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Assays for tyrosine phosphorylation in human cells

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Abstract

Tyrosine kinases are important for many cellular processes and disruption of their regulation is a factor in diseases like cancer, therefore they are a major target of anticancer drugs. There are many ways to measure tyrosine kinase activity in cells by monitoring endogenous substrate phosphorylation, or by using peptide substrates and incubating them with cell lysates containing active kinases. However, most of these strategies rely on antibodies and/or are limited in how accurately they model the intracellular environment. In cases in which activity needs to be measured in cells, but endogenous substrates are not known and/or suitable phosphospecific antibodies are not available, cell-deliverable peptide substrates can be an alternative and can provide information on activation and inhibition of kinases in intact, live cells. In this chapter, we review this methodology and provide a protocol for measuring Abl kinase activity in human cells using enzyme-linked immunosorbent assay (ELISA) with a generic anti-phosphotyrosine antibody for detection.

Keywords

tyrosine kinase assay; Abl kinase; cell penetrating peptide; K562 cells; kinase substrate

1. Introduction

1.1 Therapeutic importance of tyrosine kinases.

Protein kinases are post-translational modification enzymes that transfer the terminal phosphate from ATP onto amino acid side chains of their substrates. While many nucleophilic side chains can be phosphorylated and have been observed in different biological contexts, serine, threonine and tyrosine are the most studied and best understood due to their chemical stability and broad biological importance. At last count there are more than 90 known protein tyrosine kinases and at least four times as many protein serine/threonine kinases (Manning, Whyte, Martinez, Hunter, & Sudarsanam, 2002). Protein tyrosine kinases perform a myriad of roles in living cells, including regulating growth and response to the cell's environment, stimulating or inhibiting proliferation, modulating cell adhesion and migration, and controlling cell decisions about death through apoptosis or other mechanisms (Hubbard & Till, 2000). Because of these important signaling roles,

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kinase dysregulation can have serious consequences for cellular regulation, and many kinases have been found to drive pathologies including cancer, autoimmune diseases, and neurodegeneration. The most well-studied example of this is the Philadelphia chromosome, which creates the BCR-ABL oncogene (Bartram et al., 1985). This mutation replaces the regulatory region of c-Abl with BCR, leaving Abl kinase constitutively active to promote immature hematopoietic progenitor cell proliferation in chronic myeloid leukemia (CML) (Druker, O'Brien, Cortes, & Radich, 2002). This kinase was the first to be successfully targeted with a small molecule inhibitor, imatinib a.k.a. Gleevec™, which revolutionized both cancer chemotherapy and cancer biology starting in about the 1990s (Druker et al., 2001; Druker et al., 1996). This has made kinases attractive targets for therapeutics, and indeed, billions of dollars are spent every year on drug discovery targeting kinases—in fact, by 2018 almost every major pharmaceutical company had a program in kinase inhibitor drug discovery (Carles, Bourg, Meyer, & Bonnet, 2018). Further, tyrosine kinases have been the most popular targets to pursue, perhaps due to their overrepresentation in the set of receptor kinases that are the first line of response mechanisms between the cell and its environment. Notably, the majority of FDA approved kinase inhibitors are for tyrosine kinases (Carles et al., 2018; P. Wu, Nielsen, & Clausen, 2015).

1.2. Importance of cell-based tyrosine kinase activity measurement.

While efforts to inhibit dysregulated kinases have shown therapeutic promise, there is still a large gap between drug discovery efforts and translation—only about 5% of kinase inhibitor leads ever end up in the clinic (Moreno & Pearson, 2013). Further, even for inhibitors that are approved and used in treatment, a significant portion of patients are inhibitor resistant, sometimes for reasons that depend on the particular kinase target, but sometimes for broader reasons including other kinase upregulation or more generic multidrug resistance mechanisms (Druker et al., 2006; La Rosee et al., 2004; Radich et al., 2006). Target-based resistance is often correlated with lack of target inhibition (Pratz et al., 2009; White et al., 2007), while target-independent resistance may be mediated by activity of other kinases or pathways (Hantschel, 2015; Lin et al., 2014; Noel et al., 2019; X. Zhang et al., 2017). While pharmacokinetics and drug delivery can be systemic issues, uptake of inhibitor into cells and the complicated signaling mechanisms that cells use to adapt to different environments can also have big effects on sensitivity to drugs cell-by-cell. Therefore, to better understand the signaling that is happening in response to inhibitors, it is important to measure activity of the target kinase in the context of its cellular environment. Enzyme activity and inhibitor binding are affected by the local environment. Cellular conditions such as salt concentration and macromolecular crowding can affect kinase enzyme conformational dynamics (Xiao, Liddle, Pardi, & Ahn, 2015), and factors like scaffolding proteins and other protein-protein interactions are also crucial (Meng, Pond, & Roux, 2017). This means that assays should either be cell-based, or assay conditions to measure kinase biology and/or inhibitor effects should mimic the native environment of the cell as closely as possible (R. Zhang & Wong, 2017).

1.3. Measuring tyrosine kinase activity with antibodies against endogenous, known substrates

The most common way of measuring kinase activity in cells in most laboratory settings is to use a phosphospecific antibody to detect phosphorylation levels of endogenous substrates. These can be detected with immunocytochemistry imaging or flow cytometry (PhosphoFlow or CyTOF) (Bendall et al., 2011; Irish, Kotecha, & Nolan, 2006; Irish et al., 2010; Sachs, Perez, Pe'er, Lauffenburger, & Nolan, 2005) in intact fixed/permeabilized cells, or from cell lysates by Western blotting. Antibodies against a different epitope on the substrate (for total substrate level) as well as a phosphospecific antibody can also be used with the methods above or with cell lysate-based read-outs, including standard enzyme-linked immunosorbent assays (ELISAs) in which the substrate is captured out of cell lysate onto a plate or beads using one antibody and the phosphorylation is detected with a second anti-phospho antibody. These can also be detected with a range of FRET/luminescent proximity assays such as DELFIA, AlphaScreen, Luminex, or other bead-based ELISA-type assays (Jia, Gu, Brinker, & Warmuth, 2008). These cell-based, phosphosite-specific antibody approaches have several advantages: the kinase reaction that is being analyzed occurred in the most native setting possible, in the context of the cell and with an inherently biologically relevant substrate. However, the key limitations of this approach are its requirements that a relevant substrate and phosphosite are already known, and that a sufficiently selective and sensitive antibody against that site is available. In practice, these limitations substantially reduce the scope of kinases that can be studied in this way. These approaches are further confounded by the redundancy built-in to many signaling pathways—many kinases can compensate for each others' loss either by re-routing signaling through their own substrates or by cross-phosphorylating other substrates, but the particular substrates and sites that are prone to this are not well elucidated. These issues make it difficult to unambiguously attribute phosphorylation of a particular site to a particular kinase, and impossible to measure kinase activity for those that don't have any sites known and/or antibodies available.

1.4. Measuring tyrosine kinase activity when substrates aren't known or antibodies aren't available

Proteomics approaches can be instrumental in measuring kinase activity in situations where suitable substrates are not known and/or no antibodies are available. Mass spectrometry-based proteomics of endogenous proteins is one of the most comprehensive ways to profile phosphorylation sites. Tyrosine phosphorylation is lower abundance than serine/threonine phosphorylation, and several optimized strategies have been reported for enriching and detecting tyrosine phosphopeptides (Abe, Nagano, Tada, Adachi, & Tomonaga, 2017; Rush et al., 2005; Yao et al., 2019). However, the existing tyrosine phosphoproteome of a cell does not necessarily provide a clear-cut description of which kinases were active, since, as described above, not all phosphorylation sites are exclusively modified by a single enzyme, and unexpected factors such as protein-protein interaction can enable phosphorylation even of sites that are poor fits to a kinase's preference motif. Further, the existing steady-state of phosphorylation under a given condition does not always allow the determination of active vs. inactive kinases, since protein phosphorylation dynamics are affected by other factors like phosphatase activity.

Measuring activity in a pulse-chase-type manner rather than inferring by existing phospho-modifications can be an alternative approach for identifying active kinases in cells. This has been done on single or small sets of substrates using cell lysate, for example with a fluorescent reporter for detection (Wang et al., 2010), or on a broader proteomic scale using a range of methods including metabolic labeling with stable isotope labeled ATP or thiophosphorylated ATP (Allen, Lazerwith, & Shokat, 2005; Molden, Goya, Khan, & Garcia, 2014). Activity can also be assessed on exogenous sets of substrates. Microarrays or mini-libraries of potential substrates can be exposed to cell lysates or immunoprecipitated kinase from cells, and inferences can be made about the activation of particular kinases from which substrates were phosphorylated. These arrays can be solution-based, e.g. peptides linked to cDNAs, enriched using generic anti-phosphotyrosine antibodies or other phosphoenrichment after the kinase reaction and analyzed by deep sequencing (Kozlov et al., 2012), or sets of known substrate peptides spiked into a cell lysate kinase reaction and analyzed by mass spectrometry e.g. via the KAYAK strategy (Kubota et al., 2009). More commonly, they are immobilized and analyzed by radioactive ATP or generic anti-phosphotyrosine antibodies (Y. Deng et al., 2014; Yang Deng & Turk, 2016; Lai, Winkler, Zhang, & Pelech, 2016). While these kinds of strategies are excellent for determining substrate specificity of kinases using e.g. immunoprecipitated or recombinant enzyme, they are less clear-cut when cell lysates are used, and so far have not shown clinical benefit in predicting tyrosine kinase inhibitor (TKI) treatment success (albeit in a small study that employed *in vitro* inhibitor treatment that may have been inconsistent with inhibitor levels *in vivo*) (Labots et al., 2018). On the other hand, studies measuring kinase activity in intact cells by monitoring an endogenous substrate via Western blot have shown a relationship between phosphorylation level and outcomes (White et al., 2007), supporting the idea that while they can be informative for some aspects of kinase biology, lysate-based kinase reactions may not model the drug/target interaction environment adequately enough to make clinical decisions.

1.5. Cell-based tyrosine kinase assays using exogenous substrates

In order to address the issues inherent in measuring kinase activity from cell lysate or immunoprecipitated kinase, we and others have developed strategies to deliver defined tyrosine kinase substrates to cells and monitor their phosphorylation in response to various perturbations that should activate or inhibit the kinase of interest (Figure 1). A classic example of this type of strategy uses genetically engineered substrate constructs that include a substrate sequence, a phosphoaffinity domain (such as an SH2 domain) known to bind to the phosphorylated form of the substrate sequence, and two fluorophores that can be brought together by that interaction to give a FRET signal (e.g. EGFP as donor and mCherry as acceptor) upon phosphorylation by active kinase (Jia et al., 2008). These methods work well when expression of the construct is straightforward and robust, however not all cells are amenable to that type of workflow. As an alternative, substrate peptides can be conjugated with cell penetrating peptides and delivered to cells without the need for expression of a genetically engineered sensor construct. Phosphorylation of the substrate peptide can then be measured using many types of read-outs, including single cell electrophoresis (Soughayer et al., 2004; Turner et al., 2016), mass spectrometry (Placzek, Plebanek, Lipchik, Kidd, & Parker, 2010; Yang, Eissler, Hall, & Parker, 2013), fluorescence lifetime imaging

(Damayanti, Parker, & Irudayaraj, 2013), or generic antiphosphotyrosine antibodies (Lipchik, Killins, Geahlen, & Parker, 2012; Ouellette, Noel, & Parker, 2016), depending on the particular substrate design and application. A unique advantage of these kinds of synthetic substrates is the ability to incorporate non-natural amino acids and other chemical moieties that would not be compatible with genetic encoding into sensor proteins, such as D amino acids to improve peptide stability (Proctor, Wang, Lawrence, & Allbritton, 2012a) or constrained phosphotyrosine analogs that are resistant to dephosphorylation (Turner et al., 2016). In this chapter, we present a method to measure tyrosine kinase activity using an exogenous kinase substrate taken up in live cells, followed by extracting the substrate and quantifying phosphorylation by ELISA. We also describe the considerations that should be taken into account when designing such an assay using a peptide substrate, such that others might pursue this approach when appropriate to their biological questions and system of study.

2. Experimental design considerations for cell-based kinase assays using delivered synthetic peptide substrate

Cell-deliverable peptide substrate assays require consideration of a number of factors that may be different than in traditional kinase assays. The principles underlying the design choices and controls to address these factors, however, could inform and improve the rigor of traditional assays as well. In order to be confident in interpreting the data obtained, specificity of the substrate used and inclusion of proper positive and negative controls are crucial. The substrate needs either to be selective for the kinase of interest, or a solid understanding of and strategies to address off-targets must be in place. Without those assurances, the source of the signal obtained is not guaranteed and could be an artifact of other pathways upregulated in the cells. Even with these controls and considerations, selectivity caveats often remain and should be remembered when interpreting the results. Ultimately, optimizing these assays is a balancing act (Figure 2), and the following guiding questions should be addressed by any researcher designing or implementing these assays:

- Specificity/selectivity
 - Will the target kinase phosphorylate it efficiently?
 - Will other kinases phosphorylate it?
 - Can those two things be distinguished?
- Uptake, localization/distribution
 - Can it access the target kinase?
 - Can it get into the relevant subcellular compartment?
- Stability
 - Will it be degraded by protease(s)/peptidase(s) in the cell?
 - Will it be dephosphorylated by phosphatase(s)?
- Physiological relevance

- Is the substrate phosphorylated in response to known stimuli of the target kinase?
- Does the substrate interfere with endogenous signaling of the target kinase?

While some trade-offs between convenience and the above issues may be necessary and limitations should always be kept in mind, attention to these factors will provide the best chance of obtaining relevant results. We will discuss below several of the key factors to delineate when designing this type of experiment.

2.1. Synthetic peptide substrates: sequence selection and design

2.1.1. Substrate sequence—The kinase substrate peptide chosen for this type of assay is the most important component of the design, and may be the most challenging part to select. The sequence needs to be efficient enough that it is fairly rapidly phosphorylated in cells, but also selective enough that the experimenter can have confidence that is not phosphorylated appreciably by other tyrosine kinases in the cells during the course of the assay. This does not always mean that no other kinase can phosphorylate it at all, but rather that there is a sufficient difference in the rate of phosphorylation by the target kinase vs. other kinases expressed in the same cells. While not impossible to achieve, this is a challenge for tyrosine kinases, which often share similar substrate preference motifs. Substrate peptides derived from native sequences are often a poor choice on their own, since the efficiency and specificity of their phosphorylation is typically driven by their protein context (e.g. scaffolding or protein-protein interaction domains). There are many native sequences that are not phosphorylated at all when tested as isolated peptides. Combinatorial screening has identified a number of useful sequences for efficient assays for many kinases, however many of these are not sufficiently specific for cell-based use, since screening strategies rarely counter-screen for off-targets. We have employed a combination prediction/design workflow using known substrate information that may be useful to others in identifying a sequence to use, but for which specificity would still need to be determined on a peptide-by-peptide basis (Lipchik et al., 2015; Perez, Blankenhorn, Murray, & Parker, 2019). Accordingly, there is no one-size-fits-all solution, and researchers will need to put time and effort into identifying the best substrate for their kinase target and cell-based application. Alternatively, it may be simplest to use a substrate already characterized by others if one is available, such as those we have previously reported for Abl and Syk family kinases (Damayanti et al., 2013; Lipchik et al., 2012; Ouellette et al., 2016; Placzek et al., 2010). Overall, while identifying a suitable substrate is not trivial and requires an empirical process, ultimately it pays off in ease-of-use and can provide clear, interpretable results in cell-based assays.

2.1.2. Cell-penetrating peptide sequence or other cell delivery method—

Peptide substrates can be delivered to cells in a number of ways. Studies have used microinjection (Meredith, Sims, Soughayer, & Allbritton, 2000) and cell-penetrating peptide (CPP) sequences such as the TAT sequence derived from an HIV protein (-RKKRRQRRR-) (Placzek et al., 2010; Soughayer et al., 2004). Other general CPPs are also available (Copolovici, Langel, Eriste, & Langel, 2014), including poly-arginine (-RRRRRRRR-),

penetratin, model amphipathic peptide (MAP), or constrained versions such as stapled or cyclized CPPs (Bode & Lowik, 2017). Consideration of uptake mechanism may be useful in deciding which type to use. It was initially believed that internalization of CPPs was mediated almost exclusively via clathrin mediated endocytosis (Thoren et al., 2003). In subsequent years, it became clear that both endocytosis and direct translocation (via inverted micelle formation) across the plasma membrane can occur at the same time (Guterstam et al., 2009). In addition, recent studies suggest that a receptor-mediated entry is not ruled out for some CPP-conjugates (Ezzat et al., 2012). In any case, the uptake and distribution of the substrate-CPP (or other delivery tag) peptide needs to be characterized before a new substrate is used in a cell-based assay, and anytime a previously developed substrate is used in a new setting or cell line. Methods based on labeling e.g. fluorescence microscopy, flow cytometry (or even radiolabeling) have been used to quantify cellular uptake of CPPs. Mass spectrometry enables direct quantification of peptides inside the cells by inferring the amount of peptide per μg of cell lysate analyzed (Burlina, Sagan, Bolbach, & Chassaing, 2005; T. Y. Yang et al., 2013). Indirect methods such as detection of the biological activity of the cargo (Holm, Andaloussi, & Langel, 2011) have also been employed, but are not ideal. In our hands, TAT has been sufficient for most applications, however CPP uptake and intracellular distribution are cell-type specific and, as for the substrate sequence, need optimization per application.

2.1.3. Linkers and spacer segments—Spacer residues or other linker components can be incorporated into the substrate design in order to maintain distance between the biochemically relevant portion of the substrate (the kinase substrate sequence) and tags/CPP segments. We often use short stretches of glycines for this purpose (Damayanti et al., 2013; Lipchik et al., 2012; Placzek et al., 2010; D. Wu, Sylvester, Parker, Zhou, & Kron, 2010).

2.1.4. Affinity capture tag—Depending on the read-out method that will be employed in the assay, an affinity capture tag may be beneficial. For single cell read-outs (Damayanti et al., 2013; Meredith et al., 2000; Sims & Allbritton, 2003; Souhayer et al., 2004), analysis is typically via capillary electrophoresis or imaging, for which a fluorescent tag is more appropriate (as described below). However, for any more bulk-scale analyses involving lysis of cells, affinity enrichment of the substrate after cell lysis can greatly enhance the signal to background in the analysis, and/or enable the use of a generic anti-phospho antibody (which gets around the challenges associated with availability of site-specific antibodies for novel substrate probes). Biotin is one of the most straightforward tags to employ for this purpose, since it is easily incorporated during peptide synthesis using commercially available monomers (e.g. Fmoc-biotinyl-*L*-lysine) or via conjugation to e.g. cysteine using biotin-maleimide, and its extremely high affinity for avidin can be exploited using a wide range of streptavidin- or avidin-coated surfaces and reagents. Other tags such as the peptide Strep-tagTM (Skerra & Schmidt, 2000) (AWRHPQFGG) can also be used as streptavidin affinity modules and are easily incorporated during synthesis. These may also be preferable over biotin itself for read-outs such as mass spectrometry, for which elution is required prior to analysis—they can be eluted from the affinity media (e.g. polymer resin or magnetic nanoparticles) via mild denaturation or competed off using biotin, whereas biotinylated peptides are very difficult to fully elute due to the extremely high affinity of the biotin/

streptavidin interaction. Regardless of which affinity capture tag is chosen, the kinase substrate linked to the tag should always be evaluated via *in vitro* assays prior to employing it in a cell-based assay, to ensure that placement of the tag does not disrupt the biochemical performance of the substrate. Generally, depending on the bulk of what is attached on either end, we have found that kinase recognition is most efficient when the substrate portion makes up the free N-terminal portion of the peptide (D. Wu et al., 2010)—however, if needed, different tag positions and arrangements (C-terminal vs. N-terminal to the substrate portion, distance away from the substrate portion separated by e.g. polyglycine linker, etc.) can be tested.

2.1.5. Fluorescent labeling or conjugation with other probes—Labeling for characterization of uptake, distribution, and in some cases phosphorylation read-out is beneficial in cell-deliverable kinase substrate peptide design. Labels can be incorporated during synthesis, e.g. by coupling 5-carboxyfluorescein (5-FAM) or by adding a cysteine to the peptide and subsequently conjugating a fluorophore-maleimide (e.g. Cy5-maleimide). As for other additions to the peptide, the full peptide with fluorophore or other labels should be validated *in vitro* to make sure the additional components have not affected the biochemical efficiency or recognition of the substrate overall.

2.1.6. Other considerations: stability of substrate and product—Degradation by peptidases and proteases is a significant issue to consider in cell-based peptide substrate assays, and is dependent on cell type and peptide sequence. The TAT sequence in particular is highly prone to degradation, since it consists of mostly R and K residues, which serve as recognition sites for many proteases. One possibility is to protect the peptides through alternative formulations, similar to those employed for *in vivo* protein therapeutics. Recent studies have investigated the stability and activity of TAT sequences anchored to micelles and liposomes against proteolytic cleavage in EL-4, HeLa, and B16-F10 cells. Inhibition of TAT degradation occurred when sequences were modified by the addition of longer PEG-PE blocks, indicating an effective shielding of TAT from proteolysis by these blocks (Koren, Apte, Sawant, Grunwald, & Torchilin, 2011). In some cases, however, TAT degradation may be beneficial. TAT has the potential to direct localization of peptide cargoes, which may be undesirable for reaching the target kinase—therefore, early studies on cell-deliverable peptide substrates used disulfide chemistry to enable the substrate peptide to detach from TAT upon entering the cell (Soughayer et al., 2004). We have also found that C-terminal TAT sequences are degraded quickly in a human chronic myeloid leukemia (CML) cell line (K562), but that the substrate and other biochemically relevant portions of the sequence persisted for longer, enabling measurement of kinase activity independent of any effects that might come from TAT's effects on localization (T. Y. Yang et al., 2013). In order to prevent degradation of the substrate portion, the Allbritton and Lawrence labs have designed substrates incorporating unnatural amino acids that retain reasonable biochemical efficiency (Proctor et al., 2012a; Proctor, Wang, Lawrence, & Allbritton, 2012b; S. Yang et al., 2013). Another issue (discussed further in section 2.2.2.1 below) is that phosphatases typically can act on the phosphorylated products in the cell. Again, depending on the cell type and conditions, phosphatase activity may outstrip kinase activity and make detection of any phosphorylated product challenging. One option is to use phosphatase inhibitors (as

described below in section 2.2.2.1); a recent alternative was reported by the Allbritton and Lawrence labs using an unnatural tyrosine analog that is intrinsically resistant to dephosphorylation (Turner et al., 2016). Overall, taking these factors into account and using chemical biology principles to design cell-based substrates is a promising avenue for increasing stability, with the caveat that each substrate would still need to be individually validated as efficient and specific enough to the target kinase.

2.2. Biological system characterization and considerations

2.2.1. Cell lines or primary cells—Cell lines and primary cells require some different considerations for these types of assays. Cell lines are widely used by researchers as they are highly proliferative and easier to culture and transfect. Most cell lines have been in culture for decades and are well adapted to the two-dimensional culture environment. However, they are often less biologically relevant, since they have lost the true characteristics of the original tissue from which they were isolated. Serial passaging is known to cause genotypic and phenotypic variation in cell lines (Alge, Hauck, Priglinger, Kampik, & Ueffing, 2006; Pan, Kumar, Bohl, Klingmueller, & Mann, 2009). Signaling pathways can be significantly affected by these types of changes, and by the lack of physiological microenvironment around cultured cells (especially in 2D). In addition, misidentified and contaminated cell lines can be an issue, so cell lines should be obtained from reliable sources and verified using e.g. STR profiling prior to use (ASN-, 2010; Lorsch, Collins, & Lippincott-Schwartz, 2014). Nevertheless, cell lines are still a mainstay of biological signaling research because they are easy to use and maintain. Primary cells are isolated directly from tissues, so they often better model physiologically-relevant signaling biology—however they have a finite lifespan and limited expansion capacity. Primary cells can better mimic normal cell morphology and maintain many of the important markers and functions seen *in vivo* (Alge et al., 2006; Pan et al., 2009). Endothelial cell lines, for example, lack various functional markers, while primary endothelial cells retain these critical features. On the downside, they are extremely sensitive and require specialized growth media for optimal cell survival and growth. Traditionally, serum is included in cell culture media to provide the growth factors, hormones, lipids and other nutrients to support cellular growth. In case of primary cells, however, high serum levels can lead to differentiation or promote growth of other cells from the impure tissue isolate like fibroblasts. Other practices, such as seeding primary cells on more physiologically relevant substrates instead of synthetic polymers, or 3D cell culture conditions, can significantly improve primary cell attachment, growth, and purity. When performing primary cell culture, researchers may seek to examine the unique clinical parameters of the individual donor, not just the cells. Several factors such as age, medical history, race, and sex can be considered when building an experimental model. With a growing trend towards personalized medicine, such donor variability and tissue complexity can only be achieved with primary cells and are difficult to replicate with cell lines. Cells isolated from patient-derived xenograft (PDX) tumors may represent a “middle ground” between cell lines primary cell culture. PDX tumors are formed by subcutaneously implanting a small fragment of primary tumor into an immunocompromised mouse (Garber, 2009). PDX cells can then be passaged over time through serial animals to form a renewable tissue resource. PDX models recapitulate the human tumor microenvironment and maintain both the gene-expression and genomic profiles as well as the morphology of the original

human tumor (Hidalgo et al., 2011; Proctor et al., 2014). Detailed IHC and allelotyping analysis of PDA-derived xenografts have demonstrated that the degree of differentiation and nuclear polymorphisms in the original tumor sample are maintained immediately after establishment and following serial passage from one nude mouse to another (Hahn et al., 1995). Accordingly, PDX cell cultures may provide useful models for studying signaling using cell-deliverable tyrosine kinase substrates.

2.2.2. Positive and negative controls for kinase activity—Following the choice of biological system, it is crucial to establish conditions to provide a positive control where the kinase is as active as possible and substantial phosphorylated product can accumulate, and a negative control where the kinase is inactive or not present. This provides the right framework to interpret phosphorylation of the cell-deliverable substrate in the context of other possible off-target kinase activities in the cells.

2.2.2.1. Inducing kinase activation and/or preventing dephosphorylation to provide maximal positive signal: In many cases, conditions to induce kinase activation will be necessary, and will depend on the system to be studied. In some cases, a native pathway can be exploited to stimulate the target kinase's activity. Receptor kinases can be activated by their ligands, such as growth factors or small proteins, which induces receptor crosslinking and activation (Hubbard & Miller, 2007). Non-receptor kinases can be activated by a variety of different stimuli, but usually an upstream receptor can be activated to induce activity (Gocek, Moulas, & Studzinski, 2014). For example, the nonreceptor kinase Syk can be stimulated in B-lymphocytes by the addition of anti-IgM (Peters, Furlong, Asai, Harrison, & Geahlen, 1996). The anti-IgM causes aggregation and activation of the B-cell receptor, initiating downstream signaling that leads to the activation of Syk (Keshvara, Isaacson, Harrison, & Geahlen, 1997; Peters et al., 1996). In some cases, an activator may not be needed, such as when cell lines contain a constitutively active kinase. For example, the chronic myeloid leukemia (CML) cell line K562 is characterized by the constitutively active fusion protein BCR-ABL (Grosveld et al., 1986). As we have previously demonstrated, it is possible to measure BCR-ABL activity in K562 cells using a peptide substrate without any additional stimulation (Ouellette et al., 2016; T. Y. Yang et al., 2013). Additionally, in many cellular settings, inhibition of phosphatases will also be critical and can assist in verifying phosphorylation of the substrate. Many phosphatases are thought to have broad specificity (Fontanillo & Kohn, 2016), and while there do seem to be more complex factors governing phosphatase substrate targeting that originally thought (Powers, Melesse, Eissler, Charbonneau, & Hall, 2016; Rowland, Harrison, & Deeds, 2015), they are clearly able to dephosphorylate peptide kinase substrates in cells. This has been observed by 1) reduction in apparent phosphopeptide signal upon kinase inhibitor addition (Damayanti et al., 2013), 2) increase in phosphopeptide signal upon incubation with phosphatase inhibitor (Lipchik et al., 2012; T. Y. Yang et al., 2013), and 3) stabilization of phosphopeptide signal when using a phosphatase-resistant tyrosine analog in the substrate (Turner et al., 2016). For cell-based tyrosine kinase assays, pervanadate can be used as a general phosphatase inhibitor, however it is toxic to most cells and so incubation time should be kept short (<5–10 min). Typically, in practice, it is best to pre-incubate the cells with substrate for an optimized time (as discussed in section 2.2.3.2) followed by a final pulse with pervanadate approximately 5 min

before lysing the cells for analysis. It also may be possible to narrow down the specific phosphatase responsible for peptide substrate dephosphorylation by using more specific inhibitors (Fontanillo & Kohn, 2016).

2.2.2.2. Inhibiting or knocking down kinase activity as a negative control: When using cell-deliverable kinase substrates, it is also important to establish a system where the kinase is inactive. This is necessary for evaluating the specificity and selectivity of the substrate for the kinase of interest in the chosen cell context. This can be done using knock out cell lines or specific kinase inhibitors. Knock out cell lines can be generated by a variety of techniques, from RNAi to CRISPR (Smith et al., 2017). However, the generation and validation of these cell lines can in itself be a time-consuming process. An alternative approach is to use an inhibitor specific to the kinase of interest, if available. This is quicker than generating a new cell line or establishing RNAi knockdown, albeit some optimization of inhibitor concentration and incubation time is always needed. However, off-target effects of inhibitors must be considered as well: few (if any) inhibitors are fully selective to a single kinase (Anastassiadis, Deacon, Devarajan, Ma, & Peterson, 2011; Davis et al., 2011), which is a major caveat to using inhibitors as negative controls in these kinds of experiments. If inhibitors are used, the potential for off-target effects must be carefully evaluated.

2.2.3. Optimizing cell deliverable substrate incubation: concentration and time—In order to obtain sufficient signal within a reasonable amount of time that reflects the activity of the kinase in the cells as best as possible, it is important to identify optimal cell-deliverable peptide substrate concentration and incubation time. Ideally, concentration is as low as possible in order to avoid disruption to the native signaling of the kinase in the cells (by competing with other substrates for kinase occupancy, and/or disrupting kinase activity via substrate or product inhibition), and incubation time is as short as possible to balance against degradation and capture the kinase activity in a defined window of time.

2.2.3.1. Concentration: Uptake of cell-deliverable peptides will vary depending on the CPP, properties of the cargo substrate, cell type and culture conditions (among other experiment-specific factors), therefore there is no specific setpoint at which to start, however we have found that testing concentrations in the μM range is a good place to start. Higher concentrations of peptide inside the cell may not be desirable, since peptides could disrupt signaling if the kinase is saturated with substrate. For example, we found that in HEK293 cells expressing an engineered Abl kinase construct, $\sim 25\text{--}50\ \mu\text{M}$ was optimal, whereas $>50\ \mu\text{M}$ and longer times resulted in apparent kinase inhibition (Placzek et al., 2010). The intracellular concentration is not easy to determine—the fraction of peptide taken into cells relative to its media concentration is not predictable *de novo* and may not scale linearly with media concentration, therefore intracellular will need to be determined on a case-by-case basis when possible. In our experience, for example, incubation of K562 cells with $25\ \mu\text{M}$ cell-deliverable Abl substrate resulted in $\sim 30\text{--}50\ \text{fmol}$ of total peptide detected per μg of cell lysate, which corresponded to approximately 10,000 cells. Estimating that each K562 cell has a volume of $\sim 5\ \text{pL}$ (Jiang et al., 2016), this would correspond to low μM intracellular concentrations (T. Y. Yang et al., 2013), which is in the range of the K_M of that substrate, however a) this is likely specific to this peptide and cell type and b) the distribution of

concentration may not be uniform across the cell. In other experiments, we found that 1–2.5 μM Abl substrate in K562 cells provided sufficient fluorescent lifetime microscopy (FLIM) signal or ELISA signal. The Abl substrate we used is particularly biochemically efficient for Abl kinase, and does not disrupt the CPP activity. Kinase substrates with more acidic residues may exhibit reduced uptake due to ionic interactions between e.g. the TAT sequence and the substrate sequence, so may require higher concentrations. Overall, we recommend starting with titrating 1–100 μM cell deliverable substrate in media and narrowing the optimal concentration from there. “No substrate” controls should always be incorporated in the experiment, in order to ensure the signal detected is sufficiently above background from the cell lysate itself.

2.2.3.2. Incubation time: Optimal incubation time depends both on the properties of the cells and cell-deliverable substrate and on the kinase activity measurement that is being performed. Biochemically efficient substrates that are readily taken up by cells can produce sufficient signal to detect phosphorylated product by ELISA within 5 min of incubation, which is very advantageous for cell types that have high degradation activity as well. On the other hand, in some cases if the peptide is stable enough, incubation with cells can be 15 min to >1 hour, allowing for thorough uptake and distribution before stimulating the cells to activate the target kinase. As for concentration, incubation time should be varied to identify the minimal time point at which signal from a positive control is strong enough to reliably distinguish from negative control and no-substrate background.

3. Measuring Abl kinase activity in human CML cells (K562) with ELISA detection

3.1. Equipment

- 37°C, humidified incubator at 5% CO_2
- Plate reader with capabilities of measuring:
 - Absorbance at 280 nm
 - Absorbance at 590 nm
 - Fluorescence at 590 nm after excitation at 532 nm
- Microcentrifuge tubes
- 15 and/or 50 mL conical tubes
- Vortex
- Graduated cylinders
- Weigh paper
- Analytical balance
- pH meter
- Cell culture flasks, 6- or 12-well plates

- Probe sonicator
- 96-well clear plate
- Multichannel pipettors capable of 1–250 μ L
- Multichannel pipettor reagent reservoirs
- Pipettes capable of 0.5–1000 μ L
- Pipette tips for 0.5–1000 μ L
- Platform shaker
- Pierce™ Streptavidin Coated High Capacity Plates, Black, 96-Well (Thermo Fisher cat. # 15503)

3.2. Chemicals

- Cell-deliverable peptide substrate for Abl kinase:
EAIYAAPFAKKBGCGAPTYSPPPPGGRKKRRQRRRL (Placzek et al, 2010)
- Synthetically phosphorylated version of cell-deliverable peptide substrate (positive control for antiphospho-antibody specificity)
- Phosphate buffered saline (PBS)
- Urea
- Thiourea
- Dithiothreitol (DTT)
- Triethylammonium bicarbonate (TEAB)
- Complete™, EDTA-free Protease Inhibitor Cocktail (Sigma-Aldrich cat. #11873580001)
- Phosphatase inhibitor (Thermo Scientific cat. #88667)
- Acetonitrile
- Sodium orthovanadate
- Hydrogen peroxide, 30%
- Inhibitor for kinase-of-interest (optional)
- Bovine serum albumin (BSA) (MilliporeSigma cat. #A7906)
- Ultrapure deionized water
- Advanced Protein Assay Reagent 5x (Cytoskeleton cat. #ADV01-A)
- Sodium chloride
- Tris-Base (e.g. Fisher Scientific cat. #BP152)
- Tween-20

- Monobasic sodium phosphate
- Mouse 4G10 anti-phosphotyrosine antibody (EMD Millipore cat. #05–1050)
- Anti-mouse HRP-conjugated antibody (Abcam cat. #ab6728)
- Amplex Red reagent (Thermo Fisher Scientific cat. #A12222)

4. Protocol

4.1. Stock solutions (specific sources for key components are provided in section 3.2. Chemicals)

1. 20X Cell-deliverable peptide substrate (can be aliquoted and stored at -80°C for most peptides)
 - a. 1 to 5 mM peptide (depending on concentration)
 - b. PBS, pH 7.2
2. Pervanadate
 - a. 50 mM hydrogen peroxide
 - b. 10 mM sodium orthovanadate
 - c. Ultrapure deionized water
3. Urea Lysis Buffer, pH 8.0
 - a. 7 M urea
 - b. 2 M thiourea
 - c. 4 mM DTT
 - d. Protease inhibitor
 - e. Phosphatase inhibitor
 - f. 20% acetonitrile
4. 10X Tris-buffered saline (TBS), pH 7.6
 - a. 1.5 M NaCl
 - b. 0.25 M Tris-Base
 - c. Ultrapure deionized water
5. Blocking Buffer (suggest preparing 250 ml per 96-well streptavidin plate to be used)
 - a. 1X TBS
 - b. 0.05% Tween-20
 - c. 1% BSA or 5% fat-free milk powder
6. Sodium Phosphate Buffer, pH 7.4

- a. 50 mM monobasic sodium phosphate
 - b. Ultrapure deionized water
 - c. HCl to appropriate pH
7. Primary antibody dilution (prepare 100 μ l per ELISA well)
 - a. 1 part mouse 4G10 anti-phosphotyrosine antibody
 - b. 5000 parts blocking buffer
8. Secondary antibody dilution (prepare 100 μ l per ELISA well)
 - a. 1 part anti-mouse HRP-conjugated antibody
 - b. 1000 parts blocking buffer
9. Developing buffer (prepare 100 μ l per ELISA well)
 - a. 100 μ M Amplex Red reagent
 - b. 2.22 mM hydrogen peroxide
 - c. Sodium phosphate buffer

4.2. Cell culture

Culture conditions for the cells to be used in the assay are extremely important to measuring relevant signaling. Cells must be in the middle of log-phase of growth; we have found that cells that have not yet reached log phase or that have already reached their maximum density, and/or that have used up all the nutrients in their media, will not give reproducible signaling in these types of assays. Typically for ELISA detection from a suspension cell assay, $\sim 0.5\text{--}1 \times 10^6$ cells are needed per experimental replicate, per condition or timepoint. We recommend no fewer than triplicates be performed for each condition, so it is important to estimate in advance how many cells you will need overall for an experiment. Plan for conditions to include:

- “No substrate” controls to standardize the background in the ELISA detection
- Experiments with and without appropriate kinase activation conditions (if necessary)
 - With and without phosphatase inhibitor
 - With and without kinase inhibitor
 - ◆ Using DMSO (or other vehicle) controls in the “without” kinase inhibitor experiments
- During optimization:
 - Experiments with concentrations of substrate between $\sim 1\text{--}100 \mu\text{M}$
 - Experiments that can accommodate removal of up to 3–6 aliquots at different incubation timepoints

Cells should then be serially cultured and expanded until sufficient cell numbers are available for the assays.

Tip: The cell number may need to be optimized for each particular cell type you want to use; K562 cells are suspension cells, adherent cells may require different culture handling. Log-phase of growth can be determined by counting cells daily and plotting cell number per day. Signaling behavior for tyrosine kinases in cultured cells can be examined by Western blot with generic anti-phosphotyrosine antibody (e.g. 4G10, Millipore). We have found that $\sim 0.8\text{--}0.9 \times 10^6$ cells/ml is the inflection point at which K562 cells leave log-phase, and that overall tyrosine kinase signaling is relatively stable between 0.6 and 0.8×10^6 cells/ml, so cells should be harvested at $\sim 0.7 \times 10^6$ cells/ml to use in a cell-deliverable substrate assay.

4.3. Cell deliverable substrate assay

1. Culture human CML K562 cells to sufficient cell numbers at log phase growth as described in the *Cell Culture* section above.
2. The day of the experiment, prepare the cell-deliverable Abl substrate and pervanadate stock solutions. Pervanadate must be prepared fresh every time and should be kept on ice. Prepare PBS and lysis buffer and also keep on ice.

Tip: Lyophilized peptides are difficult to handle and weigh accurately at sub-10 mg amounts. Absorbance at 280 nm can be used to determine peptide stock concentration more precisely by taking advantage of aromatic residues, such as tyrosine or tryptophan. Peptide extinction coefficients can be estimated using online calculators (e.g. <https://web.expasy.org/protparam/>).

3. Prepare triplicate assay wells as shown in the plate map below, including no substrate controls, experimental wells, pervanadate treatment, kinase inhibitor (imatinib), and DMSO controls.

Tip: When designing these types of experiments reagents to be tested (such as inhibitors, siRNA, or DMSO controls) can be pre-incubated with the cells in a culture flask in bulk, or set up in a 6- to 12-well tissue culture plate or deep-well 96-well filter plate (Ouellette et al., 2016) at 2X concentration in media, depending on the number and nature of conditions to be tested, volume of media, and number of cells being used in the assay. Imatinib stock concentration is designed to result in 0.05% v/v DMSO in each assay, thus an equivalent volume of DMSO is used as a vehicle control in non-inhibitor wells. For experiments testing multiple conditions, deep-well 96-well filter plates (which can hold up to 1 ml per well) can facilitate reproducible handling. When using multi-well plates, the layout of conditions should be randomized in order to avoid systematic biases in plate position.

4. Immediately prior to setting up the experiment, count cells, condense by centrifugation ($600 \times g$) and bring up in media to 2X the concentration needed for the assay (for this implementation, use 2×10^6 cells/ml, for a final assay concentration of 1×10^6 cells/ml). Distributed 0.5 ml cells into the wells of the assay plate to give final concentrations of 1X for cells and pre-treatment reagents (inhibitor, vehicle control).

Tip: For time-course experiments, assays should be set up with larger volumes in culture flasks or 6- to 12-well plates, enabling the removal of aliquots containing $1-8 \times 10^6$ cells at each timepoint.

5. Incubate covered plate at 37°C in cell culture incubator with 5% CO_2 on shaker platform with gentle mixing for 1 hour to achieve sufficient kinase inhibitor exposure.

Tip: While there should not be any flow through, if using a 96-well filter plate, place the plate on top of a regular 96-well plate to capture any drips.

6. Add pervanadate from step 1 to relevant wells. Add cell-deliverable substrate (Abl substrate, $2.5 \mu\text{M}$ final concentration) to all wells (other than the no substrate control wells) to initiate the cell-based kinase assay. Pipette up and down to mix and return the plate to the shaker platform in the incubator for 5 min.

Tip: For short incubation times, as in this experiment, time spent pipetting needs to be efficient without compensating the accuracy and precision of the volume dispensed.

7. After 5 min incubation, transfer samples from wells to microcentrifuge tubes and pellet cells in a refrigerated centrifuge (4°C) at $600 \times g$ for 1 minute. Aspirate media and add 1 ml of ice cold PBS to each pellet to wash, gently mix, and repeat centrifugation and PBS aspiration for a total of two washes.

Tip: When using a 96-well filter plate, media and PBS washes can be removed using vacuum collection or centrifugation on a plate rotor into a standard 96-well plate.

8. Add ice cold urea lysis buffer to cells. Vortex or pipette up and down to mix and place on ice.

Tip: For every 10 million cells, add $160 \mu\text{l}$ lysis buffer added with a minimum volume of $30 \mu\text{l}$.

9. Hold samples on ice and sonicate each lysate with a probe sonicator (20 seconds with 30% power). Clarify lysate at 4°C by centrifugation at $7500 \times g$ for 15 min.

Tip: Ideally the probe sonicator should be set up in a cold room. If a probe sonicator is not available, samples should either be vortexed thoroughly or sheared by transferring up and down through a fine gauge needle. If using a 96-well filter plate, lysate is collected by vacuum or

centrifugation into a standard 96-well plate and no additional clarification is necessary. Take care to avoid cross-contamination of wells.

10. Measure protein concentrations using 1x Advanced Protein Assay from Cytoskeleton Inc. according to the manufacturer's instructions.

Tip: Other protein concentration assays can be used, however we find that bicinchoninic acid (BCA) based methods are the most reliable for the concentration range obtained in these experiments.

11. Use 100 µg aliquots of lysate for ELISA as described in section 4.4 below. Save remaining lysate for analysis by Western blot or other method, storing at -80 °C if necessary.

Tip: It is critical to use Western blot to validate pathway activation when first implementing a cell-deliverable substrate assay in a new setting.

4.4. Enzyme-Linked Immunosorbent Assay

1. Prepare primary and secondary antibody solutions. Dilute 4G10 anti-phosphotyrosine primary antibody 1:5000 in blocking buffer (described in section 4.1). Dilute anti-mouse HRP-conjugated secondary antibody 1:1000 in blocking buffer.
2. In the streptavidin plate: incubate wells to be used for analysis with 100 µl of blocking buffer with gentle shaking at 500 RPM for 1 hr.
3. For each sample, add 100 µg of lysate directly to the 100 µl blocking buffer already in the wells, and bring all wells to an equal volume with a minimal amount of urea lysis buffer. Incubate at room temperature with gentle shaking at 500 RPM for 1 hr.

Tip: Synthetically phosphorylated substrate can be used as a positive control for ELISA detection conditions by including triplicate incubation of synthetic phosphopeptide (~200 pmol/well) in blocking buffer in control wells alongside the lysate incubation. To provide controls for antibody specificity, also include triplicate incubation of synthetic substrate peptide (same as used as the substrate in the assay as described in step 2 of section 4.3 above), which should not give any signal from the 4G10 antibody. The "no substrate" control from the assay in section 4.3 above will also serve as an antibody specificity control since it contains no phosphorylated Abl substrate peptide.

4. Remove supernatant from wells (*see tip below*) and wash with 250 µl blocking buffer per well, pipetting up and down 15 times. Discard wash by aspiration or pouring into waste container, taking care not to cross-contaminate wells. Repeat for a total of 3 washes.

Tip: Supernatant containing 100 µg lysate can be collected and stored at 4 °C until analysis is complete, or discarded. After each step, ensure

liquid is completely removed from wells as best as possible, but do not leave the wells dry for long periods of time.

5. Add 100 μ l primary 4G10 antibody to each well and incubate at room temperature with gentle shaking at 500 RPM for 1 hr. Empty wells by pouring into waste and wash three times as described in step 4.
6. Add 100 μ l secondary HRP-conjugated antibody to each well and incubate at room temperature with gentle shaking at 500 RPM for 1 hr. Empty wells by pouring into waste and wash three times as described in step 4.
7. While secondary antibody is incubating with wells, prepare Amplex Red developing solution (described in section 4.1). After washing away excess secondary antibody, add 100 μ l developing solution to each well and incubate for 30 min in the dark.

Tip: Plates with developing solution can be protected from light by incubating in a drawer or wrapped in foil.

8. Use a plate reader to measure Amplex Red fluorescence signal according to manufacturer's instructions (excitation between 530–560 nm, emission 590 nm).

5. Data analysis and interpretation

To analyze the data from the assay described above, signal from the substrate-containing wells should be normalized to the background signal from wells that lacked substrate by calculating the fold increase in signal from substrate wells relative to background from antibody and cell lysate. These values can then be compared for A) baseline substrate phosphorylation in vehicle only cells vs. inhibitor-treated cells, B) phosphatase inhibitor-treated vs. non-phosphatase inhibitor-treated, and C) vehicle vs. kinase inhibitor in phosphatase inhibitor-treated cells. When multiple conditions are being compared, differences should be evaluated using ANOVA with multiple comparisons. Alternatively, multiple pairwise t-tests may be appropriate depending on the statistical design of your experiment, e.g. how many conditions are being compared. An outline of the analysis strategy is provided in sections 5.1 and 5.2 below. The scale of the difference is important—very small differences between substrate-containing and no-substrate cells are likely not meaningful, and most likely represent cells that had little/no active kinase or insufficient substrate concentration. Often the phosphatase-treated cells will give very strong phosphorylation signal—this indicates active kinases have phosphorylated the cell-deliverable substrate. However, if the phosphatase-treated cells do not show substantially decreased phosphorylation in the presence of kinase inhibitor or knockdown of the kinase, it is highly likely that the substrate is not selective enough to report the target kinase activity. In that case, either a different substrate design will be needed, or, if the “off-target” kinases can be identified and if they share a common inhibitor, the substrate may still have value as a sensor of that kinase inhibitor's activity.

5.1. Normalizing assay signal to background

- Calculate the mean fluorescence from the “no substrate” control wells.

- Divide all other wells' fluorescence values by the mean "no substrate" fluorescence to give fold signal over background.

5.2. Statistical analyses and presentation of results

- Enter values into tables using e.g. GraphPad Prism software.
- Analyze variability by calculating coefficients of variation (CVs) per experimental condition, and differences between conditions using ANOVA with multiple comparisons testing (e.g. Tukey's multiple comparisons test) to evaluate differences between particular pairs of conditions.
- Plot normalized fluorescence values per condition and show full range of variability
 - We recommend using box-whisker plots that also show each replicate as an individual point for full representation of the variability in the assay.
- Note: Degree of phosphorylation can vary from experiment to experiment, however we have found that CV for each condition is typically <20–30% in well-controlled cell-deliverable substrate assays with optimized conditions. Experiments exhibiting CVs >30%, or showing insufficient differences between conditions that should represent active kinase and conditions that should represent inactive kinase, most likely have not been successful and require further optimization of cell handling, peptide substrate choice, and/or peptide incubation conditions (time, concentration).

6. Summary

Overall, cell-based tyrosine kinase assays using cell-deliverable peptide substrates can be a useful way to measure kinase activity in intact cells without requiring knowledge of or antibodies for an endogenous substrate. The key features to develop in advance involve the substrate itself, designing it for the desired degree of specificity and selectivity to make it fit-for-purpose—once a suitable substrate is available, this approach could be accessible to any biomedical laboratory. With the simple read-out described here, ELISA with streptavidin-biotin enrichment and generic anti-phosphotyrosine antibody, this strategy can be readily implemented—and as long as the inherent caveats are taken into consideration, these assays can provide relevant measurements of active or inhibited kinase in live cells.

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Figure 1. Cell deliverable peptide kinase substrate design and assay strategies.

(A) Peptide kinase substrates for cell-based assays are designed with a kinase substrate sequence, a cell-penetrating peptide sequence, and other functionality to facilitate handling and read-out. (B) When incubated with cells, CPP-containing peptide substrates are taken up into the cell, phosphorylated according to the activation state of the kinase, and analyzed either using single cell or bulk cell approaches. Imaging can enable analysis of intact cells, while other methods require lysis and analysis of the cell contents.

Balancing act: Establish substrate and conditions in which on-target phosphorylation and peptide stability outweigh off-target effects and degradation

Figure 2. Balancing competing factors in cell-deliverable substrate kinase assay design.

In an optimal scenario, the delivered kinase substrate would be taken up and distributed easily by the cells, it would be stable enough to provide sufficient signal, and phosphorylated rapidly and efficiently by the desired kinase and slowly/poorly (if at all) by any others. There are a number of potential pitfalls to address, including issues of peptide/phosphopeptide stability and activity with the target kinase vs. others, all of which could lead to ambiguity in the measurement. Careful mitigation of pitfalls enables reliable, reproducible measurements of kinase activity.

Figure 3. Plate map and timeline for Abl kinase assay in K562 cells.

(A) Example plate map for 12-well plate with each of six conditions (including controls) in duplicate. Randomizing conditions around the plate will help avoid systematic biases from plate position, therefore additional replicates should shuffle these positions. (B) Experiment timeline for planning purposes.