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Peptide reporters of kinase activity in whole cell lysates

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Abstract

Kinase assays are used to screen for small-molecule inhibitors that may show promise as targeted pharmaceutical therapies. Using cell lysates instead of purified kinases provides a more accurate estimate of inhibitor sensitivity and selectivity in a biological setting. This review summarizes the range of homogeneous (solution-phase) and heterogeneous (solid-supported) formats available for using peptide substrates to monitor kinase activities in cell lysates. With a focus on heterogeneous kinase assays, the peptide substrate Abtide is used as a model to optimize presentation geometries and the modular arrangement of short sequences for kinase recognition. We present results from peptides immobilized on two- and three-dimensional surfaces such as hydrogels on 96-well plates and glass slides, and fluorescent Luminex beads. We discuss methods to increase assay sensitivity using chemifluorescent ELISAs, antibody-based recognition, and label-free mass spectrometry. Monitoring the activity of specific kinases in cell lysates presents challenges that can be overcome by manipulating peptide substrates to optimize assay conditions. In particular, signal-to-background ratios were improved by 1) adding long branched hydrophilic linkers between the substrate and the surface, 2) changing the orientation of peptides relative to the surface, and 3) including peptide ligands *in cis* or *in trans* to recruit kinases to the surface. By improving the accessibility of immobilized peptide substrates to kinases in solution, the apparent rate of phosphorylation increased and assays were more sensitive to changes in endogenous kinase activities. These strategies can be generalized to improve the reactivity of most peptide substrates used in heterogeneous kinase assays with cell lysates.

Keywords

Kinase assay; peptide substrate; cell lysates; ELISA; Luminex; MALDI

The human genome encodes approximately 500 kinases that regulate intracellular signal transduction, gene transcription, and cell development¹. Changes in protein kinase activities, including changes in the selectivity and frequency of substrate phosphorylation, are implicated in human diseases ranging from autoimmunity to malignancies^{2–4}. Given the association between changes in kinase activity and disease, many targeted pharmaceutical

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therapies are based on the modulation of kinase activities with small-molecule inhibitors⁵⁻⁹. Small-molecule inhibitors are initially screened for activity against kinases in large chemical libraries. Large libraries require efficient assay techniques for comparing inhibitor efficacies. A significant challenge in the development of high-throughput screening technologies is the measurement of altered kinase activities in their native cellular environment^{10, 11}. *In vitro* assays using purified recombinant kinases that are truncated or epitope-tagged do not provide the molecular or cellular context to observe regulatory mechanisms affecting inhibitor efficacy. On the other hand, *in vitro* assays using cell lysates are both amenable to high-throughput formats and provide native kinases in an environment that approximates the complexity of intracellular conditions.

Substrate design is an important consideration in kinase assay development. In its most simplified form, a kinase assay monitors the phosphorylation of a protein or peptide substrate in the presence of a kinase and ATP. Kinase catalytic domains typically recognize a substrate tyrosine, serine, threonine, or histidine residue in the context of a short recognition motif composed of 3-20 amino acids¹². These short motifs can be reproduced as independent peptides and used in place of intact protein substrates. Protein substrates can be difficult to produce in large amounts and peptides offer several advantages that simplify assay construction¹³⁻¹⁵. Compared to proteins, peptides are 1) relatively inexpensive to synthesize in large amounts, 2) easy to store, handle, and characterize, and 3) easily modified for immobilization or fluorescent detection. Peptide libraries are used to experimentally determine substrate sequences that provide the highest activity and specificity for a particular kinase *in vitro*^{16, 17}. Although substrate specificity is generally more difficult to achieve with short linear sequences, peptides provide efficient tools for monitoring kinase activity^{18, 19}. Peptide substrates are designed using experimentally derived consensus motifs²⁰ and random peptide libraries^{21, 22} that are screened to identify substrate sequences with the highest rate of reactivity and degree of specificity. Peptide ligands for non-catalytic kinase domains²³ have been presented in combination with peptide substrates to promote selective substrate phosphorylation. These developments have resulted in a recent increase in the use of highly multiplexed peptide arrays to investigate kinase activity in cell lysates²⁴⁻²⁷. Working with a small group of related peptides, this review focuses on strategies that optimize the design and presentation of peptide substrates to monitor kinase activity in cell lysates.

Whether derived from native recognition sequences or synthetically optimized, peptide substrates are used to screen for small-molecule inhibitors against kinases that are associated with disease (Table 1)^{17, 28-39}. Abltide (EAIYAAPFAKKK)¹⁷ is one example of an optimized substrate that was selected by screening a degenerate peptide library against purified kinases *in vitro*. Abltide demonstrated a higher rate of reactivity with Abl kinase than with closely related kinases such as Src¹⁷ and is now routinely used to monitor Abl kinase activity *in vitro*. Abltide is preferentially phosphorylated by the Abelson subfamily, which includes Abl (ABL1) and Arg kinases (ABL2)⁴⁰, as well as the oncogenic fusion protein Bcr-Abl⁴¹⁻⁴⁴ that characterizes chronic myelogenous leukemia (CML). Accordingly, Abltide can be used to report on Bcr-Abl kinase activity in cell lysates, possibly providing an activity-based molecular diagnostic for CML. The specificity of Abltide as a reporter for Bcr-Abl activity can be confirmed by both the selective inhibition of Bcr-Abl and comparisons against cell extracts that demonstrate little Abl or Bcr-Abl kinase activity. Indeed, Abltide phosphorylation is quantitatively reduced with the addition of the Bcr-Abl inhibitor imatinib, the first FDA-approved tyrosine kinase inhibitor for the treatment of CML⁴⁵ (sold as Gleevec by Novartis, East Hanover, NJ, USA). Similarly, cell lines such as the bone marrow-derived BaF3 line⁴⁶, demonstrate that Abltide is minimally phosphorylated when Bcr-Abl is not expressed⁴⁴. Assays that use Abltide as a reporter and imatinib as an

inhibitor provide a model system for deriving general principles for the design of other peptide-based kinase assays.

The optimal modification of a peptide substrate depends in large part on the type of kinase assay in which it is used. Kinase assays can be broadly categorized into homogeneous (solution-phase)^{47, 48} and heterogeneous (solid-phase) technologies⁴⁹⁻⁵¹. Homogeneous assays are typically small volume reactions using a purified enzyme and do not include post-reaction wash steps to reduce background^{52, 53}. Heterogeneous kinase assays immobilize substrates on a two- or three-dimensional surface and detect phosphorylated products after multiple wash and isolation steps. Few homogeneous assays have been developed for use with cell lysates because signal-to-background ratios become prohibitively low due to non-specific interactions with intracellular proteins. However, unique applications of homogeneous assays using cell lysates have presented peptides as direct reporters of kinase activity that do not require additional reagents, such as antibodies, to detect phosphorylation⁵⁴. In two examples peptide substrates were modified with fluorophores that react with the phosphorylated amino acid⁵⁵ or chelate co-factor metal ions present at the kinase active site during catalysis⁵⁶. Heterogeneous assays, on the other hand, have the advantage of increasing the sensitivity for a specific reaction in cell lysates by isolating the product from other phosphorylated proteins in the cell.

Peptide substrates can be modified in a variety of ways for use in heterogeneous assays, although different immobilization strategies require specific considerations. Supporting surface geometries for peptide immobilization range from microarray chips and multi-well plates to micro- and nanoparticles. Peptides can be synthesized *in situ* on solid surfaces with photolithography or SPOT technology^{57, 58}. Functional groups on peptide side chains have also been exploited for covalent immobilization on prepared surfaces. For example, primary amines have been immobilized on surfaces coated with epoxy, aldehyde, and N-hydroxysuccinimide functional groups. Similarly, amino-terminal cysteines have been used to react with glyoxylyl glass, forming a thiazolidine ring⁵⁹, and with thioesters for native chemical ligation products⁶⁰. Non-covalent attachment involves the use of biotin or poly-histidine tags⁶¹, while covalent methods have included click chemistry, such as Staudinger ligation⁶² or 1-3 dipolar cycloaddition⁶³, and Diels-Alder reactions to photo-activated self-assembled monolayers on gold surfaces⁶⁴ (also reviewed in^{65, 66}). In contrast to proteins, which are difficult to immobilize in their active conformation^{67, 68}, peptides are well suited to chemical modification and resistant to rigorous washes. Kinase assays using cell lysates require the addition of protease inhibitors to prevent the degradation of endogenous intracellular proteins and exogenous peptide substrates. Amino-terminal acetylation of peptides with acetic acid also provides improved stability in assays that use cell lysates⁶⁹. Depending on the particular scheme that is chosen for peptide immobilization various modifications can be considered to improve peptide stability and accessibility.

ELISA-based heterogeneous kinase assays

The most common format for heterogeneous kinase assays is as an enzyme-linked immunosorbent assay (ELISA). Kinase assays with an ELISA format are easily constructed and performed without expensive equipment. In a typical ELISA, the protein or peptide substrate is immobilized on the surface of a multi-well plate either before or after reaction with a kinase. The phosphorylated substrate is probed with a phospho-specific primary antibody and an enzyme-conjugated secondary antibody. Upon addition of a suitable substrate for the conjugated enzyme, the chromogenic, luminescent, or fluorescent readout is quantified⁷⁰. This method of labeling phosphorylated substrates can include the use of fluorescently labeled antibodies⁷¹ as well as the use of biotinylated reagents and their recognition with fluorescently labeled streptavidin. For example, fluorescence imaging using

biotinylated Phos-tag and streptavidin has been used to detect the phosphorylation of peptide substrates immobilized to amino-modified glass via amino-terminal cysteines with glutaraldehyde crosslinkers⁷². Chemifluorescent detection, based on the oxidation of Amplex Red with hydrogen peroxide by horseradish peroxidase, has also been used to measure the relative phosphorylation of a peptide immobilized onto a hydrogel surface⁷³. In this case, Abltide (CEAIYAAPFAKKK)¹⁷ was covalently attached by Michael addition of the amino-terminal cysteine to acrylamide-presenting hydrogel surfaces (Figure 1). C-Abl and Bcr-Abl kinase activities were monitored in cell lysates treated with a focused library of small-molecule inhibitors with pyridopyrimidine-like structures resembling imatinib⁷³. Phosphorylated Abltide was detected with an anti-phosphotyrosine antibody, a horseradish peroxidase (HRP)-conjugated secondary antibody, and chemifluorescent imaging. The significant signal amplification that accompanies immuno-detection with enzyme-conjugates capable of producing fluorescent products yields an assay that is sensitive to small amounts of substrate phosphorylation but proportional to kinase activity.

Despite the general utility of the basic ELISA format, several features can be improved to increase the signal-to-background ratio in kinase assays using cell lysates. Surfaces often require passivation to decrease non-specific interactions with abundant proteins in cell lysates and peptide substrates require distance from the surface to improve kinase accessibility. This review will summarize the effects of increasing peptide substrate accessibility while decreasing non-specific interactions for improved reaction conditions on solid surfaces in kinase assays using cell lysates.

Surface passivation and substrate accessibility

Surface passivation generally involves blocking the interaction surface with non-reactive molecules, such as BSA, casein, detergents and/or hydrophilic polymers, or capping un-conjugated functional groups such as reactive thiols with sodium acrylate. However, by using bioinert hydrophilic linkers to increase the distance between the peptide substrate and the surface, substrate accessibility can be improved while simultaneously decreasing non-specific interactions. For instance, a molecular layer of BSA⁷⁴ or long polyethylene glycol linkers⁵⁹ are commonly used to distance peptides from a surface. Branched linkers, which present chemical functional groups at multiple distal termini, improve the signal-to-background ratio by increasing substrate loading capacity. Comparisons between di-, tri-, and tetra-acrylate polyethylene glycol cross-linkers (Sartomer, Exton, PA, USA) demonstrated that the longest linker with three functional groups (SR415), yielded the strongest signal in a chemifluorescent ELISA using peptide substrates⁷³. By increasing the density of peptide substrates on the hydrogel surface, the triacrylate linker increased the effective concentration of substrate in the hydrogel and improved readout sensitivity (Figure 2).

When immobilized, the relative rate of peptide phosphorylation is dependent on the accessibility of the substrate residues to kinases in solution. The core sequence of Abltide was optimized for maximal recognition by the catalytic sites of c-Abl and Bcr-Abl. However, when Abltide was immobilized in the kinase assay described above, the tyrosine was not optimally oriented for phosphorylation by approaching kinases (Figure 3A). To investigate the effects of distancing the substrate tyrosine from the site of immobilization, additional flexible and non-reactive amino acids, such as glycine and serine, were inserted near the site of immobilization (Figure 3B). Peptides C-4X-AT, C-6X-AT, C-8X-AT were synthesized with four, six, or eight amino acids between the amino-terminal site of immobilization and the tyrosine to be phosphorylated (Table 2). Although the maximum level of tyrosine phosphorylation increased relative to the original sequence, the signal did not increase proportionately with additional amino acid spacers (Figure 4). An eight amino

acid spacer and a four amino acid spacer resulted in the same relative degree of tyrosine phosphorylation. To investigate the influence of Abltide orientation in the context of a passivated hydrogel, the site of immobilization was reversed from the amino-terminus to the carboxyl-terminus. Peptides were synthesized with cysteines at the carboxyl-terminus (AT-C) and compared to peptides with four additional amino acids at the distal end of the peptide (4X-AT-C) (Figures 3C and 3D). In both cases, there was increased phosphorylation relative to Abltide immobilized at the amino-terminus (Figure 4). From these examples, two general principles can be derived to improve the degree of substrate phosphorylation in heterogeneous assays: 1) increase the relative distance between the substrate and the site of immobilization, and 2) present the substrate tyrosine in an orientation that allows kinase accessibility.

Co-immobilization of kinase ligands

C-Abl and Bcr-Abl are structurally related to the Src family of kinases and use SH2 and SH3 domains to regulate inter- and intra-molecular interactions⁷⁵. While the catalytic kinase domain is responsible for the phosphorylation of substrates, SH2 and SH3 domains guide the kinase to its substrates and regulate its active state⁷⁶. By providing only a short amino acid sequence that is recognized exclusively by the kinase catalytic site, peptide substrates often lack the selectivity shown to protein substrates. Adding a peptide ligand for the SH2 or SH3 domains can significantly improve substrate recognition thereby increasing the relative rate of substrate phosphorylation^{77, 78}. In heterogeneous kinase assays, where peptide substrates are immobilized in relatively inert environments, the inclusion of peptide ligands for SH2 and SH3 domains effectively recruits cellular kinases to the surface. In the ELISA format previously described using cell lysates to phosphorylate Abltide on a hydrogel surface, the co-immobilization of a high-affinity peptide ligand for the SH3 domain of c-Abl (p40; CGGAPTYSPPPPLL)⁷⁹ significantly increased Abltide phosphorylation. Although Abltide peptide substrate and the p40 SH3 ligand were not synthesized in tandem but were immobilized in parallel on the hydrogel surface (*in trans*), the presence of the SH3 ligand appeared to increase the effective concentration of kinase at the hydrogel surface⁷³. Similarly, by expressing Abltide in tandem with p40 (*in cis*) as a carboxyl-terminal tail to glutathione-S-transferase and immobilizing on glutathione agarose beads⁴⁴, Abltide phosphorylation was increased. In theory, the combination of kinase ligands and peptides *in trans* or *in cis* can be applied to any kinase with multiple recognition domains⁷⁶. Using current synthesis techniques such as native chemical ligation^{80, 81}, it is possible to assemble libraries of optimized substrates⁸², or “super-substrates,” constructed from the modular rearrangement of specialized peptides for recognition by particular domains.

Solution-phase heterogeneous kinase assays

Solution-phase heterogeneous kinase assays aim to improve the reactivity of solid-supported peptide substrates by providing greater access to reactants in solution. Despite improvements in the presentation of peptides on a solid surface, the reaction rate between immobilized peptides and kinases in solution can be slow due to the limits of diffusion at the interface^{83, 84}. In contrast to many two-dimensional plate-based assays, three-dimensional bead-based assays can use mechanical mixing to increase the frequency of molecular interactions at the interface between solid and solution phases. Uniformly distributed in a solution of reactants, beads bearing peptide substrates display faster apparent reaction rates with kinases in solution. Furthermore, a dense suspension of sub- μm beads can have a larger total surface area per volume than a flat two-dimensional surface overlaid with solution ($>1 \mu\text{l}$ to wet 1 cm^2) and each bead can be loaded to higher capacity with peptide substrates due to bead surface curvature. Because the density of immobilized substrate in a given reaction volume limits the maximum signal obtainable in a heterogeneous assay, higher substrate

loading with better accessibility results in a higher signal-to-background ratio. Using immobilization strategies similar to those used for plate-based assays, but with shorter processing times and easier handling procedures, beads offer the advantages of high loading capacity, increased apparent reaction rates, and improved substrate accessibility to kinases in solution.

Multiple peptide substrates can be presented in a shared reaction volume using beads that can be individually distinguished by internal fluorescence (e.g. Luminex liquid bead arrays, Luminex, Austin, TX, USA). Peptide-conjugated beads can be detected by a flow cytometer after labeling with fluorescent phospho-specific antibodies. For example, a mixture of purified kinases was used to phosphorylate biotinylated peptide substrates immobilized on streptavidin-conjugated fluorescent beads⁸⁵. This strategy of using uniquely identifiable beads to detect multiple substrates was broadened to investigate the competitive phosphorylation of substrates by a kinase in cell lysates. Using the same chemistry that was developed for two-dimensional hydrogel surfaces, Abltide was immobilized in two orientations (C-AT and AT-C) on carboxyl-coated fluorescent beads via Michael addition of a cysteine to an amino-acrylamide linker. The peptide ligand for the Abl kinase SH3 domain, p40 (AL), was included *in cis* at the amino or carboxyl-terminus of Abltide (C-ALAT and C-ATAL). To measure the degree of substrate phosphorylation, four peptide standards were included in each reaction volume after the kinase reaction and before labeling (Figure 5). Antibody-based fluorescence intensity was translated to the percentage of substrate phosphorylation by non-linear regression from well-specific internal standard curves (Figure 6A). Presented in a single reaction volume, substrates that included the p40 c-Abl SH3 ligand sequence were competitively phosphorylated by Bcr-Abl in cell lysates over peptides that presented Abltide alone (Figure 6B). Assay conditions can be optimized to improve sensitivity and the limits of detection by using a combination of peptide ligands and internal peptide standards. The advantage of methods such as fluorescently encoded bead arrays that allow the multiplexed presentation and detection of peptide substrates is that substrate efficiency can be quantitatively compared in a single reaction.

Label-free detection methods for heterogeneous kinase assays

Whether applied to peptides immobilized on hydrogel surfaces or Luminex beads, ELISA-based methods depend on the availability of antibodies or similar reagents for the selective recognition of distinct peptide sequences or phosphorylated residues. On the other hand, label-free methods provide readouts that are independent from additional reagents used for detection. For example, mass spectrometry (MS) uses a readout based on the abundance of ions to detect distinct differences in peptide mass before and after substrate phosphorylation. This can be applied to the detection of peptides in solution using liquid chromatography (LC)-MS²⁷ or to immobilized peptides using matrix-assisted laser desorption ionization (MALDI) MS⁸⁶. In one example using peptide substrates immobilized on gold surfaces, self-assembled monolayers for MALDI (SAMDI)⁶⁴ provided a semi-quantitative and unambiguous measure of substrate phosphorylation. As an alternative to MALDI detection with higher sensitivity for surface-based analyses, time-of-flight secondary ion MS (TOF-SIMS) was used to monitor peptide phosphorylation by purified kinases *in vitro*⁸⁷. Conjugating peptides to gold nanoparticles clustered on amine-coated glass increased loading capacity and substrate accessibility on the two-dimensional glass surface⁸⁸. This demonstrates that despite the absence of additional labeling reagents, heterogeneous kinase assays using label-free methods benefit from optimized peptide immobilization and surface passivation.

Most MALDI-based methods do not include a separate step for the release of immobilized peptides, instead relying on energy imparted by the laser to desorb peptides from the solid

surface. However, to control the release of immobilized peptide substrates prior to analysis with label-free MS an ultraviolet (UV)-cleavable substrate was included⁸⁹. Using a modular approach to substrate synthesis based on native chemical ligation, a photocleavable linker was incorporated at the amino-terminus of Abltide followed by p40, the peptide ligand for the Abl SH3 domain. This UV-cleavable substrate was co-polymerized with bis-acrylamide to construct a hydrogel matrix of Abltide-functionalized polymers. With three-dimensional substrate loading capacity, hydrogel matrices swell upon the addition of soluble reactants, thereby providing a sturdy and inert environment for biochemical reactions. After substrate phosphorylation the linker was cleaved by UV light and Abltide was released for MALDI-TOF detection. Compared to peptide recognition in an ELISA format, the benefit of using mass spectrometry is the unambiguous detection of substrate mass. This allows multiple peptides with distinct masses to be analyzed in a single sample. Therefore by including an internal “heavy” peptide control, the relative intensities of phosphorylated and un-phosphorylated Abltide were quantitatively determined from a single sample using label-free detection (Figure 7A, unpublished data, Xiangfu Shi).

With label-free detection, multiple peptide substrates can be used to monitor different kinase activities from purified mixtures or cell lysates. For example, MALDI-TOF can be used to detect the selective phosphorylation of two peptides immobilized on a hydrogel surface by two kinases in a mixture. Un-phosphorylated and phosphorylated forms of each peptide are detected, allowing a straightforward qualitative analysis of reaction components (Figure 7B, Xiangfu Shi). To more accurately compare MALDI detection to chemifluorescent ELISA, a dual-detection kinase assay was developed using biotinylated Abltide immobilized on streptavidin-conjugated magnetic beads (unpublished data). Used to screen for small-molecule inhibitors, the dual-detection kinase assay eliminates false positives and negatives by validating results using two separate detection and readout methods. More complex signaling networks may be investigated using label-free methods to detect multiple peptide substrates on a shared surface in kinase assays using cell lysates. These applications may lay the foundations for using intracellular signaling profiles to personalize targeted therapies with kinase inhibitors in clinical settings⁹⁰.

Conclusion

Kinase assays that use cell lysates instead of an isolated kinase of interest more accurately approximate *in vivo* responses. To investigate the efficacy of a small-molecule inhibitor, it is necessary to account for environmental cues that regulate kinase activity. Kinase assays using cell lysates are complicated by the difficulty of monitoring a specific enzyme activity within a complex biological sample. Heterogeneous formats facilitate the separation of products from extraneous cell components and offer the advantage of reducing non-specific background with wash steps. Using peptide substrates in heterogeneous kinase assays simplifies substrate immobilization strategies and assay analysis. Changes in chemical modifications and physical orientations can be used to optimize substrate presentation for improved sensitivity and specificity in kinase assays using cell lysates.

This review summarizes several chemical approaches to peptide immobilization and emphasizes our efforts to optimize assay sensitivity in a variety of formats, including chemifluorescent ELISAs on hydrogel surfaces, antibody recognition on fluorescent beads, and with label-free methods such as MS. Chemical modifications that affect the orientation and distance of a peptide from a passivated surface result in improved substrate accessibility and recognition. This allows kinases to phosphorylate a higher concentration of substrates and produces better signal-to-background ratios. In several examples varying the orientation of peptide substrates on a surface and using long, branched hydrophilic linkers increased the degree of substrate phosphorylation and decreased non-specific interactions at the surface.

In addition, the inclusion of peptide ligands for non-catalytic kinase domains such as the p40 ligand for the Abl kinase SH3 domain improved substrate phosphorylation by directing kinases from solution to the surface. Indeed, on both hydrogels and beads, modular rearrangements of the Abltide and p40 sequences increased Abltide phosphorylation by Bcr-Abl kinase in cell lysates. To reveal kinase substrate preferences, peptides were multiplexed using fluorescent Luminex beads and competitively phosphorylated by cell lysates. The inclusion of internal peptide standards for quantitative analysis strengthened results obtained from a single multiplexed experiment. Separate beads can be used to immobilize unique peptides reporters for the simultaneous measurement of multiple phosphorylation events.

Label-free detection with MS provided a more flexible platform for the development of multiplexed assays. Although previously applied to purified kinase activities *in vitro*, promising developments include the use of MALDI-TOF MS to monitor kinase activity and inhibition in cell lysates with peptides immobilized on magnetic beads or glass slides⁸⁹. With the advantage of unambiguous results, reversible linkers and internal peptide controls can be included to quantitatively analyze kinase activity by MALDI-TOF. Toward this end, a dual-detection kinase assay was developed using both MALDI-TOF and chemifluorescent ELISA to compare readouts and eliminate false positives and negatives. Dual-detection methods may speed up the screening process thereby lowering the cost of high-throughput screens.

Although this review highlights the detection of Bcr-Abl activity in a model of CML using the peptide substrate Abltide, the strategies discussed in this review can be generalized for most peptide substrates in heterogeneous kinase assays using cell lysates. Advances in peptide synthesis technology and robotics for rapid handling will undoubtedly influence the popularity of peptide substrates in heterogeneous kinase assays. However, more compelling developments may be brought about by improved immobilization strategies that allow the presentation of optimized peptide substrates in high density for small volume reactions with cell lysates.

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References

1. Manning G, Whyte DB, Martinez R, Hunter T, Sudarsanam S. The protein kinase complement of the human genome. *Science*. 2002; 298(5600):1912–1934. [PubMed: 12471243]
2. D'Aura Swanson C, Paniagua RT, Lindstrom TM, Robinson WH. Tyrosine kinases as targets for the treatment of rheumatoid arthritis. *Nat Rev Rheumatol*. 2009; 5(6):317–324. [PubMed: 19491913]
3. Saglio G, Cilloni D. Abl: the prototype of oncogenic fusion proteins. *Cell Mol Life Sci*. 2004; 61(23):2897–2911. [PubMed: 15583852]
4. Gray N, Detivaud L, Doerig C, Meijer L. ATP-site directed inhibitors of cyclin-dependent kinases. *Curr Med Chem*. 1999; 6(9):859–875. [PubMed: 10495356]

5. Sawyers C. Targeted cancer therapy. *Nature*. 2004; 432(7015):294–297. [PubMed: 15549090]
6. Madhusudan S, Ganesan TS. Tyrosine kinase inhibitors in cancer therapy. *Clin Biochem*. 2004; 37(7):618–635. [PubMed: 15234243]
7. Sawyer TK. Novel oncogenic protein kinase inhibitors for cancer therapy. *Curr Med Chem Anticancer Agents*. 2004; 4(5):449–455. [PubMed: 15379702]
8. Ventura JJ, Nebreda AR. Protein kinases and phosphatases as therapeutic targets in cancer. *Clin Transl Oncol*. 2006; 8(3):153–160. [PubMed: 16648114]
9. Cohen P. Protein kinases--the major drug targets of the twenty-first century? *Nat Rev Drug Discov*. 2002; 1(4):309–315. [PubMed: 12120282]
10. Russello SV. Assessing cellular protein phosphorylation: high throughput drug discovery technologies. *Assay Drug Dev Technol*. 2004; 2(2):225–235. [PubMed: 15165518]
11. Whitney JA. Reference systems for kinase drug discovery: chemical genetic approaches to cell-based assays. *Assay Drug Dev Technol*. 2004; 2(4):417–429. [PubMed: 15357923]
12. Kennelly PJ, Krebs EG. Consensus sequences as substrate specificity determinants for protein kinases and protein phosphatases. *J Biol Chem*. 1991; 266(24):15555–15558. [PubMed: 1651913]
13. Cretich M, Damin F, Pirri G, Chiari M. Protein and peptide arrays: recent trends and new directions. *Biomol Eng*. 2006; 23(2-3):77–88. [PubMed: 16527536]
14. Schutkowski M, Reineke U, Reimer U. Peptide arrays for kinase profiling. *Chembiochem*. 2005; 6(3):513–521. [PubMed: 15742386]
15. Reimer U, Reineke U, Schneider-Mergener J. Peptide arrays: from macro to micro. *Curr Opin Biotechnol*. 2002; 13(4):315–320. [PubMed: 1232352]
16. Songyang Z, Cantley LC. The use of peptide library for the determination of kinase peptide substrates. *Methods Mol Biol*. 1998; 87:87–98. [PubMed: 9523263]
17. Songyang Z, Carraway KL 3rd, Eck MJ, Harrison SC, Feldman RA, Mohammadi M, Schlessinger J, Hubbard SR, Smith DP, Eng C, et al. Catalytic specificity of protein-tyrosine kinases is critical for selective signalling. *Nature*. 1995; 373(6514):536–539. [PubMed: 7845468]
18. Hardie DG. Peptide assay of protein kinases and use of variant peptides to determine recognition motifs. *Methods Mol Biol*. 2000; 99:191–201. [PubMed: 10909086]
19. Thiele A, Zerweck J, Schutkowski M. Peptide arrays for enzyme profiling. *Methods Mol Biol*. 2009; 570:19–65. [PubMed: 19649588]
20. Dane KY, Gottstein C, Daugherty PS. Cell surface profiling with peptide libraries yields ligand arrays that classify breast tumor subtypes. *Mol Cancer Ther*. 2009
21. Wu J, Ma QN, Lam KS. Identifying substrate motifs of protein kinases by a random library approach. *Biochemistry*. 1994; 33(49):14825–14833. [PubMed: 7993909]
22. Songyang Z, Blechner S, Hoagland N, Hoekstra MF, Pwnica-Worms H, Cantley LC. Use of an oriented peptide library to determine the optimal substrates of protein kinases. *Curr Biol*. 1994; 4(11):973–982. [PubMed: 7874496]
23. Kuriyan J, Cowburn D. Modular peptide recognition domains in eukaryotic signaling. *Annu Rev Biophys Biomol Struct*. 1997; 26:259–288. [PubMed: 9241420]
24. Hayashi M, Fearn C, Eliceiri B, Yang Y, Lee JD. Big mitogen-activated protein kinase 1/ extracellular signal-regulated kinase 5 signaling pathway is essential for tumor-associated angiogenesis. *Cancer Res*. 2005; 65(17):7699–7706. [PubMed: 16140937]
25. Ritsema T, Joore J, van Workum W, Pieterse CM. Kinome profiling of Arabidopsis using arrays of kinase consensus substrates. *Plant Methods*. 2007; 3:3. [PubMed: 17295910]
26. Shults MD, Kozlov IA, Nelson N, Kermani BG, Melnyk PC, Shevchenko V, Srinivasan A, Musmacker J, Hachmann JP, Barker DL, Lebl M, Zhao C. A multiplexed protein kinase assay. *Chembiochem*. 2007; 8(8):933–942. [PubMed: 17471478]
27. Kubota K, Anjum R, Yu Y, Kunz RC, Andersen JN, Kraus M, Keilhack H, Nagashima K, Krauss S, Pawletz C, Hendrickson RC, Feldman AS, Wu CL, Rush J, Villen J, Gygi SP. Sensitive multiplexed analysis of kinase activities and activity-based kinase identification. *Nat Biotechnol*. 2009; 27(10):933–940. [PubMed: 19801977]

28. Bozinovski S, Cristiano BE, Marmy-Conus N, Pearson RB. The synthetic peptide RPRAATF allows specific assay of Akt activity in cell lysates. *Anal Biochem.* 2002; 305(1):32–39. [PubMed: 12018943]
29. Bence K, Ma W, Kozasa T, Huang XY. Direct stimulation of Bruton's tyrosine kinase by G(q)-protein alpha-subunit. *Nature.* 1997; 389(6648):296–299. [PubMed: 9305846]
30. Yamadori T, Baba Y, Matsushita M, Hashimoto S, Kurosaki M, Kurosaki T, Kishimoto T, Tsukada S. Bruton's tyrosine kinase activity is negatively regulated by Sab, the Btk-SH3 domain-binding protein. *Proc Natl Acad Sci U S A.* 1999; 96(11):6341–6346. [PubMed: 10339589]
31. Lew J. MAP kinases and CDKs: kinetic basis for catalytic activation. *Biochemistry.* 2003; 42(4): 849–856. [PubMed: 12549901]
32. Orlicky S, Tang X, Willems A, Tyers M, Sicheri F. Structural basis for phosphodependent substrate selection and orientation by the SCFCdc4 ubiquitin ligase. *Cell.* 2003; 112(2):243–256. [PubMed: 12553912]
33. Haycock JW, Ahn NG, Cobb MH, Krebs EG. ERK1 and ERK2, two microtubule-associated protein 2 kinases, mediate the phosphorylation of tyrosine hydroxylase at serine-31 in situ. *Proc Natl Acad Sci U S A.* 1992; 89(6):2365–2369. [PubMed: 1347949]
34. Moreno FJ, Munoz-Montano JR, Avila J. Glycogen synthase kinase 3 phosphorylation of different residues in the presence of different factors: analysis on tau protein. *Mol Cell Biochem.* 1996; 165(1):47–54. [PubMed: 8974080]
35. Li Z, Xu M, Xing S, Ho WT, Ishii T, Li Q, Fu X, Zhao ZJ. Erlotinib effectively inhibits JAK2V617F activity and polycythemia vera cell growth. *J Biol Chem.* 2007; 282(6):3428–3432. [PubMed: 17178722]
36. Flotow H, Purton TJ, Whitaker DP, Williams DH, Wilkinson SE. Substrate specificity and inhibitor profile of human recombinant p56lck from a baculovirus expression vector. *Inflamm Res.* 1996; 45(8):412–415. [PubMed: 8872515]
37. Leverrier Y, Okkenhaug K, Sawyer C, Bilancio A, Vanhaesebroeck B, Ridley AJ. Class I phosphoinositide 3-kinase p110beta is required for apoptotic cell and Fcgamma receptor-mediated phagocytosis by macrophages. *J Biol Chem.* 2003; 278(40):38437–38442. [PubMed: 12869549]
38. Kemp BE, Graves DJ, Benjamini E, Krebs EG. Role of multiple basic residues in determining the substrate specificity of cyclic AMP-dependent protein kinase. *J Biol Chem.* 1977; 252(14):4888–4894. [PubMed: 194899]
39. Chen SJ, Klann E, Gower MC, Powell CM, Sessoms JS, Sweatt JD. Studies with synthetic peptide substrates derived from the neuronal protein neurogranin reveal structural determinants of potency and selectivity for protein kinase C. *Biochemistry.* 1993; 32(4):1032–1039. [PubMed: 8424932]
40. Kruh GD, Perego R, Miki T, Aaronson SA. The complete coding sequence of arg defines the Abelson subfamily of cytoplasmic tyrosine kinases. *Proc Natl Acad Sci U S A.* 1990; 87(15): 5802–5806. [PubMed: 2198571]
41. Ben-Neriah Y, Daley GQ, Mes-Masson AM, Witte ON, Baltimore D. The chronic myelogenous leukemia-specific P210 protein is the product of the bcr/abl hybrid gene. *Science.* 1986; 233(4760):212–214. [PubMed: 3460176]
42. Skaggs BJ, Gorre ME, Ryvkin A, Burgess MR, Xie Y, Han Y, Komisopoulou E, Brown LM, Loo JA, Landaw EM, Sawyers CL, Graeber TG. Phosphorylation of the ATP-binding loop directs oncogenicity of drug-resistant BCR-ABL mutants. *Proc Natl Acad Sci U S A.* 2006; 103(51): 19466–19471. [PubMed: 17164333]
43. Kondo N, Nishimura S. MALDI-TOF mass-spectrometry-based versatile method for the characterization of protein kinases. *Chemistry.* 2009; 15(6):1413–1421. [PubMed: 19115309]
44. Wu D, Nair-Gill E, Sher DA, Parker LL, Campbell JM, Siddiqui M, Stock W, Kron SJ. Assaying Bcr-Abl kinase activity and inhibition in whole cell extracts by phosphorylation of substrates immobilized on agarose beads. *Anal Biochem.* 2005; 347(1):67–76. [PubMed: 16236241]
45. Druker BJ, Tamura S, Buchdunger E, Ohno S, Segal GM, Fanning S, Zimmermann J, Lydon NB. Effects of a selective inhibitor of the Abl tyrosine kinase on the growth of Bcr-Abl positive cells. *Nat Med.* 1996; 2(5):561–566. [PubMed: 8616716]

46. Collins MK, Downward J, Miyajima A, Maruyama K, Arai K, Mulligan RC. Transfer of functional EGF receptors to an IL3-dependent cell line. *J Cell Physiol.* 1988; 137(2):293–298. [PubMed: 3263970]
47. Seethala R, Menzel R. A fluorescence polarization competition immunoassay for tyrosine kinases. *Anal Biochem.* 1998; 255(2):257–262. [PubMed: 9451512]
48. Ullman EF, Kirakossian H, Switchenko AC, Ishkanian J, Ericson M, Wartchow CA, Pirio M, Pease J, Irvin BR, Singh S, Singh R, Patel R, Dafforn A, Davalian D, Skold C, Kurn N, Wagner DB. Luminescent oxygen channeling assay (LOCI): sensitive, broadly applicable homogeneous immunoassay method. *Clin Chem.* 1996; 42(9):1518–1526. [PubMed: 8787723]
49. Braunwaler AF, Yarwood DR, Hall T, Missbach M, Lipson KE, Sills MA. A solid-phase assay for the determination of protein tyrosine kinase activity of c-src using scintillating microtitration plates. *Anal Biochem.* 1996; 234(1):23–26. [PubMed: 8742077]
50. Lam KS, Liu R, Miyamoto S, Lehman AL, Tuscano JM. Applications of one-bead one-compound combinatorial libraries and chemical microarrays in signal transduction research. *Acc Chem Res.* 2003; 36(6):370–377. [PubMed: 12809522]
51. Rychlewski L, Kschischo M, Dong L, Schutkowski M, Reimer U. Target specificity analysis of the Abl kinase using peptide microarray data. *J Mol Biol.* 2004; 336(2):307–311. [PubMed: 14757045]
52. Wesche H, Xiao SH, Young SW. High throughput screening for protein kinase inhibitors. *Comb Chem High Throughput Screen.* 2005; 8(2):181–195. [PubMed: 15777182]
53. Olive DM. Quantitative methods for the analysis of protein phosphorylation in drug development. *Expert Rev Proteomics.* 2004; 1(3):327–341. [PubMed: 15966829]
54. Turk BE. Measuring kinase activity: finding needles in a haystack. *Nat Methods.* 2005; 2(4):251–252. [PubMed: 15782214]
55. Chen CA, Yeh RH, Yan X, Lawrence DS. Biosensors of protein kinase action: from in vitro assays to living cells. *Biochim Biophys Acta.* 2004; 1697(1-2):39–51. [PubMed: 15023349]
56. Shults MD, Janes KA, Lauffenburger DA, Imperiali B. A multiplexed homogeneous fluorescence-based assay for protein kinase activity in cell lysates. *Nat Methods.* 2005; 2(4):277–283. [PubMed: 15782220]
57. Fodor SP, Read JL, Pirrung MC, Stryer L, Lu AT, Solas D. Light-directed, spatially addressable parallel chemical synthesis. *Science.* 1991; 251(4995):767–773. [PubMed: 1990438]
58. Reineke U, Volkmer-Engert R, Schneider-Mergener J. Applications of peptide arrays prepared by the SPOT-technology. *Curr Opin Biotechnol.* 2001; 12(1):59–64. [PubMed: 11167074]
59. Falsey JR, Renil M, Park S, Li S, Lam KS. Peptide and small molecule microarray for high throughput cell adhesion and functional assays. *Bioconjug Chem.* 2001; 12(3):346–353. [PubMed: 11353531]
60. Lesaichere ML, Uttamchandani M, Chen GY, Yao SQ. Developing site-specific immobilization strategies of peptides in a microarray. *Bioorg Med Chem Lett.* 2002; 12(16):2079–2083. [PubMed: 12127508]
61. Aina OH, Sroka TC, Chen ML, Lam KS. Therapeutic cancer targeting peptides. *Biopolymers.* 2002; 66(3):184–199. [PubMed: 12385037]
62. Kohn M, Wacker R, Peters C, Schroder H, Souleire L, Breinbauer R, Niemeyer CM, Waldmann H. Staudinger ligation: a new immobilization strategy for the preparation of small-molecule arrays. *Angew Chem Int Ed Engl.* 2003; 42(47):5830–5834. [PubMed: 14673911]
63. Lin PC, Ueng SH, Tseng MC, Ko JL, Huang KT, Yu SC, Adak AK, Chen YJ, Lin CC. Site-specific protein modification through Cu(I)-catalyzed 1,2,3-triazole formation and its implementation in protein microarray fabrication. *Angew Chem Int Ed Engl.* 2006; 45(26):4286–4290. [PubMed: 16739151]
64. Houseman BT, Huh JH, Kron SJ, Mrksich M. Peptide chips for the quantitative evaluation of protein kinase activity. *Nat Biotechnol.* 2002; 20(3):270–274. [PubMed: 11875428]
65. Uttamchandani M, Yao SQ. Peptide microarrays: next generation biochips for detection, diagnostics and high-throughput screening. *Curr Pharm Des.* 2008; 14(24):2428–2438. [PubMed: 18781992]

66. Kohn M. Immobilization strategies for small molecule, peptide and protein microarrays. *J Pept Sci.* 2009; 15(6):393–397. [PubMed: 19308932]
67. Butler JE. Solid supports in enzyme-linked immunosorbent assay and other solid-phase immunoassays. *Methods.* 2000; 22(1):4–23. [PubMed: 11020313]
68. Predki PF. Functional protein microarrays: ripe for discovery. *Curr Opin Chem Biol.* 2004; 8(1):8–13. [PubMed: 15036150]
69. Luo Y, Smith RA, Guan R, Liu X, Klinghofer V, Shen J, Hutchins C, Richardson P, Holzman T, Rosenberg SH, Giranda VL. Pseudosubstrate peptides inhibit Akt and induce cell growth inhibition. *Biochemistry.* 2004; 43(5):1254–1263. [PubMed: 14756561]
70. Sadick MD, Sliwkowski MX, Nuijens A, Bald L, Chiang N, Lofgren JA, Wong WL. Analysis of heregulin-induced ErbB2 phosphorylation with a high-throughput Kinase receptor activation enzyme-linked immunosorbant assay. *Anal Biochem.* 1996; 235(2):207–214. [PubMed: 8833330]
71. Han X, Yamanouchi G, Mori T, Kang JH, Niidome T, Katayama Y. Monitoring protein kinase activity in cell lysates using a high-density peptide microarray. *J Biomol Screen.* 2009; 14(3):256–262. [PubMed: 19211777]
72. Shigaki S, Yamaji T, Han X, Yamanouchi G, Sonoda T, Okitsu O, Mori T, Niidome T, Katayama Y. A peptide microarray for the detection of protein kinase activity in cell lysate. *Anal Sci.* 2007; 23(3):271–275. [PubMed: 17372367]
73. Wu D, Mand MR, Veach DR, Parker LL, Clarkson B, Kron SJ. A solid-phase Bcr-Abl kinase assay in 96-well hydrogel plates. *Anal Biochem.* 2008; 375(1):18–26. [PubMed: 18194660]
74. MacBeath G, Schreiber SL. Printing proteins as microarrays for high-throughput function determination. *Science.* 2000; 289(5485):1760–1763. [PubMed: 10976071]
75. Harrison SC. Variation on an Src-like theme. *Cell.* 2003; 112(6):737–740. [PubMed: 12654240]
76. Miller WT. Determinants of substrate recognition in nonreceptor tyrosine kinases. *Acc Chem Res.* 2003; 36(6):393–400. [PubMed: 12809525]
77. Pellicena P, Stowell KR, Miller WT. Enhanced phosphorylation of Src family kinase substrates containing SH2 domain binding sites. *J Biol Chem.* 1998; 273(25):15325–15328. [PubMed: 9624111]
78. Scott MP, Miller WT. A peptide model system for processive phosphorylation by Src family kinases. *Biochemistry.* 2000; 39(47):14531–14537. [PubMed: 11087407]
79. Pisabarro MT, Serrano L. Rational design of specific high-affinity peptide ligands for the Abl-SH3 domain. *Biochemistry.* 1996; 35(33):10634–10640. [PubMed: 8718852]
80. Dawson PE, Kent SB. Synthesis of native proteins by chemical ligation. *Annu Rev Biochem.* 2000; 69:923–960. [PubMed: 10966479]
81. Johnson EC, Durek T, Kent SB. Total chemical synthesis, folding, and assay of a small protein on a water-compatible solid support. *Angew Chem Int Ed Engl.* 2006; 45(20):3283–3287. [PubMed: 16596681]
82. Kent S. Novel forms of chemical protein diversity -- in nature and in the laboratory. *Curr Opin Biotechnol.* 2004; 15(6):607–614. [PubMed: 15560989]
83. Halling PJ, Ulijn RV, Flitsch SL. Understanding enzyme action on immobilised substrates. *Curr Opin Biotechnol.* 2005; 16(4):385–392. [PubMed: 16005203]
84. Nayak S, Yeo WS, Mrksich M. Determination of kinetic parameters for interfacial enzymatic reactions on self-assembled monolayers. *Langmuir.* 2007; 23(10):5578–5583. [PubMed: 17402753]
85. Bernstein DJ, Roman DL, Neubig RR. In vitro protein kinase activity measurement by flow cytometry. *Anal Biochem.* 2008; 383(2):180–185. [PubMed: 18796290]
86. Kinumi T, Niki E, Shigeri Y, Matsumoto H. Affinity-tagged phosphorylation assay by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (ATPA-MALDI): application to calcium/calmodulin-dependent protein kinase. *J Biochem.* 2005; 138(6):791–796. [PubMed: 16428308]
87. Belu AM, Graham DJ, Castner DG. Time-of-flight secondary ion mass spectrometry: techniques and applications for the characterization of biomaterial surfaces. *Biomaterials.* 2003; 24(21):3635–3653. [PubMed: 12818535]

88. Kim YP, Oh E, Oh YH, Moon DW, Lee TG, Kim HS. Protein kinase assay on peptide-conjugated gold nanoparticles by using secondary-ion mass spectrometric imaging. *Angew Chem Int Ed Engl.* 2007; 46(36):6816–6819. [PubMed: 17665386]
89. Parker LL, Brueggemeier SB, Rhee WJ, Wu D, Kent SB, Kron SJ, Palecek SP. Photocleavable peptide hydrogel arrays for MALDI-TOF analysis of kinase activity. *Analyst.* 2006; 131(10): 1097–1104. [PubMed: 17003856]
90. Parker LL, Kron SJ. Kinase activation in circulating cells: opportunities for biomarkers for diagnosis and therapeutic monitoring. *Expert Opin Med Diagn.* 2008; 2(1):33–46.
91. DeLano, WL. Delano Scientific; Palo Alto, CA, USA: 2002. <http://www.pymol.org>

A. Immobilization of peptide onto surface

B. Kinase reaction and detection

Figure 1. Strategy for immobilization and immuno-detection

A. Method for the immobilization of peptides on a two-dimensional hydrogel. B. Scheme for the detection of phosphorylated peptides in an ELISA-based format.

Figure 2. A branched hydrophilic linker increases substrate density and distance from the surface

A. The hydrogel surface was activated and coated with either bis-acrylamide or triacrylate (SR415). Equimolar mixtures of Abltide and Abl ligand were immobilized in duplicate wells at the indicated concentrations and phosphorylated by purified recombinant c-Abl kinase. Anti-phosphotyrosine antibody, followed by an HRP-conjugated secondary antibody, was used for chemiluminescent detection. B. The chemical structures of bis-acrylamide and SR415.

Figure 3. Molecular models predict the effect of peptide orientation and relative distance from the site of immobilization to the phosphorylated tyrosine

PyMOL⁹¹ was used to model the orientation of the phosphorylated tyrosine (indicated with a white arrow) relative to the site of immobilization (located at the bottom left hand corner of each figure). Carbon atoms are represented in green, nitrogen in blue, oxygen in red, and sulfur in yellow. A. Abltide with an amino-terminal cysteine (C-AT, CEAIYAAPFAKKK). B. Abltide with an amino-terminal cysteine and the inclusion of 4 amino acid spacers proximal to the site of immobilization (C-4X-AT, CSGGGKGEAIYAAPFAKKKG). C. Abltide with a carboxyl-terminal cysteine (AT-C, KGEAIYAAPFAKKKGC). D. Abltide with a carboxyl-terminal cysteine and 4 amino acids distal to the site of immobilization (4X-AT-C, SGGGKGEAIYAAPFAKKKGC).

Figure 4. Amino acid spacers increase substrate distance and accessibility at the surface
 Equimolar mixtures of a peptide substrate and the p40 SH3 ligand were immobilized on a hydrogel surface at 10 μ M concentrations. Substrate phosphorylation by purified c-Abl after various time points was detected by chemiluminescence using anti-phosphotyrosine antibody followed by HRP-conjugated secondary antibody.

Figure 5. Scheme for the multiplexed presentation of peptide substrates on Luminex beads Four peptides were immobilized on distinct Luminex beads (bead regions 27, 34, 53, 65) and distributed to each well of a 96-well plate. These four peptides were treated simultaneously with cell lysates and ATP. After termination of the kinase reaction, four peptide standards (bead regions 42, 45, 61, 73) were added to each well of a 96-well plate and labeled sequentially with the biotinylated anti-phosphotyrosine clone 4G-10 and streptavidin-conjugated phycoerythrin. Peptide standards were constructed from synthetic phospho-Abltide and Abltide, mixed at molar ratios corresponding to 0%, 15%, 30% and 45% phosphorylation.

Figure 6. Modular recognition domains increase peptide reporter sensitivity

A. A Boltzmann-sigmoidal curve was used to relate increases in fluorescence intensity to the percentage of phosphorylation on standard beads. These well-specific internal standard curves were used to calculate the percentage of substrate phosphorylation by cell lysates. B. Four peptide substrates were added to each well in one half of a 96-well plate and reacted with K-562 cell lysates for up to 60 minutes. Peptides containing the p40 peptide ligand of the Abl kinase SH3 domain (C-ALAT and C-ATAL) were preferentially phosphorylated and reached the limits of quantitative detection after only 10 minutes. Abltide immobilized by the amino- (C-AT) or carboxyl-terminus (AT-C) was not phosphorylated to the same extent that it was when presented alone. Boltzmann-sigmoidal curves provided the best fit for the data, emphasizing a dose-response relationship. Points and bars represent the mean fluorescence intensity and 99% confidence interval for a minimum of 100 beads per peptide per well.

Figure 7. Label-free detection of phosphorylated peptides cleaved from a hydrogel surface by UV light

A. Representative spectra of peptides after 30-minute and 60-minute kinase reactions. Peptide substrates constructed from Abltide with a photocleavable linker and p40, the Abl SH3 ligand, were immobilized on a hydrogel surface and released after phosphorylation by K-562 cell lysates. Peak A represents un-phosphorylated Abltide, peak B represents phosphorylated Abltide, and peak C represents “heavy” Abltide used as an internal phosphorylated control (EAlpYAAGPFA). B. The scheme for using MALDI-TOF to detect multiplexed kinase activities. Unique peptide substrates can be immobilized on a hydrogel surface and specifically phosphorylated by kinases in cell lysates. Peptides can be released by the reaction of UV light with the photocleavable linker and detected by mass spectrometry.

Table 1

A selection of peptide substrates that have been used in kinase assays.

Kinase	Peptide substrate	Small-molecule inhibitor
Akt	RPRAATF ²⁰	SR13668
Btk	KKVVALYDYMPMN ^{21,22}	LFM-A13
c-Abl	EAIYAAPFAKKK ¹⁹	Imatinib
CDK1	HATPPKKRK ^{23,24}	BMS-387032
Erk-1	KQAEAVTSPR ²⁵	U0126
GSK-3	YRRAAVPPSPSLSRHSSPH ²⁶	AR-A014418
JAK2	PQDKEYYKVKE ²⁷	Erlotinib
Lck	AEEIYGVLFAKKK ²⁸	A-420983
PI3K	KVNWLAHNVSKDNRQ ²⁹	LY294002
PKA	LRRASLG ³⁰	H-89
PKC	AALIQASFRGHMARKK ³¹	Rottlerin
Src	AEEIYGEFEAKKKK ¹⁹	PP2

Table 2
A group of peptides based on the Abltide sequence, with additional spacers or altered orientation relative to the surface

Cysteine is underlined to represent the site of immobilization. Amino acids in italic are spacers.

Peptide substrate	Sequence
C-Abltide	<u>C</u> KGEAIYAAPFAKKKG
C-4X-Abltide	<u>C</u> <i>SGGG</i> KGEAIYAAPFAKKKG
C-6X-Abltide	<u>C</u> <i>GGSGGG</i> KGEAIYAAPFAKKKG
C-8X-Abltide	<u>C</u> <i>GGGGSGGG</i> KGEAIYAAPFAKKKG
Abltide-C	KGEAIYAAPFAKKKG <u>C</u>
4X-Abltide-C	<i>SGGG</i> KGEAIYAAPFAKKKG <u>C</u>