Published in final edited form as:

Biochem Pharmacol. 2017 October 15; 142: 39–45. doi:10.1016/j.bcp.2017.06.127.

A high throughput assay to identify substrate-selective inhibitors of the ERK protein kinases

Chad J. Miller, Yagmur Muftuoglu, and Benjamin E. Turk*

Department of Pharmacology, Yale University School of Medicine, New Haven, CT 06520, United States

Abstract

Extracellular signal-regulated kinases 1 and 2 (ERK1/2) phosphorylate a variety of substrates important for survival and proliferation, and their activity is frequently deregulated in tumors. ERK pathway inhibitors have shown clinical efficacy as anti-cancer drugs, but most patients eventually relapse due to reactivation of the pathway. One factor limiting the efficacy of current therapeutics is the difficulty in reaching clinically effective inhibition of the ERK pathway in the absence of on-target toxicities. Here, we describe an assay suitable for high throughput screening to discover substrate selective ERK1/2 inhibitors, which may have a larger therapeutic window than conventional inhibitors. Specifically, we aim to target a substrate-binding pocket within the ERK1/2 catalytic domain outside of the catalytic cleft. The assay uses an AlphaScreen format to detect phosphorylation of a high-efficiency substrate harboring an essential docking site motif. Pilot screening established that the assay is suitably robust for high-throughput screening. Importantly, the assay can be conducted at high ATP concentrations, which we show reduces the discovery of conventional ATP-competitive inhibitors. These studies provide the basis for high-throughput screens to discover new classes of non-conventional ERK1/2 inhibitors.

Graphical abstract

Keywords

Protein kinase; Phosphorylation; Kinase inhibitor; High-throughput screening; Mitogen-activated protein kinase

*Corresponding author at: P.O. Box 208066, 333 Cedar St., New Haven, CT 06520, United States. Tel.: +1 203 737-2494. ben.turk@yale.edu.

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Conflict of Interest Statement

The authors confirm that there are no conflicts of interest associated with this manuscript.

1. Introduction

The extracellular signal-regulated kinase (ERK) mitogen-activated protein kinase (MAPK) pathway (Fig. 1A) is a three-component phosphorylation cascade with widespread roles in cell proliferation, survival, migration, and differentiation in animals [1]. In humans, the core cascade consists of MAPK kinase kinases of the RAF family (ARAF, BRAF and CRAF), which phosphorylate and activate the MAPK kinases MEK1 and MEK2, which in turn phosphorylate and activate the MAPKs ERK1 and ERK2. ERK MAPKs phosphorylate a large number of substrates that act as effectors of the pathway, including transcription factors, enzymes, scaffolds, and other kinases [2]. Given its key role in promoting cell survival and proliferation, the ERK pathway is frequently deregulated in human cancer, most commonly through mutation or amplification of upstream regulators. For example, driver mutations in BRAF have been identified in multiple tumor types [3–6]. In particular, BRAF mutations occur frequently in malignant melanoma, with about 50% of tumors harboring the BRAF-V600E mutation that results in high level constitutive activity [7].

The prevalent role for the ERK pathway in cancer has allowed for successful therapeutic targeting of the pathway, particularly in the setting of BRAF-mutant melanoma [1]. However, current therapies, including FDA-approved BRAF and MEK1/2 inhibitors, suffer from serious limitations, including a lack of durable response and potentially dose-limiting toxicities [8, 9]. Acquired resistance to BRAF and MEK inhibition has been ascribed to multiple molecular mechanisms, but a common feature is reactivation of ERK signaling despite the presence of inhibitor. ERK reactivation during treatment is partly attributable to disruption of negative feedback loops that restrain activity at multiple steps in the pathway (Fig. 1A) [10, 11].

Both BRAF and MEK1/2 inhibitors function by blocking activation of ERK, preventing phosphorylation of the full repertoire of ERK substrates. This general inhibition of ERK substrate phosphorylation thus underlies the therapeutic efficacy, as well as toxicity and feedback reactivation, associated with inhibition of the pathway. Likewise, ATP-competitive inhibitors of ERK1/2 currently in development [12, 13] are expected to generally block substrate phosphorylation and thus suffer from the same shortcomings. Theoretically, selective blockade of ERK activity could overcome these limitations, for example by inhibiting phosphorylation of oncogenic substrates while sparing substrates important for tissue homeostasis or those involved in feedback inhibition.

One potential avenue for generating substrate-selective ERK inhibitors is through compounds that block docking interactions, or physical interactions between the kinase and substrate occurring outside of the catalytic cleft. ERK has two distinct, well-defined regions within its catalytic domain involved in docking interactions (Fig. 1B) [14, 15]. One region, the common docking groove, interacts with a docking site motif termed the D-site that is found in many ERK substrates, including RSK1, a kinase implicated in feedback regulation [16, 17]. In addition, ERK regulators such as MEK1/2 and MAPK phosphatases also harbor D-sites that are essential for function. The other region is a hydrophobic pocket located adjacent to the catalytic cleft that interacts with “DEF-sites” (*D*ocking sites for *ERK*, *FXF*)

[18]. DEF-sites (also called F-sites) are short Phe-x-Phe/Tyr sequence motifs that are found in some, but not all ERK1/2 substrates [19–21]. ERK substrates harboring the DEF motif include key oncogenic transcription factors (Elk1, c-Fos, Fra1, and c-Myc), as well as at least one substrate important for cell survival (Bim_{EL}) [20–22]. In comparison to WT ERK2, an ERK2 mutant that cannot bind to DEF-sites was able to phosphorylate RSK1, yet provided reduced phosphorylation of c-Fos and Elk1 in cultured cells [23]. Furthermore, mutation of the DEF-interaction pocket decreased ERK2-dependent cell survival, migration, invasion and epithelial-mesenchymal transition [24]. A small molecule blocking the DEF-site interaction pocket may therefore inhibit oncogenic signaling while sparing substrates associated with toxicity and feedback reactivation of ERK.

Despite their potential utility as therapeutics or as tool compounds, substrate-selective kinase inhibitors are difficult to discover. Protein kinase inhibitors are typically identified either by elaboration of known scaffolds or by high-throughput screening (HTS) using short peptide or generic protein substrates. These approaches are strongly biased towards the discovery of canonical ATP-competitive inhibitors. Inhibitors that disrupt D-site interactions with the MAPK JNK (c-jun N-terminal kinase) were identified by high-throughput screening using a competitive binding assay, and similar inhibitors of p38 α MAPK were identified in assays using a full-length protein substrate [25, 26]. Inhibitors of both D-site interactions and DEF-site interactions with ERK have been identified by in silico screening, and improved through medicinal chemistry [27–31]. We anticipate that activity-based high-throughput screening will likely provide additional scaffolds, potentially leading to higher potency inhibitors of docking interactions. Here, we describe an assay suitable for the identification of DEF-site competitive inhibitors of ERK and its adaptation to a high-throughput format.

2. Materials and Methods

2.1 Reagents

Biotinylated peptides were prepared by standard Fmoc synthesis procedures, purified by RP-HPLC, and verified by mass spectrometry analysis. Peptides for radiolabel assays were synthesized in the Turk laboratory and were re-synthesized on larger scale for HTS assay development and pilot screening at Tufts University Core Facility (Boston, MA). Peptides were dissolved in DMSO, and stock concentrations were calculated from A₂₈₀ values of aqueous dilutions. AlphaScreen General IgG (Protein A) kit (6760617, Perkin Elmer, Waltham, MA), which includes streptavidin-coated donor beads and Protein A-conjugated acceptor beads, was used for the primary HTS screen. The phospho-c-Jun (Ser63) antibody (#9261) was purchased from Cell Signaling Technology (Danvers, MA). Cold ATP, BSA, imidazole, DNaseI, and Igepal CA-630 were from Sigma-Aldrich (St. Louis, MO), MgCl₂ was from J.T. Baker (Phillipsburg, NJ), and Coomassie Brilliant Blue R-250 was from Bio-Rad (Hercules, CA). Radiolabeled [γ -³³P]ATP was from Perkin Elmer (Waltham, MA). All other chemical reagents were from American Bioanalytical (Natick, MA).

Active ERK2 was prepared as described previously [32], with the following modifications. BL21(DE3)pLysS *E. coli* were co-transformed with plasmids expressing His₆-tagged rat ERK2 and a constitutively active allele of human MEK1. Log phase cultures were induced with 0.4 mM IPTG and grown at 30°C for 6 hrs. Cells were pelleted, resuspended in lysis

buffer (20 mM Tris, pH 8.8, 140 mM NaCl, 10 mM imidazole, 0.4% Igepal CA-630, 13 mM MgCl₂, 200 µg/mL lysozyme, 10 µg/mL pepstatin A, 10 µg/mL leupeptin, 3 mM β-mercaptoethanol, 1 mM PMSF), sonicated, and incubated with 0.03 U DNase at 4°C for 30 min. After clarification of the lysate, ERK2 was isolated by affinity chromatography using TALON metal affinity resin (Clontech, Mountain View, CA) and eluted in high imidazole buffer (20 mM Tris, pH 8.8, 140 mM NaCl, 500 mM imidazole, 7.4, 10 µg/mL leupeptin). The eluate was dialyzed overnight at 4°C into storage buffer (10 mM HEPES, pH 7.4, 100 mM NaCl, 1 mM DTT, 10% glycerol). To deplete residual MEK1, the dialysate was incubated with glutathione Sepharose 4B slurry (GE Healthcare, Chicago, IL) for 1 h at 4°C and filtered to remove beads. Protein concentration and purity were assessed by SDS-PAGE and staining with Coomassie Brilliant Blue using a BSA standard curve.

2.2 Peptide kinase assays

For radiolabel kinase assays, peptide substrate (5 or 10 µM) and active ERK2 (10 ng/µL) were mixed in assay buffer (20 mM HEPES, pH 7.5, 10 mM MgCl₂, 1 mM DTT). Reactions were initiated by the addition of ATP (to 10 µM, including 0.25 µCi/µl [γ -³³P]ATP). At 5 min increments, 2 µL aliquots were spotted onto streptavidin-coated membrane (Promega SAM2 biotin capture membrane), which was quenched and washed as previously described [33, 34]. Radiolabel incorporation was quantified by phosphor imaging. Phosphorylation rates were linear over substrate concentration in this range, and phosphorylation efficiencies were calculated from reaction rates by the formula: $k_{cat}/K_m = V/[E][S]$.

2.3 Primary screening assay

Though modifications were made throughout optimization, the general AlphaScreen procedure was as follows. All components were diluted in reaction buffer (50 mM HEPES, 10 mM MgCl₂, 0.1% BSA, 0.01% Triton X-100, mM DTT). 5 µL of ERK2 was dispensed into a 384-well white low volume assay plate (Corning 3673) using a Thermo Multidrop Combi 836 Reagent Dispenser in all but two columns, to which only buffer was added for controls. 20 nL of the screening compound DMSO stocks (or neat DMSO, as a control) were added to the enzyme by pin tool (final compound concentration was 29 µM), manipulated by a Tecan Aquarius, and incubated for 15 min at room temperature. Four columns in compound plates contained only DMSO as controls, which were used to calculate Z' factors. 1 µL peptide solution was added by Multidrop, followed by 1 µL ATP solution. The 7 µL reaction was incubated at 30 °C and then quenched with 1 µL EDTA (final concentration 25 µM) and Phospho-c-Jun (Ser63) II Antibody mixture added via Multidrop. The quenched reaction was allowed to incubate at room temperature for at least 20 min. In a green light room, 2 µL of a 1:1 mixture of AlphaScreen General IgG (Protein A) acceptor and Streptavidin donor beads were added to a final volume of 10 µL and incubated in low light for one hour at room temperature. After incubation, the plates were read using a PerkinElmer EnVision plate reader using the AlphaScreen module and reading emission at 570 nm (100 nm bandwidth, 550 ms measurement time, 180 ms excitation time).

2.4 Secondary screen

ERK2 was diluted to 200 nM in reaction buffer (50 mM HEPES, 10 mM MgCl₂, 0.1% BSA, 0.01% Triton X-100, 1 mM DTT), and 20 nL compound or DMSO was added by pin tool to

a final concentration of 33 μ M. After 15 min. incubation, a kinase reaction was started by the addition of ATP (final concentration 50 μ M) and DEF peptide (final concentration 41 μ M). The reaction was incubated for 2 hrs. at room temperature, and depletion of ATP was measured using the ADP-Glo kit (Promega) according to the manufacturer's instructions. Plates were read using a PerkinElmer EnVision plate reader using the ADPGlo module (450–700 nm emission filter, 6.5 mm height, 0.3 sec time).

2.5 Radiolabel Peptide Secondary screen

ERK2 was diluted to 25 nM (for DEF peptide reaction) or 50 nM (for FD peptide reaction) in reaction buffer (50 mM HEPES, pH 7.4, 10 mM MgCl_2 , 0.1% BSA, 1 mM DTT, in 0.1% Tween 20). Compounds (or DMSO control) were added to a final concentration of 50 μ M and incubated at room temperature with kinase for 30 min. Peptide substrate was added to a final concentration of 5 μ M (DEF peptide) or 10 μ M (FD peptide). The reaction was started by addition of [γ - ^{33}P]ATP (8 Ci/mmol) to a final concentration of 10 μ M. After incubation at 30 °C for 30 min (DEF peptide) or 4 hours (FD peptide), 2 μ L of the reaction was spotted onto streptavidin-coated membrane (Promega SAM2 biotin capture membrane, Madison WI), and quenched with 0.1% SDS/TBS. Additional washes were performed as previously described [33, 34], and radiolabel incorporation was quantified by phosphor imaging.

3. Results

To develop an assay suitable for identifying docking site inhibitors, we designed a peptide substrate incorporating an ERK phosphorylation site tethered to a DEF motif (Fig. 2A). We incorporated the sequence surrounding Ser63 of c-Jun, an established MAPK phosphorylation site for which a high-affinity phosphospecific antibody was available. We positioned an ERK optimal DEF-site motif (FEYP), previously identified from peptide library screening, six residues downstream of the phosphorylation site, spacing that affords efficient docking [35]. Our substrate incorporates a C-terminal biotin tag to facilitate use in multiple assay formats. Initial evaluation by radiolabel kinase assay showed that the DEF peptide substrate is phosphorylated ~90-fold more efficiently than a peptide in which the Phe residue within the DEF motif was substituted with Asp (FD peptide, Fig 2B). The substantial dependence on the DEF motif for phosphorylation of this peptide suggests that it should be suitable for discovering DEF-site inhibitors.

For our primary assay, we used an AlphaScreen format, which provides a luminescent readout based on transfer of singlet oxygen from proximal donor and acceptor beads (Fig. 2C). Following incubation of the biotinylated peptide substrate with recombinant ERK2 and ATP, we add phosphospecific antibody, streptavidin-coated donor beads, and protein A-conjugated acceptor beads to the reaction well. Binding of the phosphorylated peptide substrate to the antibody brings the donor and acceptor beads into close proximity. Excitation of the donor beads thereby allows singlet oxygen transfer to proximal acceptor beads, causing them to emit light at a shorter wavelength. In the presence of inhibitor, energy transfer does not occur because donor and acceptor beads are not in close proximity. This assay format was chosen in part because it could be conducted at high [ATP], which should bias against the discovery of canonical ATP-competitive inhibitors.

To optimize the AlphaScreen signal, donor and acceptor beads were titrated simultaneously with a positive control peptide (Fig. 2A) harboring phosphoserine (pSer) at an antibody concentration of 1 nM. We observed inhibition with increasing pSer peptide concentration (Fig. 3A), a characteristic of multicomponent complexes when the bridging component (peptide) is in excess [36]. No signal was observed in the absence of the pSer peptide or beads. We chose 20 $\mu\text{g/mL}$ of each bead as our working concentration for further optimization because it showed sufficient signal over background while limiting reagent consumption. We next simultaneously titrated the antibody concentration within a lower pSer peptide concentration range. This gave a pSer peptide-dependent increase in signal for each antibody concentration (Fig. 3B), indicating that it was an appropriate peptide range for the assay. To conserve assay costs, we chose a final concentration of 1 nM antibody for further optimization.

To identify suitable conditions for an ERK2 enzymatic assay, we initially titrated the DEF peptide substrate at two ERK2 concentrations, with ATP at 1.4 mM and a reaction time of 60 min. The signal increased with both ERK2 and DEF peptide concentrations (Fig. 3C). The highest signal to background was observed with 7.1 nM ERK2 and either 57.1 or 28.6 nM peptide. In a separate assay, we found that the lower peptide concentration (28.6 nM) gave a more dynamic signal over time, with a slope almost 2-fold greater than the higher peptide concentration (Fig. 3D). At 15 min kinase reaction time, the signal was also approximately linear over ERK2 concentration (Fig 3E), suggesting that it would be sensitive to ERK inhibition. To improve assay statistics after we increased our plate throughput, we slightly increased the ERK2 concentration to 10.7 nM. Under these conditions, we confirmed that peptide phosphorylation was dependent on the DEF site, as evidenced by ~6-fold decrease in signal using the FD substituted peptide (Fig. 3F).

To establish the utility of this assay for HTS, we screened two small compound collections, each comprising two 384-well plates. Compounds were added by pin tool transfer from DMSO stocks to a final concentration of 29 μM prior starting reactions by addition of ATP and peptide substrate. Z' values based on the plate controls ranged from 0.63 – 0.67, and the average signal to background ratio was 26 ± 2 , indicating robust assay performance. Among these libraries, we identified 20 hit compounds at a threshold of 2σ below the vehicle control wells, of which 10 were confirmed in a repeat AlphaScreen assay. Three of these compounds were verified to inhibit ERK2 in a secondary assay based on detection of ADP generated during the reaction (ADP-Glo, data not shown). To test whether these compounds selectively inhibited DEF-dependent substrates, assay conditions were found where phosphorylation of the FD peptide could be robustly detected. The three compounds inhibited ERK2 phosphorylation of both the DEF peptide and the FD substituted peptide as a substrate (data not shown), suggesting that these compounds are unlikely to be substrate-selective.

The identification of DEF site-independent inhibitors of ERK2 from our pilot screens suggested that, despite being conducted at high [ATP], our screen can identify ATP-competitive inhibitors. To verify that our assay conditions do bias against ATP-competitive compounds, we screened a set of compounds from a focused collection of known kinase inhibitors at both a low ATP concentration (71 μM) in the general range of reported K_m values for ERK2 and at the high (1071 μM) saturating ATP concentration used in our

standard assay conditions. At low [ATP], 44 of the 320 compounds screened scored as hits. As anticipated, most (33) of these compounds did not significantly inhibit ERK2 when assayed in the high ATP condition (Fig. 4B). These results suggest that while high affinity ATP-competitive inhibitors will substantially inhibit ERK under these conditions, most such inhibitors will have insufficient potency to score as hits.

4. Discussion

Limitations of current BRAF and MEK1/2 inhibitors have led to the development of ERK1/2 inhibitors targeting the most downstream component of the cascade [1]. By targeting the sole effectors of the pathway, ERK1/2 inhibitors theoretically should have similar efficacy to MEK1/2 inhibitors, yet may have potential advantages over current therapy. For example, ERK1/2 inhibitors reportedly have activity in the setting of BRAF/MEK inhibitor resistance [12, 37] in melanoma cells. ERK1/2 inhibitors also inhibit the growth of cells intrinsically resistant to BRAF inhibitors, such as those harboring RAS mutations [12]. ERK1/2 inhibitors may therefore have application, either alone or in combination with other agents, in clinical areas outside melanoma.

Several ATP-competitive ERK1/2 inhibitors have been discovered through standard approaches involving HTS followed by structure-guided design to improve potency, selectivity, and pharmacokinetic properties. These compounds include ulixertinib (BVD-523, an analog of the earlier generation compound VTX-11e) and raxoxertinib (GDC-0994) currently in early clinical trials [13, 38] as well as other preclinical compounds [39–44]. Another ATP-competitive inhibitor, SCH772984, was discovered through elaboration of a hit compound identified to bind to the unphosphorylated inactive form of ERK2 using a novel mass spectrometry-based approach [12, 45–47]. SCH772984 is a type II inhibitor with a “dual mechanism” of inhibition by virtue of its ability to downregulate ERK1/2 phosphorylation in cells as well as block substrate phosphorylation [48]. A structural analog of this compound, MK-8353, is currently in phase I clinical trials [1].

One factor that may limit the utility of ERK1/2 inhibitors is the difficulty in achieving a dose high enough to eradicate tumors without disrupting essential functions of the pathway required to maintain tissue homeostasis [1]. Distinct MEK1/2 inhibitors are reported to cause atypical dose-limiting side effects, including visual disturbances [49–51], and general inhibition of ERK activity may have similar dose-limiting toxicities. As proposed here, several groups have been exploring means by which to inhibit only a subset of ERK substrates, potentially maintaining efficacy against tumors while sparing those substrates required for normal physiology. One mechanism for achieving selective substrate inhibition is through control of ERK localization. For example, Herrero *et al.* developed a small molecule that disrupts ERK dimerization, reducing phosphorylation of cytoplasmic substrates (e.g. RSK1) while increasing phosphorylation of some nuclear substrates (e.g. MYC and ELK1) [52]. Interestingly, this inhibitor was only mildly toxic to mice. Another group used a cell-penetrating peptide to block ERK1/2 interaction with importin-7, preventing its translocation to the nucleus. This peptide specifically blocked phosphorylation of nuclear ELK1 and reduced tumor growth in mouse xenotransplantation models [53].

Another approach to substrate-selective ERK inhibition is through blocking ERK1/2 substrate docking interactions. Shapiro and co-workers initially described a series of compounds that block interactions with D-sites discovered through an *in silico* screen [27] followed by optimization through medicinal chemistry [28, 30, 54, 55]. These compounds block phosphorylation of ELK1 and RSK1 and inhibit cell proliferation in a panel of tumor cell lines including, but not restricted to, melanoma cells harboring BRAF mutations. Despite targeting a groove common to multiple MAPKs, these inhibitors did not block phosphorylation of ATF2, a p38 MAPK substrate harboring an essential D-site [29]. The same group also used *in silico* screening to discover substrate-selective inhibitors that bind to the ERK DEF pocket [31]. Analogs of initial hit compounds were synthesized with low μM potency *in vitro* and in cultured cells. These compounds blocked phosphorylation and activation of oncogenic transcription factors such as c-FOS, yet as anticipated did not affect RSK1 phosphorylation. Interestingly, the compounds were selectively toxic to melanoma cell lines, including a BRAF inhibitor-resistant line. Collectively, these studies suggest that targeting of distinct docking interactions on ERK1/2 (D-site or DEF-site) is likely to have differential activity depending on the tumor cell type.

Here, we have described an HTS strategy aimed at identifying DEF-site inhibitors of ERK1/2 using a peptide substrate harboring a tethered docking site. By conducting the screen at high ATP concentration, we could reduce the number of ATP-competitive compounds that score as hits. This strategy should allow for a lower threshold for hit selection, while reducing the risk of rediscovering weak ATP-competitive inhibitors. One potential advantage of this class of inhibitors over canonical ATP-competitive inhibitors is that, by targeting a pocket found in few other protein kinases, the potential for off-target inhibition of other kinases is reduced. While both ERK and p38 MAPKs have DEF interaction pockets, they recognize distinct sequence motifs, and related kinases such as JNK1 do not utilize DEF sites [35]. These observations suggest that it should be possible to identify small molecules that specifically target the DEF pocket of ERK. We expect that screens for small molecule inhibitors of the ERK1/2 docking site will generate novel scaffolds from which to build potent inhibitors with an expanded therapeutic window that ultimately may provide a new class of drugs to treat cancer.

Acknowledgments

We thank Peter Gareiss at the Yale Center for Molecular Discovery for assistance in assay development and screening. We thank Natalie Ahn for sharing coordinates for the ERK2-DEF peptide model. This work was supported by the Yale SPORE in Skin Cancer (National Institutes of Health grant P50 CA121974) and the Yale Center for Molecular Discovery pilot project program. C.J.M. was supported by National Institutes of Health training grant T32 GM007324.

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Figure 1.

Pharmacological targeting of the ERK pathway. A, ERK MAPK pathway scheme including feedback pathways disrupted by inhibitors. B, Structure of ERK2 showing interactions with docking sites. At left is a structural model of ERK2 bound to an extended peptide substrate (green) with an appended DEF motif (magenta)[18]. The DEF binding pocket is highlighted in yellow. At right is the structure of ERK2 bound to a D-site peptide derived from MKP-3 (green) on the face opposite from the substrate-binding cleft [56].

Figure 2. HTS assay to discover DEF docking site inhibitors of ERK1/2. A, Sequence of peptide substrate with a tethered DEF sequence (DEF peptide), negative control (FD peptide), and positive control product (PS peptide). B, Radiolabel assay showing DEF-site dependent phosphorylation of peptide. C, AlphaScreen assay scheme.

Figure 3.

HTS assay development. A, Simultaneous titration of AlphaScreen beads and product peptide, showing autoinhibition. B, Simultaneous titration of antibody and product peptide, with 20 μg/mL AlphaScreen beads. C, A 1 h kinase reaction at varying peptide concentrations with 1.4 mM ATP, 1 nM antibody, and 20 μg/mL AlphaScreen beads. D, Time course showing that a lower substrate concentration gives a more dynamic signal over time. Conditions used were as in panel C with 7.1 nM ERK2. E, An ERK2 titration showing that the signal is roughly linear at reduced ERK2 concentrations. Conditions were as in panel C with a 15 min reaction time and 28.6 μM peptide. F, Using optimized assay conditions (see text), WT and FD peptides show a large difference in signal. All error bars represent SD of at least 3 replicates. Curves in B and C fit to a simple, one site binding model $[Y = (B_{max} * X) / (K_d + X)]$, and D and E were fit to a line using GraphPad Prism.

Figure 4.

Assay pilot screens. A, Signals from control wells on the piloting plates, showing Z' factors (overall average = 0.66 ± 0.02) and signal/background ratios (overall average = 26 ± 2).

Error bars represent SD of 32 control replicates per plate. B, Pie chart reporting the number of ATP-competitive hits from a screen of 320 compounds in the Selleckchem Kinase Inhibitor Library under low ATP (71 μ M) conditions and the portion of these that passed the hit threshold (2σ) under high ATP (1071 μ M) conditions.