Large Scale Kinase Profiling and Screening Using HTRF Technology

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Abstract

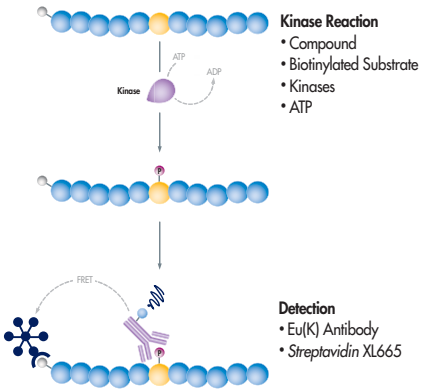
Kinase profiling has become an essential early drug discovery step as a test for functionality and specificity of potential inhibitors. As the pace of lead generation has quickened and data analysis tools have become more sophisticated, large data sets generated from, for example, profiling compound libraries against targeted kinase panels have become increasingly valuable contributions toward critical drug development decisions. Although many technologies are available for either in-house or outsourced large-scale kinase profiling, the challenge has been to select a technology that is both reliable and cost effective. Millipore has partnered with CisBio to develop a homogeneous time-resolved fluorescence (HTRF®) kinase assay technology for high-throughput screening and large-scale kinase profiling. Here, we present and analyze data generated from Millipore's **Kinase**Profiler™ XL service, which uses HTRF KinEASE™ to measure enzyme activity and inhibition to rapidly generate thousands of data points at a fractional cost of conventional labor and time-intensive methods. These data sets were analyzed to uncover complex relationships within and among kinase families that can lead to a better understanding of potential inhibitor effects. Furthermore, the unique properties of HTRF technology are demonstrated to produce robust, reproducible data that are far less susceptible to the probabilities of compound-related false positives or negatives that can hinder the effectiveness of other fluorescence-based technologies.

HTRF Technology and Assay Format

HTRF is based on time-resolved fluorescence resonance energy transfer (TR-FRET) chemistry, which utilizes long-lived lanthanide donor fluorescence to allow for measurement of acceptor energy emission approximately 100 µseconds after most background fluorescence will have occurred.

The HTRF KinEASE assay's unique properties include the use of Europium, a lanthanide with an extremely long emission half-life; the conjugation of Eu3+ to cryptate for increased assay stability; and the use of a patented ratiometric measurement that allows for assay interference correction

Millipore's **Kinase**Profiler XL service uses a set of biotinylated peptide substrates in the HTRF KinEASE assay to rapidly screen compounds for inhibitory effects against up to 118 kinases in the general assay format depicted below.



Kinases Validated in HTRF Screening Service

The following tables list the 118 kinases currently validated for HTRF assays in the **Kinase**Profiler XL service. These kinases include representatives from all major protein kinase families, and several important tyrosine kinase mutants. Further validation is currently underway to expand the kinase panel.

Tyrosine Kinases *(34 Available)*

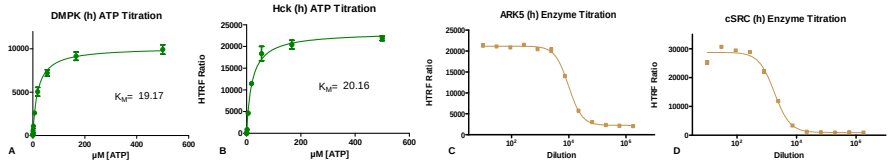
Bmx (h)	EphA2 (h)	IR (h)
c-Kit (D816H) (h)	ErbB4 (h)	JAK2 (h)
c-Kit (h)	FAK (h)	JAK3 (h)
c-Kit (D816V) (h)	FGFR1 (h)	Lck (h)
c-Kit (V560G) (h)	FGFR2 (h)	Lyn (h)
CSK (h)	FGFR2 (N549H) (h)	MER (h)
cSrc (h)	FGFR4 (h)	Met (h)
EGFR (h)	Fgr (h)	Ron (h)
EGFR (L861Q) (h)	Flt3 (h)	TrkB (h)
EGFR (L858R) (h)	Flt3 (D835Y) (h)	ZAP-70 (h)
EGFR (T790M) (h)	Flt4 (h)	
EGFR (T790M, L858R) (h)	Hck (h)	

Ser/Thr Kinases *(84 Available)*

ARK5 (h)	LKB1 (h)	p70S6K (h)	PKG1a (h)
ASK1 (h)	LOK (h)	PAK2 (h)	PKG1b (h)
Aurora-A (h)	MAPKAP-K2 (h)	PAK3 (h)	Plk3 (h)
BrSK1 (h)	MARK1(h)	PAK4 (h)	PRAK (h)
BrSK2 (h)	MELK (h)	PAK6 (h)	PRK2 (h)
CaMKIIγ (h)	MINK (h)	PASK (h)	PrKX (h)
CaMKIV (h)	MLCK (h)	PhKg2 (h)	ROCK-II (h)
CHK1 (h)	Mnk2 (h)	Pim-1 (h)	Rsk2 (h)
CHK2 (h)	MRCKa (h)	Pim2 (h)	Rsk3 (h)
CLK3 (h)	MRCKb (h)	PKA (h)	Rsk4 (h)
DAPK1 (h)	MSK1 (h)	PKBγ(h)	SGK (h)
DAPK2 (h)	MSK2 (h)	PKCa (h)	SGK2 (h)
DCAMKL2 (h)	MSSK1 (h)	PKCδ(h)	SGK3 (h)
DMPK (h)	MST1 (h)	PKCε(h)	Snk (h)
DRAK1 (h)	MST2 (h)	PKCγ(h)	STK33 (h)
DYRK2 (h)	NEK2 (h)	PKCη(h)	TBK1 (h)
GRK6 (h)	NEK6 (h)	PKCι(h)	TSSK1 (h)
HIPK1 (h)	NEK11 (h)	PKCμ (h)	TSSK2 (h)
HIPK2 (h)	NEK3 (h)	PKCθ(h)	WNK2 (h)
HIPK3 (h)	NEK7 (h)	PKCζ(h)	WNK3 (h)
IKKβ(h)	NLK (h)	PKD2 (h)	ZIPK (h)

HTRF Assay Development

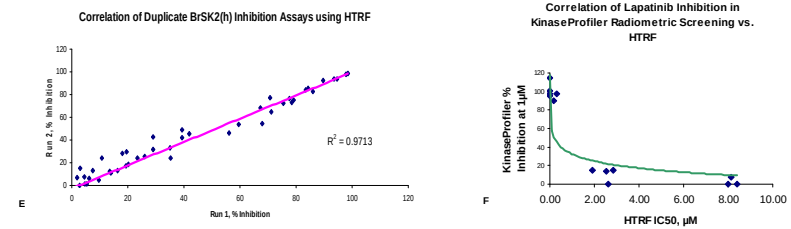
K_M(ATP) and optimal enzyme dilutions for each kinase in the HTRF KinEASE assay were determined using 12-point, 3-fold dilution curves for ATP (A, B) and enzyme (C, D). Depicted are assay development curves for human serine/threonine kinases DMPK (A) and Ark5 (C); and human tyrosine kinases Hck (B) and cSrc (D). Each point represents the mean of triplicate data points.



HTRF Assay Validation

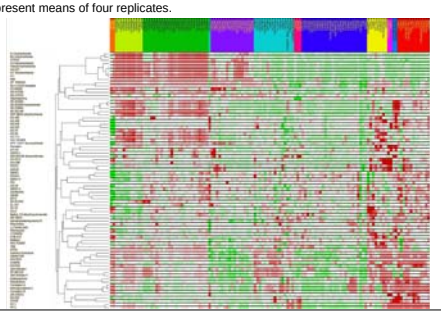
Duplicate HTRF assays were run with panels of clinical inhibitor compounds against single kinases and plotted to assess reproducibility. Representative data for BrSK2 (h) is depicted in Figure E.

Inhibition results of Lapatinib at 1µM in HTRF assays and radiometric **Kinase**Profiler assays were compared (Figure F) to demonstrate compatibility between the assay formats. Results indicate good correlation of relative inhibition rankings between the two platforms.



Large Scale Profiling: Cluster Analysis Using HTRF Technology

127 kinases (89 STKs and 38 TKs) were profiled with 77 compounds at K_M(ATP) using Millipore's **Kinase**Profiler XL HTRF assays. Results were color coded based on inhibition levels and grouped based on related inhibition profiles. Data points represent means of four replicates.



Further Characterization of Lapatinib in HTRF Assays

IC_{50} Determinations

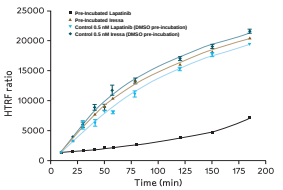
Duplicate 10-point serial dilutions of Lapatinib were assayed against recombinant human kinases at K_M(ATP) using HTRF technology to determine IC_{50} values. Results indicate significant specificity toward wild type and mutant EGFR, and ErbB4.

Lapatinib IC_{50} (nM)

EGFR (h)	0.90	cSrc (h)	>10000	JAK2 (h)	>10000
EGFR (L858R) (h)	0.40	EphA2 (h)	>10000	JAK3 (h)	>10000
EGFR (L861Q) (h)	0.45	FAK (h)	>10000	Lyn (h)	>10000
EGFR (T790M) (h)	177.27	FGFR1 (h)	>10000	Met (h)	>10000
EGFR (T790M,L858R) (h)	298.42	FGFR2 (h)	>10000	Ron (h)	>10000
ErbB4 (h)	5.81	FGFR4 (h)	>10000	TrkA (h)	>10000
c-Kit (h)	>10000	Flt4 (h)	>10000	TrkB (h)	>10000
CSK (h)	>10000	IR (h)	>10000	ZAP-70 (h)	>10000

Slow Off-Rate Measurement

EGFR (h) was pre-incubated with 500 nM Lapatinib, Iressa, or DMSO for 30 mins on ice. Each pre-incubation was diluted 1/1000 into an HTRF assay (0.5 nM final assay concentration). Control assays included the same final compound concentrations, but were pre-incubated with DMSO alone.



Summary

- Large scale profiling of compound libraries against kinases offers rapid advancements in inhibitor development projects
- HTRF technology is a robust technology for large scale kinase inhibitor screening, and for advanced mechanism of action studies
- HTRF technology provides reliable results with a direct enzyme assay format while avoiding compound fluorescence interference issues
- Millipore's **Kinase**Profiler XL service has commercialized large scale HTRF profiling of 118 protein kinases, with comparable results to radiometric screening at a considerably reduced cost

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