KINASE PROFILING & SCREENING

Choosing a Biochemical Assay Platform

INTRODUCTION

Protein kinases have emerged as a major drug target over the past two decades. Since 2001, over 50 kinase inhibitors were approved for the treatment of cancers and inflammatory diseases.[1] The market continues to grow, with global sales of kinase inhibitor drugs forecasted to reach $46.4 billion in 2018 and is projected to expand at an annual growth rate of ~4 % from 2019 to 2027.[2]

The human kinome comprises 518 known protein kinases and approximately 20 lipid kinases. To date, most protein kinase inhibitors target the highly conserved ATP-binding site.[3] Though several promiscuous inhibitors have proven effective against cancer cells via the deregulation of multiple kinase-dependent pathways, highly selective drugs are of interest—especially for the treatment of chronic diseases—due to their superior safety profiles.

In the push to identify highly selective inhibitors, high-throughput screening (HTS) of compounds against comprehensive kinase panels has become the standard approach for lead discovery.[4] To this end, a variety of assay platforms have been developed.

COMMON BIOCHEMICAL KINASE ASSAY PLATFORMS

Biochemical kinase assays can be divided into two classes: activity assays and binding assays. Activity assays directly or indirectly quantify the catalytic product (i.e., the phosphorylated substrate) and include radiometric, fluorescence-based, luminescence-based, and mobility shift platforms. Binding assays quantitatively measure the binding of small molecules to the ATP-binding site.

Radiometric Activity Assays

The radiometric activity assay is considered the gold standard for kinase profiling. Highly validated for drug discovery[16-20] and used to validate non-radiometric assay formats,[21] it is the only format that directly detects the true product without the use of modified substrates, coupling enzymes, or detection antibodies. Test or control compounds are incubated with kinase, substrate, cofactors, and radioisotope-labeled ATP (^{32}P-γ-ATP or ^{33}P-γ-ATP). Reaction Biology employees two different methods, HotSpot℠ and ^{33}PanQinase™, to detect the radioisotope-labeled catalytic product.

The miniaturized HotSpot℠ assay is based on the traditional filter binding approach, with reaction mixtures spotted onto filter papers, which bind the radioisotope-labeled catalytic product. Unreacted phosphate is removed via washing of the filter papers.

The plate-based ^{33}PanQinase™ assay relies on incubation of the reaction mixture in scintillant-coated polystyrene microtiter plates. Unbound phosphate is also removed via washing.

Advantages of radiometric HotSpot℠ and ^{33}PanQinase™ assays

- Detect the true catalytic product
- No substrate tags required for capturing
- Compatible with protein or peptide substrates
- Homogenous reaction
- Tolerate fluorescent compounds
- HTS friendly

Limitations of radiometric HotSpot℠ and ^{33}PanQinase™ assays

- Radioisotope management
- Require wash step
- Peptide substrates should have a MW >10 KDa (^{33}PanQinase™)

Bead-based Scintillation Proximity

Like radiometric assays, bead-based scintillation proximity assays are activity-based formats utilizing radioisotope-labeled ATP. In this format, the tagged substrate is bound to scintillation beads. When the radioisotope is brought into proximity with the scintillant-filled beads via binding to the substrate, scintillation is triggered. Scintillation proximity is a "mix and read" format involving no wash steps, but substrate modification is required.

- Advantages of the Bead-based Scintillation Proximity Assay
  - Detects the true catalytic product
  - Homogenous reaction
  - No wash step required

- Limitations of the Bead-based Scintillation Proximity Assay
  - Radioisotope management
  - Substrate requires modification for capture
  - Difficult to adapt for use with protein substrates
  - High ATP concentration may interfere with the signal-to-noise ratio

Fluorescence Resonance Energy Transfer

Fluorescence resonance energy transfer (FRET) involves the transfer of non-radiative energy from a donor fluorophore to a close-proximity acceptor fluorophore. One type of FRET assay employs a protease-coupled reaction. In the primary reaction step, synthetic peptide substrate, labeled with donor and acceptor fluorophores, is incubated with kinase, ATP, and the test or control compound. In the second reaction step, protease is added to the reaction mixture, resulting in cleavage of the unphosphorylated substrate, thereby separating the donor and acceptor fluorophores. Upon excitation, only uncleaved, phosphorylated substrate will exhibit FRET signal.

The advantages of FRET are its homogenous format and simple application to HTS. However, this format requires screening of the compounds for inhibitory action against the coupling enzyme. Additionally, fluorescent compounds may cause signal interference, though this concern may be addressed by performing radiometric measurements. A limited number of synthetic substrates are typically used due to the constraints involved in designing substrate sequences for protease-coupled FRET.

- Advantages of FRET
  - HTS friendly
  - Homogenous reaction

- Limitations of FRET
  - Uses synthetic peptide substrate
  - Requires counter screening against coupling enzyme
  - Demonstrates a high false-positive rate for fluorescent compounds
  - Substrate requires modification for fluorophore labeling

Time-Resolved Fluorescence Resonance Energy Transfer

Time-resolved fluorescence resonance energy transfer (TR-FRET) is based on the same principle as FRET but uses fluorophores with long decay times, thereby allowing measurement of enzyme kinetics in real time and avoiding interference from compounds with short fluorescence lifetimes. TR-FRET technology is used for both binding assays and activity assays. One type of activity-based TR-FRET assay employs a peptide substrate labeled with an acceptor fluorophore, and an anti-phosphopeptide detection antibody labeled with a donor fluorophore.

As a result, only the phosphorylated substrate will exhibit TR-FRET. Due to the challenges of developing specific detection antibodies, only peptide substrates are compatible with this format. Another TR-FRET activity assay is based on antibody detection of ADP.

- Advantages of TR-FRET
  - HTS friendly
  - Homogenous reaction
  - Measures real-time kinetics

- Limitations of TR-FRET
  - Antibody-based detection
  - Demonstrates medium to low false-positive rates for fluorescent compounds
  - Substrate requires modification for fluorophore labeling

Luminescence Detection

Luminescence-based assays measure the amount of ATP using luciferase. In the presence of ATP, luciferase converts luciferin to oxyluciferin, resulting in the emission of light. Though this format can demonstrate low sensitivity with low ATP concentrations, this effect can be counteracted by using a two-step detection method involving *(1)* stopping the kinase reaction and depleting the remaining ATP, and *(2)* adding reagent to convert ADP to ATP, which is measured using the coupled luciferase reaction. Luminescence detection methods accommodate fluorescent compounds but require screening of compounds for inhibitory activity against luciferase.[13, 14]

- Advantages of Luminescence Detection
  - HTS friendly
  - Homogenous reaction
  - Accommodates fluorescent compounds
- Limitations of Luminescence Detection
  - Requires counter screening against coupling enzyme
  - May exhibit low sensitivity at low ATP concentrations

Mobility Shift

Mobility shift assays use fluorophore-labeled substrate and employ electroporation to separate the more-negatively charged phosphorylated product from unphosphorylated substrate. The product is quantified by measuring fluorescence intensity. Because mobility shift platforms are highly dependent on the charge difference between substrate and product, specially developed peptide substrates, are typically used.

- Advantages of Mobility Shift
  - HTS friendly
  - Homogenous reaction
  - Measures real-time kinetics

- Limitations of Mobility Shift
  - Uses peptide substrates only
  - Requires special electroporation instrument and analysis
  - Substrate requires modification for fluorophore labeling

Competition Binding

Competition binding assays quantify the binding of small molecules to the kinase active site, rather than measuring catalytic product. Typically, a standard active site–binding inhibitor is immobilized on a solid support or conjugated to a tracer molecule. This standard inhibitor competes with the test compound for binding to the protein kinase domain. Competition binding assays are performed in the absence of ATP and substrate. Thus, these assays are generally unable to detect substrate-specific inhibitors,[5, 6] compounds that are of special interest for their selectivity[7-9] and their therapeutic potential in tumor cells that are resistant to ATP-competitive inhibitors.[10, 11] Binding assays are also unlikely to detect inhibitors that interact with domains other than the kinase domain, including the pleckstrin homology domain, which has emerged as a highly selective anti-cancer target.[12]

- Advantages of Competition Binding
  - Amenable to partially purified kinases
  - Measures binding to inactive kinases

- Limitations of Competition Binding
  - Does not measure the catalytic product
  - Requires probes or tracer molecules
  - ATP and substrate are not used
  - Phage-displayed protein may fold differently than the purified protein
  - Does not typically detect inhibitors that are substrate-specific or bind to domains other than the kinase domain

CONCLUSION

Each platform for kinase profiling and screening has distinct advantages and limitations. Reaction Biology prefers the radiometric activity assays, the radioisotope filter binding assay HotSpot[SM] and the ^{33}PanQinase[TM] scintillation-based assays, which remain the gold standard for kinase screening and profiling.

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Comparison of Common Kinase Profiling Platforms

	Measures catalytic activity	*Detects substrate-specific inhibitors*	*No counter screening required*	*Accommodates both peptide and protein substrates*	*No modified substrates*
Radiometric Activity Assays	√	√	√	√	√
Bead-based Scintillation Proximity	√	√	√	√	X
FRET*	√	√	X	X	X
TR-FRET†	√	√	X	X	X
Luminescence Detection	√	√	X	√	√
Mobility Shift	√	√	√	X	√
Competition binding	X	X	√	N/A	N/A

* Protease-coupled format

† Anti-phosphopeptide–based detection format

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