Novel Kinase Assays for HTS

Michael Tsionsky,
Tatiana Plissova, Eli N. Glezer,
James L. Wilbur, George B. Sigal,
Hans A. Biebuyck and Jacob N. Wohlstadter

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Abstract

Activity assays for Tyrosine Kinase and Serine/Threonine Kinase are highly desirable in drug discovery. There are few satisfactory binding assays for measuring kinase activity, in part because there are few selective and high-affinity antibodies that bind to the phosphorylated products of kinases. Assay methods that use harsh conditions or aggressive wash steps can disrupt weakly bound antibodies and compromise the sensitivity and reliability of the assay. Some homogeneous technologies, which avoid washes, suffer from unacceptable levels of background signal. In this poster, we present kinase assays conducted on a new assay platform developed by Meso Scale Discovery™ (MSD™) that overcomes the difficulties listed above. This platform combines array technologies and electrochemiluminescence detection to achieve sensitive kinase assays that have a simple no-wash format. The assays have been optimized for both 96 and 384 well microplates. Model screens were run on a 10,000 compound library, demonstrating robust assay performance for both Tyrosine and Serine/Threonine Kinases.
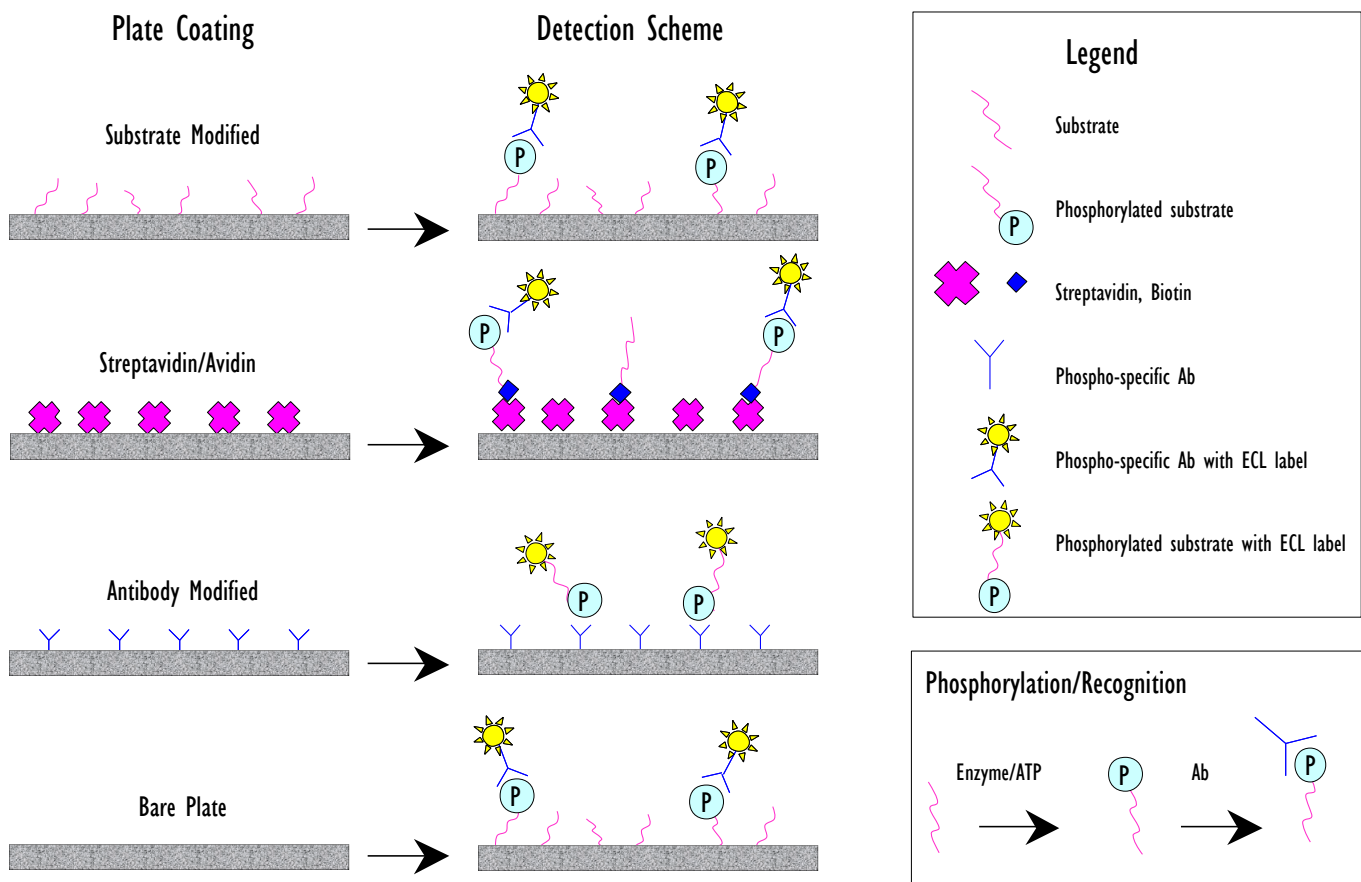
Introduction to Kinases

Protein phosphorylation is a critical regulatory mechanism found in cells. Cells often respond to such diverse stimuli as mitogens, inflammatory cytokines, growth factors, and toxins via protein kinases and phosphatases. Modulation of these signaling cascades is often the mechanism for therapies directed against cancer, inflammation, immunology and neurodegenerative disorders[1]. Assays that screen for kinase inhibitors are very popular oncology drug targets[1, 2].

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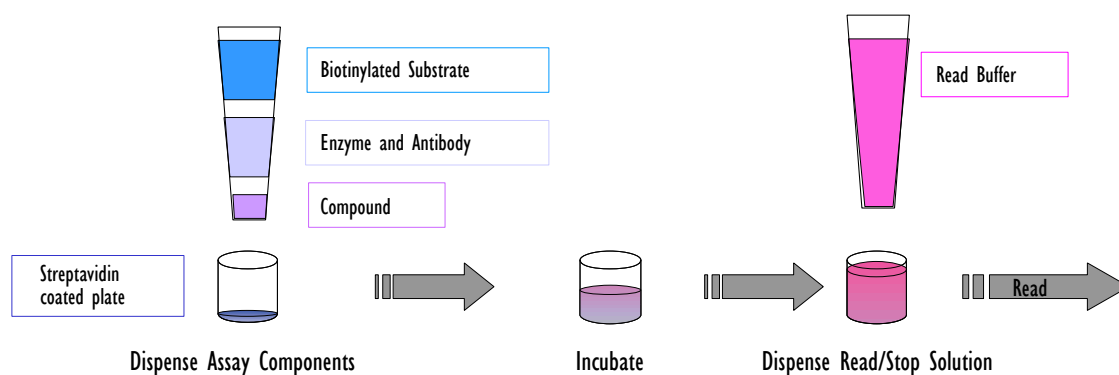A division of Meso Scale Diagnostics®, LLC.

Versatility of Assay Formats

Plate Coating

Detection Scheme

Substrate Modified

Streptavidin/Avidin

Antibody Modified

Bare Plate

Legend

Substrate

P Phosphorylated substrate

Streptavidin, Biotin

Phospho-specific Ab

Phospho-specific Ab with ECL label

Phosphorylated substrate with ECL label

Phosphorylation/Recognition

Enzyme/ATP   P   Ab   P

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Protocol for Tyrosine Kinase Assay on Streptavidin Plates

Biotinylated Substrate

Enzyme and Antibody

Compound

Read Buffer

Streptavidin coated plate

Dispense Assay Components

Incubate

Dispense Read/Stop Solution

Read

HTS-compatible format
- Only 2 dispense steps
- No wash steps
- Flexible scheduling - only one timed incubation

Methods

Plates
Plates were coated by micro-dispensing 2.5 μL/well of 0.5 mg/mL Streptavidin solution using Cartesian's PixSys^{TM} 4200 dispensing system. Plates were then blocked with 5% (w/v) BSA for at least 2 hours. The blocked plates were washed and dried prior to use.
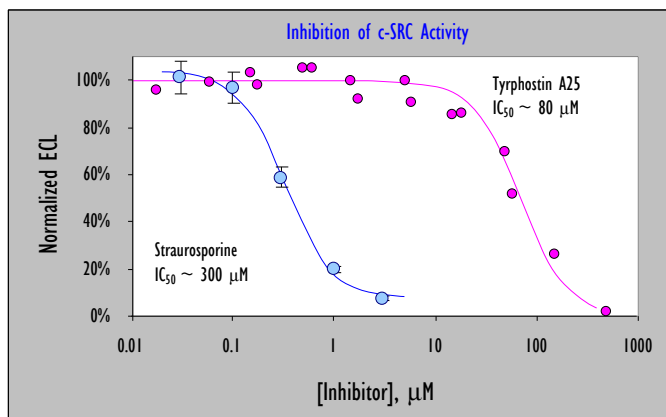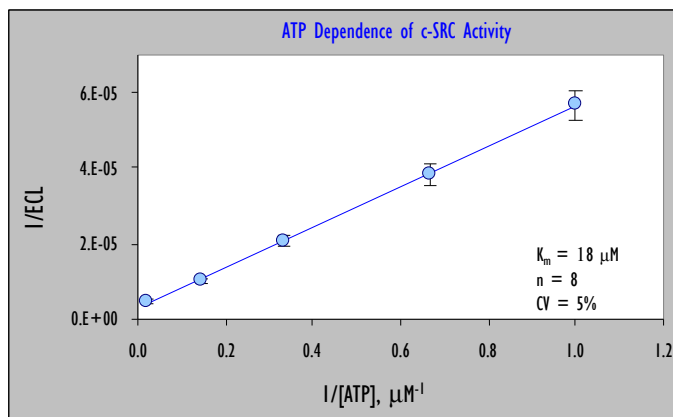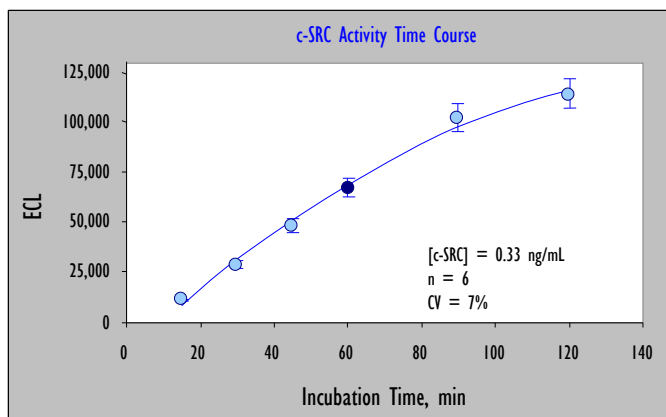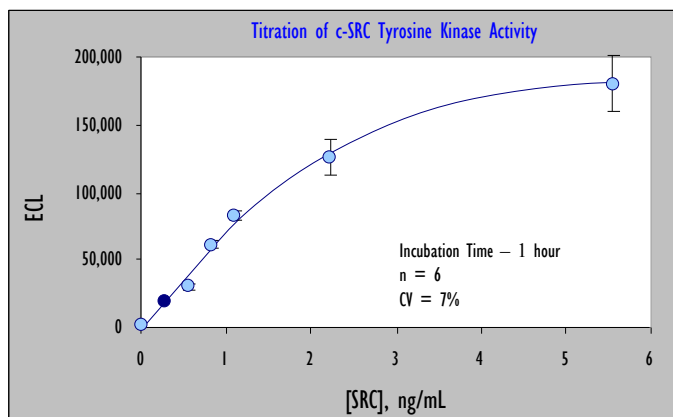
Assay
Biotinylated substrate solution (25 μL), enzyme and labeled Ab solution (25 μL), and compound from the library (2.5 μL) were dispensed on Zymark's RapidPlate$^{®}$. After 1-hour incubation, each well received 200 μL of Read/Stop Buffer solution.

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Tyrosine Kinase Assay on 96-Well Substrate Modified Plates

Titration of c-SRC Tyrosine Kinase Activity

ECL

[SRC], ng/mL

Incubation Time – 1 hour
n = 6
CV = 7%

c-SRC Activity Time Course

ECL

Incubation Time, min

[c-SRC] = 0.33 ng/mL
n = 6
CV = 7%

ATP Dependence of c-SRC Activity

1/ECL

1/[ATP], μM^{-1}

K_m = 18 μM
n = 8
CV = 5%

Inhibition of c-SRC Activity

Normalized ECL

[Inhibitor], μM

Tyrphostin A25
IC_{50} ~ 80 μM

Straurosporine
IC_{50} ~ 300 μM

Selected Conditions: [c-SCR] = 0.33 ng/mL, [ATP] = 50 mM, labeled Ab = 0.5 μg/mL, poly(Glu,Tyr) 4:1 modified plates, Incubation time = 1 hour
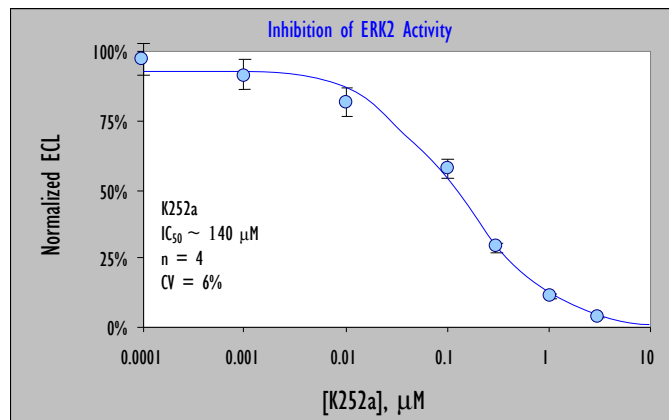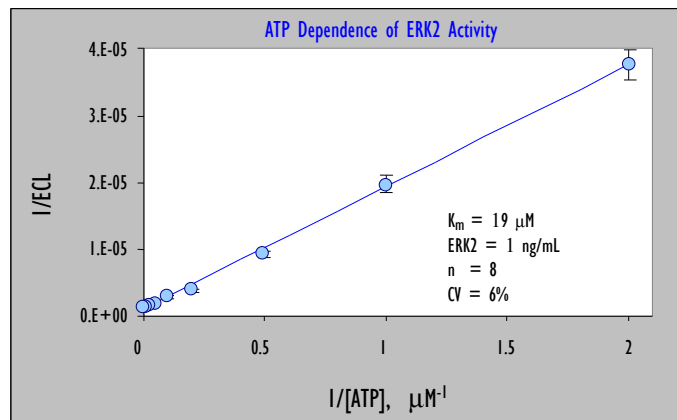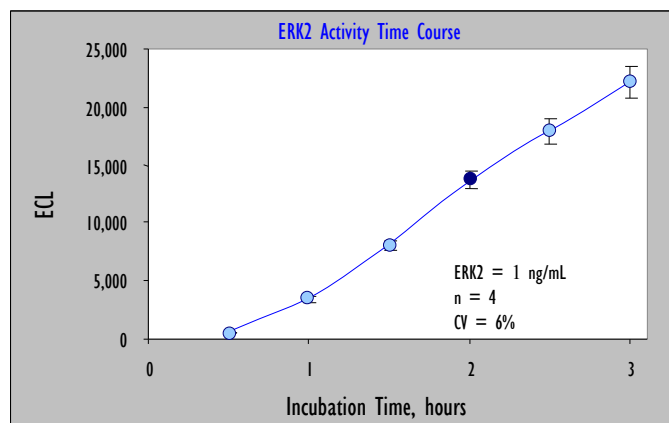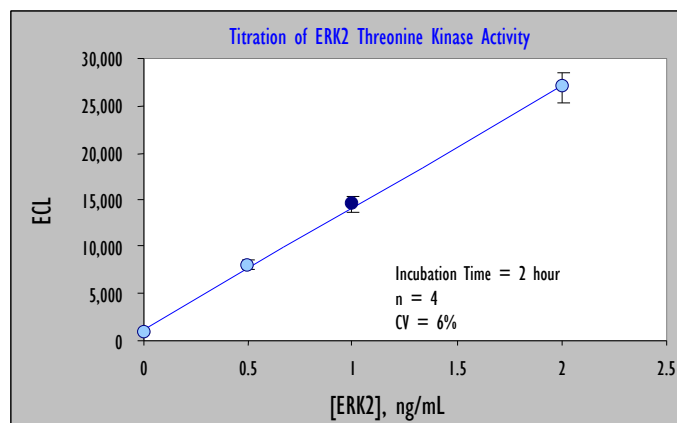
Assay Performance: Signal/Background = 60, CV < 10%, Z-factor >0.6

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Threonine Kinase Assay on 96-well Streptavidin Modified Plates

Titration of ERK2 Threonine Kinase Activity

Incubation Time = 2 hour
n = 4
CV = 6%

x-axis: [ERK2], ng/mL
y-axis: ECL

ERK2 Activity Time Course

ERK2 = 1 ng/mL
n = 4
CV = 6%

x-axis: Incubation Time, hours
y-axis: ECL

ATP Dependence of ERK2 Activity

$K_m = 19$ μM
ERK2 = 1 ng/mL
n = 8
CV = 6%

x-axis: 1/[ATP], μM^{-1}
y-axis: 1/ECL

Inhibition of ERK2 Activity

K252a
$IC_{50} \sim 140$ μM
n = 4
CV = 6%

x-axis: [K252a], μM
y-axis: Normalized ECL

Selected Conditions: [ERK2] = 1 ng/mL, [ATP] = 50 μM, [bMBP] = 100 nM, primary Ab = 2 nM, secondary Ab = 5 nM, Incubation time = 2 hours
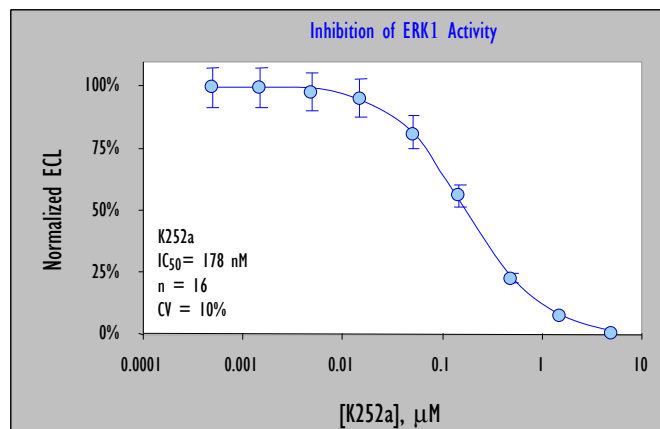
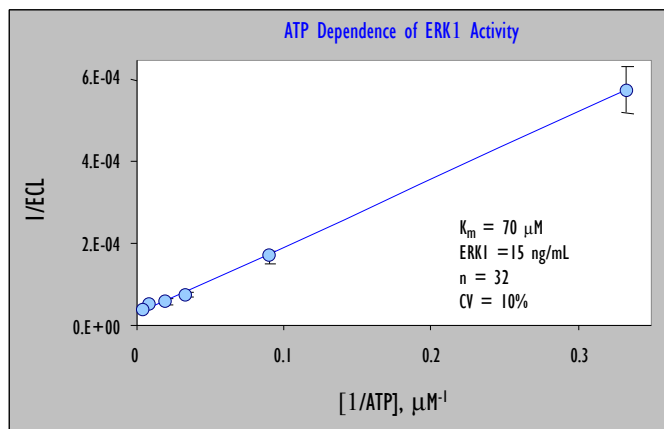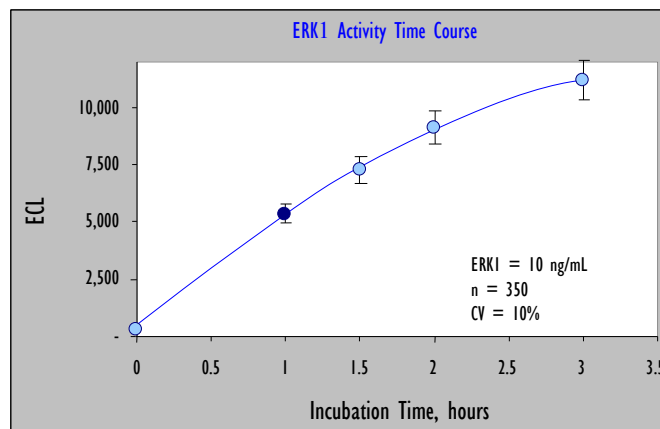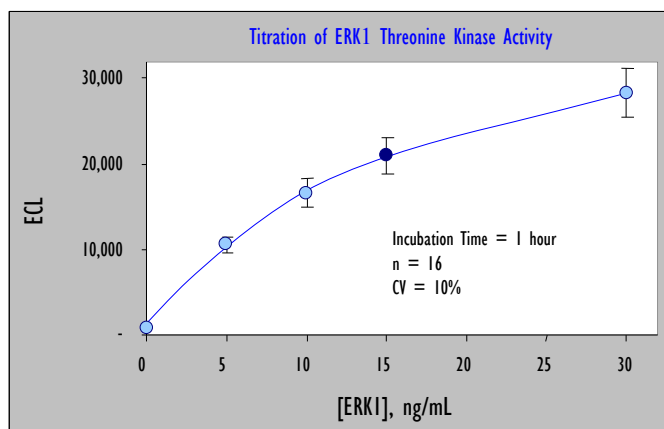**Assay performance:** Signal/Background = 30, CV <10%, Z-factor > 0.6

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Threonine Kinase Assay on 384-well Bare Plates

Titration of ERK1 Threonine Kinase Activity

ECL vs [ERK1], ng/mL

Incubation Time = 1 hour
n = 16
CV = 10%

ERK1 Activity Time Course

ECL vs Incubation Time, hours

ERK1 = 10 ng/mL
n = 350
CV = 10%

ATP Dependence of ERK1 Activity

1/ECL vs [1/ATP], μM^{-1}

K_m = 70 μM
ERK1 = 15 ng/mL
n = 32
CV = 10%

Inhibition of ERK1 Activity

Normalized ECL vs [K252a], μM

K252a
IC_{50} = 178 nM
n = 16
CV = 10%

Selected Conditions: [ERK1] = 15 ng/mL, [ATP] = 100 μM, [MBP] = 600 nM, primary Ab = 10 nM, secondary Ab = 25 nM, Incubation Time = 1 hour

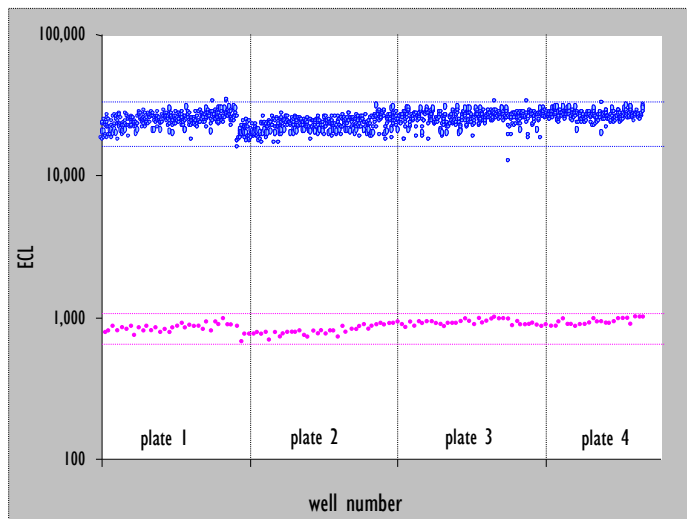**Assay Performance:** Signal/Background = 29, CV = 10%, Z-factor > 0.6

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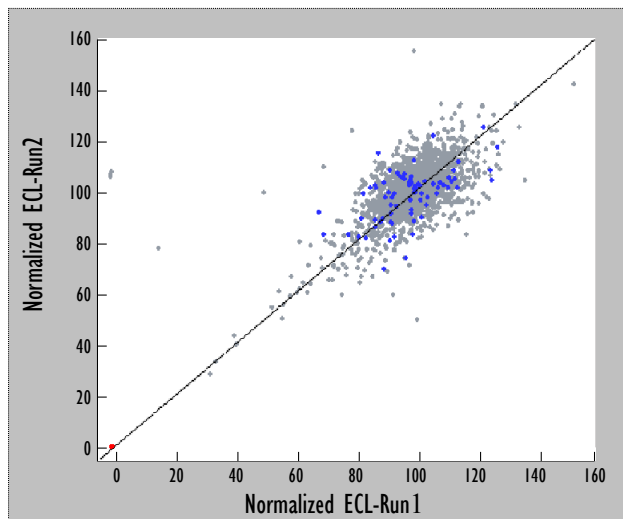
Novel Kinase Assays for HTS

Assay Performance in HTS applications

Threonine Kinase Controls
Bare 384-well plates

Signal to Background = 29
Z-factor = 0.63
Positive Control CV = 12%
Negative Control CV = 8%
Lines represent 3 SD

Tyrosine Kinase Screen in Duplicates
Substrate-modified 96-well plates

Signal to Background = 60
Z-factor = 0.75
Positive Control CV = 8%
Negative Control CV = 7%
1,800 compound run shown

Conclusion

Tyrosine and Serine/Threonine Kinase assays were developed on a novel platform from Meso Scale Discovery. This platform uses Multi-Array technology that combines array technology with electrochemiluminescent detection.

Multi-Array technology provides a sensitive, robust, simple, non-radioactive means to assay protein kinase activity.

Assay protocols were designed for HTS – no-wash format, short incubation time, automation-friendly protocol.

High quality compound screening data were obtained in 96 and 384-well plates – S/B>30, CVs – 10-12%, Z-factor > 0.6.

>100-fold reduction in enzyme consumption results from highly sensitive detection (relative to other technologies[3,4]).

Several assay formats were shown to be effective.

References:
1. Society for Medicines Research Committee (1999). Drug News Perspect. 12: 247-251.
2. Levitzki, A. and Gazit, A. (1995). Science 267: 1782-1788.
3. Amersham Pharmacia Biotech Catalog, 2000, p.76
4. Packard BioScience-Alpha Screen c-Src Kinase Assay. AN003-ASc Application Note, www.packardbioscience.com.

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