Cell-Based Assays for Enhanced Safety, Activity, and Target Specificity Determination

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Cell-Based Assays for Enhanced Safety, Activity, and Target Specificity Determination

Your Moderator

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Managing Editor
Genetic Engineering & Biotechnology News
Cell-Based Assays for Enhanced Safety, Activity, and Target Specificity Determination

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and the PKI team

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Major causes of Attrition

Safety continues to be a major factor!

In order for industry to effectively deliver new therapies in a cost efficient manner, tools must be developed to detect these safety liabilities at earlier stages of development.

J. McKim, Comb. Chem & HTS 13:188, 2010
Origins of Adverse Safety Events

Primary pharmacology
- PDE-4 inhibitors are linked to emesis and vasculitis
- D-1 activity is linked to tremors

Chemical structure
- Clozapine causes agranulocytosis and forms reactive metabolites

Secondary pharmacology
- Lipophilic basic compounds at risk of:
  - Phospholipidosis; hepatotoxicity
  - QT interval prolongation

Pharmacokinetic properties
- Chemical structure
- Metabolism
- Adverse effects

Chemical structure
- Clozapine
- Ariflo

Graphical representation
- Hit threshold (%M) vs. D1 and tremor percentage of hits
Compound Profiling

Profiling compounds across a panel of assays helps to assess promiscuity and safety liabilities.

Potential CV safety
Cytotoxicity,
Genetic risk,
Biological Promiscuity

Structural Toxicophore alerts
PhysChem properties

PhysChem properties
Kinase Project Goals

For kinase target projects: can we supply kinase-specific profiling to assess selectivity?

**Goal:** Identify an external collaboration partner:

- To develop and validate a panel of functional, cellular kinase assays for use in compound testing and de-risking
- Provide on-going, fee-for-service capability for compound testing
Kinase Drug Discovery

- Protein kinases phosphorylate serine, threonine or tyrosine residues

![Chemical structures](image)

- Ser, Thr, Tyr
- ATP
- ADP
- Protein Kinase
- Mg\(^{2+}\)
- pSer, pThr, pTyr

- >500 human protein kinases
  - 2% of human genome, modify up to 30% proteins
Kinases as drug targets

- 14 kinase small molecule drugs on the market *

* Not counting the 3 Rapamycin derivatives
Highly conserved catalytic domain and ATP binding site
• most Ki bind in/near this site

This leads to specific challenges
• Selectivity vs the kinome.....safety issues
• Cellular potency can be difficult to achieve
  because of high cellular ATP levels (1-5 mM)
What we are facing

- Biology is complex, with an incomplete understanding of pathways and actors
- Selectivity is hard to measure, and how much is good enough?
- The importance of other ATP-binding proteins is not known
- Toxicities may be on-target or off-target and difficult to predict
- We lack in vitro to in vivo correlations
Kinome promiscuity and safety

Example: THLE assay: Human liver epithelial cell line

- General cytotoxicity assay measuring proliferation (72 hr treatment, Cell-Titer Glo assay)
- Inhibition in this assay is associated with an increased risk of toxicity in a preclinical rat safety study

An increase in kinome promiscuity correlates with increased risk in THLE assay, predicts increased risk for *in vivo* toxicity

Conclusion: Promiscuity is undesirable

Optimize compound selectivity
Kinase Toolbox: Cell-based assays

- Identified by kinase project teams as a gap
  - does biochemical activity against purified enzyme at $K_m$ ATP concentrations translate to cellular activity?
  - can the results be used to aid in assessing potential safety liabilities and promiscuity of kinase inhibitors?

Monitor substrate phosphorylation

Technology: Preference for direct assessment of kinase cellular activity vs reporter assay
Technology Overview

- Bead-based flow cytometry
- Phospho-specific detection of specific substrate
- Ratiometric and homogenous cell assay format
- Treatment of intact cells with inhibitor, followed by cell lysis, incubation with beads + Ab, then detection
- Capabilities for multi-plex format
## PKI Cell-based Kinase Assays

<table>
<thead>
<tr>
<th>Kinase</th>
<th>Substrate</th>
<th>Cell Line</th>
<th>Potential safety liability</th>
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</thead>
<tbody>
<tr>
<td>ABL</td>
<td>Auto PO\textsubscript{4}</td>
<td>Human epidermoid carcinoma (A431)</td>
<td>immune, skeletal muscle</td>
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<tr>
<td>AKT</td>
<td>PRAS40</td>
<td>Human prostate carcinoma (LnCaP)</td>
<td>CNS, cardio, immune</td>
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<td>Aurora</td>
<td>Histone H3</td>
<td>Cervix adenocarcinoma (HeLa)</td>
<td>anti-proliferative</td>
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<td>EGFR</td>
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<td>skin, bone</td>
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<td>JAK1</td>
<td>STAT3</td>
<td>T lymphocyte, leukemia (Jurkat)</td>
<td>Immune</td>
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<tr>
<td>p38</td>
<td>HSP27</td>
<td>Lung carcinoma (A549)</td>
<td>skin, liver</td>
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<tr>
<td>GSK3β</td>
<td>Tau</td>
<td>Recombinant</td>
<td>GI, liver, heart</td>
</tr>
<tr>
<td>VEGFR2</td>
<td>Auto PO\textsubscript{4}</td>
<td>Recombinant</td>
<td>hypertension</td>
</tr>
</tbody>
</table>
KinaseScreen Assay Development

Example: p38 Assay

Concentration dependence of IL-1β treatment on HSP27 phosphorylation in A549 cells

Time course of IL-1β treatment on HSP27 phosphorylation in A549 cells

For each assay: optimize plating, ligand stimulation (time and concentration), S/N, and validate with known inhibitors.
p38 Assay Validation

Assays show good reproducibility with control inhibitors

<table>
<thead>
<tr>
<th>Compound</th>
<th>Biochemical IC50, nM</th>
<th>PKI Kinase IC50, nM</th>
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<tbody>
<tr>
<td>PF-616</td>
<td>0.5</td>
<td>9.5</td>
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<tr>
<td>SC-80036</td>
<td>36</td>
<td>163</td>
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<tr>
<td>SB-203580</td>
<td>NA</td>
<td>590</td>
</tr>
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</table>
**VEGFR assay**

Effect of Sunitinib on VEGFR2 phosphorylation in PAE.KDR cells

- IC50 = 5.7 nM
- HillSlope = -1.317
- Log [Sunitinib] (M)
- VEGFR2 phosphorylation (% of VEGF-treated control)

Effect of EMD Inhibitor III on VEGFR2 phosphorylation in PAE-KDR cells

- IC50 = 44 nM
- HillSlope = -1.330
- Log [EMD Inhibitor III] (M)
- VEGFR2 phosphorylation (% of VEGF-treated control)

**VEGFR assay**

- Compound: PF-889
- Biochemical % inh at 1 uM: 97%

Results show no inhibition in functional VEGFR assay → biochemical does not translate to cellular activity
**Aurora Assay**

Plate cells | 24 hr | Add nocodazole | 16 hr | Add compounds | 2 hr | Lyse, Histone phospho-S10 ELISA

Assay detects compounds with anti-proliferative potential
In collaboration with PKI, a panel of cellular kinase assays that measure specific substrate phosphorylation have been developed.

These assays are robust and reproducible, and have been validated with known inhibitors for each kinase.

The Functional KinaseScreen assays offer an opportunity to determine biochemical to cellular translation of kinase activity, and to profile compounds for kinase selectivity.

These assays are available as a fee-for-service capability for drug discovery projects.

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Cell-Based Assays for Enhanced Safety, Activity, and Target Specificity Determination

Vincent Dupriez, Ph.D.
Principal Scientist
PerkinElmer
AlphaScreen® SureFire® for Homogeneous cell-based Kinase assays

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Principal Scientist
PerkinElmer Discovery and Research Reagents
Vincent.dupriez@perkinelmer.com
Agenda

- Why functional assays are needed
- Introduction to Alpha
- Use of Alpha in cellular assays
- AlphaScreen SureFire for cellular Kinase assays
Functional Assays Reveal Pharmacological ‘Texture’

Type of modulation
- FULL AGO
- PARTIAL AGO
- NEUTRAL ANTAGO
- INVERSE AGO

Collateral efficacy:
- Receptor activation (1)
- Receptor occupancy
- Protein binding
- Internalization
- Phosphorylation

Some molecules may stimulate all the pathways in parallel
Others NOT

Permissive Antagonism:
- Receptor activation (1)
- Receptor occupancy
- Protein binding
- Internalization
- Phosphorylation

Some molecules may block all the pathways in parallel
Others NOT

Allosteric modulators

Some molecules may stimulate all the pathways in parallel
Others NOT

Others NOT

Figure 1. Types of allosteric modulator. Allosteric ligands can affect receptor function in three general ways. (1) Allosteric modulation of orthosteric-ligand-binding affinity. (2) Allosteric modulation of orthosteric ligand efficacy. (3) Direct allosteric agonism.
What is Alpha Technology?

- Homogeneous bead-based platform
- Allows highly sensitive detection of biomolecules & biomolecular interactions over a broad dynamic range
- No cumbersome wash-steps
- Detects virtually any molecule from large endogenous protein complexes to very small peptides
- Works in a variety of different sample types including serum, plasma, cell lysates & cell supernatant

All in ONE WELL and NO WASH STEPS

*Amplified Luminescent Proximity Homogeneous Assay*
How does **Alpha Technology** work?

- Bead based proximity assay
- Streptavidin coated Donor beads capture an analyte specific biotinylated antibody or binding protein
- Acceptor beads are conjugated with a second antibody or protein which recognizes the analyte
- The Donor beads are excited with a laser at 680nm resulting in the release of singlet oxygen
- Singlet oxygen excites an amplified fluorescent signal in the Acceptor bead producing an emission of light between 520 and 620nm,
- Emission occurs only if the beads are brought within 200nm proximity by the capture of the analyte by both the antibodies
- The amount of light emission is proportional to the amount of analyte present

**Amplified Luminescent Proximity Homogeneous Assay**
Biomarkers in various therapeutic areas

- Sensitivity
- Accuracy & precision
- Serum, plasma & tissue samples
# Biomarkers in various therapeutic areas

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<thead>
<tr>
<th>Angiogenesis</th>
<th>Biologics &amp; Bioprocess</th>
<th>Cancer</th>
<th>Angiogenesis</th>
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<td>EPO</td>
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<td>AFP</td>
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<td>β-NGF</td>
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<td>VEGF</td>
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<td>IgA</td>
<td>CXCL11/I-TAC</td>
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<td>IgG</td>
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<td>NSO-P</td>
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<td>PERC6 HCP</td>
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<td></td>
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<td>Renin/Prorenin</td>
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<td>Aβ 1-40</td>
<td>Adiponectin</td>
<td>IL1β (mouse)</td>
<td>IL11</td>
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<td>Aβ 1-40 (mouse/rat)</td>
<td>Adiponectin (mouse)</td>
<td>IL1β (rat)</td>
<td>IL12 (p70)</td>
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<td>Aβ 1-42</td>
<td>Albumin (mouse)</td>
<td>IL2</td>
<td>IL13</td>
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<td>C-peptide</td>
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<td>IL15</td>
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<td>IL15 (mouse)</td>
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<td>Aβ Oligomers</td>
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<td>IL4</td>
<td>IL17</td>
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<td>IL6</td>
<td>IL17A (mouse/rat)</td>
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<td>GLP-1</td>
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<td>IL18</td>
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<td>IGF1</td>
<td>IL7</td>
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<td>(mouse/rat)</td>
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<td>IL8</td>
<td>CCL5/RANTES (mouse)</td>
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<td>sAPPβ (high sensitivity)</td>
<td>Leptin</td>
<td>IL10</td>
<td>CXCL1/GRO-α</td>
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<td>Tau</td>
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<td>Prolactin</td>
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<td>IL6</td>
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<td>IL6 (mouse)</td>
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<td>IL10</td>
<td>CXCL9/MIG</td>
</tr>
<tr>
<td>IL10 (mouse)</td>
<td>Coming soon</td>
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AlphaLISA Epigenetic Cellular Detection kits

**ALL-IN-ONE WELL PROTOCOL**

**PREPARATION**
- Overnight

**EXTRACTION**
- 25 min

**DETECTION**
- 90 min

Read Plate on EnVision® or EnSpire®

**Steps**

- **Seed cells in plate**
  - 4 hours

- **Treat cells – overnight**

- **Add Cell-Histone Lysis Buffer**
  - 15 min

- **Add Cell-Histone Extraction Buffer**
  - 10 min

- **Add Acceptor Beads and Biotinylated Anti-H3**
  - 60 min

- **Add Acceptor Beads and Biotinylated Anti-H3**

- **Add Donor Beads**
  - 30 min
H3K27me3 levels in B cell lymphoma cell lines

- No compound so far for modulating the H3K27me3 mark
- B cell lymphoma cell lines show different levels of H3K27me3
  - SU-DHL-6
    - Heterozygous EZH2 HMT mutation (Y641N) affecting substrate selectivity
    - Accumulation of H3K27me3
  - OCI-LY-19
    - Wild type for EZH2
    - Accumulates mostly H3K27me2
**AlphaScreen® SureFire® Kits**
- Lysis Buffer
- Activation Buffer
- Reaction Buffer
- Postive & Negative Control Samples: unstimulated cell lysate, stimulated cell lysate

**AlphaScreen Protein A**
- Anti-IgG (Protein A) Acceptor beads
- Streptavidin Donor Beads
- Biotinylated-rabbit IgG
- 10 X buffer

**Phospho-Protein**
- Endogenous
- Cellular
- Most Mammalian Cell
- Transfection Not Required
AlphaScreen® SureFire® Cellular Kinase Assays

Run Immunoassay
Mix Cell Lysate + Reagent Mix + Acceptor Beads

- Non-Phosphorylated Analyte
- Analyte
- Biotin Ab
- Protein A-conjugated Acceptor Bead

1. Incubate (2h, RT°, dark)
2. Add Donor Beads
3. Streptavidin-coated Donor Bead
4. Incubate (2h-O/N, RT°, dark)

Prepare cells
- Seed cells (incubate)
- Add Inhibitor (incubate)
- Add stimulant (incubate)
- Add cell lysis buffer (shake)

Excitation 680 nm

\[ ^1O_2 \]

Emission 520-620 nm
**AlphaScreen® SureFire® ... Homogenous Western Blots**

Excitation 680 nm

Emission 520-620 nm

Streptavidin coated Donor Beads

phospho ERK

Protein A coated Acceptor Beads

Excitation

S**ureFire Total AKT**

**SureFire p- AKT (Ser473)**

**SureFire p-GSK3α (Ser21)**

**SureFire p-G6 RP (Ser240/244)**

**Bar Graphs:**

- **SureFire Total AKT**
  - X-axis: ng of lysate/well
  - Y-axis: SureFire Signal (counts)
  - Data points 3400, 1700, 850, 425, 212, 106, 53, 27, 14

- **SureFire p- AKT (Ser473)**
  - X-axis: ng of lysate/well
  - Y-axis: SureFire Signal (counts)
  - Data points 3400, 1700, 850, 425, 212, 106, 53, 27, 14

- **SureFire p-GSK3α (Ser21)**
  - X-axis: ng of lysate/well
  - Y-axis: SureFire Signal (counts)
  - Data points 3400, 1700, 850, 425, 212, 106, 53, 27, 14

- **SureFire p-G6 RP (Ser240/244)**
  - X-axis: ng of lysate/well
  - Y-axis: SureFire Signal (counts)
  - Data points 3400, 1700, 850, 425, 212, 106, 53, 27, 14

**Western Blots:**

- Each lane represents different samples or conditions.
... Insulin receptor signaling pathway screen for PI3K / AKT / mTOR

AlphaScreen SureFire: p70 S6K Phosphorylation in MCF-7 Cells

AlphaScreen SureFire – using Kinase Inhibitors
Multi-target analysis of MCF7 cells

Measured 12 phosphorylation events from a single 96-well plate:

### 96-well Plate Map

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>rapamycin</th>
<th>wortmannin</th>
<th>LY294002</th>
<th>UCN-01</th>
<th>staurosporine</th>
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<tbody>
<tr>
<td>Basal phosphorylation</td>
<td>Untreated cells</td>
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<td></td>
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<tr>
<td>Activation timecourse</td>
<td>Insulin 5 min</td>
<td>Insulin 10 min</td>
<td>Insulin 25 min</td>
<td>Insulin 45 min</td>
<td>Insulin 60 min</td>
</tr>
</tbody>
</table>

AlphaScreen SureFire can be used in a “ParaPlexing” mode
One culture well – multiple assays: Effects of inhibitors
One culture well – multiple assays: Time course of insulin activation
Phospho-ERK, Akt and CREB as a Readout of GPCR Activation

... through multiple G-proteins
Streamlined protocol for compound testing using frozen cells

**Using Fresh (cultured) cells**

- **Detach cells (PBS-EDTA)**
- **Re-suspend in culture medium (10% FBS)**
- **Seed at 40,000 cells/well**
- **Let cells adhere for 4-7h**
- **Replace medium with 100 µL serum-free medium**
- **Incubate overnight (serum-starvation)**
- **Replace medium with 100 µL Agonist-containing serum-free medium**
- **Incubate for 10 min at RT**
- **Replace medium with 50 µL 1 x lysis buffer**
- **Plate shaker for 10 min**
- **Use in parallel aliquots of 4 µL of cell lysate for:**
  - p-ERK analysis
  - p-Akt analysis
  - p-CREB analysis
- **Read Alpha Signal with Envision®**

**Using Frozen cells**

- **Thaw cells**
- **Detach cells (AequoZen, cAMPZen)**
- **Re-suspend in culture medium (10% FBS)**
- **Seed at 40,000 cells/well**
- **Let cells adhere for 4-7h**
- **Replace medium with 100 µL serum-free medium**
- **Incubate overnight (serum-starvation)**
- **Replace medium with 100 µL Agonist-containing serum-free medium**
- **Incubate for 10 min at RT**
- **Replace medium with 50 µL 1 x lysis buffer**
- **Plate shaker for 10 min**
- **Use in parallel aliquots of 4 µL of cell lysate for:**
  - p-ERK analysis
  - p-Akt analysis
  - p-CREB analysis
- **Read Alpha Signal with Envision®**

**Timecourse in CHO-GAL1 cells (G\textsubscript{ai}-coupled GPCR)**
Same response in Frozen and Cultured cells

Comparison of phospho-ERK response obtained from cultured cells and γ-irradiated frozen cells. CHO-Gal1 cells, agonist response.

<table>
<thead>
<tr>
<th>pERK</th>
<th>rGal 2-29</th>
<th>M32</th>
<th>M35</th>
<th>M40</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cultured</td>
<td>8.79</td>
<td>8.31</td>
<td>9.33</td>
<td>8.48</td>
</tr>
<tr>
<td>Frozen</td>
<td>8.70</td>
<td>8.22</td>
<td>9.09</td>
<td>8.27</td>
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</table>

<table>
<thead>
<tr>
<th>pEC50</th>
<th>S/B</th>
<th>Z'</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cultured</td>
<td>15.0</td>
<td>19.4</td>
</tr>
<tr>
<td>Frozen</td>
<td>21.7</td>
<td>28.2</td>
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<tr>
<td>Cultured</td>
<td>0.81</td>
<td>0.85</td>
</tr>
<tr>
<td>Frozen</td>
<td>0.88</td>
<td>0.77</td>
</tr>
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</table>

Testing of 95 GPCRs using cAMP Zen and AequoZen cells

...Demonstrating the potential of SureFire for selectivity profiling

Poster available at www.perkinelmer.com/ask
→ AlphaScreen SureFire
→ Applications
→ GPCR Studies
AlphaScreen SureFire signaling targets

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**Therapeutic Areas**

- **Cancer**
- **Immunity**
- **Inflammation**
- **CNS & Neuro-degeneration**
- **Diabetes**
- **MEK-1**

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**Key Targets**

- **Caspase 9**
- **p53**
- **RPS6**
- **4EBP1**
- **ATF1**
- **α-Catenin**
- **Total p53**
- **Total Akt1**
- **Total Akt 1/2/3**
- **Akt 1/2/3**
- **IKKα**
- **IKKβ**
- **NFκB p65**
- **Total NFκB p65**
- **Total Akt1**
- **Total Akt 1/2/3**
- **Akt 1/2/3**
- **mTOR**
- **p70S6K**
- **CREB**
- **BAD**
- **GSK3α**
- **GSK3β**
- **BAD**
- **HSP27**

---

**Additional Targets**

- **C-Jun**
- **MKK3/6**
- **ATF2**
- **MEK**
- **ERK ½/2**
- **Total ERK ½**
- **Elk-1**
- **Total Akt1**
- **Total Akt 1/2/3**
- **Total Akt1**
- **Total Akt 1/2/3**
- **Akt 1/2/3**
- **IKKα**
- **IKKβ**
- **NFκB p65**
- **Total NFκB p65**
- **Total Akt1**
- **Total Akt 1/2/3**
- **Akt 1/2/3**
- **mTOR**
- **p70S6K**
- **CREB**
- **BAD**
- **GSK3α**
- **GSK3β**
- **BAD**
- **HSP27**

---

**Additional Features**

- **Internal Standard**
- **Total GAPDH**

---

**NB: The therapeutic areas classification is indicative and certainly not restrictive!**
Summary

- Functional assays are now widely available to characterize in cellular models the action of drugs in development.
- The alpha technology enables the development of homogeneous cell-based assays for detection of phosphorylation events.
- The same assay platform enables to analyze other types of cell's responses, such as epigenetic modifications.
- Transfection of cells is not needed: detection of endogenous protein modifications.
- The Alphascreen SureFire platform was validated on a wide basis of receptors and cell lines, demonstrating its usefulness for compound selectivity profiling.

Many Thanks ! To:
Ron Osmond,
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Michael F. Crouch

Eric Morreale,
Janet Park,
Martin Loignon,
Lenka Rihakova,
And many others!

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Cell-Based Assays for Enhanced Safety, Activity, and Target Specificity Determination

Q&A
Cell-Based Assays for Enhanced Safety, Activity, and Target Specificity Determination

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Thank You For Attending

Cell-Based Assays for Enhanced Safety, Activity, and Target Specificity Determination

Broadcast Date: Tuesday, October 2, 2012
Time: 1:00 pm EDT

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