



Quantitative methods for analysis of protein phosphorylation in drug development

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Most signal transduction and cell signaling is mediated by protein kinases. Protein kinases have emerged as important cellular regulatory proteins in many aspects of neoplasia. Protein kinase inhibitors offer the opportunity to target diseases such as cancer with chemotherapeutic agents specific for the causative molecular defect. In order to identify possible targets and assess kinase inhibitors, quantitative methods for analyzing protein phosphorylation have been developed. This review examines some of the current formats used for quantifying kinase function for drug development.

Expert Rev. Proteomics 1(3), (2004)

Normal cell growth is characterized by tightly regulated signal transduction pathways consisting of complex sets of co-ordinated cellular signals that modulate or alter cell function. In contrast to the normal cell, the defining feature of all neoplasms is deregulated cell growth. In addition, malignant neoplasms have the ability to invade normal tissue as well as metastasize to, and grow at body sites distant from the original neoplasm [1]. At the heart of deregulated cell growth observed in cancer cells are aberrant changes in signaling pathways controlling cellular growth, division, differentiation and apoptosis [2–4].

Protein kinases have emerged as important cellular regulatory proteins in many aspects of neoplasia. Genetic mutations in protein kinase-mediated signaling processes frequently occur in the initiating events that result in disruption of the normal cell signaling pathways. This creates a survival advantage for the cancer cell and allows it to ignore the usual control signals [1,5–10]. Protein kinases are enzymes that covalently attach a phosphate group to the side chain of tyrosine, serine, or threonine residues found in proteins. Phosphorylation changes the activity of important signaling proteins. By controlling the activity of these proteins, kinases control most cellular processes including metabolism, transcription, cell cycle progression, cytoskeletal rearrangement, cell movement, apoptosis and differentiation [11].

With the completion of the human genome sequence, it is estimated that there are approximately 500 protein kinases encoded within the genome [11,12]. This represents approximately 1.7% of all human genes [11]. Most of the 30 known tumor suppressor genes and more than 100 dominant oncogenes are protein kinases [13]. Somatic mutations in this group of genes play a role in a significant number of human cancers. Therefore, protein kinases offer an abundant source of potential drug targets at which to intervene in cancer.

The remarkable success of imatinib mesylate (Gleevec[®], Novartis) in the treatment of chronic myelogenous leukemia has stimulated every major pharmaceutical company to focus on developing kinase inhibitory drugs. Imatinib specifically inhibits the activity of the p210^{BCR-ABL} kinase that is formed during a chromosomal translocation characteristic of the disease [14]. Imatinib has also shown the ability to inhibit the protein kinases c-kit and platelet-derived growth factor receptor. These are causative factors in the development of gastrointestinal stromal cell tumors and metastatic dermatofibrosarcoma protuberans [15,16].

Protein kinase inhibitors offer the opportunity to target cancer chemotherapy to the specific causative molecular defect. It is therefore hoped that targeted therapy of this

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KEYWORDS:
pathway, phosphorylation,
protein kinase, signal
transduction

kind will result in more effective treatment with fewer negative side effects than those that are currently associated with generalized chemotherapy.

This review discusses some of the most commonly used methods as well as some of the newer formats for quantifying kinase activity for drug development.

Role of protein kinases in cancer & drug development

Several important signaling pathways and proteins have already been identified as significant in the development of a variety of cancers, and efforts to develop kinase-inhibiting drugs are at various stages of the clinical trial process (TABLE 1). One of the most widely studied targets is epidermal growth factor receptor (EGFR). Two thirds of all solid tumors derived from epithelial cells overproduce EGFR and its ligands [17]. Approximately half of the 130,000 cases of colorectal cancer diagnosed in the USA each year show over expression of EGFR [17]. Overexpression of EGF ligands and EGFR have been shown to promote cell proliferation and growth, metastasis, angiogenesis, inhibition of apoptosis, and resistance to standard cytotoxic therapies [18–23]. Inhibitors of EGFR can suppress these adverse effects while inducing either tumor stasis or regression. EGFR inhibitors in development include anti-EGFR monoclonal antibodies such as cetuximab (Erbix[®], Imclone Systems Inc.) and small molecule inhibitors such as

gefitinib (Iressa[®], AstraZeneca) and erlotinib HCl (Tarceva[™], OSI Pharmaceuticals). At least ten different drugs that target EGFR are currently in clinical trials [12,17].

The Ras family of small G-proteins play a crucial role in relaying signals from activated growth factor receptors such as EGFR to downstream members of several signaling pathways. Ras activation of the Raf/MEK/ERK pathway modulates the activity of nuclear transcription factors such as c-Fos, Jun, and AP-1, which regulate the transcription of genes involved in cell proliferation, a common oncogenic characteristic [12]. Ras mutations have been identified in 30% of all cancers [12,17,24–26].

The Raf family of kinases have shown significant involvement in cancer. This family of kinases triggers the MEK/ERK kinase pathway [27]. The Raf kinase family is composed of three related serine/threonine protein kinases, Raf-1, A-Raf, and B-Raf, all of which act as downstream effectors of the Ras kinase [28]. Mutations in kinase signaling proteins of the Raf/mitogen-activated protein kinase (MEK)/extracellular signal-regulated kinase (ERK) pathway have been described. B-Raf mutations are found in 66% of malignant melanomas as well as at a lower frequency in other cancers and constitutively mutated activated Raf can transform cells *in vitro* [12,17,27,29]. Raf may play a wider role in oncogenesis as it can be activated independent of Ras through protein kinase C- α and can promote expression of the multidrug resistant gene *MDR1* [30,31].

Table 1. Examples of kinase-inhibiting drugs and their clinical trial status.

Target	Drug	Type	Company	Regulatory status
EGFR	Erbix	MAB1	Imclone	Approved
	Tarceva	SMI2	OSI-Pharmaceuticals	Approved
	Iressa	SMI	AstraZeneca	Approved
	ABX-EGF	MAB	Abgenix	Phase II
	EMD72000	MAB	Merck	Phase I
	MDX447	MAB	Medarex/Merck	Phase I
	CI-1033	SMI	Pfizer	Phase II
	EKB-569	SMI	Wyeth	Phase I
	GW2016	SMI	GlaxoSmithKline	Phase I
	GW572016	SMI	GlaxoSmithKline	Phase II
Raf	PKI166	SMI	Novartis	Phase II
	ISIS5312	Anti-S3	Isis	Phase II
	L-779	SMI	Merck	Phase I
Mek	BAY 43-9006	SMI	Bayer	Phase II
	CI-1040	SMI	Pfizer	Phase II
PI3K	CEP-701	SMI	Cephalon	Phase II

EGFR: Epidermal growth factor receptor; PI3K: Phosphatidylinositol-3-kinase.

Raf activates the dual specificity serine/threonine and tyrosine kinases, MEK1 and MEK2, which then activate ERK1 and ERK2 [32]. As previously stated, ERK activates the transcription of genes involved in cellular proliferation through phosphorylation and activation of c-Fos, c-Jun and AP-1 transcription factors. While MEK has not been directly implicated as a cause of oncogenesis, it is a crucial point of convergence through which several different Ras-activated pathways act. This makes it an attractive target for anticancer drug development.

Phosphatidylinositol-3-kinase (PI3K) phosphorylates phosphoinositides that then bind Akt and phosphoinositide-dependent kinase (PDK)1, anchoring them to the cell membrane. PDK1 can then activate Akt by phosphorylation. PTEN, a tumor suppressor phosphatase, is a negative regulator of Akt. The PI3K/PDK1/Akt pathway has also been experimentally implicated as playing a major role in oncogenesis [33,34]. The PI3K, PDK1 and Akt kinases are important in the regulation of cell survival and proliferation, most notably by decreasing the cell's ability to respond to apoptosis. Growth factor receptor tyrosine kinases, integrin-dependent cell adhesion molecules, and G-protein coupled receptors (GPCRs) activate PI3K either directly or indirectly through adapter molecules. Loss of PTEN, amplification of PI3K and overexpression of Akt are common to many malignancies [33]. Furthermore, overexpression of the PI3K/Akt pathway is one of the mechanisms responsible for resistance to the EGFR family inhibitors AG1478 and trastuzumab (Herceptin[®], Genentech) [35,36]. Thus the PI3K/Akt pathway is also an attractive target for anticancer drug development as successful agents may inhibit proliferation, and reverse both the repression of apoptosis and the resistance of cancer cells cytotoxic therapy.

It is clear that disruption of the function of signaling pathways, particularly those that involve phosphorylation cascades, has a strong relationship to the development of cancer. Dysfunctional phosphorylation cascades also play a role in other disease processes including diabetes, Alzheimer's disease and Parkinson's disease [17]. Therefore, the ability to quantitatively monitor the status of these pathways may provide information as to the disease process, the identity of potential drug targets, and drug responsiveness in an individual patient. Therapeutics targeted at inhibiting specific protein kinases and their ability to phosphorylate their targets require quantitative methods to evaluate the efficacy of new drug candidates.

Kinase & signaling pathway assays in drug development

Assessment of kinase inhibition due to candidate drug compounds can be performed in a variety of formats depending upon the number of drug compounds to be screened and the criteria desired for hit selection. In the past, radiometric assays such as scintillation proximity have been used for high-throughput screening (HTS). However, radiometric methods have largely been replaced by approaches employing fluorescent measurements.

One of the factors driving the development of fluorescent assays has been the rapid growth in the number of phosphoantibodies available. These phosphospecific antibodies can be directed against phosphotyrosine, phosphoserine and phosphothreonine

residues. The rapid expansion of the variety of phosphoantibodies has enabled the development of phospho-enzyme-linked immunosorbent assays (ELISAs), fluorescence polarization assays, fluorescence resonance energy transfer (FRET) assays, time-resolved fluorescence (TRF) assays, and cell-based assays. Both homogeneous and nonhomogeneous assays are used for assessment of kinase-inhibiting drugs.

Quantitative biochemical methods for analysis of protein phosphorylation in drug development

Fluorescence immunoassays

Sandwich ELISAs for detecting phosphoproteins have commonly been used to quantitate kinase function and can be performed in two configurations. In the first configuration, polyclonal antibodies directed against the structural part of the protein and away from the phosphorylation site (panprotein) are coated onto the bottom of a microwell plate. A cell lysate containing the phosphorylated target protein is added to the well, allowed to bind and the excess lysate is removed by washing. A monoclonal antibody of either mouse or rabbit origin, specific for the phosphorylated form of the protein, is added followed by an enzyme-labeled secondary antibody specific for the monoclonal antibody species. A chromagen is added and the color is quantified spectrophotometrically. In the second configuration, the capture antibody is directed against the phosphoantibody and the detection antibody is an antibody directed against the panprotein. The latter configuration is sometimes preferred as the amount of phosphoprotein present may be small compared to the total amount of the panprotein. In this situation, the large amount of nonphosphorylated panprotein can outcompete the phosphorylated protein for binding to the microwells. This decreases the overall sensitivity of the assay for the phosphoprotein. Using a phosphospecific capture antibody enriches for the desired target and significantly increases the sensitivity of the assay.

Phospho-ELISAs can be used to screen drug candidates targeting a purified kinase or, alternatively, can be used to assess kinase activity in cell lysates. Angeles and coworkers have described two versions of an ELISA for quantitation of trkA receptor phosphorylation from cell cultures [37]. The difference between the two assays was the label readout. In the first configuration, a horseradish peroxidase-conjugated secondary antibody was used to develop a colorimetric signal that was measured by absorbance. In the second configuration, the secondary antibody was conjugated to europium, a lanthanide, and the readout was TRF, an approach similar to the dissociation-enhanced lanthanide fluorescence immunoassay (DELFLIA assay[®], Perkin Elmer, Inc.) described below. Both assays showed similar sensitivities and gave the same rank order of potency on a series of kinase inhibitory compounds.

Phospho-ELISA kits are available from a number of vendors including Biosource, Cell Signaling Technology, Assay Designs and Calbiochem for many of the proteins found in the major cancer-related signaling pathways, such as c-Kit, Mek1, ERK 1/2, Akt and EGFR.

FRET & TRF assay formats

TRF assays employ lanthanide fluorescent labels, the fundamental characteristic of which is their long fluorescent decay times. The fluorescence of these lanthanides persists for a considerable time interval in the assay mixture before decaying. In contrast, background fluorescence generally has a shorter lifetime, and decays or fades rapidly in the assay mixture. The long decay times enable measurements of the fluorescent signal to be made after a time interval during which nonspecific fluorescent background is no longer present. The DELFIA illustrated in FIGURE 1 employs lanthanide chelate labels with long decay times and large Stokes shifts [101]. Four different lanthanides with mutually exclusive emission spectra can be used in the assay. These include terbium (emission: 545), dysprosium (emission: 572), europium (emission: 613) and samarium (emission: 643). In its simplest form, DELFIA is performed like an ELISA assay. A lanthanide-labeled secondary antibody replaces the enzyme-labeled antibody for detection. Upon binding of the lanthanide-labeled secondary antibody, an enhancement solution of low pH is added to the sample microwells. The low pH enhancer dissociates the lanthanide which forms a stable fluorescent chelate inside a protective micelle in

the enhancer solution. The signal is then read on a suitable microplate reader. DELFIA assays have been reported to have a wider dynamic range and greater sensitivity than traditional ELISA assays. In addition, the availability of four different lanthanide labels with distinct emission wavelengths, allows target multiplexing. DELFIA assays can be read on a number of different microwell plate readers with TRF capability.

Waddleton and coworkers developed assays for quantitating phosphorylation of the insulin and epidermal growth factor receptors using DELFIA technology. [38] Phosphorylation was measured using a europium-labeled antiphosphotyrosine antibody. Using *in vitro* phosphorylated partially purified human insulin receptor, the DELFIA assay had a linear range of 10^5 with a sensitivity of 0.1 pmole in 200 μ l. Similarly, the degree of EGFR phosphorylation in A431 cells could be accurately assessed using the same assay format. The inhibitory concentration of 50% (IC_{50}) values for trkA inhibitors using either the colorimetric or TRF readout were comparable. The TRF assay format had good throughput, sensitivity, and reproducibility.

The LANCE[®] assay format (Perkin Elmer, Inc.) shown in FIGURE 1 combines TRF with FRET. Similar to the AlphaScreen[®] assay (Perkin Elmer, Inc.) described below, a biotinylated peptide

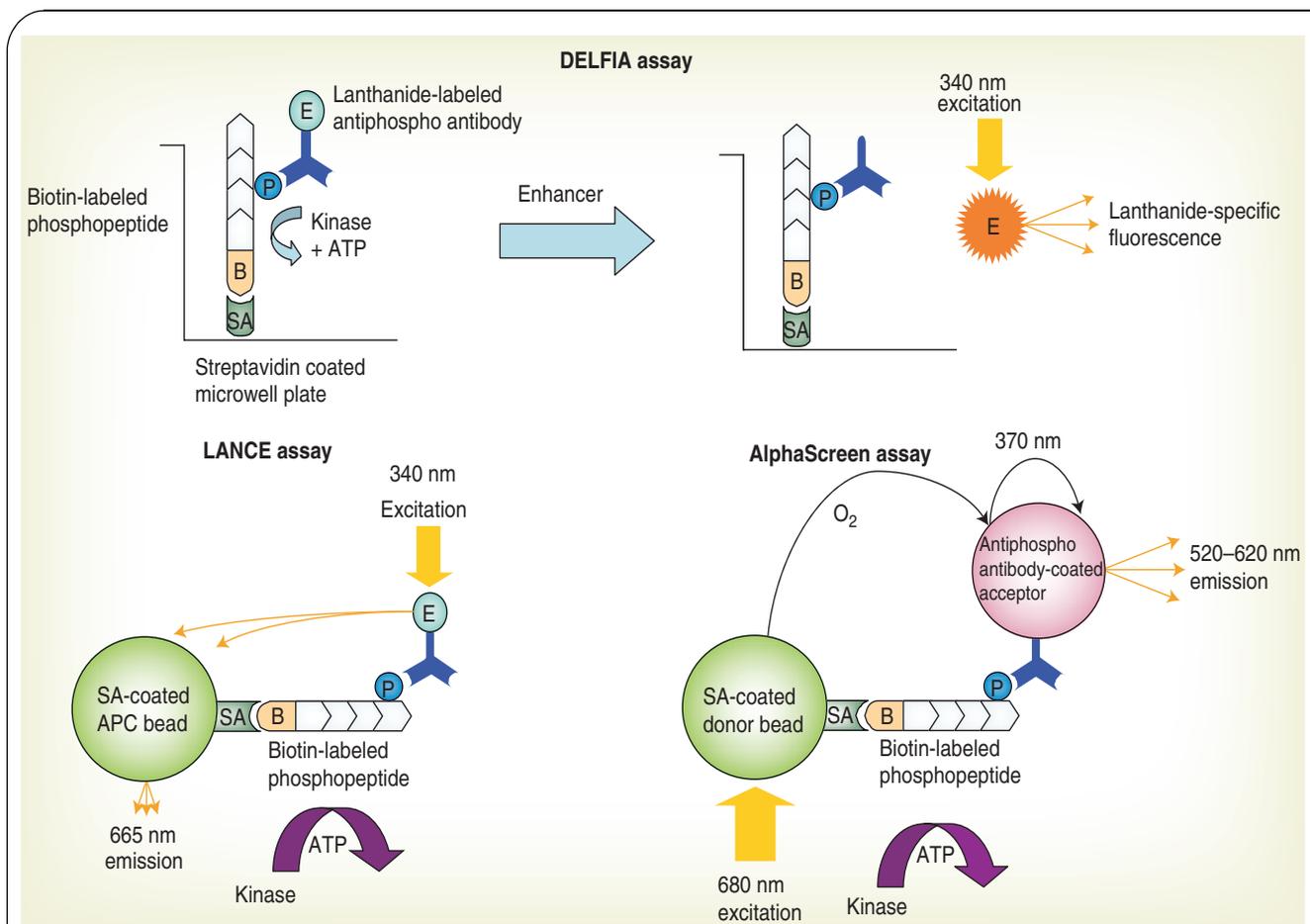


Figure 1. Illustration of the DELFIA[®] (Perkin Elmer, Inc.), LANCE[®] (Perkin Elmer, Inc.) and AlphaScreen[®] (Perkin Elmer, Inc.) assays for protein phosphorylation and kinase function.

Ab: Antibody; B: Biotin; E: Europium; DELFIA: Dissociation-enhanced lanthanide fluorescence immunoassay; P: Phosphate group; SA: Streptavidin.

kinase substrate is bound to an allophycocyanin–streptavidin acceptor conjugate. The kinase to be examined is added to the target peptide along with ATP and a donor europium-labeled antiphosphopeptide antibody. If the target peptide becomes phosphorylated, the europium-antiphosphopeptide antibody binds to the phosphorylated peptide, bringing the europium label in close proximity to the allophycocyanin. Upon excitation at 340 nm, the europium-labeled donor transfers its excited energy to the allophycocyanin acceptor complex, which emits fluorescence at 665 nm. Again, the use of lanthanide chelates with long excited lifetimes avoids interference and background caused by non-specific short-lived emissions.

The AlphaScreen assay shown in FIGURE 1 is an easily automatable homogeneous FRET assay. The AlphaScreen assay employs two main reagents: a biotinylated peptide substrate bound to a donor streptavidin-coated fluorescent dye-containing bead, and a second fluorescent dye-containing bead coated with an antiphosphotyrosine antibody. A candidate kinase is added and, if phosphorylation of the peptide occurs, the donor bead is brought into close proximity with the acceptor bead through the binding of the phosphospecific antibody on the acceptor bead. The mixture is subjected to excitation at 680 nm, which induces the formation of singlet oxygen at the surface of the donor bead following conversion of ambient oxygen to a more excited singlet state by a photosensitizer present in the donor bead. The singlet oxygen molecules diffuse and react with a thioxene derivative present in the acceptor bead, generating chemiluminescence emitting at 370 nm. The chemiluminescence excites fluorophores contained in the acceptor bead that emit at 520–620 nm. The signal generated has a half-life measured in seconds which allows signal measurement to be time gated, thereby eliminating short-lived fluorescent background. The AlphaScreen assay differs from time-resolved (TR)-FRET in that the signal is significantly amplified by the generation of the diffusible singlet oxygen. Binding events can be detected over a distance of 200 nm, whereas TR-FRET is nonamplified and limited to a binding distance of 9 nm [39]. If the assay is performed in the presence of a candidate kinase inhibitory drug, the decrease in fluorescent signal can be measured quantitatively and an IC_{50} determined.

Glickman and coworkers have compared the DELFIA (TRF), LANCE (TR-FRET) and AlphaScreen formats in a model assay for the nuclear bile acid receptor, farnesyl X receptor (FXR), a regulator of cholesterol homeostasis [40]. While this assay is not aimed at quantitation of phosphorylation, it does serve as a basis to compare the performances of each format relative to each other. In terms of sensitivity, the DELFIA format was the least sensitive. The LANCE assay format showed an acceptable dynamic range and low assay variability. The AlphaScreen assay was the most sensitive and had the greatest dynamic range along with good assay reproducibility. Signal to background was tenfold greater in the AlphaScreen assay than either of the other formats. Due to the high signal to background, the AlphaScreen assay would require considerably less reagent, a feature that could yield significant cost savings.

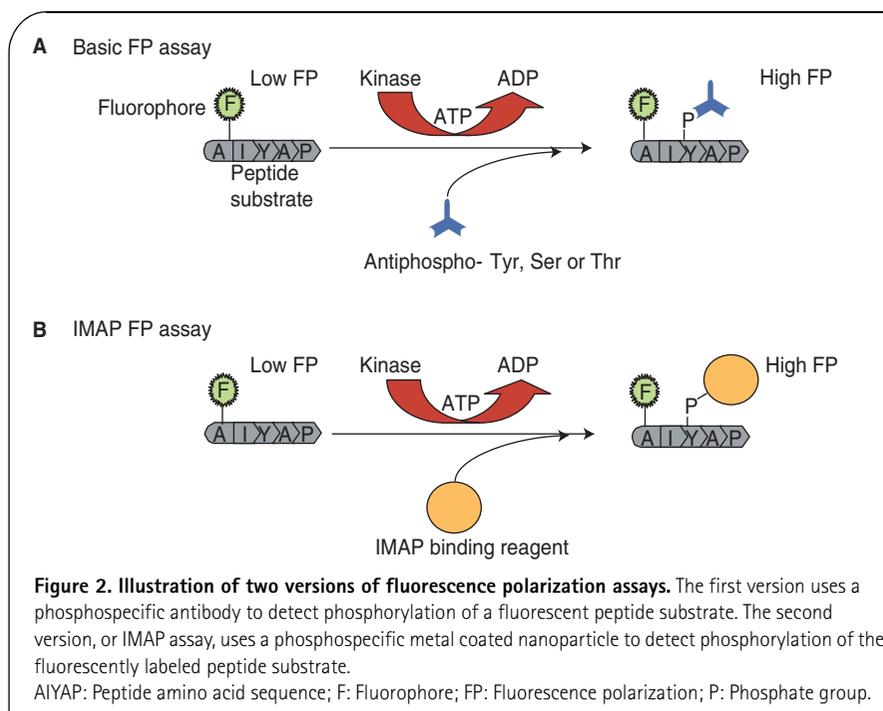
Zhang and coworkers have described a statistical factor, Z' , which is indicative of assay quality and robustness [41]. Values for Z' of 1.0 indicate a perfect assay. Generally, assays with Z' values greater than 0.5 are considered to have very good performance. In a 384-well format, the LANCE, AlphaScreen and DELFIA assays had Z' values, of 0.8, 0.9 and 0.6 respectively. These values indicate that each format is quite suitable for kinase analysis in terms of assay robustness and data quality. The choice of assay will depend on available instrumentation and cost. In this respect, DELFIA is generally the lowest cost per data point.

Fluorescence polarization formats

Fluorescence polarization assays have become a very attractive format for assessing kinase activity. Burke and coworkers have extensively reviewed the principle and application of fluorescence polarization assays to drug discovery [42]. Fluorescence polarization is based on the principle that smaller molecules rotate faster than large molecules when in solution. Thus fluorescence polarization assays can be used to monitor molecular interactions such as the binding of an antibody to a fluorescently labeled phosphorylated peptide substrate. In the absence of phosphorylation, the fluorescently labeled peptide is a small molecule that rotates relatively quickly. However, if phosphorylation occurs due to the action of a kinase, it results in the binding of an antiphosphopeptide antibody, and the peptide–antibody complex rotates more slowly due to its larger size. The rotational change can be measured and quantified.

Fluorescence polarization assays are attractive for the quantitative analysis of kinase inhibitors for several reasons. First, the assays are homogeneous, that is, there are no washing steps required. Rather the reaction components are combined and the fluorescence polarization measurements made with reference to appropriate standards. One-step assays generally have better precision than multistep assays. Second, they are generally less expensive to perform. Lastly, they are amenable to miniaturization and high-throughput screening. Traditional fluorescence polarization assays are manufactured by a number of companies including PanVera/Invitrogen and Chromagen, and each of these companies has its own assays for quantification of protein phosphorylation.

While several fluorescence polarization assay formats exist, the basic format is as depicted in FIGURE 2. A variation of this format is based upon the competitive binding of a labeled phosphopeptide tracer, a phosphospecific antibody, and a substrate protein or peptide that becomes phosphorylated as a result of the action of an added kinase. The phosphoantibody binds to the labeled phosphopeptide tracer forming a complex with a high polarization value. When this complex is added to a kinase reaction in which a kinase can act on a substrate protein or peptide, the unlabeled phosphorylated protein can compete with the labeled tracer, displacing it from the phosphoantibody, causing the polarization value to decrease. The decrease in polarization is proportional to the amount of kinase activity.



Fluorescence polarization formats are widely used for quantifying phosphorylation due to kinase activity. Turek and coworkers developed a competitive fluorescence polarization assay for the serine/threonine kinase, Akt [43]. In a comparison of the competitive fluorescence polarization assay to a radioactive flashplate assay, comparable IC_{50} values were generated. The Z' factor was 0.7 for the competitive fluorescence polarization assay and 0.55 for the Flashplate assay.

A major drawback to fluorescence polarization assays employing visible wavelength fluorophores is high background and interference from compound fluorescence [44,45]. Fowler and coworkers examined the performance of competitive fluorescence polarization assays for quantitating phosphorylation due to protein kinase C (PKC) and c-jun N-terminal kinase (JNK)-1 [46]. Both assays showed significant interference from compound fluorescence resulting in a high false-positive rate. In approaching the problem of compound fluorescence, the authors converted both assays to a fluorescence lifetime format. Although the lifetime-discriminated polarization decreased the amount of interference, the apparent false-positive rate was still significantly higher than a radioactive filter binding assay used as a standard.

Red-shifted fluorophores such as BODIPY-TMR have been used in an attempt to improve assay performance by shifting the emission and excitation of the labeling fluorophore out of the emission region of most interfering substances. In a limited examination of assay parameters, Banks and coworkers have shown that BODIPY-TMR-labeled ligands appear to be less susceptible to compound fluorescence [44].

Vedvik and coworkers have recently described a new red-shifted fluorescent tracer compatible with fluorescence polarization assay formats that shows less interference from light scatter and autofluorescence of compound libraries [47]. The

new redshifted tracer has a fluorescence lifetime similar to fluorescein and excitation/emission optima above 600 nm. The redshifted tracer was tested in parallel with fluorescein and TAMRA for compound interference from individual members a commercially available library of 1280 compounds of known biological activity (LOPAC¹²⁸⁰, Sigma-Aldrich). The red-shifted tracer showed interference from only three of the 1280 compounds compared with 19 and eight compounds for fluorescein and TAMRA, respectively. While the red-shifted tracer has not been validated in actual compound screening, the results are very promising.

The IMAP[®] assay (Molecular Devices, Inc.) is a variation of fluorescence polarization that employs nanoparticles bearing immobilized trivalent metal co-ordination complexes that bind specifically to phosphate groups (FIGURE 2). Fluorescently labeled peptide substrates are combined

with the target kinase in a microwell format. In the absence of an inhibitor, the fluorescent peptide becomes phosphorylated and can bind to the IMAP nanoparticles derivatized with the metal co-ordination complex. The binding causes a change in the motion of the peptide and a resultant increase in fluorescence polarization. In the presence of an inhibitory compound, the fluorescence polarization is decreased proportionately to the degree of kinase inhibition.

A significant advantage of the IMAP assay is that antibodies are not required. This makes the assay applicable to a wide variety of tyrosine and serine/threonine kinases for which high quality antibodies may not be available. The lack of a requirement for phosphospecific antibodies has made the IMAP assay one of the more frequently used formats for quantifying phosphorylation and kinase inhibition in drug development [48–52]. Beasley and coworkers have examined compound interference effects on the performance on the IMAP technology in a null screen of a four million-member compound collection [48]. Using a mixture of fluorescein-labeled phosphopeptide and the nonphosphorylated version of the same peptide, they reported assay interference from compound fluorescence for the IMAP format at a frequency equivalent to standard fluorescence polarization assays. Although the IMAP assay uses an approximately tenfold higher concentration of fluorescent peptide than other fluorescence polarization assays, the requirements of a pH of 5.5 for efficient metal-phosphate binding decreased the fluorescence intensity of the fluorescein by a factor of ten, thereby negating the potential gain from the higher concentration of fluorophore.

Turek-Etienne and coworkers developed a prototype IMAP assay for quantitating phosphorylation of Akt [49]. A fluorescein-labeled peptide was validated using six known kinase inhibitors and gave IC_{50} values comparable to those reported in

the literature. The Akt IMAP assay formatted for 384-well plates had a Z' value of 0.75. In contrast to the results of Beasley and coworkers [48], they saw no evidence of compound interference following screening of a 640-member biologically active LOPAC sample set.

Lu and coworkers have also used an IMAP format to quantify PDK1 mediated phosphorylation of Akt [51]. The PDK1/Akt assay was validated by determining the IC_{50} value for staurosporine in comparison with a radiometric assay. Both assays gave comparable IC_{50} values. Similar to Turek-Etienne, screening the 640-compound LOPAC library in a 384-well format yielded a Z' value of 0.73, indicating good assay robustness.

Gaudet and coworkers have demonstrated the versatility of the IMAP assay format and have used it to examine serum- and glucocorticoid-induced kinase (SGK)1, Akt, mitogen-activated protein kinase-activated protein kinase (MAPKAPK)-2, stress-activated protein kinase (p38 β 2) and Eph kinase [52]. The assay format was easily adaptable to quantifying the function of a broad range of targets including both serine/threonine and tyrosine kinases. IC_{50} values for known inhibitors of SGK, Akt, MAPKAPK-2 and p38 β 2 were in agreement with the values reported in the literature. The fluorescence polarization response was substantially greater than other FP assays. Furthermore, the assay quality was reflected by the fact that the Z' values were 0.97 for both the optimized SGK and Akt assays, 0.89 for MAPKAPK-2, 0.70 for p38 β 2, and 0.55 for Eph kinase.

Fluorescence-quenching assays

The Antibody Beacon™ tyrosine kinase assay is a fluorescence-quenching assay employing a small molecule tracer ligand labeled with Oregon Green® 488 dye (Invitrogen, Inc.) (FIGURE 3) [102]. In the presence of an antiphosphotyrosine antibody, the tracer ligand binds to form an antibody beacon complex in which the fluorescence is quenched. In the presence of a phosphotyrosine-containing peptide the antibody beacon complex is disrupted and the Oregon Green-labeled tracer is released, relieving the antibody-induced quenching effect. The assay is conducted by combining the target kinase, the Oregon Green-labeled tracer, and the peptide substrate. In the absence of an inhibitory drug compound, fluorescence signal is detected and quantified. Adding a kinase inhibitory compound decreases the amount of fluorescence by some amount proportional to the effectiveness of the inhibition.

Trivalent metal ions, when complexed with specific co-ordinating ligands, can selectively bind to phosphate groups [53]. Metal ion co-ordinating complexes have been widely used with immobilized metal affinity chromatography for separation and purification of

phosphoproteins in drug discovery research. The IMAP technology described above uses trivalent metal ions bound to nanoparticles to selectively bind phosphorylated peptides in a fluorescent polarization assay.

McCauley and coworkers described a new homogeneous assay format, the IQ™ assay (Pierce Biotechnology, Inc.), that measures phosphorylation of fluorescently labeled substrates (FIGURE 4) [54]. In the IQ assay, a peptide comprised of an amino acid sequence recognized by the desired kinase is synthesized with a fluorophore end-label. The peptide is combined with ATP, the kinase to be tested, and a proprietary trivalent metal phosphate binding/quenching agent. In the absence of phosphorylation, the binding/quenching reagent cannot bind the peptide and the fluorescent emission of the peptide can be detected. However, if the kinase phosphorylates the peptide, the quenching agent attaches to the phosphate group and quenches the fluorescence. Morgan and coworkers reported a detailed characterization of the assay [55]. The maximum distance between the phosphoryl group and the fluorophore at which 95% quenching would occur was examined and found to be greater than 20 amino acids. This is almost double the distance limit for traditional FRET. When compared with data from radiometric filter binding assays for PKA, PKC and Src tyrosine kinase, the IQ assay yielded comparable IC_{50} values for all of the tested inhibitors. The IQ assay is robust ($Z' = 0.7$), homogeneous and sensitive. Since the assay uses a phosphospecific quenching reagent rather than antibodies, the format is applicable to virtually any kinase.

The QTL Lightspeed™ kinase assay platform (QTL Biosystems) uses a similar principle to the IQ assay [56]. However, in the Lightspeed assay, signal is generated from a polystyrene microsphere that is coated with a modified fluorescent polyelectrolyte (FIGURE 4). The proprietary copolymer surface contains charged side groups that can complex trivalent metal ions via polyelectronic self assembly [56]. The metal ions, while tightly bound to the surface of the

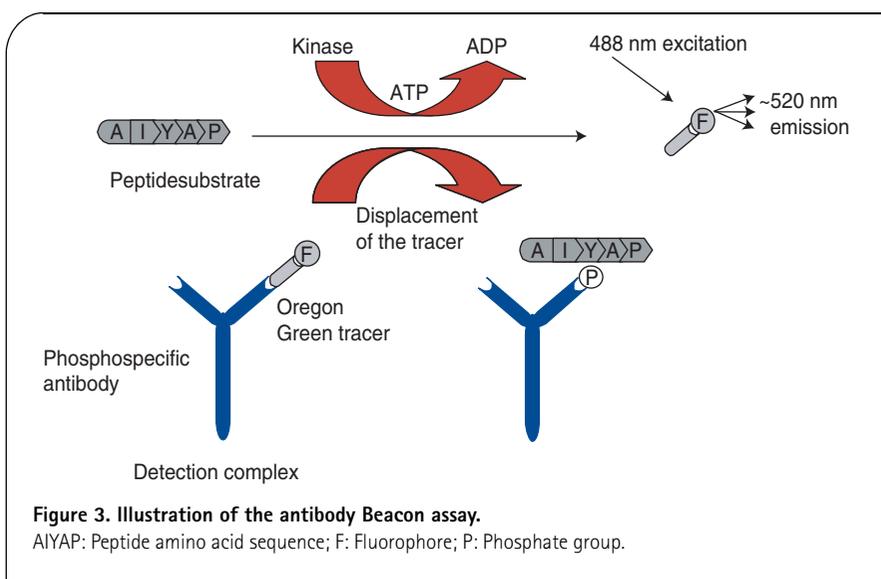
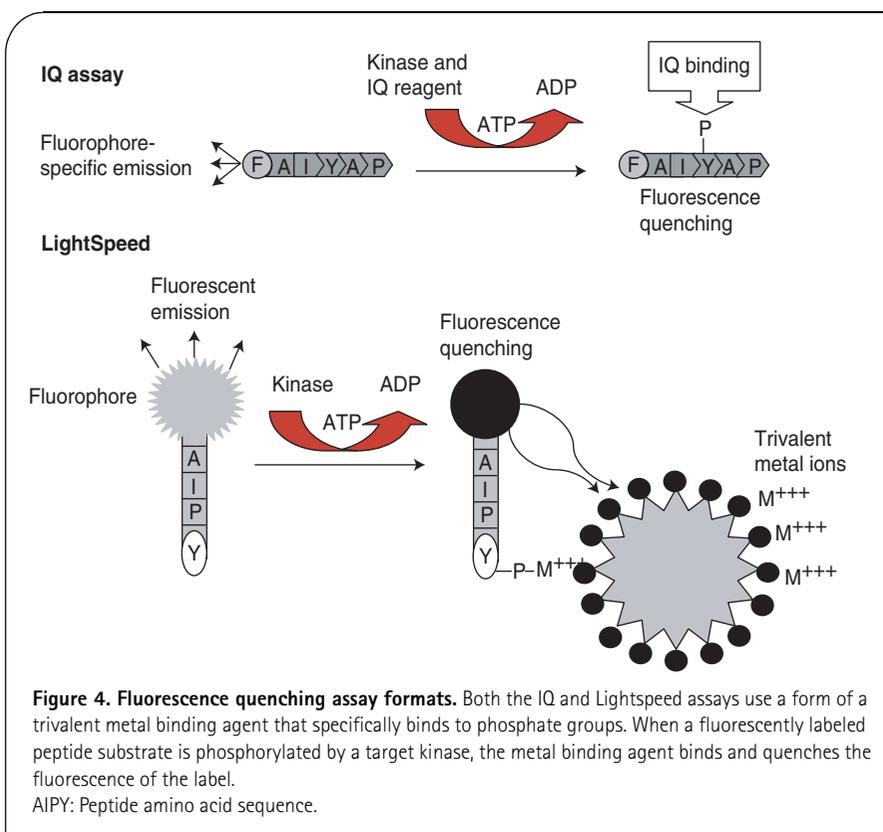


Figure 3. Illustration of the antibody Beacon assay.

AIYAP: Peptide amino acid sequence; F: Fluorophore; P: Phosphate group.



fluorescent polyelectrolyte-coated polystyrene, can simultaneously complex with phosphate groups.

Lightspeed consists of a peptide substrate for the kinase of interest conjugated to a quencher molecule that has been selected for high efficiency superquenching with the fluorescent polymer on the nanoparticle. Quencher peptides that become phosphorylated as a result of the action of a kinase become bound to the nanoparticle through the trivalent metal ions, effectively quenching the fluorescence of the nanoparticle. The net charge of the nanoparticle surface is tuned to yield maximum contrast between phosphorylated and nonphosphorylated peptides.

The authors were able to effectively measure phosphorylation by Akt and Rsk2 [57]. In a comparison with Z'-Lyte (Invitrogen, Inc.), a commercially available fluorescence polarization assay, the Lightspeed assay required a 15-fold lower enzyme concentration of Akt to achieve the same limit of detection and IC₅₀ as the fluorescence polarization assay.

For both the IQ and Lightspeed assays, the use of visible fluorophores for signal generation will potentially make the assays susceptible to compound interference. In the case of the IQ assay, the use of higher concentrations of fluorescent peptide may result in less interference, but this will need to be explored more fully.

Mobility shift assays

The phosphorylation sites on target substrates for protein kinases can be mapped and short peptides comprising only the phosphorylation site synthesized. When these peptides are phosphorylated and subjected to electrophoresis, their mobility

changes due to the added charge of the phosphate group. Caliper Life Sciences has exploited this feature to create microfluidics chip-based kinase assays [57,58,103]. The assays have been designed in two continuous flow formats in which a fluorescently labeled peptide substrate and its phosphorylated product are separated electrophoretically and subsequently detected. In the first format, the kinase under investigation is combined with its peptide in the presence of ATP in a microtiter plate. After the phosphorylation reaction occurs, the reaction mixture is transferred onto a microfluidics chip on which separation of the product and substrate occurs by electrophoresis. The product and substrate are quantified by fluorescence detection of the leading and trailing peaks. In a second format, the kinase reaction takes place directly on a microfluidics chip followed by electrophoretic separation and detection. In both formats, inhibitory compounds can be introduced and evaluated for their effect on the target kinase. Both of these assay formats are used with the Caliper instrument systems.

In a study by Dunne and coworkers, the on-chip assay consistently yielded lower inhibition values [58]. Although the two assays differed in the level of inhibition, there was a 70% overlap between the inhibitory hits identified. Both assays showed good reproducibility. The advantage of the on-chip format is the smaller reaction volumes which lead to a lower requirement for reactants and a concomitant lower cost. Both formats are in use for both primary and secondary screening.

Bead-based array detection

Luminex, Inc. has developed a unique bead-based multiplex technology for quantification of phosphoproteins [104]. Their xMAP[®] technology uses 5.6- μ m polystyrene microspheres that contain a mixture of a red and a near infrared (NIR) fluorophore (FIGURE 5). Using different ratios of the two dyes, distinct sets of beads can be created, each with a unique spectral signature characteristic of the red/infrared dye ratio. It is claimed that up to 100 different beads can be distinguished by their spectral signatures. The surface of each bead set can be coated with an antibody specific for a particular target protein such as phosphorylated Akt, ERK or MEK1. The coated beads are combined with the target ligand and bound target ligand is detected by addition of a biotinylated detection antibody and dye-labeled streptavidin. In practice, multiple types of coated beads are combined with an unknown sample along with detection antibodies and fluorescently labeled streptavidin in a single well of a microwell plate. After binding of the detector molecules, the beads are passed via a microfluidic device in single file

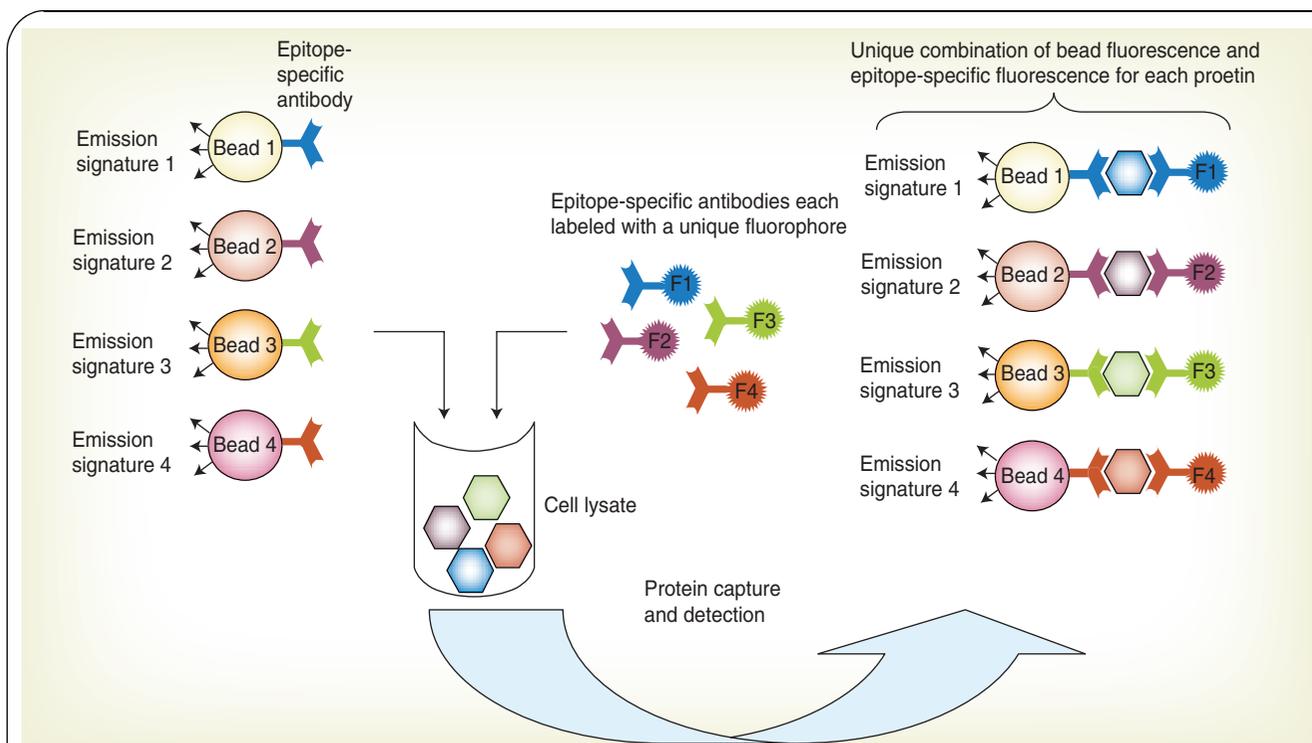


Figure 5. The Luminex bead-based multiplex assay. Beads are filled with a mixture of two fluorescent dyes such that each bead has a unique signature fluorescent emission. Each bead is coated with a specific phospho- or panspecific antibody. The beads are mixed and added to a cell lysate whereupon the target proteins are captured. A set of target-specific antibodies, each labeled with a different fluorophore, are added, thereby creating a sandwich with a unique set of fluorescent emissions due to the bead-detector antibody combination.
F: Fluorophore.

through a detection chamber. As the beads pass through the detection chamber they are illuminated by two lasers. A red laser excites the red/NIR dyes contained in the beads, identifying the particular assay being conducted (the classification channel), while a green laser excites the label on the surface reporting the amount of ligand present (the reporter channel). Theoretically, 100 different assays can be multiplexed at one time. However, nonspecific interactions between antibodies coated on the bead surfaces limit the number to considerably fewer assays per well. Generally, eight to 12 analytes are analyzed per well.

Quantitative cell-based methods for analysis of protein phosphorylation

While biochemical assays for protein phosphorylation are easily carried out, they cannot duplicate the cellular environment. Cellular phosphorylation cascades are multidirectional pathways rather than single biochemical reactions. Although a particular member of a signaling pathway may be effectively inhibited by a candidate drug, the pathway itself may remain unaffected due to alternative signaling routes that bypass the targeted kinase. Therefore, the behavior of a drug in a biochemical assay may not correlate with the behavior in either a whole cell or an animal. To more precisely assess the effects of a drug compound on a kinase-mediated pathway, cell-based assay formats can be used to validate the inhibitory effects of a

drug candidate on both the target and the targeted pathway. Cell-based methods enable assessment of entire pathways or even multiple pathways to fully evaluate the functional effects of the drug compound. Whole cell formats also allow a simultaneous assessment of drug penetration and toxicity.

While these assays are more complex to perform and may have higher costs associated with them, the benefit is a more accurate assessment of the candidate drug's effects in the biological system for which it is intended. The overall hope is that cell-based assays will lead to fewer failures further on in the clinical development process.

Chen and Olive reported on a method for using adherent cells in a microplate format [59]. The in-cell western (ICW) system consists of a NIR assay chemistry and a microplate scanner (Odyssey or Aeries; LI-COR Biosciences) employing two near infrared lasers and detectors for excitation and detection of fluorescent signals. The assay is based on standard immunocytochemical methods. However, the NIR fluorescence technology enables extremely sensitive and quantitative analysis of protein signaling pathways in cultured cells in a higher throughput manner. The assay has broad applicability for the analysis of protein signaling pathways, cell-based determinations of IC_{50} concentrations for lead optimization, as well as the examination of the effects of drug compounds on multiple points within one or more signaling pathways [59,60].

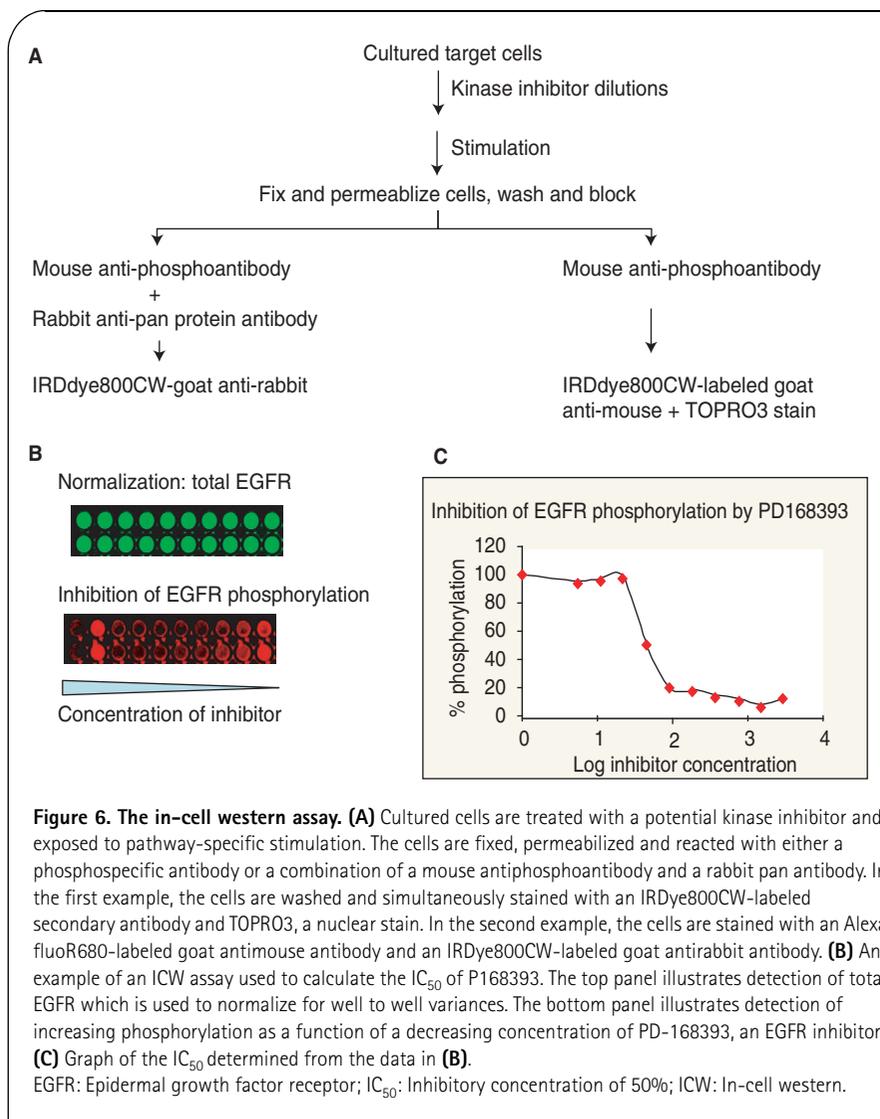
The ICW assay is performed in 96- or 384-well plates and is illustrated in FIGURE 6. Cells are pretreated with candidate compounds and the desired signal transduction pathways are stimulated with a suitable ligand such as epidermal growth factor. The cells are fixed and stained with phospho- and pan-antibodies from different species followed by species-specific goat antibodies labeled with one of two NIR dyes. Alternatively, the cells can be stained with a phosphospecific antibody and TOPRO3, a nuclear stain. The use of two colors allows ratiometric normalization across the wells of the plate and yields a quantitative assessment of the amount of phosphorylation that occurred [59,60]. Multiple markers, pathways or drug compounds can be examined on each plate. As there is very little autofluorescence from either cellular materials or plastics in the NIR region, the signal-to-noise ratio is quite high.

Wong reported the use of an ICW assay for monitoring phosphorylation of ERK in response to activation of dopamine D2 and D3 receptors [61]. ERK phosphorylation is elevated by agonist-bound $G_{i/o}$ - and G_q -coupled GPCRs, which represent

more than 75% of the members of the GPCR superfamily [62,63]. This makes phosphorylation of ERK an attractive marker for monitoring ligand-induced activation of GPCRs.

The ICW assay was used to determine the functional potency of both agonists and antagonists for D2 and D3 receptors on a single 384-well plate. Besides functional K_i determinations for antagonists, the assay could be used to detect the potency, efficacy, and selectivity of both partial and full agonists. Compared with other functional assays for GPCRs, the phospho-ERK ICW offered several advantages. The phospho-ERK ICW did not require any modification of the cells such as the expression of exogenous G-protein, chimeric G-protein or reporter genes such as β -lactamase, since ERK is a naturally expressed cellular protein. Additionally, reagents such as forskolin to elevate cyclic AMP, probenacid and Ca^{2+} dye for a Ca^{2+} mobilization assay, or a dye to monitor lactamase expression were not required. Addition of these reagents or the expression of exogenous genes may alter the pharmacology of the receptor [61]. Comparing the sensitivity of the phospho-ERK ICW with other standard assays of GPCR function, neither agonist-induced Ca^{2+} mobilization (as monitored by fluorometric imaging plate reader) nor agonist-mediated inhibition of adenylyl cyclase could be detected, yet dopamine-induced phospho-ERK was elevated five- to six-fold. The ICW format has broad applicability and has been used with a variety of cell lines including HEK293, CHO, NIH3T3, HeLa and A431. In addition, quantitative assays have been developed to monitor phosphorylation of individual or combinations of markers such as EGFR, Akt, STAT3, MEK1, KSR, JNK and p53. The ICW system can also be used with robotic automation enabling throughput compatible with the needs of lead optimization.

While the ICW is aimed at analysis of adherent cells, Perez and Nolan have applied fluorescence-activated cell sorting (FACS) to the quantitative analysis of phosphorylation in nonadherent cells [64]. The authors were able to stimulate several different cell types and assess the effects of kinase inhibitors on several important signaling proteins including ERK, p38, and JNK/SAPK. Due to the multicolor capability of FACS, multiple markers could be simultaneously analyzed for phosphorylation. While the system is an excellent research tool and can assess the effects of a kinase inhibitor at multiple points across a pathway, the method is not compatible with, nor is it intended to be, a high-throughput assay.



Summary & conclusions

As discussed above, there are many assay formats for quantifying phosphorylation and assessing the effects of drug candidates on kinase targets. Phosphospecific antibodies have opened the door to the development of several new biochemical and cell-based assay formats. While these assays show good performance in terms of sensitivity, robustness and data quality, they can be limited by the availability of high quality phosphospecific antibodies. New non-antibody based technologies with the ability to quantify protein phosphorylation and kinase function have begun to appear. The non-antibody assays have shown good data quality and robust performance, indicated by Z' factors above 0.5. The variety of assay formats available for quantification of phosphorylation is very advantageous in terms of the needs of drug development. Primary drug screens in which a million or more compounds need to be examined require good assay performance but low cost per data point, for example the fluorescent polarization assay. In contrast, accurate secondary screening may be better accomplished with an assay with the highest Z' values, such as AlphaScreen or a cell-based format such as the ICW, which examines a candidate drug in a cellular context.

Many of these assays employ fluorescent readouts. One potential drawback to fluorescent assays is autofluorescent background from both biological samples and the candidate compounds. The result can be a high rate of assay false positives. Some of these problems can be minimized by assay formats that employ high concentrations of fluorescent indicators. Other formats such as fluorescent polarization assays using red-shifted fluorophores or the cell-based ICW assay, which relies on near infrared fluorophores, may completely avoid compound interference.

Non-antibody-based assay formats show great promise in terms of versatility, ease of use and assay performance. Assays such as IMAP, IQ, Lightpeed and Caliper's mobility shift assay have varying degrees of cost and throughput but their ability to quantify kinase function in the absence of a specific antiphosphoantibody makes them more adaptable to broad usage for evaluation of kinase function.

Cell-based assays such as the ICW can assess the IC_{50} of a compound as well as examine multiple points within a signaling pathway. Since, in the cell, kinases act within a network of interactive pathways, it may be important to examine drug activity in a cellular context as a final confirmation of the results of a high-throughput biochemical screen. The ICW assay is well suited to secondary screening and lead optimization.

As the knowledge of kinases and their roles in disease processes grows, the need for high quality quantitative assays for protein phosphorylation will increase. Each of the kinase assay formats described in this review have shown good quality and when known kinase inhibitors were examined for their IC_{50} values, each appeared to correlate well with published values. The assumption is often made that the same set of positive hits will be identified by assays with good sensitivity, low variability and high Z' values, regardless of the technology. However, Sills and

coworkers compared a LANCE assay, a fluorescence polarization assay and a scintillation proximity assay (SPA) for their results in high-throughput screening and found that each assay generated a nonoverlapping set of positive hits [65]. A subset of 30,000 compounds of Novartis' synthetic compound library were examined for tyrosine kinase inhibitory activity in primary screening, deconvolution and dose response experiments using optimized versions of each assay. The results of the screening identified 100 and 101 active compounds by LANCE and FP assays, respectively, and 40 compounds by SPA. Whereas all 40 of the compounds identified by the SPA assay were included in those identified by LANCE, only 35 were active in the FP assay. The IC_{50} values obtained using the LANCE and FP assays showed good correlation with each other; however, those of the SPA showed poor correlation with the other two assays. Furthermore, the rank order of lead potency differed for each of the assays. Their results suggest that the assumption that similar leads are identified regardless of the screening technology used is not valid. Therefore, even though kinase assays may have good sensitivity, low variability and high Z' values, one must be cautious as different sets of leads may be identified with each assay.

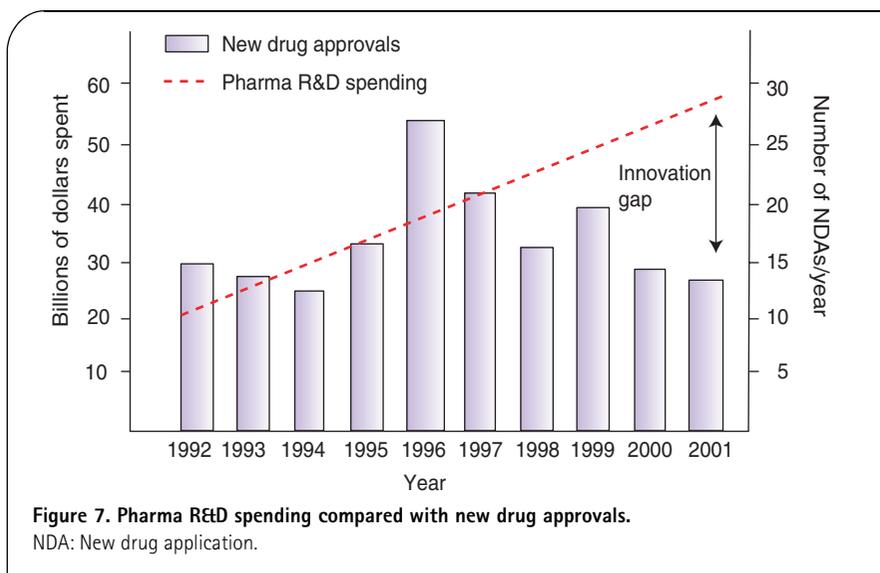
In summary, a number of good assay formats exist that are adaptable to low-, moderate- and high-throughput requirements. Many of the assays show good reproducibility and robustness. However, researchers should be aware that different assays may yield different, non-overlapping sets of qualified leads. Thus, it may be preferable to use two or more methods in order to obtain a set of leads with the highest probability of later success.

Expert opinion

Quantitative measurement of protein phosphorylation has become essential for the development of kinase inhibiting drugs aimed at anticancer therapy. Anticancer agents consumed more R&D dollars than any other disease area, accounting for one in five of all indications under development [66]. As of 2002, there were 178 anticancer drugs in development [66]. Since kinases are a major source of drug targets, assay technologies that quantify phosphorylation will continue to be in demand.

Biochemical assays that rely on antibodies for assay function are limited by the availability of phosphospecific antibodies with high affinity and specificity. While many phosphospecific antibodies exist, most are unsuitable for use in quantitative assays due to poor sensitivity or nonspecificity [67]. Alternative methods such as mobility shift, IMAP, IQ and Lightspeed assays do not rely on antibodies and allow assessment of targets for which no suitable antibodies exist. As a result, these formats will find wider usage in the future.

As illustrated in FIGURE 7, new drug approvals have not kept up with the increases in R&D spending. This is particularly significant considering the magnitude of the scale-up in screening capabilities that has occurred over the past decade. Furthermore, other than imatanib, the kinase inhibitors that have gained approval to date have shown limited efficacy in treating disease. Current kinase assay technologies are optimized for



consistency and robustness. However, the report by Sills and coworkers combined with the low return for the size of the investment in terms of new drugs coming to market would appear to indicate that perhaps these are the wrong criteria on which to base assay optimization. The definitive criterion for evaluation of a drug is successful treatment of disease in a clinical model. Late-stage failures are disastrous and costly. Therefore, it will be important to identify methods that best correlate with efficacy in either a preclinical or clinical model of disease.

Cell-based methods, although more complex to implement at the front end of the drug development process, may pay higher dividends in the form compounds with a higher probability of success in later clinical trials. Cell-based assays, with their ability to examine entire signaling networks, are expected to see increased use. Along these lines, high content screening can examine multiple parameters within a single cell. These assays can simultaneously measure multiple parameters yielding multiple criteria on which to base the quality of a drug candidate. Furthermore, although a singleplex cell-based assay may have a higher cost per test, the ability of high content assays to examine multiple parameters per test may decrease the overall cost per run. These assays are generally performed on highly complex and expensive instruments such as the Discovery-1 (Universal Imaging, Inc.), the ArrayScan VI (Cellomics, Inc.), or the In Cell Analyzer 1000 and 3000 (GE Medical Corp.). Many of these instruments are capable of analyzing individual cells which, in terms of a cell-based assay format, is the ultimate in miniaturization. Overall costs are still high and must be reduced. Currently, the price of these systems preclude their use by smaller academic laboratories. Only high-budget laboratories found principally in pharmaceutical companies can afford the systems, which can cost as much as US\$1 million.

As an alternative to the costly high content methods, simple, automated cell-based techniques may offer a practical alternative. While these methods may not screen individual cells, the level of screening needed may be satisfied by their ability to examine

populations of cells in a more cost effective manner. A 384-well microwell plate format can accommodate tests for multiple markers on a single plate, as illustrated by the agonist/antagonist experiments reported by Wong [61]. The challenge of quantitative pathway analysis is quite daunting but examining a kinase within its cellular context should give a more complete picture of the signaling processes affected by inhibitory drug compounds.

Five-year view

Protein kinase inhibitors are the largest and fastest growing category of drugs in development [17]. Kinase inhibitors currently consume 30% or approximately US\$12 billion in research each year [17]. Sales estimates of imatinib, erlotinib,

cetuximab and gefitinib are estimated to exceed US\$10 billion for 2004 [12,17]. In spite of this, the successes of these drugs have been limited. For example, gefitinib appears to work on a mutation-containing subset of EGFR. With the exception of imatinib, all of the kinase inhibitors developed thus far have very narrow clinical application. Furthermore, although many new technologies for quantifying kinase function have been developed, the rate of drug development has not significantly increased [22]. As stated above, the study by Sills and coworkers appears to pinpoint an important problem [65]. Assay chemistries are optimized for sensitivity, consistency and robustness rather than for generation of high quality, effective leads.

In order to increase the efficiency of identifying drug compounds that target protein kinases and decrease the risk of late-stage failures, pharmaceutical companies will need to move to processes that allow them to simultaneously assess multiple characteristics of a drug in a manner that is more relevant to efficacy in the whole animal. In order to accomplish this, it will be necessary to move many of the initial primary and secondary screening processes to cell-based formats. A quantitative understanding of the many interconnected networks, their interactions and regulation within cells will be critical to improving the assessment of cellular phosphorylation and signal transduction. Understanding these processes will lead to a more precise knowledge of the series of events that lead to diseases such as cancer.

Tools that allow rapid, cost-effective analysis of entire cellular signaling systems will be important in generating the necessary information for an understanding of disease processes. Technologies that allow multiple kinases and phosphorylation reactions to be monitored simultaneously within a single cell will provide a better understanding of the cellular system biology. Molecular imaging technologies such as those described by Luker and coworkers that permit the observation of protein-protein interactions in an experimental animal may aid in target validation and preclinical testing [68]. Single molecule imaging of signaling

pathways, such as activation of Ras as described by Murakoshi, may lead to a better understanding of cellular processes and the mechanisms of disease progression [69]. A better understanding of the disease process will allow more precise targeting of therapeutic agents.

Phosphorylation is important in many cellular processes, the disruption of which often leads to disease. With the success of the currently approved kinase inhibitors, methods for quantitative analysis of protein phosphorylation will be critical for years to come

Acknowledgements

The author would like to thank Amy Geschwender for critical reading of the manuscript.

Information resources

- Bead-based array reagents
www.biorad.com
(Viewed September 2004)
- Phospho-ELISAs, bead-based array reagents
www.biosource.com
(Viewed September 2004)
- Mobility shift technology
www.caliper.com
(Viewed September 2004)
- Reverse-phase arrays and validated phosphoantibody list
www.clinicalproteomics.steen.com
(Viewed September 2004)
- Phosphoantibodies
www.cellsignal.com
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www.invitrogen.com
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- Bead-based array assays
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www.molecularprobes.com
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- IQ assay technology
www.pierce.com
(Viewed September 2004)
- Bead-based array reagents
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Key issues

- High quality antibodies against phosphoproteins in the major signaling pathways are required.
- Non-antibody methods need to be improved to give better signal-to-noise ratios.
- Inexpensive cell-based assays that permit pathway analysis need to be validated in a true screening setting.
- Fluorophores and readouts that avoid autofluorescence from biological materials, plastics and compound libraries must be incorporated into current assay formats to increase signal-to-noise ratios.

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