phosphothreonine residues followed by Michael addition.[20] Our assay achieves selective phosphate labeling with a dye in a one-tube reaction, with a carboxylate blocking step prior to phosphate labeling as a key feature. The approach is simple, mild, and labels all three phosphorylated amino acid residues in a variety of peptide substrates.

The key advancements in our array-based kinase assay are solution-phase phosphorylation reactions enabled by DNA encoding of peptide substrates, a universal chemical phosphate-labeling method that avoids radioactivity, and the ability to use pre-existing DNA microarrays for readout. We characterized the phosphorylation signature for 26 different kinases acting on the mixture of 900 peptide substrates and showed that the signatures support the detection of multiple kinase activities simultaneously. In addition, analysis of kinase activities in a cell extract can be performed.

Results and Discussion

Assay strategy

The assay scheme is depicted in Scheme 1. A solution-phase phosphorylation reaction is performed with a pool of DNA–peptide conjugates and the kinase sample of interest. Then, phosphate groups are selectively labeled with an amine-functionalized dye. This labeling requires that carboxylate side chains be blocked as a first step. The blocking and labeling reactions are discussed in the following section. After labeling, the solution is hybridized to oligonucleotides attached to individual beads on a BeadArray.

The signal from the dye is amplified through a multilayer immunohistochemical stain. Dye is first detected with a monoclonal mouse anti-dye antibody, followed by a biotinylated anti-mouse secondary antibody. The biotin is detected with a streptavidin–Alexa555 conjugate, and this signal is amplified by two rounds of sandwich staining, as described previously.[21] This very sensitive amplification strategy maximizes the signal-to-background and signal-to-noise ratios.

Chemical phosphate labeling

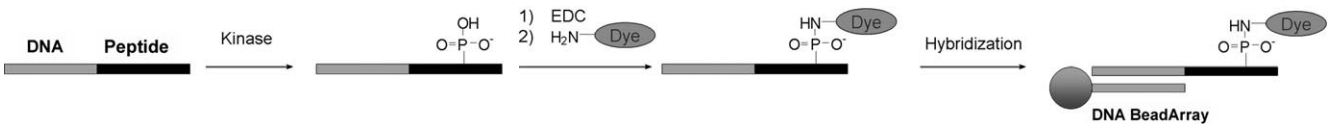
A key aspect of our approach is the method used to identify phosphate introduction by the protein kinase. We sought to covalently modify the electrophilic phosphate residue with a reporting group. In aqueous solution, amines can be coupled to electrophiles with EDC as a coupling agent.[22] We have found that the labeling of phosphate monoesters introduced by the kinase is efficient and preferential over some low-level labeling of the DNA tag that was subtracted as general assay background. However, the presence of carboxylic acid residues in a peptide sequence does compromise the selectivity of the phosphate labeling. As a method to first block carboxylates, we implemented a usually undesirable side reaction of carboxylic acids with EDC at low pH. This pretreatment induces formation of O-acyl derivatives of the carbonyls, which rearrange to form stable *N*-acylurea derivatives.[23] Subsequent amino dye addition allows for a selective EDC-mediated coupling of dye to the phosphate. This ability to selectively block carboxylate labeling is essential for this detection strategy.

Assay optimization

To validate the labeling and detection strategy, ten control peptide conjugates were prepared (Table 1). Peptides containing N-terminal aminooxyacetic acid residues were linked by known oxime chemistry to give unique aldehyde-containing 23- to 25-mer DNA sequences that are complementary to Illumina's universal Sentrix® Array Matrix. Conjugates were purified from their uncoupled components and pooled (see the Experimental Section for details). The ten conjugates incorporated 1) tyrosine-, serine-, and threonine-containing model peptides, in both unphosphorylated (C1–C3) and phosphorylated (C4–C6) states, to assess detection of phosphorylated residues, 2) peptides containing aspartate (C7) and glutamate (C8) resi-

Table 1. Prepared control peptide conjugates.

Conjugate name	Peptide sequence	Conjugate name	Peptide sequence
C1	GGGYG	C6	GGGpTG
C2	GGGSG	C7	GGGDG
C3	GGGTG	C8	GGGEG
C4	GGGpYG	C9	GGYIYGSFK
C5	GGGpSG	C10	GGALRRSLGG



Scheme 1. The three steps of the kinase assay: phosphorylation, selective labeling of phosphate groups, and hybridization. Phosphorylation is accomplished through a solution-phase reaction of a pool of DNA–peptide conjugates with a purified kinase or cell extract sample of interest. Selective labeling requires that carboxylate residues are first blocked with EDC and then phosphate residues are labeled with an amine-functionalized dye. Hybridization to complimentary DNA sequences on a microarray deconvolutes the signals from the peptides.

dues to test the blocking strategy, and 3) substrates for Src kinase (C9) and PKA (C10) to assess enzymatic phosphorylation.

We used fluoresceinylglycine amide as a labeling dye. In the initial experiments without an EDC blocking step, dye labeling easily distinguished phosphotyrosine and phosphoserine from their unphosphorylated counterparts, but not from aspartate and glutamate. When the pool was blocked with EDC for 1.5 h at pH 6 prior to labeling, the signals from the carboxylate residues were reduced to near background levels (Figure 1 A). To optimize the signal, several different blocking and labeling pH values as well as combinations thereof were attempted. We found that blocking and labeling at pH 6 was the most efficient approach. Since amines are not typically nucleophilic at pH 6, we believe that some intramolecular hydrogen bonding in fluoresceinylglycine amide aids nucleophilicity.

During the development of our assay, several different commercially available amine-containing dyes and haptens were screened for phosphate labeling, including fluorescein, biotin, and dinitrophenol derivatives. Various linkers between the dye or hapten and amine were also investigated. We found that with the exception of fluoresceinylglycine amide, all compounds exhibited suppressed labeling below pH 7.0, and suppressed labeling of phosphoserine and phosphothreonine more than that of phosphotyrosine even at pH 8. To minimize pH adjustments after blocking and obtain efficient labeling of all three phosphorylated residues, fluoresceinylglycine amide seems to be the best choice for labeling. We showed that three hours of dye labeling gave nearly equivalent labeling of all three phosphorylated amino acids (Figure 1 B).

Once assay conditions were established, the pool was incubated with Src and PKA, independently and in combination, to verify the detection of enzyme-dependent phosphorylation (Figure 1 C). As expected, each conjugate only gave a signal when its target enzyme was present. The assay response to inhibitors for each of these enzymes further verified the ability of the assay to detect enzyme activity (Figure 1 C). Finally, the labeling strategy was validated by comparison with detection

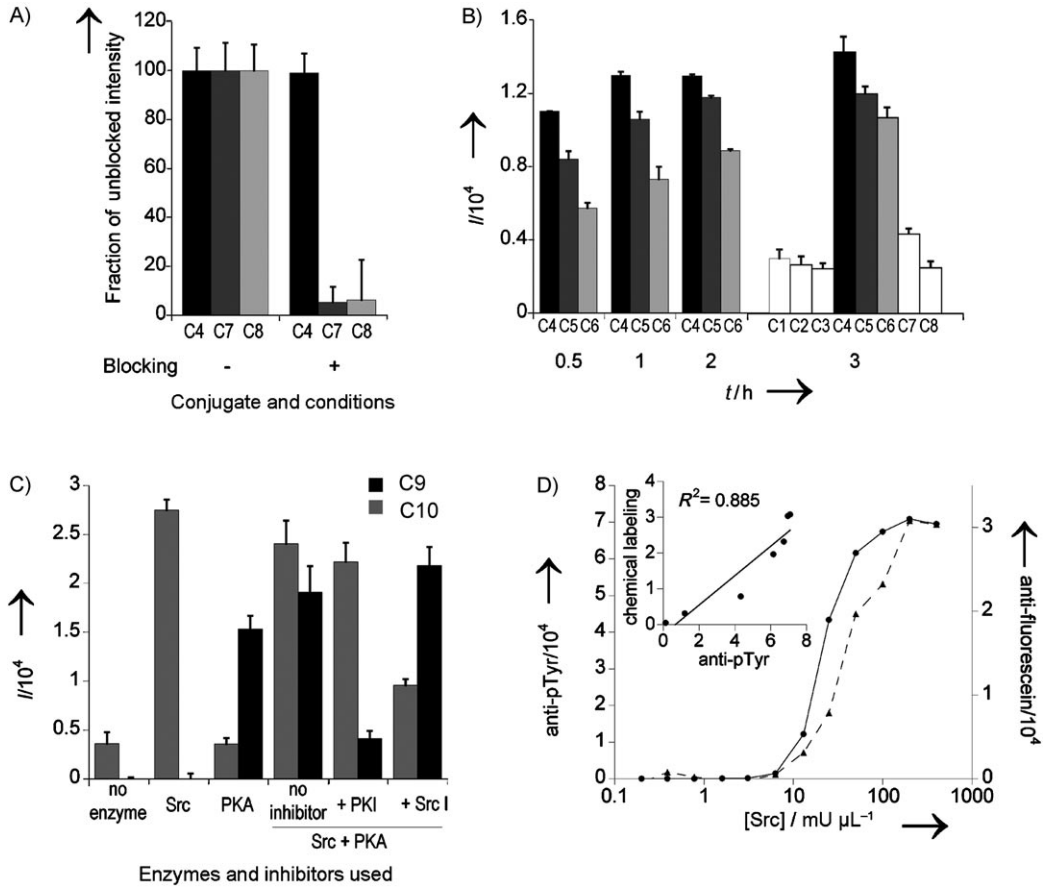


Figure 1. The assay was optimized with control conjugates to generate selective phosphate labeling, to respond to enzymatic activity, and to correlate with an antibody-based detection approach. Control conjugate sequences are listed in Table 1. A) EDC pretreatment at pH 6 is necessary for selective phosphate labeling. For this experiment, the final labeling mixture contained 50 nM of each conjugate, 200 µM dye, 0.15 M EDC, and 30% DMSO in kinase assay buffer (see the Experimental Section). B) Nearly saturated and equivalent labeling of all three phosphorylated amino acids requires 3 h with 5 mM dye and 0.4 M EDC. C) The assay strategy detects enzyme-dependent phosphorylation by Src (0.4 U µL^{-1}) and PKA (17 U µL^{-1}). These enzymes can be used in combination and selectively inhibited by 22 µM PKI or 17 µM Src I, as expected. D) Reactions containing a series of concentrations of Src were detected by two methods: unlabeled samples were detected with an antiphosphotyrosine antibody (——) and samples that had been fluorescein labeled by the approach in Scheme 1 were detected with an antibody sandwich (-----). Inset: the correlation between the methods is 89% (linear regression). *I* indicates fluorescence intensity. Error bars indicate the standard deviation of three hybridization replicates.

by an anti-phosphotyrosine antibody (Figure 1D); good correlation (89%) was calculated between the two methods by linear regression analysis (inset).

Ultimately, the phosphorylation reaction mixtures contained 10 nM of each conjugate. This concentration could be increased for improved sensitivity with poorer substrates or for assaying kinases that do not phosphorylate peptides efficiently. We verified that the assay is compatible with ATP concentrations of up to 1 mM, as well as typical concentrations of phosphatase inhibitors such as sodium orthovanadate (1 mM) and β-glycerophosphate (5 mM) utilized in the study of cellular extracts. The ability to utilize 1 mM ATP improves the limit of detection for all kinases.

The labeling strategy, including the dye used, was optimized to clearly differentiate synthetically and enzymatically phosphorylated amino acids from their unphosphorylated analogues and carboxylate-containing amino acids. Reaction conditions were kept as simple as possible: only a single pH adjustment is necessary, and the reaction mixture is simply diluted prior to hybridization to the microarray. Furthermore, the labeling strategy was validated by good correlation with a known antibody-based phosphate-detection method.

High-complexity array content

In order to build a high-complexity pool, the peptide sequence content was derived from several sources: known kinase phosphorylation consensus sequences, randomized sequences based on peptide library data or consensus sequences, and a phosphorylation site database (see Table S1 in the Supporting Information and references therein). Peptide sequences contained up to 15 amino acids for reasonable quality without purification. The final pool comprised 900 peptide substrates that targeted over 50 protein kinases. The peptides and kinases are listed in Table S1. These targeted kinases represent a large portion of the diversity of the human kinome.[24] Furthermore, 60 duplicate peptides coupled to two different oligonucleotides exist in the pool for the evaluation of DNA-sequence effects. Conjugates were purified in bulk for this pool, and successful conjugation was determined after hybridization to the array (see the Experimental Section). The flexibility of the conjugation and pooling strategy allows for any custom pool to be easily assayed on any existing DNA microarray.

In addition to the 900 peptide substrates, 102 controls were incorporated in the pool (see Table S1). These include Cy3-labeled oligonucleotides as hybridization controls (1–10), FAM-labeled oligonucleotides for normalization (11–25), unlabeled oligonucleotides as background controls (26–45), carboxylate-containing peptides as blocking controls (46–47), very simple peptides containing unphosphorylated serine, threonine, and tyrosine (48–53), all three varieties of phosphorylated peptides coupled to different oligonucleotide sequences (54–96), and peptides incapable of being phosphorylated (97–102). The controls are all vital for monitoring assay performance; in particular, the FAM-labeled oligonucleotides enable comparison between different assay samples.

Substrate pools with higher complexity would be possible. The DNA sequence content on the array, database information for peptide substrates, and substrate concentration are not limiting at this time. Only peptides that contain an amino acid that is already phosphorylated or certain peptides that give high background (see Supporting Methods) are excluded as substrates. New peptides should be screened for low background and successful conjugation. We found that 75% of the peptides we synthesized passed this screen.

In theory, new substrates could also be screened with this approach. However, the concentration of substrates used in the assay is too low to allow kinetic measurements on individual peptides. The assay intensity for each peptide will vary not only based on the amount of phosphorylation, but also based on the hybridization efficiency of its attached DNA tag. Therefore, substrate comparisons based on assay intensities are not meaningful for a single sample. A next-generation assay that normalized the response of each substrate based on the hybridization efficiency of its attached DNA tag would enable the signal to indicate substrate preferences of a given kinase. By using the assay described herein, substrate comparisons can be made when an enzyme titration curve is used (vide infra). The greatest strength of the current approach is its profiling capability.

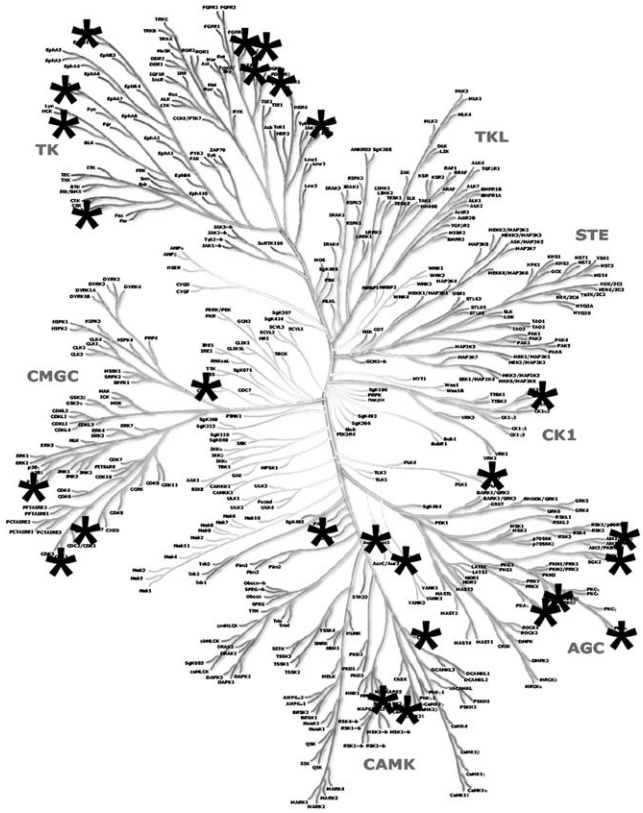


Figure 2. The human kinome tree, courtesy of *Cell Signaling Technology*. Stars indicate the kinases tested.

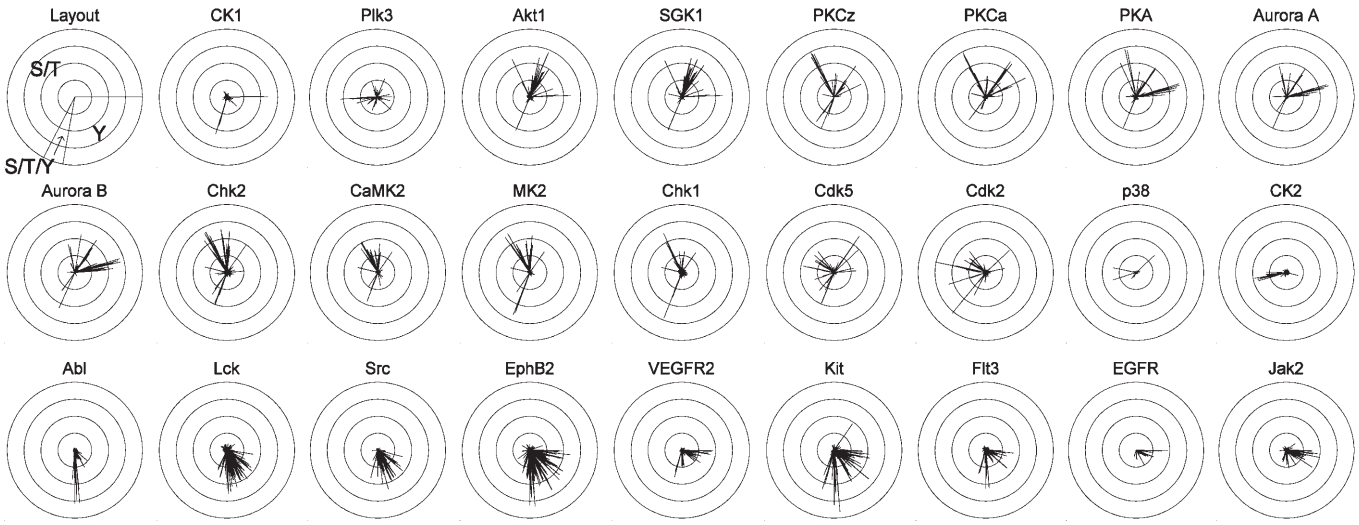


Figure 3. Unique activity signatures are observed for 26 kinases with a 900-plex substrate pool. In the radial plots pictured, each of the 900 individual substrates occupies a unique ray. The scale is linear, with the maximum value here of 2. The first plot shows that serine/threonine-containing substrates and tyrosine-containing substrates are spatially separated. The rest of the top two rows contain signatures for serine/threonine kinases, and the bottom row contains signatures for tyrosine kinases. From left to right, the kinases are organized by family, roughly clockwise around the kinome tree.[24] Similar kinases are located next to one another, and their signatures have some similar features. The kinases tested provide good coverage of the kinome. Cyclin A and p35 were the partners for Cdk2 and Cdk5, respectively. All reactions contained 10 nM of each conjugate and 1 mM ATP. Additional assay conditions are described in the Experimental Section, and enzyme amounts are listed in Supporting Methods.

Individual kinase activity profiling

The 900-plex substrate pool was subjected to phosphorylation by 26 different individual enzymes (Figure 2). Each enzyme generates a unique profile of activities, or signature. Kinases from similar families produce similar signatures, and the individual substrates that each kinase phosphorylates agree with known substrate preferences for these kinases (see peptide sequences). The layout of peptide substrates based on the residues that can be phosphorylated (Figure 3, top left) allows clear differentiation of serine/threonine kinases (Figure 3, top two rows) from tyrosine kinases (Figure 3, bottom row). The number of rays in a signature is not an indication of the promiscuity or specificity of a particular kinase, it is a reflection of its activity on the contents of our pool.

Eight of the kinases profiled were not specifically included in the pool design, but gave unique signatures with the 900-plex substrate pool. The diversity of our substrate pool will likely allow additional kinases to be screened.

Several of these enzymes require additives for proper activity. The ability to screen enzymes such as protein kinase C (PKC), epidermal growth factor receptor (EGFR), and calcium/calmodulin-dependent protein kinase-2 (CaMK2) illustrates that our assay strategy is compatible with the many additives required for kinase activity (such as lipids, additional proteins, and salts). The presence of high concentrations of these analytes can modulate the assay background, and thus, a separate "no-enzyme" control for background subtraction is performed for these cases.

When these signatures are compared with one another, specific substrates are observed for 16 of the 26 kinases tested under these conditions. In addition to specific substrates, it might be possible to examine a group of substrates that overlap with different kinases in order to identify a particular kinase. While the profiles change with changing concentration (see next section), it is possible to use pattern matching to identify a kinase from an unknown sample, especially if multiple concentrations of a purified enzyme have been tested. For precise pattern matching, methods such as training of a neural network could be used with such data. Overall, the kinase signature concept provides an important solution to the lack of substrate specificity.

Effect of enzyme concentration on signature

An additional feature of the substrate pool is that the profile for a given kinase changes with enzyme concentration, because each substrate has its own kinetic parameters associated with phosphorylation by a particular kinase. To investigate this point, titrations of four different protein kinases were performed. The change in profile with increasing concentrations of cyclin-dependent kinase-2 (Cdk2) is shown in Figure 4A. The titration curves for a few of the 900 substrates illustrate the individual behavior of each substrate, which results in the change in profile for Cdk2 (Figure 4B). Furthermore, the ability to quantify enzyme concentration over a wider range than is possible with a single substrate is apparent. The plotted substrates were chosen based on the following criteria: 1) a change in fluorescence response over the course of the titration and 2) good spacing across the range of responses from most-sensitive to least-sensitive substrate. To illustrate the spread of the titration data, all three hybridization replicates for one of the substrates are shown (Figure 4B). Titrations curves for Src (Figure 4C), PKA (Figure 4D) and serum and glucocorticoid-regulated kinase-1, or SGK1, (Figure 4E) show the

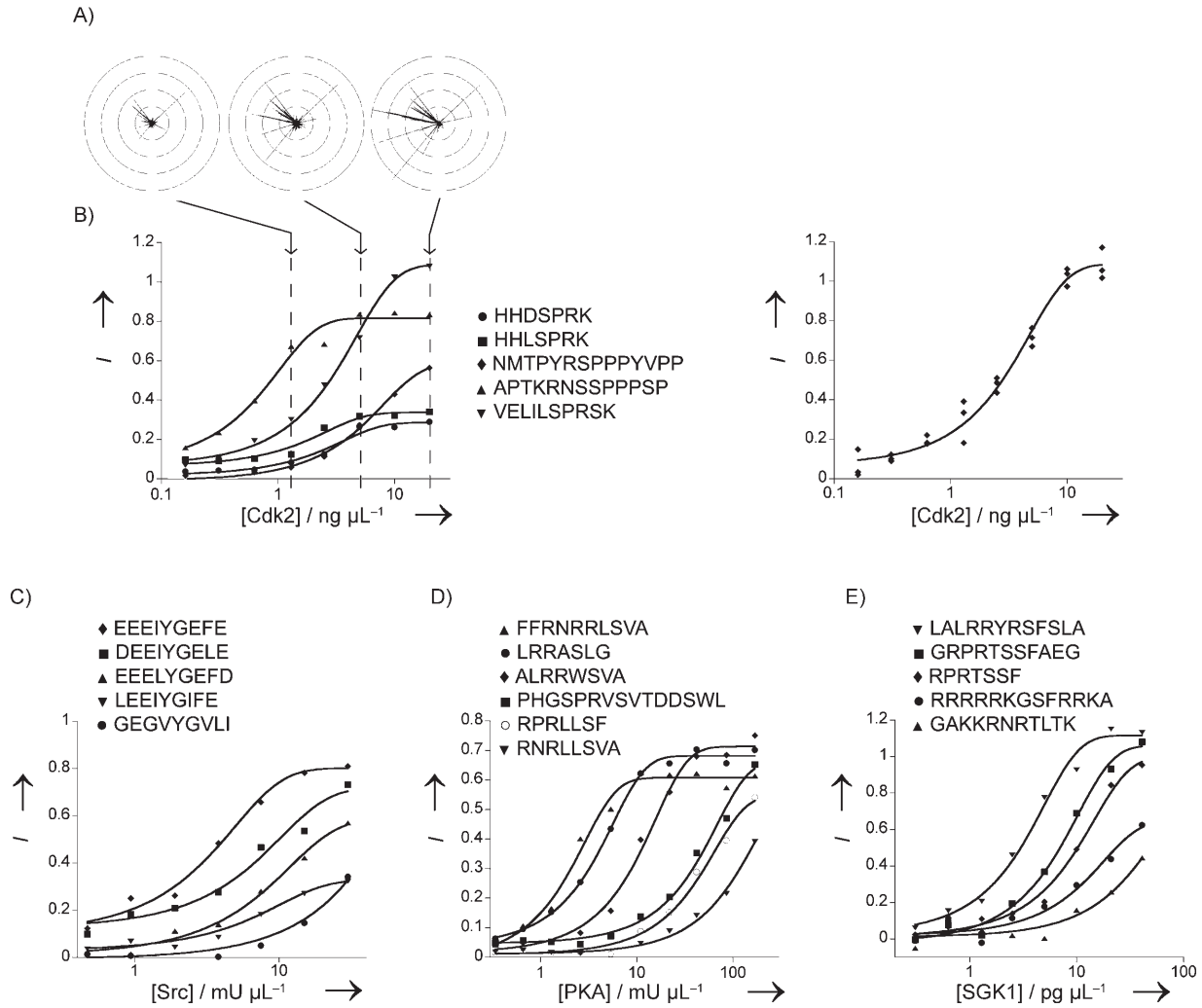


Figure 4. The multisubstrate approach generates a distinct signature over a broad range of enzyme concentrations. A) The enzyme-activity profile changes with Cdk2 concentration. B) The titration curves of individual Cdk2 substrates are different due to different kinetic parameters, and the individual response of these substrates explains the change in profile. All three hybridization replicates were utilized for curve fitting, but only the average is shown on the left for clarity. The plot on the right illustrates the variation in three hybridization replicates for an individual substrate. The titration curves of individual substrates for C) Src, D) PKA, and E) SGK1 show a similarly large dynamic range. All reactions contained 10 nM of each conjugate and 1 mM ATP. *I* indicates fluorescence intensity normalized to control dye-labeled conjugates as described in the Experimental Section.

same broad dynamic range and variation in substrate behavior. Assay-reproducibility studies with replicates prepared separately beginning with the kinase assay show phosphorylation and labeling variation (Figure S1) and indicate that hybridization itself only accounts for half of assay variability.

The fact that these conjugates do not all plateau at the same intensities is mainly due to differences in hybridization efficiency between oligonucleotide sequences, but slight differences in conjugate concentration make a small contribution. This reinforces the idea that the ratio of intensities between codes in a pool is not necessarily a measure of the relative efficacy against a kinase. However, a titration curve may be used for this purpose. Substrates that persist at lower concentrations of enzyme and have a lower half-maximal response are better substrates in the assay.

Further, the ranking of these substrates correlates well with kinase peptide library rankings when available. For Src, we found the same optimal substrate as Songyang et al., and the rest of the substrates agree with the rankings in that work.[25] Additionally, for PKA, we found Kemptide to be one of the best substrates in the assay, and the other substrates agree well with library results.[26,27]

These enzyme titrations were also used to compare the performance of identical peptide substrates conjugated to different DNA tags. Titration curves for duplicate substrates indicate that the shape of the substrate phosphorylation curve is independent of DNA sequence (see Figure S2).

The limit of detection of the assay varies depending on the kinase. This is a function of the peptide substrates that are included in the substrate pool. With better substrates, the assay becomes more sensitive.

Calibration titration curves can aid the quantification of enzyme concentration over a broad range in an unknown sample. This is a distinct advantage over assays that utilize a

single substrate. In general, the dynamic range is boundless at the top end as poorer and poorer substrates become phosphorylated by the kinase. These poorer substrates can be helpful for quantification of higher enzyme concentrations, for which phosphorylation of the best substrates is saturated. For single-substrate assays, the kinase sample might need to be diluted into the substrate's linear range; this could be problematic as enzymes can lose activity upon dilution. Further, the multisubstrate approach to quantification can reduce error due to the averaging effect of multiple substrates and can provide significant time savings to optimizing the enzyme concentration range.

As mentioned previously, identifying new substrates for kinases (especially those that are not well studied) is a powerful application of this approach. When a single enzyme concentration is used, comparison between substrates is not possible, since there is currently no way to normalize between conjugates with different hybridization efficiencies. By using enzyme titration curves, it is possible to compare new substrates.

Multiplexed kinase assays

To assess our ability to analyze mixtures of kinases in complex samples, the activity profiles of mixtures of purified kinases were investigated. As a starting point, a two-enzyme mixture of Src and casein kinase-2 (CK2) was chosen. This two-enzyme mixture showed an activity profile that is a combination of the individual signatures (Figure 5 A, top row).

Next, we attempted to combine nine purified kinases: Src, PKA, CK2, SGK1, Cdk2, Lck, Abl, PKC_{ζ}, and CaMK2. We prepared a universal assay buffer containing all required additives (phosphatidylserine, diacyl glycerol, calcium, calmodulin) and chose enzyme concentrations identical to those used for Figure 5. We first discovered that the lipids required for PKC_{ζ} activity suppressed the activity of Src, Lck and Abl. Further, CaMK2 activity did not seem to be reproducible. Thus, these two kinases were eliminated from the mixture. When the remaining seven kinases were assayed together, all signatures could be seen, but some were suppressed by half of the intensity of the kinase alone. Further, when inhibitors were screened with this seven-enzyme mixture, some of the kinases showed greater activity in the presence of an inhibitor compared to in its absence. This suggests that there are interactions between the kinases. In general, multiplexing would be limited to those kinases that can be assayed in the same solution mixture, including the presence of cofactors or components of enzyme dilution buffers. Additionally, multiplexing might also be limited if kinases phosphorylate one another in vitro. Multiplexing is also limited by the number of kinases attempted—we achieved a 45% success rate (four out of nine). In our hands, the mixture PKA +

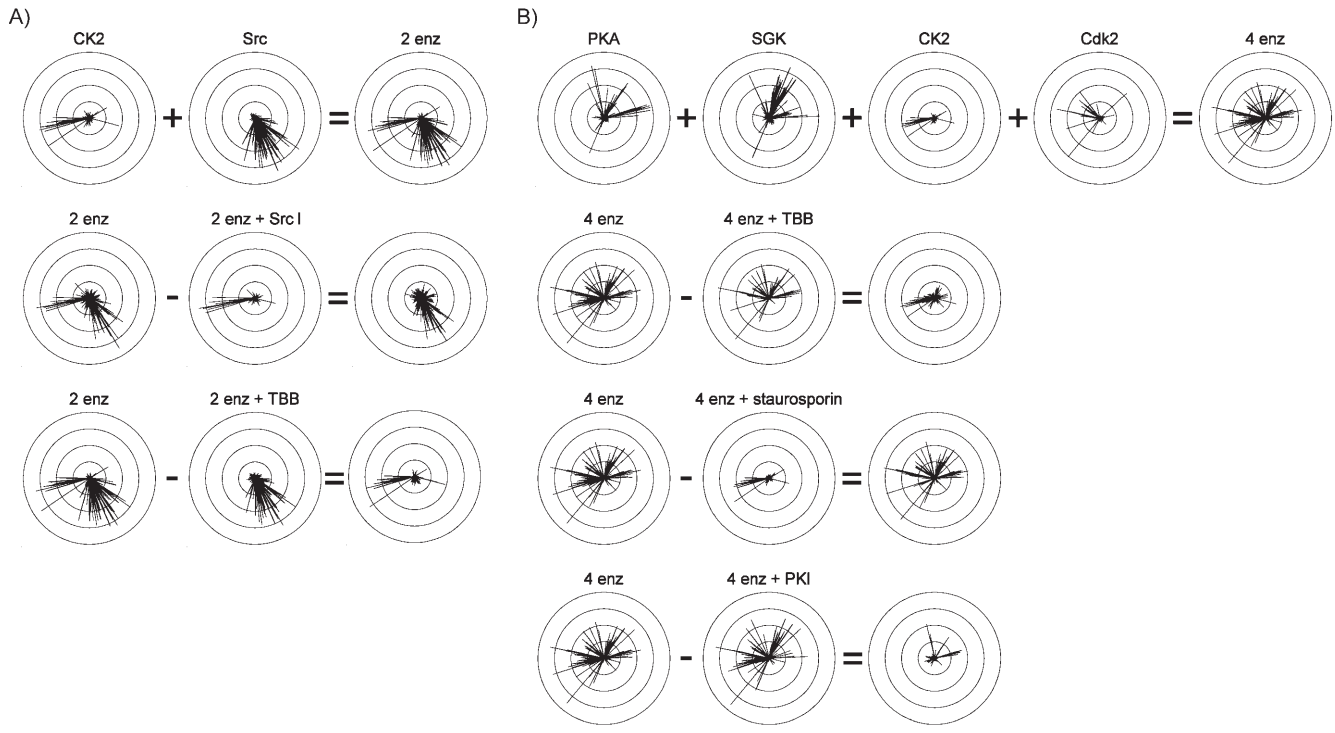


Figure 5. Enzyme mixtures can be evaluated in a single reaction with the multisubstrate array, both with and without kinase inhibitors. A) The combination of CK2 (2 ng μL^{-1}) and Src (30 mU μL^{-1}) in a single reaction results in the addition of the signatures of the two enzymes. Comparison of the two-enzyme mixture with those that have a single inhibitor per reaction shows what kinase activity was affected by that inhibitor. The kinase is identified by comparison with the individual kinase signature. In this experiment, Src I (22 μM) behaves as expected and inhibits Src activity, and TBB (100 μM) behaves as expected and inhibits CK2 activity. B) The combination of PKA (25 mU μL^{-1}), SGK1 (0.2 ng μL^{-1}), CK2 (2 ng μL^{-1}), and Cdk2 (20 ng μL^{-1}) in a single reaction results in the addition of the signatures of the four enzymes. Reactions containing a single inhibitor were compared with the four-enzyme mixture and with individual kinase signatures. As expected, TBB (100 μM) inhibits CK2 activity, PKI (17 μM) inhibits PKA activity, and staurosporine (5 μM) inhibits all kinases except CK2. All reactions contained 10 nM of each conjugate and 50 μM ATP. The radial plots here have a maximum value of 1.

SGK1 + CK2 + Cdk2 gave quantitatively reproducible results (Figure 5B).

To illustrate rapid screening of kinase inhibitors, we tested commercially available kinase inhibitors on the mixtures. Prior to the use of an inhibitor with a mixture of kinases, it was first tested to verify complete inhibition of the target enzyme. To choose inhibitor concentrations based on literature values, 50 μM ATP was used in all reaction mixtures. We also calculated an IC_{50} value for 4,5,6,7-tetrabromo-2-azabenzimidazole (TBB) inhibition of CK2, and found that it compared well with literature values (see Figure S3).

Two small-molecule kinase inhibitors,[28] TBB and 4-(4′-phenoxyanilino)-6,7-dimethoxyquinazoline[29] (Src I, a Src inhibitor), were tested individually on a mixture of CK2 and Src (Figure 5A). For each inhibitor, the signature corresponding to the targeted enzyme disappears. To more easily visualize the signature that disappears, the profile of the inhibited sample is subtracted from the profile of the uninhibited sample. This subtraction result correlates well with individual enzyme signatures.

This subtraction analysis is especially helpful on more complex mixtures, such as the four-enzyme mixture of PKA, SGK1, CK2, and Cdk2 (Figure 5B). The inhibitor TBB only inhibits CK2. The very nonspecific kinase inhibitor, staurosporine,[30] inhibits all kinases except CK2, whose resistance to staurosporine has been previously noted.[31] The subtraction profile for inhibition by PKI 6-22 amide[32] only shows some elements of the PKA signature. Because PKA and SGK1 compete for some of the same substrates, inhibition of PKA activity allows SGK1 to phosphorylate the peptides for which it does not compete effectively with PKA. Thus, inhibition with PKI only decreased phosphorylation of PKA-specific substrates in the mixture. Moreover, the reason the activities of these two enzymes in the mixture are slightly suppressed compared to their activities alone is likely due to these overlapping substrates.

The ability to test kinase inhibition against multiple kinases simultaneously provides economical screening of both novel and off-target inhibition by drug candidates. It is necessary to screen for the kinases that can be assayed reproducibly together. The ability to multiplex a few enzymes in a solution-phase, small-volume reaction, as reported here, results in an increase in throughput compared with existing methods.

The key advantage of a multisubstrate approach is in screening mixtures whose kinase activities are unknown, particularly biological samples. Purified kinase activity could be detected when spiked into commercially available total protein preparations, though the limit of detection was threefold higher in the presence of total cellular protein (data not shown). Furthermore, kinase profiles could be seen in two different commercially available cellular extracts (see Figure S4). The ability of our assay to detect kinase activity against a background of cellular protein enables further evaluation for use with biological samples. Ultimately, biological experiments and diagnostic applications could be possible.

Conclusions

Protein kinases play central roles in cellular signaling processes, and the study of these enzymes will provide important insights into overall cellular function. Moreover, many human diseases result from improper function of these enzymes.

We have introduced a microarray kinase assay platform in which multiple kinase activities can be assessed simultaneously, in a small sample volume. Encoding the peptide sequences with DNA tags allows us to perform kinase assays in solution, and decode the substrate responses on a DNA microarray. The universal phosphate labeling strategy detects all phosphorylated residues in a variety of peptide substrates without the need for radioactivity. We achieved selective phosphate labeling with an amino-functionalized dye in a one-tube reaction, with a carboxylate blocking step prior to phosphate labeling as a key feature.

With a diverse 900-member substrate pool targeted at over 50 kinases from all over the kinome, we characterized the phosphorylation signature for 26 different kinases. The analysis of several kinase substrates simultaneously circumvents some of the problems associated with kinase substrate specificity. Further, a broader range of enzyme concentrations can be quantified than possible with a single substrate. We show that these phosphorylation signatures support detection of multiple kinase activities simultaneously. The ability to test kinase inhibition against multiple kinases at once provides economical screening of both novel and off-target inhibition by drug candidates. In addition, kinase activities can be analyzed against a background of total cellular protein. The ability to detect kinase activity in complex mixtures underlines the potential of this method.

Experimental Section

Peptide–DNA conjugate preparation and characterization: Peptides were synthesized by Fmoc solid-phase peptide synthesis with an N-terminal aminooxyacetic acid residue in 96-well plates under optimized conditions as described.[33] All peptides were analyzed for purity by HPLC and spot-checked by mass spectrometry analysis (7%). Oligonucleotides were synthesized at Illumina and modified with a 5′-aldehyde moiety, similar to that previously reported.[34] Each peptide was coupled to a unique oligonucleotide through an oxime linkage, under conditions similar to those previously reported.[35] Conjugates C1–C10 were individually gel purified (15% TBE–urea), eluted (PBS), precipitated (ethanol in the presence of sodium acetate), redissolved in water (Millipore, Molecular Biology Grade), and quantified (A_{260}). Characterization data for representative conjugates can be found in the Supporting Methods. All other conjugates were gel purified in groups of 96, eluted, precipitated, and redissolved in water, then their total concentrations were quantified. Individual conjugates were assumed to be present at similar concentrations. Conjugates were screened for their presence in the final pool and for high background (see Supporting Methods). All conjugates that passed these criteria (900 out of 1250) were repurified in groups of 96 or less for the final pool.

Phosphorylation reactions with purified enzymes: Purified enzymes were obtained from Upstate and BPS Bioscience (San Diego, CA). Kinase reactions were prepared by mixing a 2× substrate mix-

ture (20 nM each conjugate, 2 mM ATP, 60 mM $MgCl_2$) in kinase assay buffer (50 mM HEPES, pH 7.5, 0.1 mM ethylenediamine tetraacetic acid (EDTA), 0.1% Brij-35) with diluted kinase (5 μL, diluted in enzyme dilution buffer (kinase assay buffer with 0.4 mg mL^{-1} bovine serum albumin (BSA), 0.2% β-mercaptoethanol)). For enzyme titrations, twofold enzyme dilutions were performed serially in enzyme dilution buffer. For background subtraction, a "no-enzyme" sample was also prepared in which only enzyme dilution buffer was added to the substrate mixture. When enzyme additives were used, a separate no-enzyme control containing each additive was prepared. Final reaction conditions were: HEPES (50 mM; pH 7.5), EDTA (0.1 mM), 0.1% Brij-35, BSA (0.2 mg mL^{-1}), 0.1% β-mercaptoethanol, each conjugate (10 nM), ATP (1 mM), $MgCl_2$ (30 mM). Reactions were allowed to proceed at 30 °C for 1 h. Enzyme amounts and additive concentrations can be found in the Supporting Methods.

Multiplex inhibition: 2× substrate mixture (5 μL) for these assays contained conjugates (20 nM each) and ATP (100 μM) in kinase assay buffer. A 10× stock of each inhibitor in DMSO (1 μL) was added to this prior to addition of the enzyme. The following inhibitors were used: PKI 6–22 amide (17 μM; EMD Biosciences), Src inhibitor I (22 μM; EMD Biosciences), staurosporine (5 μM; Upstate), and TBB (100 μM; Biaffin, Kassel, Germany). DMSO (1 μL) was also included in the no-enzyme control. Enzyme dilutions (1 μL) were added to the reaction mixture on ice (see the Supporting Methods for enzyme amounts), and reaction volumes were equalized to 10 μL with enzyme dilution buffer. Then the mixture was incubated at 30 °C for 1 h.

Phosphorylation reactions with cell extracts: Forskolin-treated PC12 nuclear extracts (1 μL, 2.5 μg, Active Motif) and HeLa nuclear extracts (1 μL, 2 μg, Upstate) were added with enzyme dilution buffer (4 μL) to a 2× substrate mixture. The 2× substrate mixture was identical to that used for purified enzymes plus β-glycerophosphate (10 mM) and sodium orthovanadate (2 mM). As a background control, a separate reaction was also prepared with a substrate mixture that lacked ATP. We confirmed that endogenous ATP from the extracts was not sufficient to observe kinase activity with our assay by spiking purified enzymes into this background control. For inhibition, enzyme dilution buffer (1 μL) was substituted with 10× inhibitor or DMSO; PKI (17 μM) and TBB (1 mM, because of the higher ATP concentration) were used. Reaction mixtures were incubated for 1 h at 30 °C.

Blocking and labeling: Reaction mixtures (10 μL) underwent blocking for 1.5 h at room temperature by addition of EDC (1.2 M, Fluka) in MES (0.7 M, 5 μL; pH 5.5). The final pH of the reaction mixture was 6.0. Reaction mixtures were subsequently labeled for 3 h at room temperature by addition of labeling solution (5 μL) containing fluoresceinylglycine amide (20 mM, Invitrogen) and EDC (1.2 M) in MES (0.2 M)/DMSO (3:2; pH 6.0).

Hybridization: Reaction mixtures were diluted to 25 pM in hybridization buffer (Illumina's commercially available WB1 containing 0.1 M cysteine and 25 pM Cy3- and fluorescein-labeled oligonucleotides as controls). The Sentrix Array Matrix (Illumina) was prepared by being washed in formamide (2×1 min) and WB1 (5 min). Diluted reaction mixtures (45 μL per bundle) were hybridized to the array at 48 °C for 14–18 h.

Staining: The following staining sequence was used: 2 min WB1, 2 min PB1 (Illumina), 2 min PBS with 0.1% Tween (PBST), 2 h anti-fluorescein (1:100, Roche), 4×2 min PBST, 1 h biotinylated anti-mouse κ-light chain (1:150, BD Biosciences, San Jose, CA), 4×2 min PBST, 10 min LMM (Illumina), 4×1 min PBST, 10 min ASM (Illumina), 4×1 min PBST, 10 min LMM, 4×1 min PBST, 10 min ASM, 4×1 min PBST, 10 min LMM, 4×1 min PBST. Imaging was performed on Illumina's BeadArray Reader (λ_{ex} = 532 nm, λ_{em} = 550–600 nm, PMT = 441).

Assay optimization: Assay conditions were slightly different for some of the data presented during assay optimization. Reaction mixtures containing peptide substrate pool (100 nM) and ATP (50 μM) in kinase assay buffer were labeled and then hybridized to the microarray at a concentration of 100 pM. Fluorescein detection for all these experiments was accomplished with 1 h biotinylated antifluorescein (1:1000, Invitrogen) and 15 min streptavidin-phycoerythrin (1:500, Invitrogen). For experiments evaluating the effect of EDC blocking, a reaction mixture (5 μL) was treated for 1.5 h with MES (1 M, 1 μL; pH 5.5), EDC (0.5 M in DMSO, 1.5 μL), or just DMSO as a control (1.5). The mixture was labeled with fluoresceinylglycine amide (670 μM), and treated with EDC (0.3 M) in DMSO (2.5 μL) overnight. For the labeling time trial, finalized blocking and labeling conditions were used. For the two-enzyme multiplex experiment and Src titration, blocking was not necessary, and final labeling concentrations were 170 μM dye and 0.13 M EDC. For the detection comparison experiment, an aliquot of the reaction mixture was hybridized to the microarray without labeling, and stained for 1 h with biotinylated anti-pTyr (1:500, Invitrogen) and 15 min streptavidin-phycoerythrin (1:500, Invitrogen). For these experiments, a "no-pool" control was used for background subtraction instead of a no-enzyme control.

Data analysis: Data was extracted by using a SentrixAnalyzer (Illumina, v2.2.9.12) and normalized to the average signal of the 15 fluorescein-labeled oligonucleotides, with the exception of that presented in Figure 1. For all data, normalized intensities from 3 hybridization replicate bundles were averaged, and an average of background readings from a similar no-enzyme (purified enzyme) or no-ATP (lysate) sample was subtracted. During optimization, background and noise in the assay were monitored with the 20 unlabeled oligonucleotide controls. Following normalization and background subtraction, profiles of intensity data were plotted as radial plots with MATLAB (v7.2.0.232 (R2006a)); here each individual peptide substrate occupies a single ray in the circle. The scale for these plots is linear, with the maximum value indicated in the figure legends. All other plots were completed with KaleidaGraph (v4.0). For enzyme titration curves, data points for all three or eight replicates of each enzyme concentration were fitted in MATLAB by using the following equation: $y = f(x) = a \times \tanh(bx) + c$, where tanh is the hyperbolic tangent.

Abbreviations

Akt = protein kinase B, CaMK = calcium/calmodulin-dependent protein kinase, Cdk = cyclin-dependent kinase, CK = casein kinase, DMSO = dimethylsulfoxide, EDC = 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, EGFR = endothelial growth factor receptor, EphB2 = ephrin receptor B2, FAM = 6-carboxyfluorescein, Fmoc = 9-fluorenylmethoxycarbonyl, Jak2 = Janus kinase-2, MES = 2-morpholinoethane sulfonic acid, MK2 = mitogen-activated protein kinase-activated protein kinase-2, PBS = phosphate-buffered saline, PKA = cAMP-dependent protein kinase, PKC = protein kinase C, PKI = protein kinase A peptide inhibitor, Plk = polo-like kinase, SGK = serum and glucocorticoid-regulated kinase, Src I = 4-(4′-phenoxyanilino)-6,7-dimethoxyquinazoline, TBB = 4,5,6,7-tetrabromo-2-azabenzimidazole, TBE = Tris borate EDTA buffer, VEGFR = vascular endothelial growth factor receptor.

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