# Opening Pandora's Box *kinase inhibitor screening*

Kinases have become a proven target class for new drugs and have created a thirst for the development of new technology platforms for inhibitor detection. We discuss some of the latest technologies and techniques along with their advantages and disadvantages.

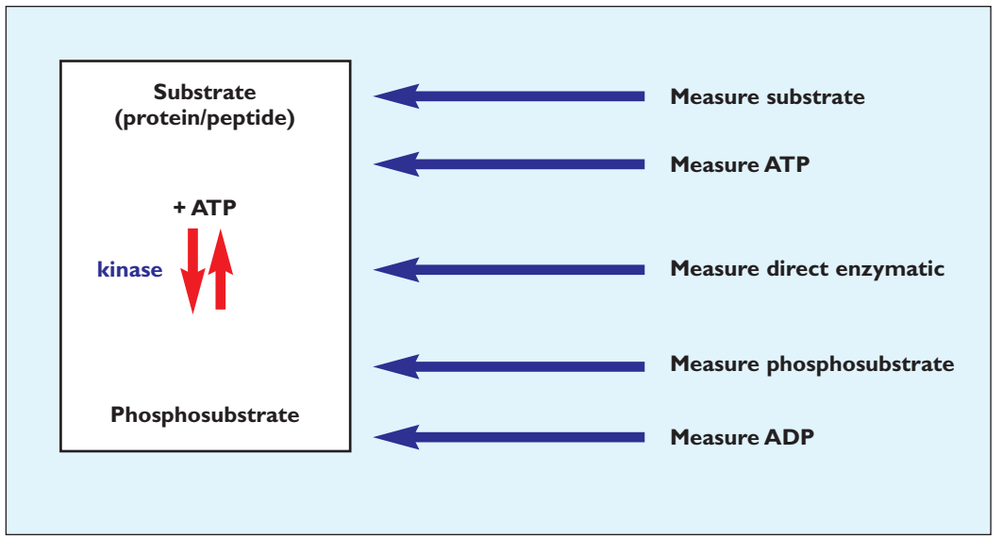
**By Dr Richard M. Eglen and Dr Roger Bossé**

Co-ordinated protein phosphorylation is controlled by kinases, a protein class that represents approximately 2% of the human genome. Kinases comprise two major subgroups: those that phosphorylate serine and/or threonine residues (Ser/Thr kinases), and those that phosphorylate tyrosine residues (Tyr kinases). Aberrant expression or function of kinases is associated with neoplasias, including metastasis, diabetes, psoriasis and liver fibrosis. Currently, kinase inhibitors such as Gleevec for the treatment of chronic myelogenous leukemia, and Iressa, Fasudil and Tarceva for the treatment of solid tumours, are either approved for clinical use or are in advanced development. Ser/Thr kinases inhibitors such as BAY 43 006, LY 333531 and CEP 1347, for the treatment of colon cancers, diabetic neuropathy and Parkinson's disease respectively, are also in clinical evaluation. Kinases are consequently a very 'druggable' target class and elicit a high interest in the development of high throughput screening (HTS) platforms for inhibitor detection (**Figures 1** and **2**).

Many kinase drug discovery programmes are aimed at enzymes that play in the etiology of cancers, although other diseases are emerging as major applications of kinase inhibitors. In oncology notably, there is increasing recognition that multiple kinase are involved in the disease. Consequently, novel inhibitors will probably need to exhibit actions across many kinases. Indeed, the concept of inhibitor 'polypharmacy' is now widely accepted principally due to the fact that several clinically active kinase inhibitors have been retrospectively shown to inhibit many kinases, over and above the target for which they were initially designed and optimised. There is, therefore, intense interest in determining the inhibitory profile of a novel compound, which now represents a major activity in several lead optimisation studies. Of note is that, unlike the primary screening campaigns undertaken to detect novel inhibitors, many drug discovery groups now outsource such secondary profiling activities to specialist companies.

Given that kinase assays are now widely used in drug discovery teams, screening groups and profiling organisations, both internal and external, it is not surprising that many technologies have been developed to meet this growing need. In terms of kinase assay development, these are discussed in more detail below. However, there are several critical points to consider when choosing a screening technology, particularly if the action of novel inhibitors is to be polypharmacologic, which necessitates determination of its activity across several kinases under equivalent experimental conditions. Since many inhibitors are designed to act at the ATP binding pocket, the concentration of ATP used in the kinase reaction is critical. In many HTS assays, low ATP concentrations are frequently used, providing assays inherently biased toward inhibitor detection and thus relatively high hit

**Figure 1**
The kinase reaction




rates. However, in kinase profiling studies, many different kinases are used, each of which has varying apparent affinity values for ATP. As a result, it is desirable to use different ATP concentrations such that each kinase is studied under conditions suitable for optimal activity (often at or above the Km value for ATP). Since different kinases have different Km values for ATP, the assay format adopted needs to be able to accommodate a range of ATP concentrations. Indeed, it is critical that observed differences in the compound potency reflect differences in pharmacological action at the kinases *per se* and do not emanate from the use of different ATP concentrations used in the assay. This point becomes more germane when the assay system employs the use of multiplexing technologies in which several kinases are studied simultaneously. In lead optimisation studies, detailed knowledge of the mechanism of action of the putative inhibitor is required. Here, kinetics of the inhibitor, often assessed using classical Michaelis-Menten techniques, all require assay techniques that can accommodate a range of ATP and substrate concentrations as well. It is probably for these reasons that the use of classical radiometric assays, which incorporate isotopic phosphorous, remains widely used, despite the availability of many non-radioactive assay formats.

Most of these considerations relate to *in vitro* studies, although the goal of finding a novel inhibitor is to obviously develop a compound with a cellular mode of action. In a living cell 40% of proteins are phosphorylated, with kinases acting as intracellular switches that govern both the extent and duration of protein phosphorylation. It is evident that kinases are arranged in biochemical pathways, each of which are highly interrelated and serve to control key cellular events, both temporally and spatially. Indeed, most kinases phosphorylate other kinases, and by this means modulate their level of activity in a controlled manner in order to govern cell function. A definitive understanding of these kinase pathways is far from complete and a major challenge in validating the kinase as a drug discovery target lies in understanding the proteins with which it interacts. A description of these 'proteomic' strategies is beyond the scope of this editorial.

However, it has relevance to kinase screening in that the more information known concerning the nature of the interaction of the substrate with the kinase, the more optimal the assay and potentially the more relevant the pharmacology of the inhibitor subsequently identified. To an extent, this concept has driven the need for robust cell-based assays for kinases, where presumably the authentic kinase substrate is phosphorylated. Even in *in vitro* studies, the nature of the substrate used in the assay is important. As mentioned above, kinases *in situ* act to phosphorylate other proteins. However, those assays that accommodate the use of proteins as assay substrates are surprisingly limited, with many assays employing peptide substrates that mimic the site of action of the kinase on the protein. Consequently, the action of inhibitors in assays in which peptide-mimetics are used may not accurately reflect their pharmacological action *in vivo*. This point has also lead many researchers to undertake lead optimisation studies using cell-based kinase assays.

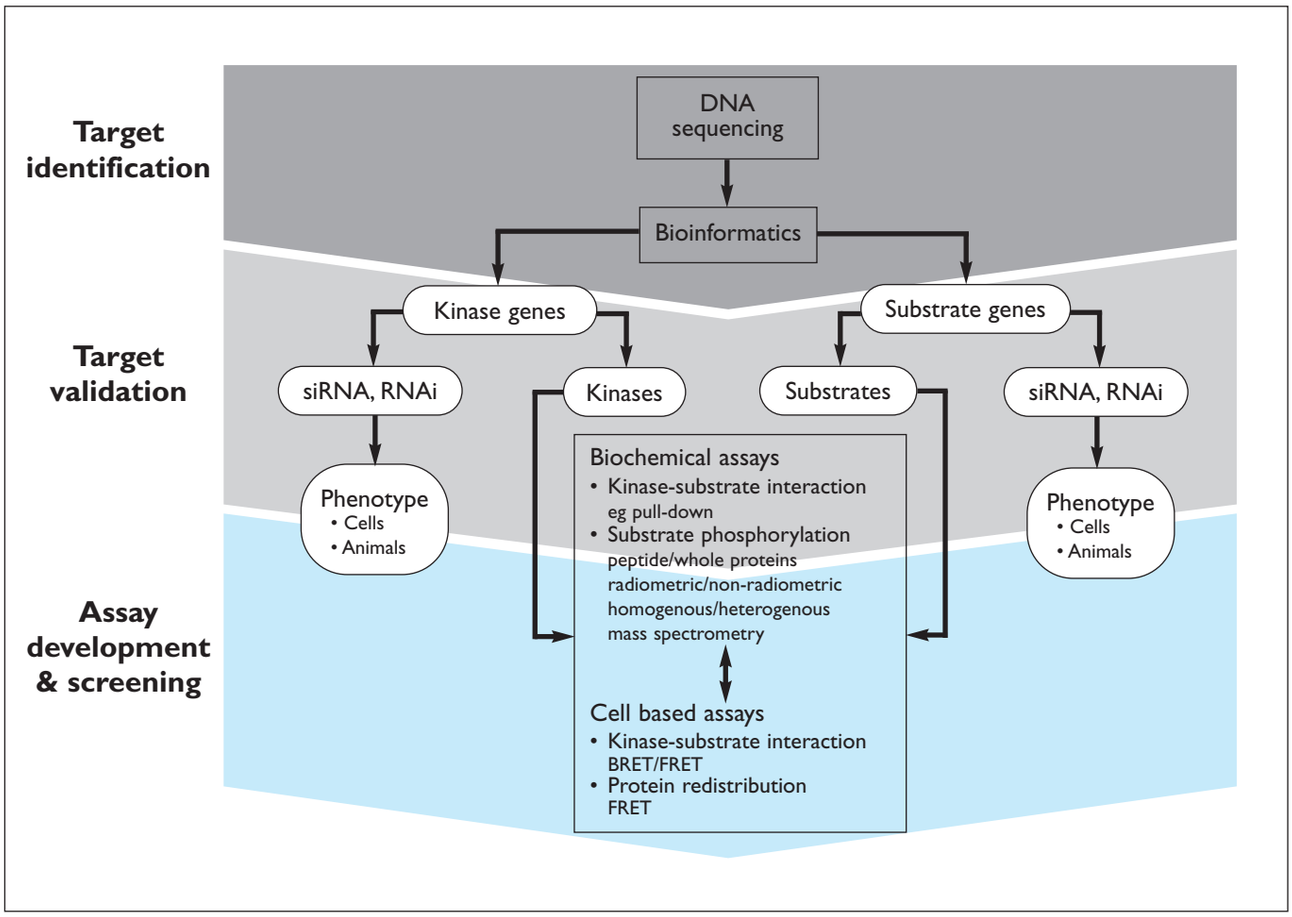
Collectively, the widespread use of primary screening assays used to identify novel kinase inhibitors has accelerated development of assay techniques amenable to automated liquid handling systems, as well as the use of detection systems with exquisite sensitivity. As discussed above, the expansion of kinase inhibitor profiling services for lead optimisation studies has driven development of assay techniques which are more generic. These are discussed below.

## High throughput kinase assays – cell free formats

Historically, kinase assays measuring the incorporation of isotopic phosphate have provided the principal method for compound screening (**Figure 1**). This approach, in the context of HTS, has been superseded by other assay technologies, principally due to difficulties of automation and the economics of radioactive waste disposal. Numerous non-isotopic alternatives are now available, many of which use competition immunoassays employing selective, high affinity antibodies to detect the phosphorylated over the non-phosphorylated form of the substrate. Antibody-based approaches, initially developed to measure activity of receptor tyrosine kinases, used enzyme-linked immunosorbent assays. However, laborious washing and separation steps could not be easily adapted to automated systems in routine use in HTS laboratories. Consequently, homogeneous assays (ie assays in which all reactions are undertaken in the same liquid phase) were widely adopted for HTS. Many homogeneous assays employing fluorescent detection are now available for kinase inhibitor screening, including time resolved fluorescence, time resolved fluorescence energy transfer, and protease sensitive fluorescence resonance energy transfer. One limitation of fluorescent-based assays is the potential for optical interference due to compounds in the screening library. Consequently, technologies have now been developed, including time resolved (TR)-FRET approaches (such as LANCE® and HTRF®), which have a reduced propensity for compound interference.

However, all these techniques require antibodies

**Figure 2**
Modular and systematic process flow to perform kinomics studies



with a good specificity for the phosphorylated substrate. Unfortunately, a limitation of these approaches is that in several cases such antibodies do not exist, thereby restricting the portfolio of assays that can be developed. Although this is less of a concern with tyrosine kinases, where there are potent and generically selective antibodies for phosphotyrosine residues, this is certainly not the case with Ser/Thr kinases, where relatively few antibodies are available for homogeneous assay development. Partly to address this issue, affinity capture techniques have been developed in which a Lewis metal is used to bind the phospho-substrate, quenching the assay fluorescent signal either directly or modulating the fluorescent polarisation of a suitable fluoroprobe. These approaches have limitations in the concentration of ATP that is used in the assay, as well as the nature of the peptides used as substrates. To remedy this situation, the approach has been combined with TR-FRET and AlphaScreen® approaches to include IMAP® TR-FRET and AlphaScreen PhosphoSensor™.

For many of the reasons discussed above, alternative kinase assay formats have been developed that do not detect formation of a specific phospho-substrate. They are designed to have a generic applicability, in that they are designed to measure universal reactants or products of any kinase reaction, specifically ATP or ADP, respectively. In the former, these approaches measure, via a chemiluminescent reporter enzyme, depletion of ATP which results from substrate phosphorylation during the kinase reaction. However, when either low concentration of kinases are used or when weakly active kinases are screened, only small decreases in ATP depletion occur, resulting in small changes in assay signal. This situation is exacerbated when high levels of ATP are required in the kinase assay, resulting in a high background signal. Consequently, alternative assays have now been developed that measure a secondary product of a kinase reaction, ADP, which accumulates in proportion to the level of substrate phosphorylation. Assays of this nature also have the theoretical advantage of permitting a wide range of ATP concentrations to be used, and allowing inhibitor screening to be conducted at the appropriate Km concentration value of the substrate and ATP. Two such methods are now available to detect ADP – one that employs a fluorescent polarisation technology and antibodies with a degree of ADP selectivity, Transcreener™, and a second that generates a fluorescent signal using a coupled enzyme system to generate an assay signal, ADP Quest™.

## High throughput kinase assays – cell based formats

There has been a growing trend towards the use of cell assay formats for kinases (**Figure 2**). These have been more problematic to develop, partly as many kinases act intracellularly, and thus the cell is destroyed during the assay procedure. Other reasons include the fact that many protein substrates are rapidly dephosphorylated following kinase action, as well as the high intracellular levels of ATP that may confound the assay signal. In the case of membrane bound kinase receptors, particularly membrane bound tyrosine receptors, several assays have measured the sequelae of phosphorylation such as ligand binding or dimerisation. In terms of intracellular kinases, the downstream effects of kinase mediated phosphorylation, such as protein translocation or degradation, are often studied. These changes often employ established techniques used for cell-based imaging approaches and high content screening protocols. However, relatively few of these are useful in primary screening campaigns, although their use in secondary screening and lead optimisation studies are clearly growing. In terms of cell-based assays for primary screening, the challenge is to selectively determine the change in the levels in a phosphorylated protein, using non-radiometric, homogenous protocols based on employing microtiter plate techniques. Historically, measurement of the proteins is undertaken using separation-based methods such as gel electrophoresis followed by identification by selective antibodies. Recently, however, several homogeneous assays (Surefire™) have been developed in which the phosphoprotein is detected in a crude cell lysate using microtiter plate protocols and are thus amenable to HTS.

## Summary

Kinase inhibitor screening *in vitro* is currently characterised by a wide availability of techniques, each of which possesses advantages and disadvantages. Some of these provide a generic approach, while others are specific to discrete kinase substrate pairs. Techniques are also emerging in which the binding of the inhibitor to the kinase is assessed, thus opening up the potential for assays that can determine the pharmacology of compounds at weakly active or inactive kinases. By contrast, kinase screening using cell-based assays remain very limited. It is instructive to compare the status of kinase screening to that of another large class of druggable proteins, G protein coupled receptors (GPCRs). Both are widely studied in drug discovery and are often taken into HTS

campaigns with the goal of identifying specific ligands. However, in the last decade GPCR screening has moved from biochemically-based ligand binding assays to functional cell-based assays. In contrast, kinase screening remains very much based upon biochemical approaches, with cell-based approaches only now emerging. One reason for the growth in cell-based GPCR assays was the advantage of screening in a physiology relevant environment. This is also a likely driver for developing cell-based assays for kinase inhibitors, particularly if the inhibitor needs to act at several kinases, all of which will contribute to overall action on the cell response. DDW

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***Dr Roger Bossé*** *joined PerkinElmer in 2000 and is currently Technology and BD Leader, Discovery and Research Reagents, Molecular Medicine. Prior to joining PerkinElmer, Roger was the Associate Director Biology of Packard Biosciences and Head of Research for the HTS platform AlphaScreen. He has also been responsible for the pipeline of G-protein coupled receptors, developed a method to immobilise cell membranes on polystyrene support as well as leading the initial evaluation of Dade-Behring's LOCI technology. During the past years, he and his team have delivered numerous products and developed more than 50 HTS assays. Roger holds three degrees from the University of Sherbrooke including a BSc in Biochemistry, an MSc and PhD in Pharmacology.*