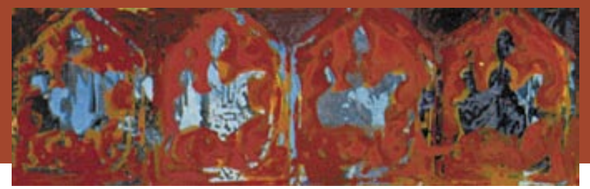

Screening and profiling for kinases: Development of a unique and versatile platform

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Abstract

To build a unique kinase platform providing reliable and quantitative data, we have designed a flexible system with well-defined yet different assay conditions for each kinase. This platform minimizes interferences, including those from test compounds, and displays a homogeneous and generic assay format. After reviewing available assay technologies, Homogeneous Time Resolved Fluorescence (HTRF) was selected as that which best met our requirements. To date, 70 kinase assays have been developed on this model. Using pertinent examples, we will illustrate how this technology supports and serves scientific drug discovery, and allows direct and obvious comparison across the panel for both high throughput compound profiling and high throughput screening.

Introduction

Following the GPCRs, kinases are one of the most interesting target families in Drug Discovery [1]. The kinases are a large family with 518 members (2 sub-families, protein tyrosine and protein serine-threonine), and they mediate a wide spectrum of actions [2].

Kinases are involved in therapeutic areas of high interest and continuous evolution including cancers [3], inflammation [4] and diabetes. Examples of Fasudil® (Asahi Kasei), Iressa® (AstraZeneca) and Gleevec® (Novartis) have already proven the drugability of the target family [5].

An interesting feature of kinases to consider when designing inhibitory compounds is the various possible ways to reach that goal: Inhibitors may be ATP-site or substrate-site directed, they may act on enzyme activation steps (phosphorylation cascade) or through an allosteric regulation [6].

Kinase inhibitors may hold activity against a spectrum of kinases, as opposed to other therapeutic areas in which highly-specific inhibitors are desired. Thus the profiling of mid- to large-size libraries is necessary to systematically detect and characterize compounds with the desired selectivity profile. A unique, generic HTS compatible format allowing comparison across and throughout the panel is therefore preferred.

Cerep's kinase platform, providing reliable and quantitative data, has thus been developed, and will be described here.

Current status

Over 20 platform technologies are available (Radioactivity, Fluorescence Polarization, Time Resolved Fluorescence…) [7], however most display important limitations for the development of a coherent screening-profiling platform. Well known drawbacks include those related to heterogeneous assay systems, limitations in ATP concentration, compound interferences and limitations of substrate size and charge [8].

HTP & HTS platform requirements

To develop a high throughput screening and profiling platform, the chosen technology and assay format must resolve specific limitations:

- Results must be reliable and allow quantitative data comparison from one kinase to another. Thus, the technology has to be unique for all kinases, with a generic assay format that also accommodates well-adapted assay conditions for each kinase.
- The selected technology must minimize interferences of test compounds and reagents as well as accommodate different substrates and various ATP concentrations.
- Finally, the assay format must be homogeneous, and miniaturizable for high throughput testing at a competitive cost/point.

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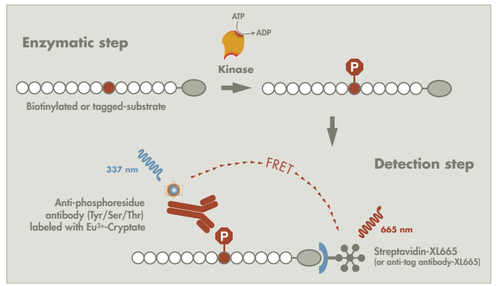
KINASE HTRF® ASSAY PRINCIPLE



HTRF® was chosen as best meeting our specifications.

HTRF® is based on a transfer of fluorescence using Europium (Eu^{3+}) cryptate and XL665 as donor and acceptor respectively. When the biotinylated (or tagged) substrate is phosphorylated by kinase, it can be recognized by the cryptate-labeled phospho-specific antibody. Addition of Streptavidin-XL665, SA-XL665, (or anti-tag antibody XL665) causes the juxtaposition of the cryptate and XL665 fluorophore, resulting in FRET (fluorescence resonance energy transfer). FRET intensity depends on the amount of bounded cryptate antibody, which is proportional to the extent of substrate phosphorylation [9].

HTRF® is a trademark of CisBio International

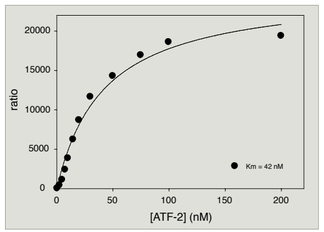
CHARACTERISTICS OF FEW KINASE ASSAYS

Target	Family	Substrate	[S]/ KmS	[ATP]/ KmATP	SNR	Z' factor
MEK1	Ser/Thr	Unactive ERK2 (GST-tagged protein)	1.0	0.6	12	> 0.8
JNK1	Ser/Thr	ATF2 (GST-tagged protein)	0.5	1.0	10	> 0.8
RSK2	Ser/Thr	biotinyl-CREB derived peptide (including RRPS^{133}YR)	1.0	1.8	14	> 0.7
CHK1	Ser/Thr	cdc25 peptide (biotinyl-βAβAβAKKKVSRSGLYRSP SMPENLNRPR)	1.1	1.0	9	> 0.7
FGFR1	Recept. Tyr	optimized peptide (biotinyl-βAβAβAAEEEYFFLFAKKK)	1.2	1.0	7	> 0.7
Lyn	Cytopl. Tyr	cdc2 peptide (biotinyl-βAβAβKVEKIGEGTYGV-VYK)	0.7	1.5	14	> 0.7

The assay parameters for six kinases are presented in the above Table. Both Serine-Threonine and Tyrosine (receptor and cytoplasmic) kinases have been successfully implemented in a platform designed for flexibility. Substrates are physiologically relevant proteins, derived peptides or optimized peptides. All activity measurements are performed with a non-saturating concentration of enzyme and at initial rate. Moreover, all assays are run with ATP and substrate concentrations [S] close to their respective Km in order to offer a sensitive assay for all type of inhibitors (both ATP and substrate site). Finally, assays are robust as attested by SNR (Signal-to-Noise Ratio) and/or Z'.

ATF-2 Km determination for JNK1

→ HTRF® is compatible with a protein as substrate



Substrate Km determination (JNK1)

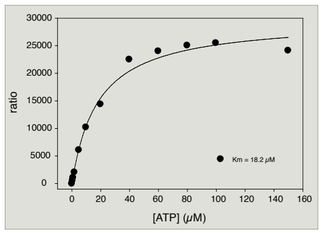
The ATF2 Km value for the human JNK1 was determined by increasing ATF2 concentration in the presence of 40 ng/mL of enzyme and 1 µM of ATP (saturating concentration). A similar experiment was performed in the absence of enzyme to determine the background signal for each substrate concentration (subtracted in graph shown).

After 15 minutes of incubation at room temperature (initial rate condition), reaction was stopped by EDTA addition. HTRF reagents (cryptate labeled anti-phospho-ATF2(Thr71) antibody and SA-XL665) were then added to the reaction medium. The substrate to SA-XL665 ratio was kept constant. Reading was carried out with a Rubystar (BMG Labtechnologies) at 665 and 620 nm after excitation at 337 nm, and 665/620 ratios were calculated.

Results are expressed as the specific measured ratio at different substrate concentrations.

ATP Km determination for TRKA

→ a wide range of useable ATP concentration



ATP Km determination (TRKA)

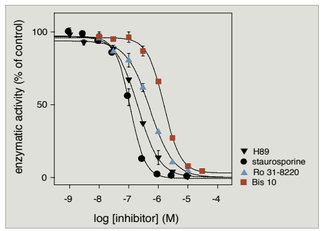
The ATP Km value was determined by increasing its concentration in the presence of 250 ng/mL of human TRKA catalytic domain and 4 µM (saturating concentration) of substrate, a biotinylated PLCγ derived-peptide.

After 20 minutes of incubation at room temperature (initial rate condition), reaction was stopped by EDTA addition. HTRF reagents (cryptate labelled anti-phospho-Tyr PT66 antibody and SA-XL665) were then added to the reaction medium. Reading was carried out with a Rubystar at 665 and 620 nm after excitation at 337 nm, and 665/620 ratios were calculated.

Results are expressed as the specific measured ratio at different ATP concentrations.

Inhibition of PKA activity

→ no interference of colored test compound (■,▲)



Short Pharmacological Profile (PKA)

The effect of selected compounds (known PKA inhibitors) was determined in the presence of 7.5 ng/mL of human PKA catalytic domain (except for negative controls), 10 nM of substrate, a biotinylated CREB derived-peptide, and 0.25 µM of ATP.

After 20 minutes of incubation at room temperature, reaction was stopped by EDTA addition. HTRF reagents (cryptate labelled anti-phospho-CREB (Ser^{133}) antibody and SA-XL665) were then added to the reaction medium. Reading was carried out with a Rubystar at 665 and 620 nm after excitation at 337 nm, and 665/620 ratios were calculated.

Results are expressed as the % of remaining enzymatic activity at different inhibitor concentrations (M).

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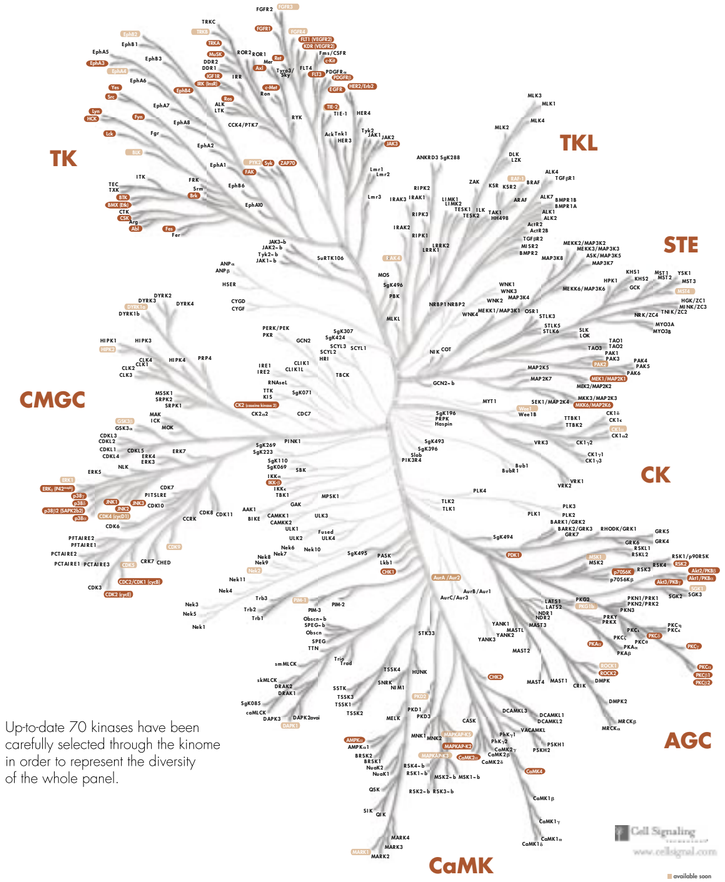
CEREP'S KINASE PANEL DIVERSITY



Up-to-date 70 kinases have been carefully selected through the kinome in order to represent the diversity of the whole panel.

CONCLUSION

Cerep's unique and versatile kinase platform:

- **Diverse panel: 70 kinases available today**
- **Single technology (HTRF®) and generic format for all the assays → reliable data comparison**
- **High assay quality: relevant parameters are well-defined for each target (0.3<Km<3 for both ATP and substrate as standard) but are also customizable**
- **High throughput for both profiling and screening: homogeneous and miniaturized assays**
- **Panel continuously increasing: 100 targets by the end 2005 based on Cerep's expertise in both kinases and HTRF assay development**
- **Cerep's kinase panel has been validated testing several known kinase inhibitors, including Gleevec®, Iressa®, Fasudil®, SB203580, (R)-roscovitine …**

Cerep's platform is not only driven by the technology, it answers scientific questions (target, type of inhibitors, mechanism of action…).