HHS Public Access

Author manuscript

Cell Rep Phys Sci. Author manuscript; available in PMC 2024 January 11.

Published in final edited form as:

Cell Rep Phys Sci. 2023 October 18; 4(10): . doi:10.1016/j.xcrp.2023.101599.

LIMS-Kinase provides sensitive and generalizable label-free *in vitro* measurement of kinase activity using mass spectrometry

Cynthia Meyer[1,2], **Melissa McCoy**[1], **Lianbo Li**[1,2], **Bruce Posner**[1,*], **Kenneth D. Westover**[1,2,3,4,*]

[1]Department of Biochemistry, The University of Texas Southwestern Medical Center at Dallas, Dallas, TX 75390, USA

[2]Department of Radiation Oncology, The University of Texas Southwestern Medical Center at Dallas, Dallas, TX 75390, USA

[3]X (formerly Twitter): @KENWESTOVER

[4]Lead contact

SUMMARY

Measurements of kinase activity are important for kinase-directed drug development, analysis of inhibitor structure and function, and understanding mechanisms of drug resistance. Sensitive, accurate, and miniaturized assay methods are crucial for these investigations. Here, we describe a label-free, high-throughput mass spectrometry-based assay for studying individual kinase enzymology and drug discovery in a purified system, with a focus on validated drug targets as benchmarks. We demonstrate that this approach can be adapted to many known kinase substrates and highlight the benefits of using mass spectrometry to measure kinase activity *in vitro*, including increased sensitivity. We speculate that this approach to measuring kinase activity will be generally applicable across most of the kinome, enabling research on understudied kinases and kinase drug discovery.

Graphical abstract

*Correspondence: bruce.posner@utsouthwestern.edu (B.P.), kenneth.westover@utsouthwestern.edu (K.D.W.).

AUTHOR CONTRIBUTIONS

Conceptualization, K.D.W.; methodology, K.D.W.; validation, formal analysis, and investigation, K.D.W., B.P., M.M., C.M., and L.L.; resources, K.D.W. and B.P.; writing – original draft, K.D.W. and C.M.; writing – review & editing, M.M., B.P., and L.L.; supervision, K.D.W. and B.P.; funding acquisition, K.D.W. and B.P.

DECLARATION OF INTERESTS

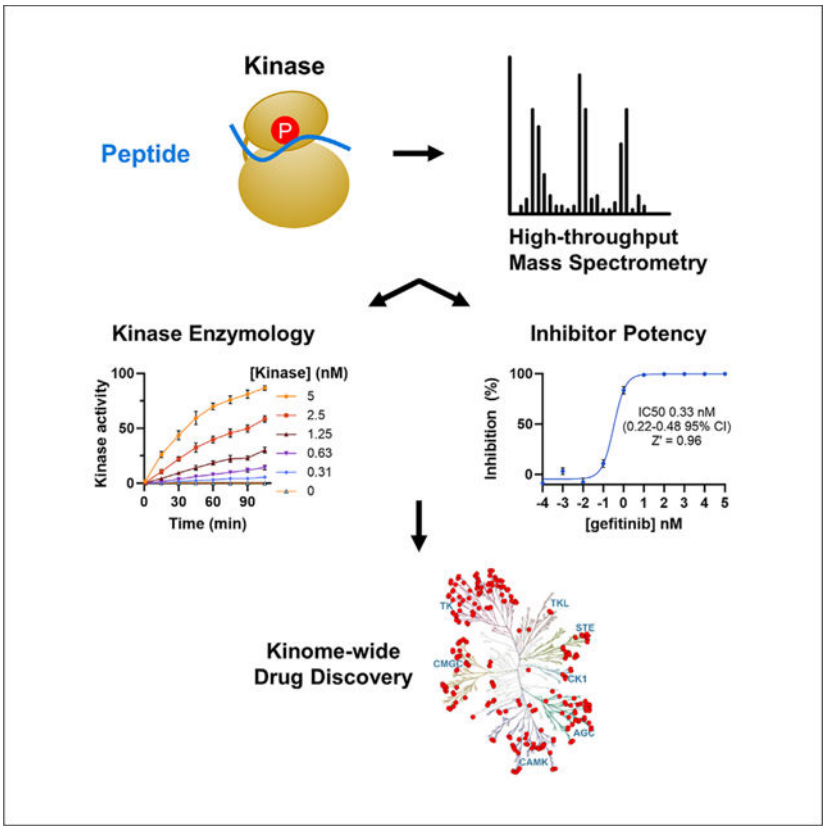
K.D.W. is an equity holder and advisor to Vibliome Therapeutics and advisor to Reactive Biosciences.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xcrp.2023.101599.

DECLARATION OF GENERATIVE AI AND AI-ASSISTED TECHNOLOGIES IN THE WRITING PROCESS

During the preparation of this work the author(s) used ChatGPT 3.5 in order to copyedit for grammar and punctuation. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the publication.



In vitro assays are commonly used to support early stages of targeted inhibitor discovery, especially in the context of kinases. Meyer et al. report a label-free mass spectrometry-based kinase assay that is generalizable to numerous high-value drug targets. This system gauges the efficacy of potent inhibitors with high sensitivity.

INTRODUCTION

Targeted therapies have greatly improved the medical outcomes of many debilitating or life-threatening conditions including inflammatory diseases and cancer.[1–3] Much of this success can be attributed to kinase-directed therapies, which target the central role of kinases as the regulators of signaling pathways that are often dysregulated in disease.[4] As of now, over 100 kinase inhibitors have been approved for the treatment of various diseases worldwide.[5] However, there are additional opportunities to address unmet medical needs through the development of inhibitors against new kinase targets and by improving drug responses for established targets, especially in cases of acquired on-target drug resistance.[6,7] Efficient, robust, and accurate tools are necessary to drive kinase inhibitor discovery.

Kinase drug discovery programs often rely on biochemical assays of individual kinase proteins for initial hit identification and structure-activity relationship analyses. These methods are suitable for rapid, high-throughput workflows, can offer better assay windows, and are less expensive than cell-based approaches.[8–10] These assays typically measure either compound binding or their ability to impair biological functions, particularly enzymatic

kinase activity. Enzymatic assays are useful for processive enzymes as they allow for signal amplification, often making them more sensitive and therefore able to discriminate between compounds in higher affinity ranges than is possible with binding assays.

Enzymatic kinase assays most often detect the transfer of the gamma phosphate of ATP to a serine, threonine, or tyrosine found on a protein or smaller peptide substrate (Figure 1A). A large number of active peptide substrate-kinase pairs have been reported.[11–13] One common, early method for measuring kinase activity used ^{32}P-labeled adenosine triphosphate to detect substrate phosphorylation, but this method is not easily adapted to high-throughput workflows and generates hazardous radioactive waste. Newer methods that are compatible with high-throughput regimes include ATP depletion methods, which utilize coupled assays to indirectly monitor ATP levels,[12,14] and methods that focus on monitoring changes in the properties of a peptide substrate. Examples include IMAP, Z-lyte, electrophoretic mobility, and CSOX peptides.[15–19] These methods have been useful for many studies, but they have limitations such as the use of proprietary components, which limits further innovation by the broader scientific community, and the use of labeled components, which can constrain assay conditions (specifically decrease the affinity of peptides for kinases) and introduce other potential artifacts.[20,21]

Mass spectrometry (MS) has been proposed as a general method for enzyme activity measurements.[22–24] This is most often applied to monitoring levels of small-molecule substrates or products, but it has also been used to monitor peptide phosphorylation.[25–29] We hypothesized that the activity of many individual kinases could be measured in purified assay systems by high-throughput MS. Further we hypothesized that assay creation could be accelerated by adapting reagents, especially peptide substrates, from other known purified kinase enzyme assay systems. Therefore, our goal was to build on prior work to refine this concept into an easy to follow, widely accessible format, facilitating its adoption into current approaches and standards of kinase inhibitor discovery. This concept has advantages over other assay methods because it directly measures the product of the kinase reaction, thus eliminating potential sources of experimental artifact, while also simplifying workflows and reducing cost. Importantly, the assay readily scales to 384-well microplates, which enables high-throughput screening as well as inhibitor lead optimization efforts.

Here, we report the development of mass spectroscopy-based kinase assays against a panel of targetable cancer-associated kinases: ABL, ALK, EGFR, MET, RET, ROS1, and SRC. We call this method label-free *in vitro* MS-based kinase assays (LIMS-Kinase). We benchmark these assays against known inhibitors and speculate on the feasibility of adapting other established substrates to this assay platform. We estimate that approximately half of the enzymatically active kinome can be immediately assayed with this method based on validated substrates, and we anticipate that a good portion of the remainder of the enzymatically active kinome will be targetable through substrate discovery.

RESULTS

Adaptation of peptide substrate designs for MS enzymology

Currently, many *in vitro* high-throughput enzymatic kinase assay platforms use labeled peptides as substrates for reactions to enable signal detection. These labels include fluorophores, affinity tags such as biotin, and additional charged residues that regulate electrophoretic mobility in capillary electrophoresis (CE) systems. CE-based mobility shift assays, with associated substrates, have been developed for at least 200 kinases. Examples of established biochemical assay substrates for ABL, ALK, EGFR, MET, RET, ROS1, and SRC are shown in Figure 1B. These substrates contain non-physiologic, charged residues to optimize peptide migration during electrophoresis and a fluorescein (FAM) tag to facilitate assay readouts. We hypothesized that MS-based enzymological methods would not have the same charge or size limitations as CE methods. To determine whether known CE substrates could easily be adapted into simplified substrates for MS purposes, we removed both ancillary charged residues and FAM (see Figure 1B). These modified sequences are designated with the prefix ‘‘MS’’ to distinguish them from the validated peptide sequences from which they were derived.

To detect peptide phosphorylation, we employed a RapidFire liquid chromatography sampler (Agilent) connected to a triple quad mass analyzer (AB Sciex’s 6500) to perform multiple reaction monitoring (MRM).[30] In this setup, quadrupole 1 (Q1) selects ions based on their mass–to-charge ratio and acts as a mass filter. The second quadrupole (Q2) is a collision cell to fragment the ion into a product ion, and the third quadrupole (Q3) acts as a final mass filter to monitor specific daughter ion fragments of the parent ion selected in Q1 (Figures 1C and S1). In LIMS-Kinase assays, product ions are measured quantitatively to follow enzyme activity. We found that all peptides we tested were detectable over a range of concentrations in a linear fashion (Figures 2A–2F and Table 1), suggesting that they were all readily ionized under the conditions of our assay and spectrometer setup.

As an initial screen of kinase activity and to find ions that could be followed in kinase activity assays, we subjected peptides to phosphorylation by enzymes that were previously reported to have activity on the original CE substrates. We used conditions that would be predicted to give a high degree of peptide phosphorylation. This typically consisted of enzyme in the low nanomolar concentration range, peptides at ~1 μM, and ATP near its typical K_m for kinases (~100 μM). We found that phosphorylation of most peptides was detectable and went to completion in many cases (Figure 2G). We also noted that some peptides, such as MS-Srctide and MS-Csktide1, showed high activity with multiple different kinases.

Assay optimization

Enzyme concentration is important for small-molecule inhibitor studies because it determines the lower limit of assay sensitivity for inhibitor potency.[31,32] To optimize reactions for high-throughput endpoint assays that can evaluate kinase inhibitors, we varied key parameters of the reaction. Our goal was to find conditions that produced linear enzymatic activity within an experimentally manageable time window while also

maintaining a wide assay window for each of the kinase targets under investigation. As expected, all kinases showed a dose-dependent increase in substrate phosphorylation with increasing enzyme (Figure 3). We used conditions that produced a linear assay progression curve and large assay window. We then proceeded to use optimized assay conditions to benchmark these assays using known inhibitors. Using zero enzyme as a negative control, Z'[33] for most assays was 0.6–0.9.

MS-Srctide is a substrate for SRC

Src is a well-known oncogene that is involved in various aspects of tumor development such as cell proliferation, adhesion, migration, and metastases. It is often overexpressed or mutated in cancers, making it a potential target for drug development. Several Src inhibitors have been developed, although no Src-directed clinical therapies have been approved for clinical use.[34] We developed an LIMS-Kinase assay for Src, using a derivative of Srctide. Srctide is a peptide of unknown biological origin that has been used as a substrate for a number of kinases, including ALK, BMX, BTK, EGFR, FER, FGFR, FRK, HER4, JAK2, JAK3, LTK, KDR, RET, ROS, SRC, SYK, TEC, and YES.[35–41] In our assay, we observed phosphorylation activity by ALK, EGFR L858R, MET, RET, ROS1, and Src (Figure 2G). For Src, a concentration of 1.25 nM enzyme achieved ~44% conversion of 1 μM initial peptide substrate to phosphorylated product after 60 min (Figure 3A). Using 1 nM Src and 100 μM ATP, we determined the K_m of the peptide to be 120 μM (Figure S2).

To validate the MS-Srctide assay for inhibitor testing, we measured the inhibitory activity of bosutinib as a reference compound. Bosutinib is an FDA-approved Src/multikinase inhibitor used to treat Ph+ chronic myelogenous leukemia (CML) and acute lymphoblastic leukemia. While Src is not a primary driver of tumor growth, Src inhibitors play an important auxiliary role in cancer treatment.[42] We evaluated the IC_{50} of bosutinib in a dose-response assay with Src at a concentration of 1 nM. The IC_{50} of bosutinib for Src has been previously measured in an ELISA format at 1.2 nM.[43] Our assay obtained a similar result, with an IC_{50} of 1.1 nM and a Z' of 0.9 (Figure 3B).

MS-Srctide is a substrate for MET

In normal physiology, the receptor tyrosine kinase (RTK) MET mediates embryogenesis and wound healing. However, it is dysregulated in numerous cancers through mutations, chromosomal rearrangement, amplification, and overexpression. Activated MET stimulates signaling pathways that promote tumor development and progression.[44,45] MET is therefore an attractive target for small-molecule inhibitors. MET, like SRC, showed strong activity with MS-Srctide (Figure 2G). Roughly 40% of MS-Srctide was phosphorylated at 1 h for 2.5 nM MET with 1 μM peptide (Figure 3C). The peptide K_m value for similar conditions (2 nM MET with 100 μM ATP) was 110 μM (Figure S2).

Multiple inhibitors with anti-MET activity have been approved for clinical use including cabozantinib, capmatinib, crizotinib, and tepotinib.[46,47] To demonstrate the potential of the LIMS-Kinase assay for MET-targeted drug discovery, we evaluated the potency of capmatinib using the LIMS-Kinase assay. Capmatinib is a selective MET inhibitor that was approved for MET exon 14 skipping mutations found in non-small-cell lung cancer

(NSCLC).[47,48] The potency of capmatinib has been measured by homogeneous time-resolved fluorescence (HTRF), with an IC_{50} value of 0.13 nM using a different peptide and a higher concentration of ATP.[49,50] In our hands using MET at 2 nM, we measured an IC_{50} of 0.95 nM with a Z′ of 0.9 (Figure 3D).

MS-Srctide is a substrate for ROS1

ROS1 encodes an orphan RTK that functions in embryological development and epithelial cell differentiation.[51,52] While ROS1 is expressed in a number of tissues, it is absent in normal lung tissue.[53] A variety of ROS1 fusions have been identified in both adults and children, across a diverse group of cancers,[54] and are an established drug target for lung cancer. ROS1 downstream signaling activates several pathways that influence cell proliferation, survival, and migration.[55] MS-Srctide exhibited very strong activity with ROS1, reaching 100% conversion in our initial assay. (Figure 2G). Further evaluation demonstrated that 1 μM MS-Srctide could be converted at a rate of ~30% with reaction concentration of enzyme at a sub-nanomolar level (0.63 nM) (Figure 3E). Under these conditions, the peptide K_m value was 33.7 μM (Figure S2).

Multikinase inhibitors that target ROS1 in NSCLC are approved, including crizotinib, brigatinib, cabozantinib, ceritinib, and entrectinib. However, no ROS1 selective inhibitors are currently available.[54–56] Crizotinib (PF-2341066) was previously reported to have an IC_{50} of 1.7 nM ina ROS1 binding assay.[57] We evaluated crizotinib inhibition of ROS1 using LIMS-Kinase at an enzyme concentration of 0.63 nM and measured an IC_{50} of 0.64 nM with a Z′ of 0.9 (Figure 3F).

MS-Axltide is a substrate for ALK

Anaplastic lymphoma kinase (*ALK*) is an RTK that can become activated through both translocation and mutation, leading to the development of cancers such as NSCLC, neuroblastoma, and lymphomas.[58] ALK-targeted drugs are commonly used for many indications and are being investigated for additional uses in the clinical setting.[59–61] We developed a substrate for ALK, MS-Axltide, based on the known substrate Axltide, which is derived from the mouse insulin receptor 1 (*IRS1*) gene. MS-Axltide has been used to measure the activities of ALK, DDR1, DDR2, FGFR1, INSR, IGF1R, IR, LOK, MST1, MST2, and Src.[41,62–66] In our initial assay, we saw strong phosphorylation of MS-Axltide by ALK (65% conversion) (Figure 2G). Further characterization using 5 nM enzyme with 1 μM peptide resulted in approximately 30% peptide phosphorylation at 60 min (Figure 3G). The peptide's K_m was measured at 70.6 μM under these conditions (Figure S2).

Multiple ALK inhibitors including crizotinib, lorlatinib, alectinib, brigatinib, and ceritinib have been approved for clinical use.[67] These drugs produce high response rates, but development of therapeutic resistance is common.[68] We used our LIMS-Kinase assay to measure the potency of ceritinib. Prior measurements of potency observed an IC_{50} of 0.2 nM using a mobility shift assay.[69] Our LIMS-Kinase assay measured the potency of ceritinib against 5 nM ALK at 0.15 nM with a Z′ of 0.8 (Figure 3H).

MS-Abltide is a substrate for ABL1

ABL kinase regulates cell proliferation and survival and is dysregulated in CML. In CML, the fusion product *BCR-ABL* constitutively activates ABL1 kinase.[70] Overexpression of ABL1 has also been found in solid tumors.[71] ABL inhibitors have been shown to alter the natural course of CML.[72] Abltide is a peptide of unknown biological origin that is a reported substrate for ABL, ARG, BTK, FGR, FLT3, KIT, MET, RET, and SRC.[41,73–75] We established an ABL1 activity assay using MS-Abltide, a modification of Abltide. LIMS-Kinase evaluation of ABL1, MET, and RET M918T with MS-Abltide yielded measurable conversion for ABL1 only (Figure 2G). Further study at a concentration of 1.25 nM ABL1 showed linear enzymatic activity over 60 min, reaching ~31% (Figure 3I). Under these conditions, MS-Abltide had a K_m value of 31.0 μM (Figure S2).

In 2001, imatinib became the first tyrosine kinase inhibitor approved by the FDA, targeting ABL for the treatment of CML. Dasatinib, a second-generation Bcr-Abl inhibitor, was developed to address on-target resistance. Dasatinib binds to both DFG-in and DFG-out conformations of Bcr-Abl, and this flexibility is believed to contribute to its effectiveness, even in the presence of conformation-altering mutations.[72] Previous studies have shown that dasatinib has an IC_{50} of 0.6 nM when measured using a radiometric ([$\gamma-^{32}$P] ATP) kinase assay.[76] In the LIMS-Kinase assay using an ALK concentration of 1.25 nM, we measured a notably lower IC_{50} of 36 pM with a Z′ of 0.6 (Figure 3J).

MS-Csktide1 is a substrate for EGFR L858R

EGFR (epidermal growth factor receptor, also known as HER1 or ErbB1) is a transmembrane glycoprotein that is overexpressed in many human tumors but normally regulates cell growth and development. EGFR is also involved in regulating cell proliferation, apoptosis, angiogenesis, and metastasis. EGFR L858R is one of the most common activating mutations in NSCLC. Several generations of small-molecule EGFR inhibitors have been developed and approved for cancer therapy.[77,78] We derived the LIMS-Kinase peptide for EGFR from Csktide, a peptide whose biological origin may be the human semaphorin 4F gene and which is reported as an effective substrate for AXL, CSK, FLT1, FLT4, FMS, KIT, MET, MSTR, PDGFR, and TRKA.[41,79,80] MS-Csktide1 showed strong activity with EGFR L858R and near complete conversion with MET, RET M918T, and ROS1 (Figure 2G). Enzyme optimization showed a concentration of 2.5 nM EGFR L858R with 1 μM MS-Csktide1 converting approximately 20% of the substrate within 60 min (Figure 3K). A full K_m study was not possible due to peptide insolubility at high concentrations.

Gefitinib, erlotinib, lapatinib, mobocertinib, vandetanib, and osimertinib are all approved EGFR-targeted agents. Many of these drugs were developed to address resistance mutations to early generation inhibitors.[81] We benchmarked the EGFR LIMS-Kinase assay using gefitinib, which was previously reported to inhibit EGFR with an IC_{50} of <0.1 nM using an *in vitro* assay.[82] In our LIMS-Kinase assay using 3.5 nM EGFR L858R, we measured an IC_{50} of 330 pM inhibition by gefitinib with a Z′ of 0.9 (Figure 3L).

MS-Csktide2 is a substrate for RET M918T

RET (rearranged during transfection) plays a crucial role in the development of the kidneys and enteric nervous system during embryogenesis, as well as in maintaining homeostasis in various tissues.[83] In cancer, RET point mutations (such as M918T)[84] are associated with multiple endocrine neoplasia and medullary thyroid carcinomas, while RET fusions are common in papillary thyroid carcinomas and NSCLC. Several multikinase inhibitors targeting RET have been approved by the FDA for the treatment of cancer.[85] In our initial screen of LIMS-Kinase peptides, MS-Csktide1 and MS-Csktide2, which differ by only one amino acid, were both highly phosphorylated by RET (Figure 2G). We further characterized MS-Csktide2 as a substrate based on slightly higher activity. Approximately 39% of MS-Csktide2 was converted by 2.5 nM RET M918T after 60 min (Figure 3M). However, we were unable to conduct a full substrate K_m study due to the peptide's insolubility at high concentrations.

RET-targeted inhibitors include the multikinase inhibitors cabozantinib and vandetanib. In 2020, selpercatinib was approved by the FDA due to its higher potency, selectivity, and improved toxicity profile.[86] Next-generation RET inhibitors are also being developed to address on-target resistance to current drugs.[87,88] We benchmarked the RET LIMS-Kinase assay using selpercatinib, which has a reported IC_{50} of nM.[86] Utilizing RET at 2.5 nM in our assay system, selpercatinib gave an IC_{50} of 0.11 nM with a Z' of 0.9 (Figure 3N).

DISCUSSION

In this study, we demonstrate that multiple established *in vitro* enzymatic kinase assays can be adapted for detection by MRM MS. LIMS-kinase assays were either comparable or more sensitive in evaluating inhibitor potency when benchmarked against methods with other readouts. Based on these results, we anticipate that LIMS-Kinase assays may be possible with many other kinases for which peptide substrates are known.

The high sensitivity of the LIMS-Kinase platform may highlight limitations of prior assay methods, differences in assay conditions, or other factors. The potency of crizotinib (PF-2341066) was nearly three times higher in the LIMS-Kinase assay compared to a previously reported value (1.7 nM vs. 0.64 nM). This may relate to technical limits of the prior assay method, which used phage-tagged enzyme as the basis for a competition binding assay between a biotin-labeled ATP binding ligand immobilized on streptavidin beads and crizotinib.[57,89] Similarly, the potency of selpercatinib (LOXO-292) for RET M918T was more than six times higher in the LIMS-Kinase assay (0.7 nM vs. 0.11 nM). This difference is potentially explainable by the previous use of a cell-based assay, which can show lower potencies because of cell permeability or other effects. Dasatinib's potency on ABL1 measured by LIMS-Kinase was more than 15 times greater than prior (0.6 nM vs. 0.036 nM), a disparity that might be attributable to differences in enzyme concentrations, which are known to set the limit of assay sensitivity.[31,32] The originally reported radiometric assay utilized a higher concentration of ABL1.[76] For EGFR/gefitinib IC_{50}s, prior estimates placed the potency of gefitinib below 0.1 nM for wild-type (WT) EGFR.[82] We were able to measure an IC_{50} of 0.325 nM, but we cannot rule out that our measure may also have been

limited by assay sensitivity. Another possible explanation is that EGFR L858R is slightly less sensitive to gefitinib than WT EGFR.

We wish to emphasize that ours is not the first report of detecting kinase activity using MS. *In vitro* assays utilizing a single purified kinase and synthetic peptide followed by MS detection of peptide phosphorylation have been reported as an accurate, selective method to evaluate kinase activity and inhibitor potency.[29,90] Nevertheless, few additional studies using this methodology have been reported, despite the wide use of other *in vitro* kinase assay readouts for inhibitor development.[11,15–17,21] A major goal of our study is to support wider adoption of this assay readout, given that this method appears to be generally applicable to many members of the kinome and provides high-quality data.

We also note that analogous methods have been used to measure the activity of kinase-mediated signal transduction pathways in cell lysates.[25] While the potential value of these methods is apparent, we distinguish LIMS-Kinase assays from these methods both in their high complexity, which likely limits their utility for high-throughput inhibitor discovery, and because these methods currently do not unambiguously measure the activity of individual kinases.[26–28] A major reason may be that many of the established peptide substrates used in *in vitro* kinase assays are non-selective, as highlighted by our data (Figure 2G). In other words, several of the substrates we studied were active with multiple kinases. If general selectivity could be improved, our LIMS-Kinase method could also be applied to more complex mixtures, such as cellular extracts to track specific kinase activity *in situ*.[91,92] This could be useful for kinases that rely on unknown factors or complex mechanisms. Moreover, LIMS-Kinase readouts could enable optimization of such selective peptide substrates. Nevertheless, we estimate that much work will likely be required to generate truly selective substrates.

Despite current limitations of peptide selectivity, our data suggest that mass spectrometry-based peptide phosphorylation assays can be generalized to a wide range of peptide substrates. Although we surveyed six peptides derived from established assay methods that rely on radiometric readouts or peptide mobility shifts in microcapillary electrophoresis, many other substrates have been reported that are also likely amenable to this approach.[11,12,14,41] In our survey of the literature, we have identified established peptide substrates for ~250 of the nearly 540 known human kinases that cover all branches of the kinome (Figure 4).[41,93]

In summary, MRM assays of kinase activity have broad potential application, especially in drug discovery efforts, where modern hit-to-lead campaigns are often driven by rapid biochemical assays. This method is advantageous over other kinase assay methods because it directly measures substrate phosphorylation, avoiding potential artifacts seen in indirect methods. It also does not require specialized or labeled peptides that can also introduce artifacts and pose a barrier to novel assay development within the scientific community. Finally, it shows high sensitivity, in some cases beyond what could be measured by previously reported methods.

EXPERIMENTAL PROCEDURES

Resource availability

Lead contact—Further information and requests for reagents and resources should be directed to and will be fulfilled by the lead contact, Ken Westover (kenneth.westover@utsouthwestern.edu).

Materials availability—All unique/stable reagents generated in this study will be provided without restriction as long as stocks remain available and reasonable compensation is provided by the requestor to cover processing and shipment.

Data and code availability—This study did not generate any unique datasets or code.

Kinase assays

Enzymes were obtained from Carna Biosciences (ABL1, #08–001; ALK, #08–518; EGFR[L858R] #08–502; MAP2K1, #07–141; MET, #08–151; RET[M918T], #08–508; ROS1, #08–163), and substrate peptides were obtained from Biomatik Corporation (Wilmington, DE, USA). SRC was expressed and purified as previously reported.[94,95] All components were equilibrated to 25°C prior to setting up reactions in 384-well microplates (White Costar 3657, 384-well, round bottom plate). All reagents were added manually except for inhibitors; see below. Final working concentrations in enzyme optimization reactions were 1 μM substrate peptide and 100 μM ATP, with enzyme concentrations ranging between 0.08 and 10 nM, each in assay buffer (100 mM HEPES pH 7.5, 0.003% Brij-35, 0.004% Tween 20, 10 mM $MgCl_2$, 2 mM DTT). Buffer, peptide, and ATP were premixed in assay wells, and then the reaction was initiated by the addition of enzyme.

For peptide optimization, final working concentrations of enzyme varied, ATP was 100 μM, and peptide concentrations started at 200–400 μM followed by 2-fold dilutions. For peptide optimization, buffer, enzyme, and ATP were premixed in assay wells, and then the reaction was initiated by the addition of peptide. For IC_{50} determinations, ten concentrations of each inhibitor test compound were added to assay wells using an Echo 655 acoustic dispenser. Buffer and enzyme were added followed by a 1-h incubation at RT. Peptide (1–10 μM) and ATP (100 μM) in buffer were then added, and the plate was incubated an additional 1 h and quenched with 1% formic acid (final). Results were evaluated by MS. Experiments were repeated two or three times, with results expressed as mean ± standard deviation.

Calculation of K_m, V_{max}, IC_{50}, and Z′

Z′ for enzyme optimization was calculated from percent phosphorylation values at 60 min with no-enzyme values as negative control (baseline) and values for the enzyme concentration producing near 30% conversion as the positive control. K_m and V_{max} were evaluated by linear regression of the first three time points at each concentration of peptide. The slope was used to represent the initial velocity in the graph of initial velocity versus concentration of peptide. Nonlinear regression using GraphPad Prism's Michaelis Mention equation was used to find K_m and V_{max}. IC_{50} values were calculated from phosphorylated peptide signals. No-inhibitor wells were negative control. Percent inhibition

was calculated as (100*(negative control – sample)/negative control). GraphPad nonlinear regression analysis, log(inhibitor) vs. response – variable slope (four parameters), was used to find IC_{50} values. Z' values for inhibitor studies were calculated from phosphorylated fragment signal values, with no-inhibitor signal values as negative control (baseline) and highest inhibitor concentration signal values as positive control (plateau).

RapidFire chromatography

Analyses of reaction samples were performed using the RapidFire 300 high-throughput SPE system (Agilent Technologies, Wakefield, MA, USA) interfaced with a Sciex 6500 (Sciex, Framingham, MA, USA). For RapidFire sample preparation, the load/wash solvent (solvent A) was water containing 0.1% formic acid. The elution solvent (solvent B) was acetonitrile/water (8:2, v/v) containing 0.1% formic acid. Samples were aspirated from 384-well assay plates and loaded onto an SPE cartridge (cartridge type A, C4) to remove buffer salts, using solvent A at a flow rate of 1.5 mL/min for 3,000 ms. The retained and purified analytes were eluted to the mass spectrometer by washing the cartridge with solvent B at mL/min for 3,000 ms. The cartridge was re-equilibrated with solvent A for 500 ms at 1.5 mL/min.

Mass spectrometry

Peptides were measured using a selective reaction monitoring protocol. The area under the daughter ion peaks (area under the curve, AUC) was quantified using RapidFire integrator software. Mass-to-charge ratios of parental and daughter ions we used to follow reactions for each assay are provided in Table 1. Other experimental parameters such as collision energies are listed in Tables S1 and S2.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

American Cancer Society Award 132205-RSG-18–039-01-DMC (K.D.W.); Cancer Prevention and Research Institute of Texas RP170373 (K.D.W.); Welch Foundation Grant I1829 (K.D.W.); NIH CA244341 (K.D.W.); CI Cancer Center Support Grant 2P30CA142543–11 (B.P.); S10 Shared Instrumentation Grant 1S10OD026758–01 (B.P.).

REFERENCES

1. Ahmed A, Merrill SA, Alsawah F, Bockenstedt P, Campagnaro E, Devata S, Gitlin SD, Kaminski M, Cusick A, Phillips T, et al. (2019). Ruxolitinib in adult patients with secondary haemophagocytic lymphohistiocytosis: an open-label, single-centre, pilot trial. Lancet. Haematol. 6, e630–e637. 10.1016/S2352-3026(19)30156-5. [PubMed: 31537486]
2. Humby F, Durez P, Buch MH, Lewis MJ, Rizvi H, Rivellese F, Nerviani A, Giorli G, Mahto A, Montecucco C, et al. (2021). Rituximab versus tocilizumab in anti-TNF inadequate responder patients with rheumatoid arthritis (R4RA): 16-week outcomes of a stratified, biopsy-driven, multicentre, open-label, phase 4 randomised controlled trial. Lancet 397, 305–317. 10.1016/S0140-6736(20)32341-2. [PubMed: 33485455]
3. Mok TS, Wu YL, Thongprasert S, Yang CH, Chu DT, Saijo N, Sunpaweravong P, Han B, Margono B, Ichinose Y, et al. (2009). Gefitinib or carboplatin-paclitaxel in pulmonary adenocarcinoma. N. Engl. J. Med. 361, 947–957. 10.1056/NEJMoa0810699. [PubMed: 19692680]

4. Manning G, Whyte DB, Martinez R, Hunter T, and Sudarsanam S. (2002). The protein kinase complement of the human genome. Science 298, 1912–1934. 10.1126/science.1075762. [PubMed: 12471243]
5. Cohen P, Cross D, and Jänne PA. (2021). Kinase drug discovery 20 years after imatinib: progress and future directions. Nat. Rev. Drug Discov. 20, 551–569. 10.1038/s41573-021-00195-4. [PubMed: 34002056]
6. Kurz CG, Preuss F, Tjaden A, Cusack M, Amrhein JA, Chatterjee D, Mathea S, Berger LM, Berger BT, Krämer A, et al. (2022). Illuminating the Dark: Highly Selective Inhibition of Serine/Threonine Kinase 17A with Pyrazolo[1,5-a]pyrimidine-Based Macrocycles. J. Med. Chem. 65, 7799–7817. 10.1021/acs.jmedchem.2c00173. [PubMed: 35608370]
7. Robichaux JP, Le X, Vijayan RSK, Hicks JK, Heeke S, Elamin YY, Lin HY, Udagawa H, Skoulidis F, Tran H, et al. (2021). Structure-based classification predicts drug response in EGFR-mutant NSCLC. Nature 597, 732–737. 10.1038/s41586-021-03898-1. [PubMed: 34526717]
8. von Ahsen O, and Bömer U. (2005). High-throughput screening for kinase inhibitors. Chembiochem 6, 481–490. 10.1002/cbic.200400211. [PubMed: 15742384]
9. Goldstein DM, Gray NS, and Zarrinkar PP. (2008). High-throughput kinase profiling as a platform for drug discovery. Nat. Rev. Drug Discov. 7, 391–397. 10.1038/nrd2541. [PubMed: 18404149]
10. Attwood MM, Fabbro D, Sokolov AV, Knapp S, and Schiöth HB. (2021). Trends in kinase drug discovery: targets, indications and inhibitor design. Nat. Rev. Drug Discov. 20, 839–861. 10.1038/s41573-021-00252-y. [PubMed: 34354255]
11. Card A, Caldwell C, Min H, Lokchander B, Hualin X, Sciabola S, Kamath AV, Clugston SL, Tschantz WR, Leyu W, and Moshinsky DJ. (2009). High-throughput biochemical kinase selectivity assays: panel development and screening applications. J. Biomol. Screen 14, 31–42. 10.1177/1087057108326663. [PubMed: 19073965]
12. Charter NW, Kauffman L, Singh R, and Eglen RM. (2006). A generic, homogenous method for measuring kinase and inhibitor activity via adenosine 5'-diphosphate accumulation. J. Biomol. Screen 11, 390–399. 10.1177/1087057106286829. [PubMed: 16751335]
13. Wu D, Sylvester JE, Parker LL, Zhou G, and Kron SJ. (2010). Peptide reporters of kinase activity in whole cell lysates. Biopolymers 94, 475–486. 10.1002/bip.21401. [PubMed: 20593469]
14. Tanega C, Shen M, Mott BT, Thomas CJ, MacArthur R, Inglese J, and Auld DS. (2009). Comparison of bioluminescent kinase assays using substrate depletion and product formation. Assay Drug Dev. Technol. 7, 606–614. 10.1089/adt.2009.0230. [PubMed: 20059377]
15. Sportsman JR, Gaudet EA, and Boge A. (2004). Immobilized metal ion affinity-based fluorescence polarization (IMAP): advances in kinase screening. Assay Drug Dev. Technol. 2, 205–214. 10.1089/154065804323056549. [PubMed: 15165516]
16. Rodems SM, Hamman BD, Lin C, Zhao J, Shah S, Heidary D, Makings L, Stack JH, and Pollok BA. (2002). A FRET-based assay platform for ultra-high density drug screening of protein kinases and phosphatases. Assay Drug Dev. Technol. 1, 9–19. 10.1089/154065802761001266. [PubMed: 15090152]
17. Shults MD, Carrico-Moniz D, and Imperiali B. (2006). Optimal Sox-based fluorescent chemosensor design for serine/threonine protein kinases. Anal. Biochem. 352, 198–207. 10.1016/j.ab.2006.03.003. [PubMed: 16600168]
18. González-Vera JA, and Morris MC. (2015). Fluorescent Reporters and Biosensors for Probing the Dynamic Behavior of Protein Kinases. Proteomes 3, 369–410. 10.3390/proteomes3040369. [PubMed: 28248276]
19. Shults MD, Janes KA, Lauffenburger DA, and Imperiali B. (2005). A multiplexed homogeneous fluorescence-based assay for protein kinase activity in cell lysates. Nat. Methods 2, 277–283. 10.1038/nmeth747. [PubMed: 15782220]
20. Hong L, Quinn CM, and Jia Y. (2009). Evaluating the utility of the HTRF Transcreener ADP assay technology: a comparison with the standard HTRF assay technology. Anal. Biochem. 391, 31–38. 10.1016/j.ab.2009.04.033. [PubMed: 19406097]
21. Zegzouti H, Zdanovskaia M, Hsiao K, and Goueli SA. (2009). ADP-Glo: A Bioluminescent and homogeneous ADP monitoring assay for kinases. Assay Drug Dev. Technol. 7, 560–572. 10.1089/adt.2009.0222. [PubMed: 20105026]

22. Liesener A, and Karst U. (2005). Monitoring enzymatic conversions by mass spectrometry: a critical review. Anal. Bioanal. Chem. 382, 1451–1464. 10.1007/s00216-005-3305-2. [PubMed: 16007447]
23. Lee ED, Mueck W, Henion JD, and Covey TR. (1989). Real-Time Reaction Monitoring by Continuous-Introduction Ion-Spray Tandem Mass-Spectrometry. J. Am. Chem. Soc. 111, 4600–4604. 10.1021/ja00195a012.
24. Bothner B, Chavez R, Wei J, Strupp C, Phung Q, Schneemann A, and Siuzdak G. (2000). Monitoring enzyme catalysis with mass spectrometry. J. Biol. Chem. 275, 13455–13459. 10.1074/jbc.275.18.13455.
25. Cutillas PR, Khwaja A, Graupera M, Pearce W, Gharbi S, Waterfield M, and Vanhaesebroeck B. (2006). Ultrasensitive and absolute quantification of the phosphoinositide 3-kinase/Akt signal transduction pathway by mass spectrometry. Proc. Natl. Acad. Sci. USA 103, 8959–8964. 10.1073/pnas.0602101103. [PubMed: 16751276]
26. Kubota K, Anjum R, Yu Y, Kunz RC, Andersen JN, Kraus M, Keilhack H, Nagashima K, Krauss S, Paweletz C, et al. (2009). Sensitive multiplexed analysis of kinase activities and activity-based kinase identification. Nat. Biotechnol. 27, 933–940. 10.1038/nbt.1566. [PubMed: 19801977]
27. Yi L, Shi T, Gritsenko MA, X'Avia Chan CY, Fillmore TL, Hess BM, Swensen AC, Liu T, Smith RD, Wiley HS, and Qian WJ. (2018). Targeted Quantification of Phosphorylation Dynamics in the Context of EGFR-MAPK Pathway. Anal. Chem. 90, 5256–5263. 10.1021/acs.analchem.8b00071. [PubMed: 29584399]
28. Reyes AJF, Kitata RB, Dela Rosa MAC, Wang YT, Lin PY, Yang PC, Friedler A, Yitzchaik S, and Chen YJ. (2021). Integrating site-specific peptide reporters and targeted mass spectrometry enables rapid substrate-specific kinase assay at the nanogram cell level. Anal. Chim. Acta 1155, 338341. 10.1016/j.aca.2021.338341.
29. Leveridge M, Collier L, Edge C, Hardwicke P, Leavens B, Ratcliffe S, Rees M, Stasi LP, Nadin A, and Reith AD. (2016). A High-Throughput Screen to Identify LRRK2 Kinase Inhibitors for the Treatment of Parkinson's Disease Using RapidFire Mass Spectrometry. J. Biomol. Screen 21, 145–155. 10.1177/1087057115606707. [PubMed: 26403521]
30. Gillette MA, and Carr SA. (2013). Quantitative analysis of peptides and proteins in biomedicine by targeted mass spectrometry. Nat. Methods 10, 28–34. 10.1038/nmeth.2309. [PubMed: 23269374]
31. Fassy F, Dureuil C, Lamberton A, Mathieu M, Michot N, Ronan B, and Pasquier B. (2017). Chapter Twenty-Six - In Vitro Characterization of VPS34 Lipid Kinase Inhibition by Small Molecules. In Methods in Enzymology, Galluzzi L, Bravo-San Pedro JM, and Kroemer G, eds. (Academic Press), pp. 447–464. 10.1016/bs.mie.2016.09.070.
32. Tonge PJ. (2019). Quantifying the Interactions between Biomolecules: Guidelines for Assay Design and Data Analysis. ACS Infect. Dis. 5, 796–808. 10.1021/acsinfecdis.9b00012. [PubMed: 30860805]
33. Zhang JH, Chung TD, and Oldenburg KR. (1999). A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays. J. Biomol. Screen 4, 67–73. 10.1177/108705719900400206. [PubMed: 10838414]
34. Zhang S, and Yu D. (2012). Targeting Src family kinases in anti-cancer therapies: turning promise into triumph. Trends Pharmacol. Sci. 33, 122–128. 10.1016/j.tips.2011.11.002. [PubMed: 22153719]
35. Ramadan M, A M M Elshaier Y, Aly AA, Abdel-Aziz M, Fathy HM, Brown AB, Pridgen JR, Dalby KN, and Kaoud TS. (2021). Development of 2'-aminospiro [pyrano [3,2-c]quinoline]-3'-carbonitrile derivatives as non-ATP competitive Src kinase inhibitors that suppress breast cancer cell migration and proliferation. Bioorg. Chem. 116, 105344. 10.1016/j.bioorg.2021.105344.
36. Gillooly KM, Pulicicchio C, Pattoli MA, Cheng L, Skala S, Heimrich EM, McIntyre KW, Taylor TL, Kukral DW, Dudhgaonkar S, et al. (2017). Bruton's tyrosine kinase inhibitor BMS-986142 in experimental models of rheumatoid arthritis enhances efficacy of agents representing clinical standard-of-care. PLoS One 12, e0181782. 10.1371/journal.pone.0181782.
37. Vasbinder MM, Alimzhanov M, Augustin M, Bebernitz G, Bell K, Chuaqui C, Deegan T, Ferguson AD, Goodwin K, Huszar D, et al. (2016). Identification of azabenzimidazoles as potent JAK1 selective inhibitors. Bioorg. Med. Chem. Lett. 26, 60–67. 10.1016/j.bmcl.2015.11.031. [PubMed: 26614408]

38. Katayama R, Shaw AT, Khan TM, Mino-Kenudson M, Solomon BJ, Halmos B, Jessop NA, Wain JC, Yeo AT, Benes C, et al. (2012). Mechanisms of acquired crizotinib resistance in ALK-rearranged lung Cancers. Sci. Transl. Med. 4, 120ra17. 10.1126/scitranslmed.3003316.
39. Eno MS, Brubaker JD, Campbell JE, De Savi C, Guzi TJ, Williams BD, Wilson D, Wilson K, Brooijmans N, Kim J, et al. (2022). Discovery of BLU-945, a Reversible, Potent, and Wild-Type-Sparing Next-Generation EGFR Mutant Inhibitor for Treatment-Resistant Non-Small-Cell Lung Cancer. J. Med. Chem. 65, 9662–9677. 10.1021/acs.jmedchem.2c00704. [PubMed: 35838760]
40. Zhou Y, Chen Y, Tong L, Xie H, Wen W, Zhang J, Xi Y, Shen Y, Geng M, Wang Y, et al. (2012). AL3810, a multi-tyrosine kinase inhibitor, exhibits potent anti-angiogenic and anti-tumour activity via targeting VEGFR, FGFR and PDGFR. J. Cell Mol. Med. 16, 2321–2330. 10.1111/j.1582-4934.2012.01541.x. [PubMed: 22304225]
41. PerkinElmer. (2012). LabChip EZ Reader Substrate Selection Guide. https://resources.perkinelmer.com/corporate/cmsresources/images/gde_labchip_ez_reader_substrate_selection.pdf.
42. Roskoski R Jr. (2015). Src protein-tyrosine kinase structure, mechanism, and small molecule inhibitors. Pharmacol. Res. 94, 9–25. 10.1016/j.phrs.2015.01.003. [PubMed: 25662515]
43. Boschelli DH, Ye F, Wang YD, Dutia M, Johnson SL, Wu B, Miller K, Powell DW, Yaczko D, Young M, et al. (2001). Optimization of 4-phenylamino-3-quinolinecarbonitriles as potent inhibitors of Src kinase activity. J. Med. Chem. 44, 3965–3977. 10.1021/jm0102250. [PubMed: 11689083]
44. Peters S, and Adjei AA. (2012). MET: a promising anticancer therapeutic target. Nat. Rev. Clin. Oncol. 9, 314–326. 10.1038/nrclinonc.2012.71. [PubMed: 22566105]
45. Zhang Y, Xia M, Jin K, Wang S, Wei H, Fan C, Wu Y, Li X, Li X, Li G, et al. (2018). Function of the c-Met receptor tyrosine kinase in carcinogenesis and associated therapeutic opportunities. Mol. Cancer 17, 45. 10.1186/s12943-018-0796-y. [PubMed: 29455668]
46. Wang SXY, Zhang BM, Wakelee HA, Koontz MZ, Pan M, Diehn M, Kunder CA, and Neal JW. (2019). Case series of MET exon 14 skipping mutation-positive non-small-cell lung cancers with response to crizotinib and cabozantinib. Anti Cancer Drugs 30, 537–541. 10.1097/CAD.0000000000000765. [PubMed: 30762593]
47. Mathieu LN, Larkins E, Akinboro O, Roy P, Amatya AK, Fiero MH, Mishra-Kalyani PS, Helms WS, Myers CE, Skinner AM, et al. (2022). FDA Approval Summary: Capmatinib and Tepotinib for the Treatment of Metastatic NSCLC Harboring MET Exon 14 Skipping Mutations or Alterations. Clin. Cancer Res. 28, 249–254. 10.1158/1078-0432.CCR-21-1566. [PubMed: 34344795]
48. Wolf J, Seto T, Han JY, Reguart N, Garon EB, Groen HJM, Tan DSW, Hida T, de Jonge M, Orlov SV, et al. (2020). Capmatinib in MET Exon 14-Mutated or MET-Amplified Non-Small-Cell Lung Cancer. N. Engl. J. Med. 383, 944–957. 10.1056/NEJMoa2002787. [PubMed: 32877583]
49. Liu X, Wang Q, Yang G, Marando C, Koblish HK, Hall LM, Fridman JS, Behshad E, Wynn R, Li Y, et al. (2011). A novel kinase inhibitor, INCB28060, blocks c-MET-dependent signaling, neoplastic activities, and cross-talk with EGFR and HER-3. Clin. Cancer Res. 17, 7127–7138. 10.1158/1078-0432.CCR-11-1157. [PubMed: 21918175]
50. Behshad E, Klabe RM, Margulis A, Becker-Pasha M, Rupar MJ, Collier P, Liu PC, Hollis GF, Burn TC, and Wynn R. (2010). Phosphorylation State-Dependent High Throughput Screening of the c-Met Kinase. Curr. Chem. Genom. 4, 27–33. 10.2174/1875397301004010027.
51. Sonnenberg E, Gödecke A, Walter B, Bladt F, and Birchmeier C. (1991). Transient and locally restricted expression of the ros1 protooncogene during mouse development. EMBO J. 10, 3693–3702. 10.1002/j.1460-2075.1991.tb04937.x. [PubMed: 1718742]
52. Jun HJ, Roy J, Smith TB, Wood LB, Lane K, Woolfenden S, Punko D, Bronson RT, Haigis KM, Breton S, and Charest A. (2014). ROS1 signaling regulates epithelial differentiation in the epididymis. Endocrinology 155, 3661–3673. 10.1210/en.2014-1341. [PubMed: 24971615]
53. Davies KD, and Doebele RC. (2013). Molecular pathways: ROS1 fusion proteins in cancer. Clin. Cancer Res. 19, 4040–4045. 10.1158/1078-0432.CCR-12-2851. [PubMed: 23719267]

54. Drilon A, Jenkins C, Iyer S, Schoenfeld A, Keddy C, and Davare MA. (2021). ROS1-dependent cancers - biology, diagnostics and therapeutics. Nat. Rev. Clin. Oncol. 18, 35–55. 10.1038/s41571-020-0408-9. [PubMed: 32760015]
55. Roskoski R Jr. (2017). ROS1 protein-tyrosine kinase inhibitors in the treatment of ROS1 fusion protein-driven non-small cell lung cancers. Pharmacol. Res. 121, 202–212. 10.1016/j.phrs.2017.04.022. [PubMed: 28465216]
56. Liu M, Dai J, Wei M, Pan Q, and Zhu W. (2022). An updated patent review of small-molecule ROS1 kinase inhibitors (2015–2021). Expert Opin. Ther. Pat. 32, 713–729. 10.1080/13543776.2022.2058872. [PubMed: 35343863]
57. Lovly CM, Heuckmann JM, de Stanchina E, Chen H, Thomas RK, Liang C, and Pao W. (2011). Insights into ALK-driven cancers revealed through development of novel ALK tyrosine kinase inhibitors. Cancer Res. 71, 4920–4931. 10.1158/0008-5472.CAN-10-3879. [PubMed: 21613408]
58. Hallberg B, and Palmer RH. (2013). Mechanistic insight into ALK receptor tyrosine kinase in human cancer biology. Nat. Rev. Cancer 13, 685–700. [PubMed: 24060861]
59. König D, Savic Prince S, and Rothschild SI. (2021). Targeted Therapy in Advanced and Metastatic Non-Small Cell Lung Cancer. An Update on Treatment of the Most Important Actionable Oncogenic Driver Alterations. Cancers 13, 804. [PubMed: 33671873]
60. Naoum GE, Morkos M, Kim B, and Arafat W. (2018). Novel targeted therapies and immunotherapy for advanced thyroid cancers. Mol. Cancer 17, 51. 10.1186/s12943-018-0786-0. [PubMed: 29455653]
61. Whittle SB, Smith V, Doherty E, Zhao S, McCarty S, and Zage PE. (2017). Overview and recent advances in the treatment of neuroblastoma. Expert Rev. Anticancer Ther. 17, 369–386. 10.1080/14737140.2017.1285230. [PubMed: 28142287]
62. Sammon D, Hohenester E, and Leitinger B. (2020). Two-step release of kinase autoinhibition in discoidin domain receptor 1. Proc. Natl. Acad. Sci. USA 117, 22051–22060. 10.1073/pnas.2007271117.
63. Wang H, Kruszewski A, and Brautigan DL. (2005). Cellular chromium enhances activation of insulin receptor kinase. Biochemistry 44, 8167–8175. 10.1021/bi0473152. [PubMed: 15924436]
64. Turunen SP, von Nandelstadh P, Öhman T, Gucciardo E, Seashore-Ludlow B, Martins B, Rantanen V, Li H, Höpfner K, Östling P, et al. (2019). FGFR4 phosphorylates MST1 to confer breast cancer cells resistance to MST1/2-dependent apoptosis. Cell Death Differ. 26, 2577–2593. 10.1038/s41418-019-0321-x. [PubMed: 30903103]
65. Iwai LK, Payne LS, Luczynski MT, Chang F, Xu H, Clinton RW, Paul A, Esposito EA, Gridley S, Leitinger B, et al. (2013). Phosphoproteomics of collagen receptor networks reveals SHP-2 phosphorylation downstream of wild-type DDR2 and its lung cancer mutants. Biochem. J. 454, 501–513. 10.1042/BJ20121750. [PubMed: 23822953]
66. Edgar AJ, Trost M, Watts C, and Zaru R. (2014). A combination of SILAC and nucleotide acyl phosphate labelling reveals unexpected targets of the Rsk inhibitor BI-D1870. Biosci. Rep. 34, e00091. 10.1042/BSR20130094.
67. Muller IB, De Langen AJ, Honeywell RJ, Giovannetti E, and Peters GJ. (2016). Overcoming crizotinib resistance in ALK-rearranged NSCLC with the second-generation ALK-inhibitor ceritinib. Expert Rev. Anticancer Ther. 16, 147–157. 10.1586/14737140.2016.1131612. [PubMed: 26654422]
68. Shaw AT, and Engelman JA. (2013). ALK in lung cancer: past, present, and future. J. Clin. Oncol. 31, 1105–1111. 10.1200/JCO.2012.44.5353. [PubMed: 23401436]
69. Marsilje TH, Pei W, Chen B, Lu W, Uno T, Jin Y, Jiang T, Kim S, Li N, Warmuth M, et al. (2013). Synthesis, structure-activity relationships, and in vivo efficacy of the novel potent and selective anaplastic lymphoma kinase (ALK) inhibitor 5-chloro-N2-(2-isopropoxy-5-methyl-4-(piperidin-4-yl)phenyl)-N4-(2-(isopropylsulfonyl)phenyl)pyrimidine-2,4-diamine (LDK378) currently in phase 1 and phase 2 clinical trials. J. Med. Chem. 56, 5675–5690. 10.1021/jm400402q. [PubMed: 23742252]
70. Ben-Neriah Y, Daley GQ, Mes-Masson AM, Witte ON, and Baltimore D. (1986). The chronic myelogenous leukemia-specific P210 protein is the product of the bcr/abl hybrid gene. Science 233, 212–214. 10.1126/science.3460176. [PubMed: 3460176]

71. Greuber EK, Smith-Pearson P, Wang J, and Pendergast AM. (2013). Role of ABL family kinases in cancer: from leukaemia to solid tumours. Nat. Rev. Cancer 13, 559–571. 10.1038/nrc3563. [PubMed: 23842646]
72. Rossari F, Minutolo F, and Orciuolo E. (2018). Past, present, and future of Bcr-Abl inhibitors: from chemical development to clinical efficacy. J. Hematol. Oncol. 11, 84. 10.1186/s13045-018-0624-2. [PubMed: 29925402]
73. Diculescu VC, and Enache TA. (2014). Electrochemical evaluation of Abelson tyrosine-protein kinase 1 activity and inhibition by imatinib mesylate and danusertib. Anal. Chim. Acta 845, 23–29. 10.1016/j.aca.2014.06.025. [PubMed: 25201268]
74. Park G, Baek S, Kim JE, Lim TG, Lee CC, Yang H, Kang YG, Park JS, Augustin M, Mrosek M, et al. (2015). Flt3 is a target of coumestrol in protecting against UVB-induced skin photoaging. Biochem. Pharmacol. 98, 473–483. 10.1016/j.bcp.2015.08.104. [PubMed: 26341390]
75. Rutaganira FU, Barks J, Dhason MS, Wang Q, Lopez MS, Long S, Radke JB, Jones NG, Maddirala AR, Janetka JW, et al. (2017). Inhibition of Calcium Dependent Protein Kinase 1 (CDPK1) by Pyrazolopyrimidine Analogs Decreases Establishment and Reoccurrence of Central Nervous System Disease by Toxoplasma gondii. J. Med. Chem. 60, 9976–9989. 10.1021/acs.jmedchem.7b01192. [PubMed: 28933846]
76. O'Hare T, Walters DK, Stoffregen EP, Jia T, Manley PW, Mestan J, Cowan-Jacob SW, Lee FY, Heinrich MC, Deininger MWN, and Druker BJ. (2005). In vitro activity of Bcr-Abl inhibitors AMN107 and BMS-354825 against clinically relevant imatinib-resistant Abl kinase domain mutants. Cancer Res. 65, 4500–4505. 10.1158/0008-5472.CAN-05-0259. [PubMed: 15930265]
77. Ayati A, Moghimi S, Salarinejad S, Safavi M, Pouramiri B, and Foroumadi A. (2020). A review on progression of epidermal growth factor receptor (EGFR) inhibitors as an efficient approach in cancer targeted therapy. Bioorg. Chem. 99, 103811. 10.1016/j.bioorg.2020.103811.
78. Rocco D, Della Gravara L, Battiloro C, Maione P, and Gridelli C. (2021). The treatment of advanced lung adenocarcinoma with activating EGFR mutations. Expet Opin. Pharmacother. 22, 2475–2482. 10.1080/14656566.2021.1957096.
79. Register AC, Leonard SE, and Maly DJ. (2014). SH2-catalytic domain linker heterogeneity influences allosteric coupling across the SFK family. Biochemistry 53, 6910–6923. 10.1021/bi5008194. [PubMed: 25302671]
80. Subramanian G, Johnson PD, Zachary T, Roush N, Zhu Y, Bowen SJ, Janssen A, Duclos BA, Williams T, Javens C, et al. (2019). Deciphering the Allosteric Binding Mechanism of the Human Tropomyosin Receptor Kinase A (hTrkA) Inhibitors. ACS Chem. Biol. 14, 1205–1216. 10.1021/acschembio.9b00126. [PubMed: 31059222]
81. Lu X, Yu L, Zhang Z, Ren X, Smaill JB, and Ding K. (2018). Targeting EGFR(L858R/T790M) and EGFR(L858R/T790M/C797S) resistance mutations in NSCLC: Current developments in medicinal chemistry. Med. Res. Rev. 38, 1550–1581. 10.1002/med.21488. [PubMed: 29377179]
82. Abdelmalek CM, Hu Z, Kronenberger T, Küblbeck J, Kinnen FJM, Hesse SS, Malik A, Kudolo M, Niess R, Gehringer M, et al. (2022). Gefitinib-Tamoxifen Hybrid Ligands as Potent Agents against Triple-Negative Breast Cancer. J. Med. Chem. 65, 4616–4632. 10.1021/acs.jmedchem.1c01646. [PubMed: 35286086]
83. Drilon A, Hu ZI, Lai GGY, and Tan DSW. (2018). Targeting RET-driven cancers: lessons from evolving preclinical and clinical landscapes. Nat. Rev. Clin. Oncol. 15, 151–167. 10.1038/nrclinonc.2017.175. [PubMed: 29134959]
84. Kato S, Subbiah V, Marchlik E, Elkin SK, Carter JL, and Kurzrock R. (2017). RET Aberrations in Diverse Cancers: Next-Generation Sequencing of 4,871 Patients. Clin. Cancer Res. 23, 1988–1997. 10.1158/1078-0432.CCR-16-1679. [PubMed: 27683183]
85. Roskoski R Jr., and Sadeghi-Nejad A. (2018). Role of RET protein-tyrosine kinase inhibitors in the treatment RET-driven thyroid and lung cancers. Pharmacol. Res. 128, 1–17. 10.1016/j.phrs.2017.12.021. [PubMed: 29284153]
86. Subbiah V, Yang D, Velcheti V, Drilon A, and Meric-Bernstam F. (2020). State-of-the-Art Strategies for Targeting RET-Dependent Cancers. J. Clin. Oncol. 38, 1209–1221. 10.1200/JCO.19.02551. [PubMed: 32083997]

87. Subbiah V, Shen T, Terzyan SS, Liu X, Hu X, Patel KP, Hu M, Cabanillas M, Behrang A, Meric-Bernstam F, et al. (2021). Structural basis of acquired resistance to selpercatinib and pralsetinib mediated by non-gatekeeper RET mutations. Ann. Oncol. 32, 261–268. 10.1016/j.annonc.2020.10.599. [PubMed: 33161056]

88. Choudhury NJ, and Drilon A. (2020). Decade in review: a new era for RET-rearranged lung cancers. Transl. Lung Cancer Res. 9, 2571–2580. 10.21037/tlcr-20-346. [PubMed: 33489819]

89. Fabian MA, Biggs WH 3rd, Treiber DK, Atteridge CE, Azimioara MD, Benedetti MG, Carter TA, Ciceri P, Edeen PT, Floyd M, et al. (2005). A small molecule-kinase interaction map for clinical kinase inhibitors. Nat. Biotechnol. 23, 329–336. 10.1038/nbt1068. [PubMed: 15711537]

90. de Boer AR, Letzel T, Lingeman H, and Irth H. (2005). Systematic development of an enzymatic phosphorylation assay compatible with mass spectrometric detection. Anal. Bioanal. Chem. 381, 647–655. 10.1007/s00216-005-3070-2. [PubMed: 15703914]

91. Li M, Luraghi P, Amour A, Qian XD, Carter PS, Clark CJ, Deakin A, Denyer J, Hobbs CI, Surby M, et al. (2009). Kinetic assay for characterization of spleen tyrosine kinase activity and inhibition with recombinant kinase and crude cell lysates. Anal. Biochem. 384, 56–67. 10.1016/j.ab.2008.07.040. [PubMed: 18762159]

92. Luković E, Vogel Taylor E, and Imperiali B. (2009). Monitoring protein kinases in cellular media with highly selective chimeric reporters. Angew. Chem., Int. Ed. Engl. 48, 6828–6831. 10.1002/anie.200902374. [PubMed: 19681083]

93. Elkins JM, Fedele V, Szklarz M, Abdul Azeez KR, Salah E, Mikolajczyk J, Romanov S, Sepetov N, Huang XP, Roth BL, et al. (2016). Comprehensive characterization of the Published Kinase Inhibitor Set. Nat. Biotechnol. 34, 95–103. 10.1038/nbt.3374. [PubMed: 26501955]

94. Gurbani D, Du G, Henning NJ, Rao S, Bera AK, Zhang T, Gray NS, and Westover KD. (2020). Structure and Characterization of a Covalent Inhibitor of Src Kinase. Front. Mol. Biosci. 7, 81. 10.3389/fmolb.2020.00081. [PubMed: 32509799]

95. Du G, Rao S, Gurbani D, Henning NJ, Jiang J, Che J, Yang A, Ficarro SB, Marto JA, Aguirre AJ, et al. (2020). Structure-Based Design of a Potent and Selective Covalent Inhibitor for SRC Kinase That Targets a P-Loop Cysteine. J. Med. Chem. 63, 1624–1641. 10.1021/acs.jmedchem.9b01502. [PubMed: 31935084]

Highlights

- Targeted mass spectrometry effectively measures phosphorylation activity *in vitro*
- LIMS-Kinase is a label-free MRM assay, applicable to many kinase drug targets
- LIMS-Kinase measures targeted inhibitor potency with high sensitivity
- Potential for expansion to segments of the kinome where assays are undeveloped

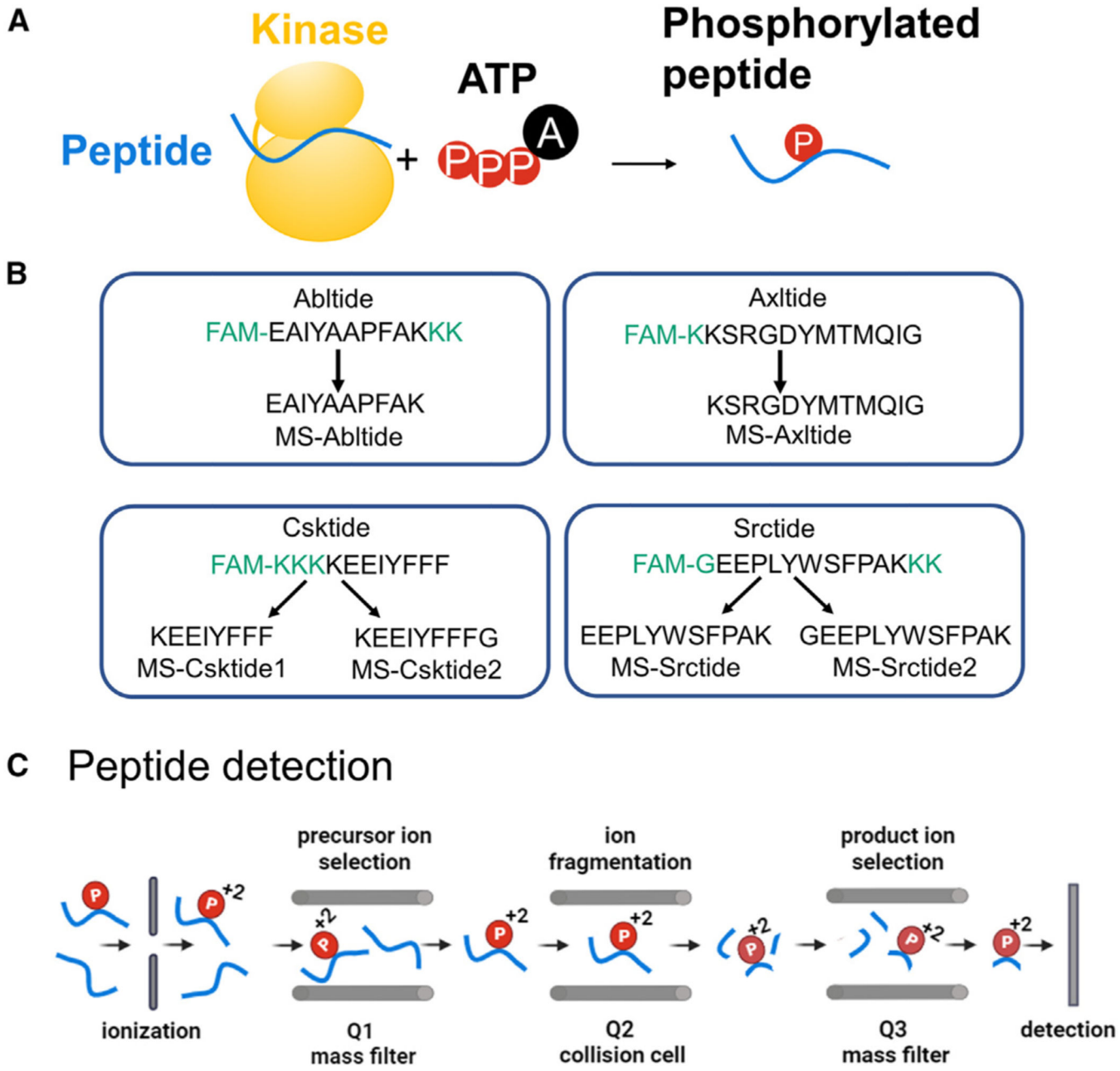
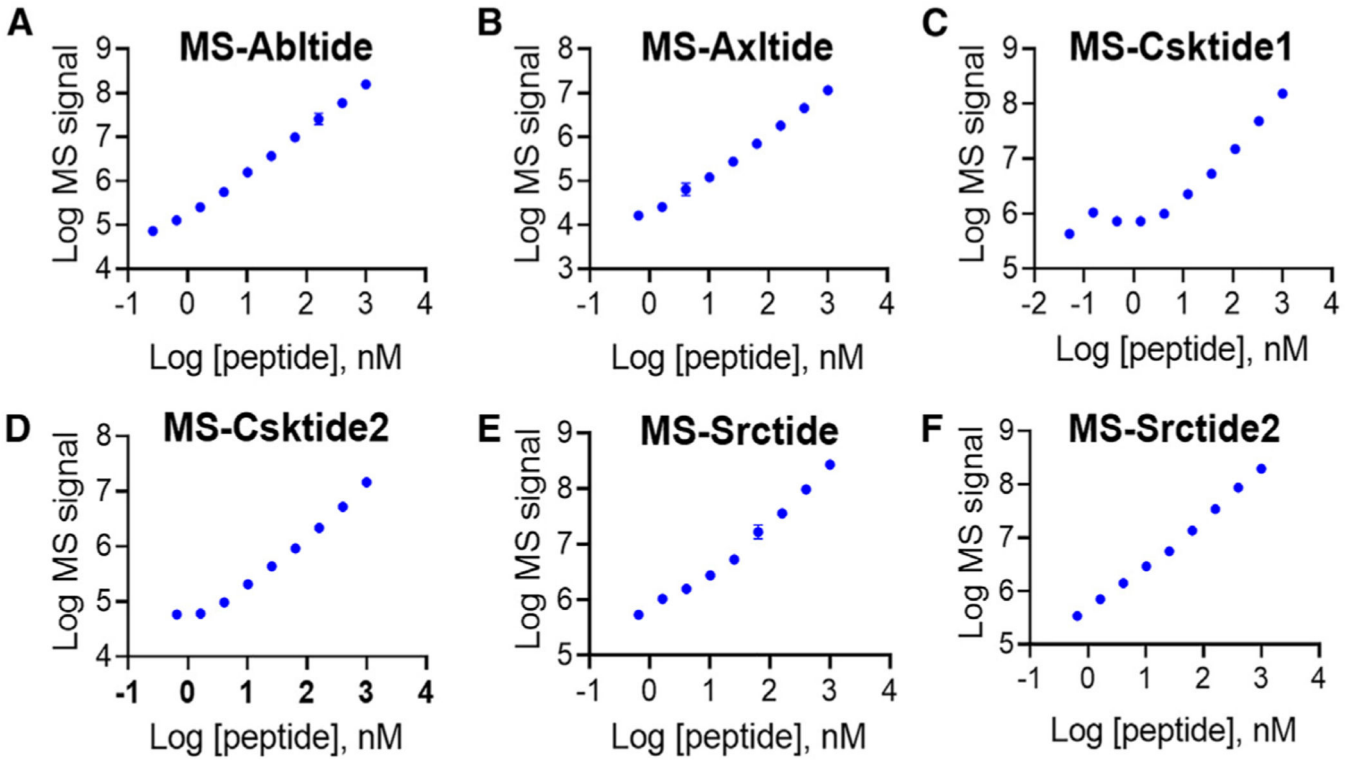


Figure 1. Mass spectrometry-based detection of kinase reactions can be generalized

(A) Schematic of kinase assay reaction. Phosphates are indicated by red spheres and the adenine nucleoside in black.

(B) Summary of peptide designs. MS-Kinase peptides were derived from established kinase substrates optimized for other detection methods.

(C) Schematic of MRM phosphopeptide detection strategy. Triple quadrupole mass spectrometer quantifies and isolates product ion.



G

	ABL1	ALK	EGFR L858R	MET	RET M918T	ROS1	SRC
MS-Abltide	29			0	0		
MS-Axltide		65					
MS-Csktide1			67	92	89	94	
MS-Csktide2					92		
MS-Srctide		26	49	94	85	100	99
MS-Srctide2		10	24	77			87

Figure 2. MS-Kinase substrate evaluation by mass spectrometry

(A–F) Intensity of detection of daughter products are plotted in log-log scale. Detection is linear over multiple logs for all peptides. Each data point represents the mean of two or three independent measurements. Error bars are the standard deviation.

(G) Phosphorylation of substrates by kinases presented as percent conversion of substrate to product. Pink shading indicates peptide with highest conversion for a given kinase.

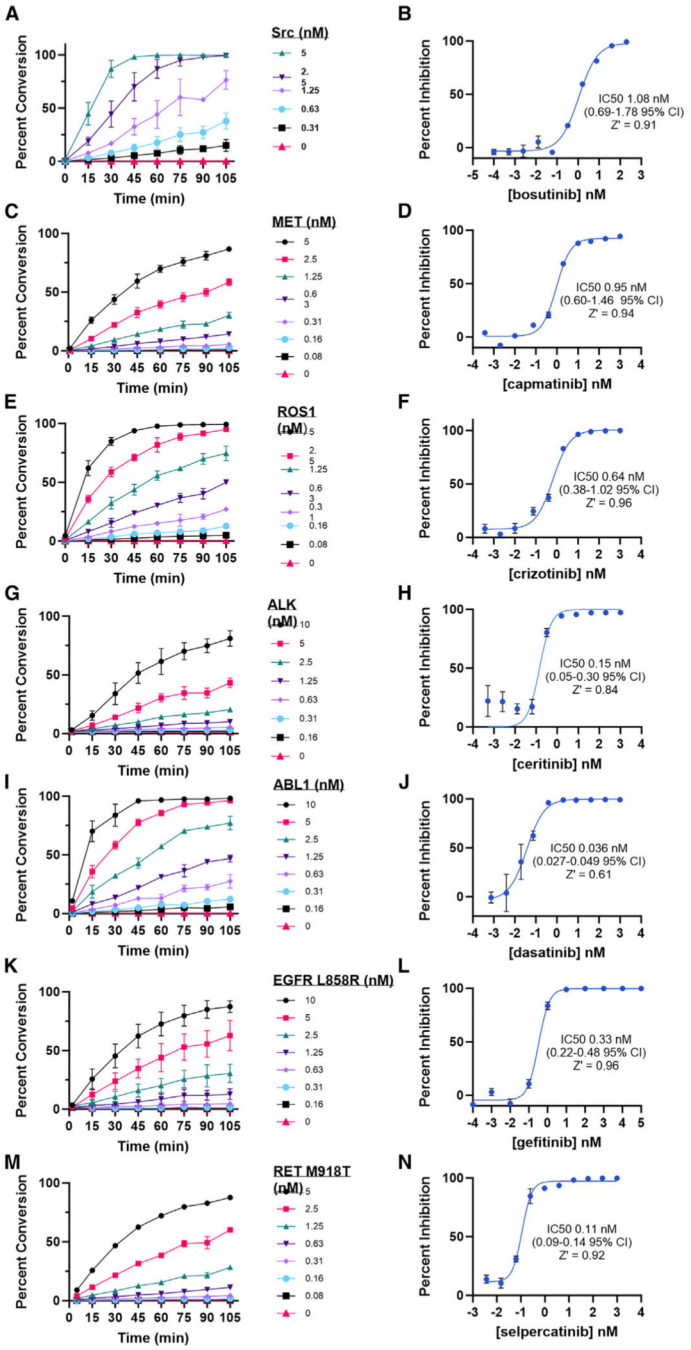


Figure 3. MS-Kinase detects enzyme activity and inhibition with high sensitivity

(A–N) For all reactions, peptides were 1 μM and [ATP] 100 μM, except ALK-ceritinib IC_{50} assay, which used 10 μM peptide. Enzyme or inhibitor concentrations are variable as indicated. Each data point represents the mean of two independent measurements (optimization assays) or three independent measurements (inhibition assays). Error bars are the standard deviation.

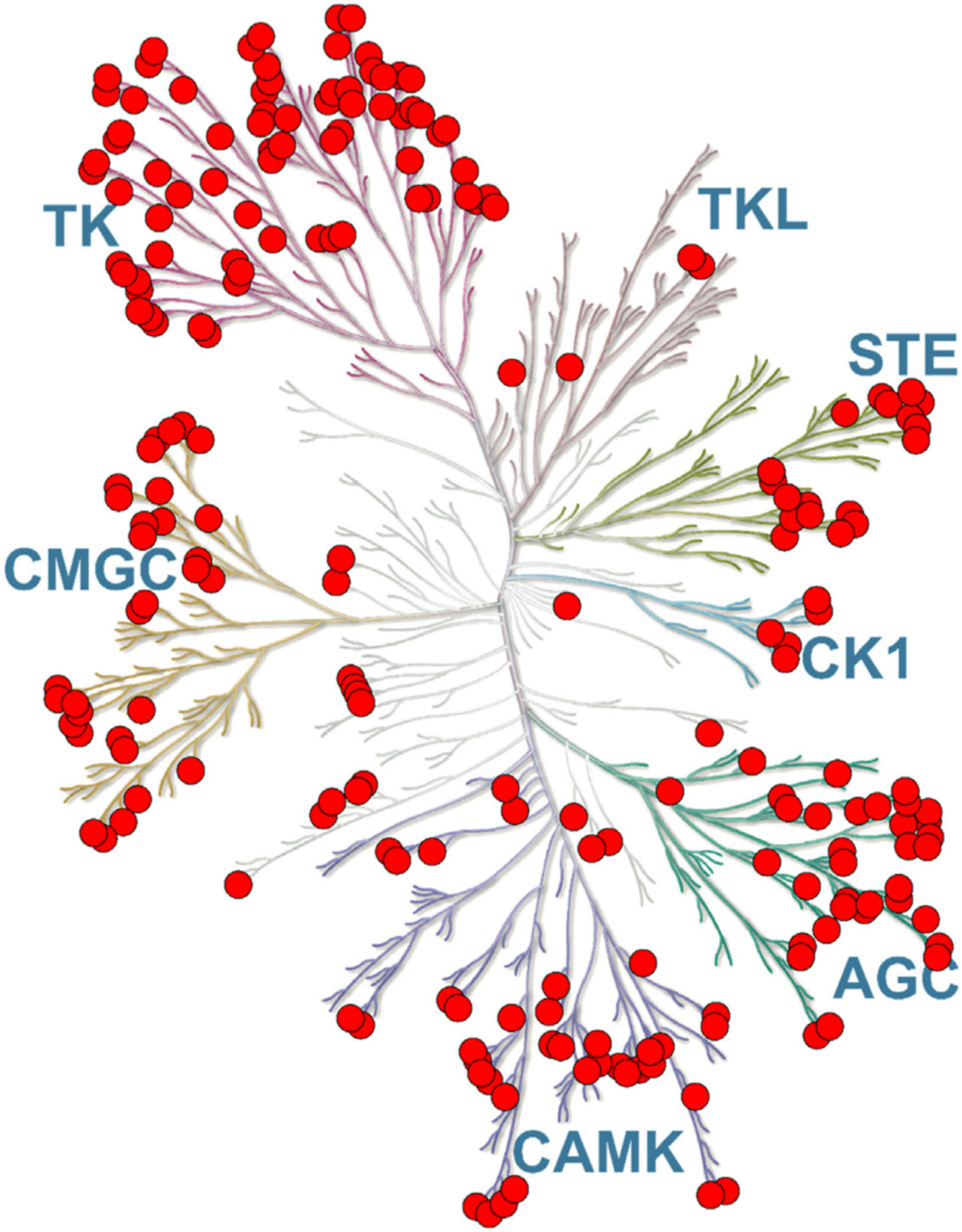


Figure 4. Established kinase assay peptide substrates within the kinome

Red circles indicate kinases for which enzymatic peptide substrates have been reported.

Table 1.

Ions used as the basis of assay measurements

		Non-phosphorylated		Phosphorylated	
Substrate	**Sequence**	**Precursor ion m/z**	**Product ion m/z**	**Precursor ion m/z**	**Product ion m/z**
MS-Abltide	EAIYAAPFAK	540.4	83.9	580.4	83.9
MS-Axltide	KSRGDYMTMQIG	693.4	84.2	733.4	84.2
MS-Csktide1	KEEIYFFF	561.5	120	601.5	120
MS-Csktide2	KEEIYFFFG	589.9	120.2	629.9	120.2
MS-Srctide	EEPLYWSFPAK	683.5	554.4	723.5	594.4[a]
MS-Srctide2	GEEPLYWSFPAK	712.1	70	752.1	70

[a]With PO_3.