# An Integrated Approach to High Throughput, High Content Screening for Regulatory Interactions in Epidermal Growth Factor Receptor Regulation

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## Abstract

We developed a suite of HTS-compatible assays for inhibitors of sequential steps in the Epidermal Growth Factor Receptor (EGFR) pathway. This poster presents assays that measure: i) binding of EGF to EGFR in cell-derived membrane fragments; ii) autophosphorylation of EGFR at specific tyrosine residues and iii)"docking" of adapter proteins to specific EGFR phosphopeptides. These assays are conducted on a single platform based on Meso Scale Discovery's Multi-Array™ technology that combines array technologies and electrochemiluminescence detection. The ligand binding assay uses immobilized membrane fragments; the immobilized membranes retain their fluid-dynamic properties and permit identification of allosteric regulators of multiple distinct classes. The autophosphorylation assay is configured as a multiplex assay using spatially distinct, immobilized antibodies specific for the phosphorylated and non-phosphorylated forms of distinct EGFR epitopes. The assay can identify inhibitors specific for unique tyrosine residues that, in turn, serve as the docking sites for different adapter proteins. The assay that measures "docking" of adapter proteins uses immobilized EGFR phosphopeptides. It can be used to screen for compounds that inhibit the binding of specific adapter proteins to phospho-EGFR. High content assays can be performed using MSD's Multi-Spot™ platform. As examples we show an assay that allow the simultaneous detection of multiple post-translational states of a protein and an assay that permits simultaneous quantification of preferential binding affinity among multiple targets.



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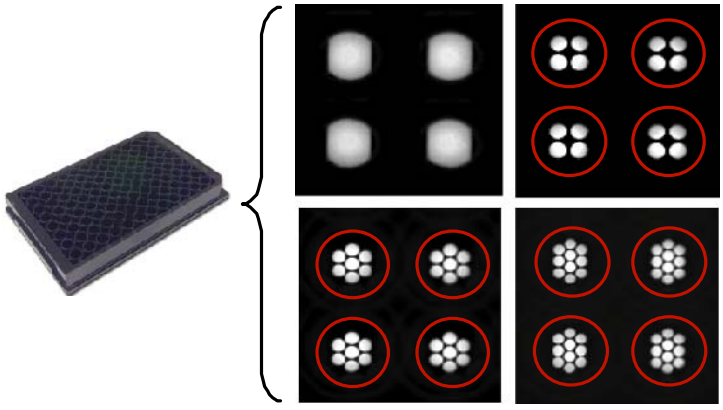
## Meso Scale Discovery™: Multi-Array Technology

### Sector HTS™ Reader Features

- Highly sensitive, ultra high-throughput
- Designed for high-throughput screening (HTS) and automated assay development
- Custom optics with telecentric lens design and CCD imaging detection
- High-speed motion control systems
- Electrochemiluminescence detection

### Plate Features

- Disposable Plates
- Carbon Electrodes with high binding capacity
- 96-, 384-, 1536-well and Multi-Spot™ plate formats
- Biocompatible: direct immobilization of avidin, IgG, membrane fragments, intact cells, etc.
- Functional Assays: simple binding reactions, GPCRs, enzyme cascades, post-translational modification, etc.



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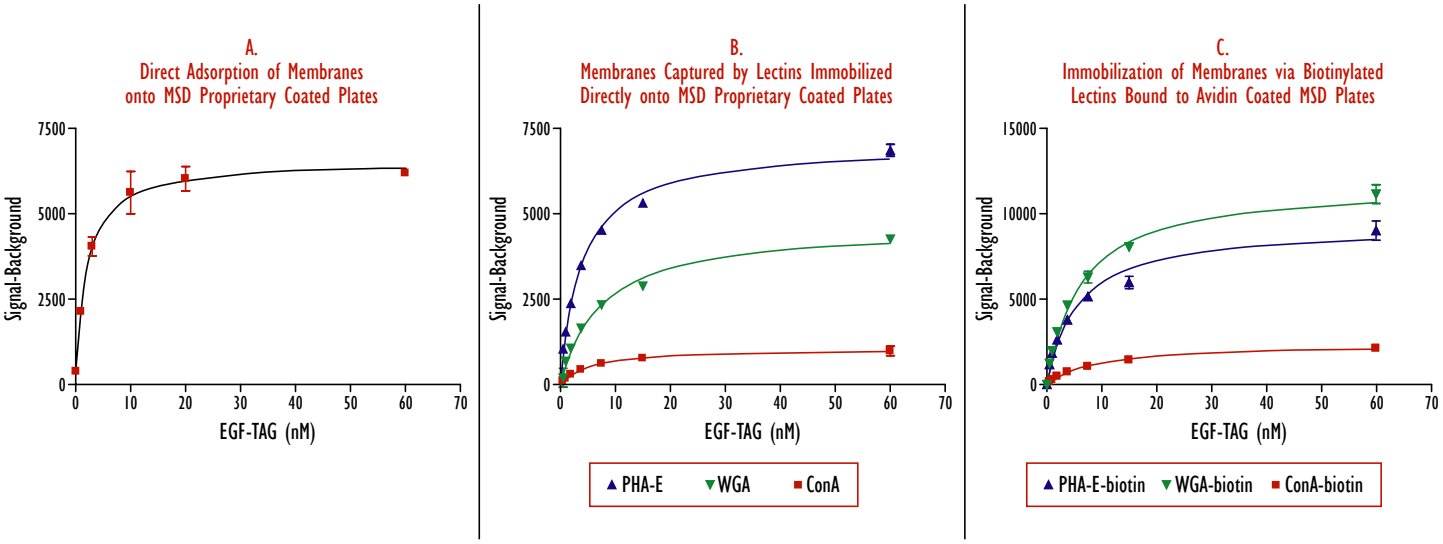
## Receptor-Ligand Binding Assay for EGFR-EGF: Versatility of Cell Membrane Immobilization




$K_D$ (nM) = 1.9 ± 0.4

| | $K_D$ (nM) |
|---|---|
| Con A | 5.2 ± 0.7 |
| PHA-E | 3.8 ± 0.9 |
| WGA | 7.2 ± 2.1 |

| | $K_D$ (nM) |
|---|---|
| Con A-biotin | 8.9 ± 1.9 |
| PHA-E-biotin | 5.6 ± 2.6 |
| WGA-biotin | 6.0 ± 1.6 |

Binding of EGF-TAG to EGFR-containing A431 membranes immobilized on MSD™ proprietary coated carbon electrodes, 96-well plates, either directly (A), via native lectins (B), or via biotinylated lectins bound to avidin. The data presented are corrected for K562 cell membrane-based background signal.

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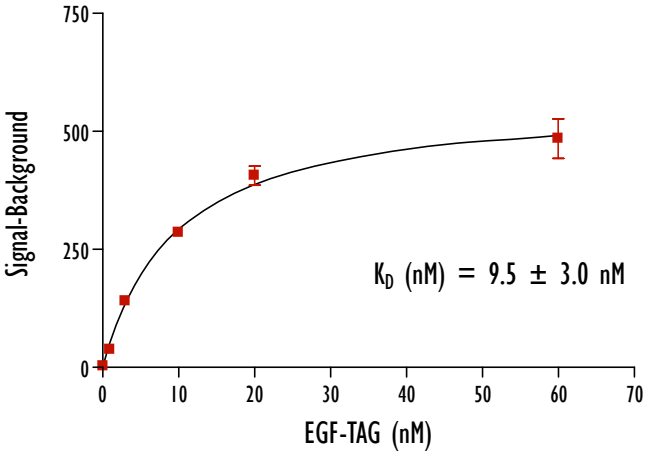
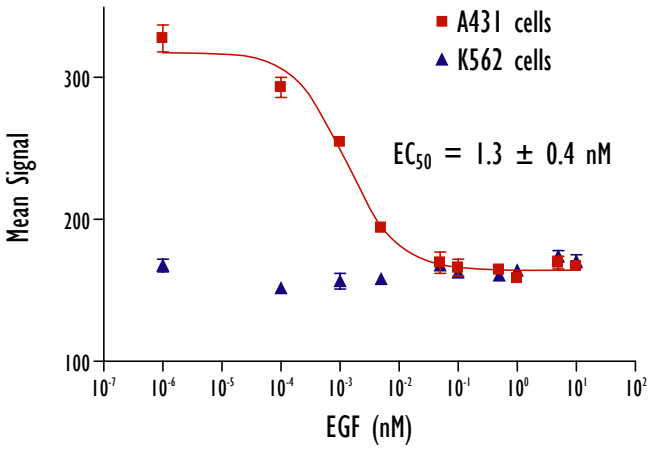
## Whole Cells Immobilized on Carbon Electrodes: Simple, Non-Washed Assay Complete in 1.5 h

Binding Characteristics are Conserved After Whole Cell Immobilization




EGF Receptor In Immobilized Whole Cells Retains Specificity




A431 or K562 Cells (1,000/well) were immobilized on MSD Multi-Array plates for 30 min → Addition of labeled ligand for 1 h → Plate imaged on MSD Sector HTS™ Reader

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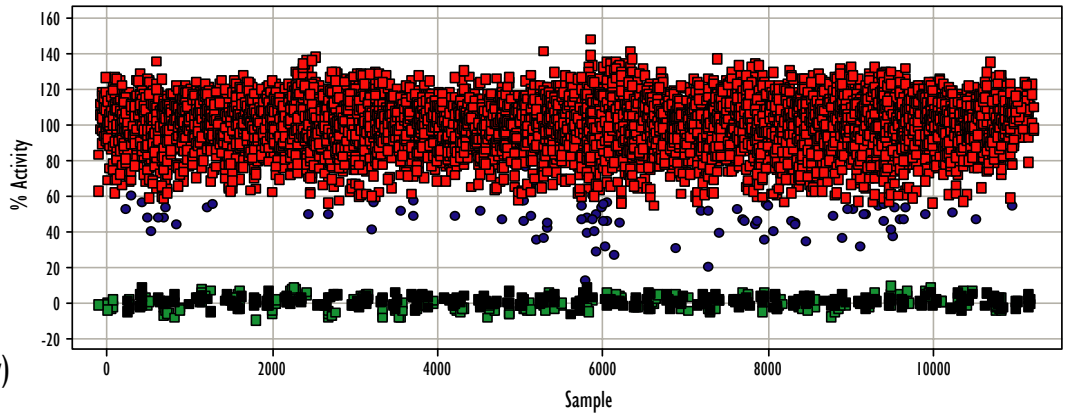
## High-Throughput Homogeneous Screen: EGFR-EGF Receptor-Ligand Binding Assay

Screen Statistics

- 3 Addition Assay Format
- 120 384-well plates/day

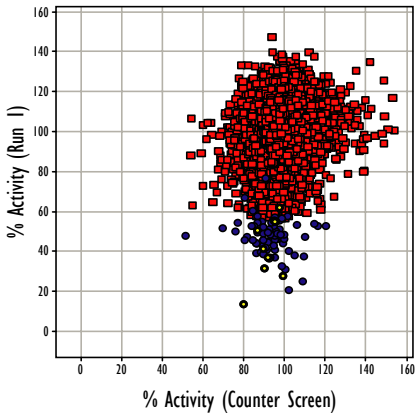
- Z-Score: 0.75
- Positive Control CV: 5.1%

- Reconfirmed Hit Rate: 0.51%
- Novel compounds with sub-micromolar $IC_{50}$
- False positive rate (<50% activity) less than 0.02%
- Hits are specific

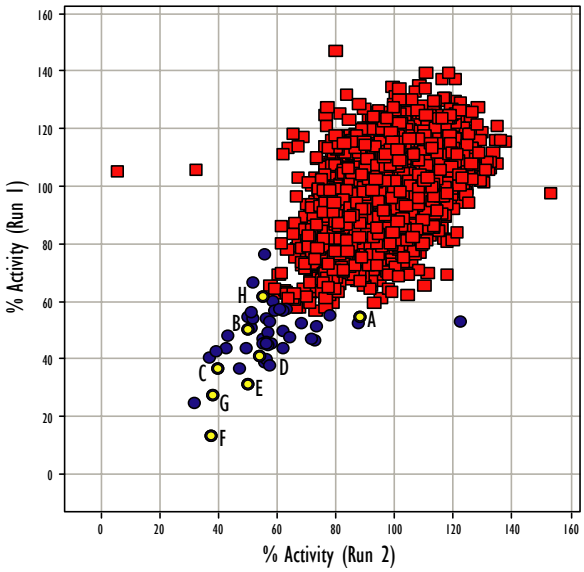



Red: Compounds Green: Known Inhibitor Black: K562 (no receptor) Blue: Selected Compounds

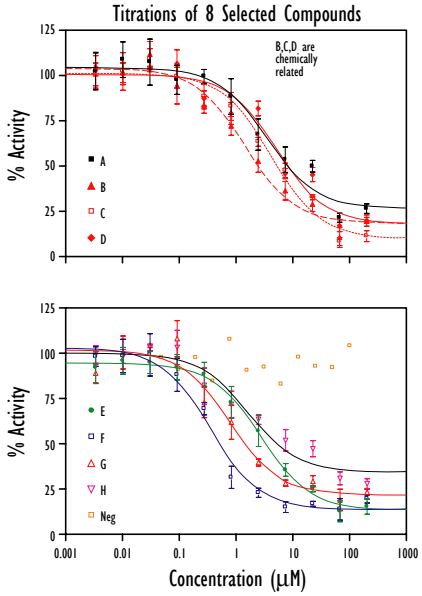
Correlation of Selected Compounds Against ECL Inhibition Counter-Screen




Correlation of 6,000 compounds Re-Screened




Titrations of 8 Selected Compounds

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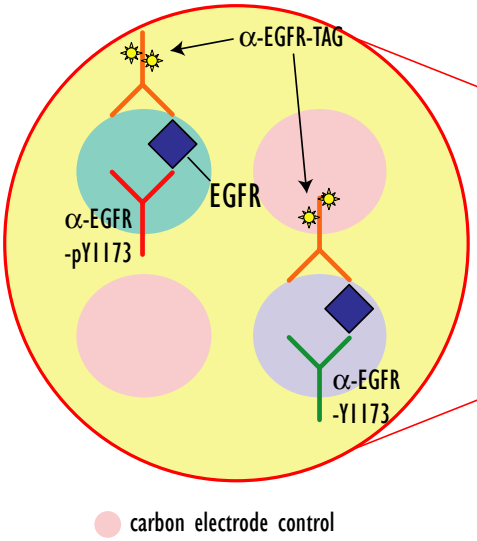
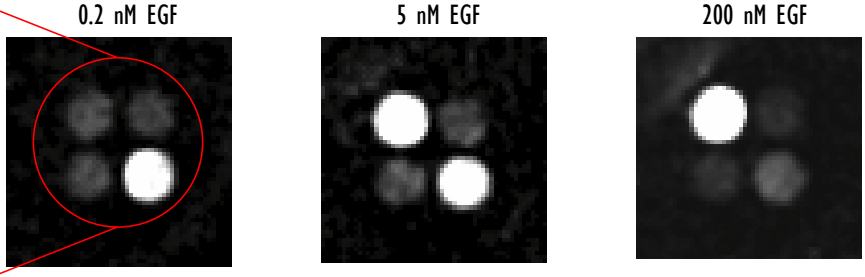
## Multiplex EGFR Autophosphorylation Assay: Detection of Phosphorylated and Nonphosphorylated Tyrosine1173 in the Same Well




EGF Induced Receptor Autophosphorylation at Tyrosine 1173
Using MSD Multi-Array 4-spot plates




The multiplex assay allows quantitation of the amount of receptor phosphorylation at tyrosine 1173. A titration of EGF shows no detectable autophosphorylation of tyrosine 1173 at 0.2 nM, 50% at 5 nM, and 90% at 200 nM.

Phosphorylated (α-EGFR-pY1173) and nonphosphorylated (α-EGFR-Y1173) antibodies are immobilized onto spatially distinct electrodes in MSD Multi-Spot plates. Stimulated and unstimulated receptor-containing lysates from A431 cells bind the appropriate antibody. The reporter α-EGFR antibody binds the extracellular domain of both receptors.

### Multi-Spot plates facilitate simultaneous detection of multiple post-translation states of a protein

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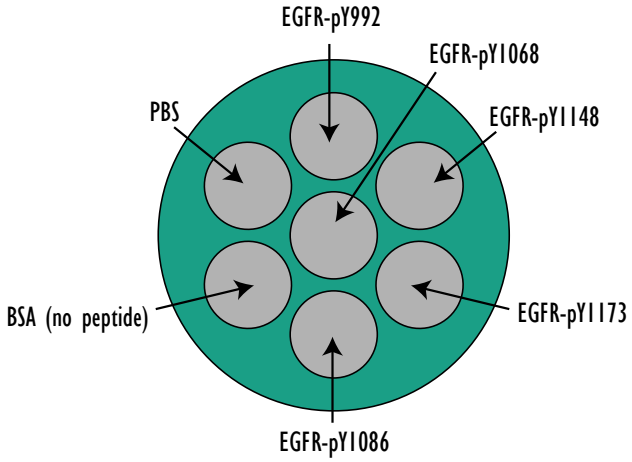
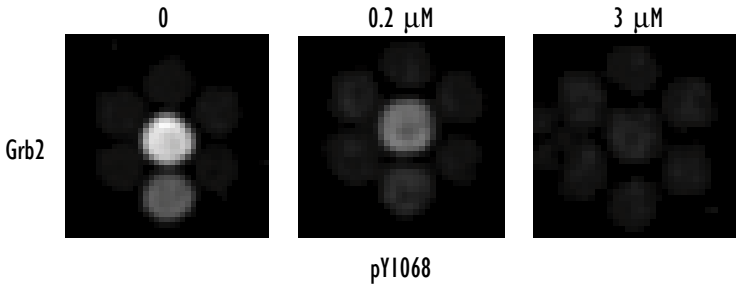
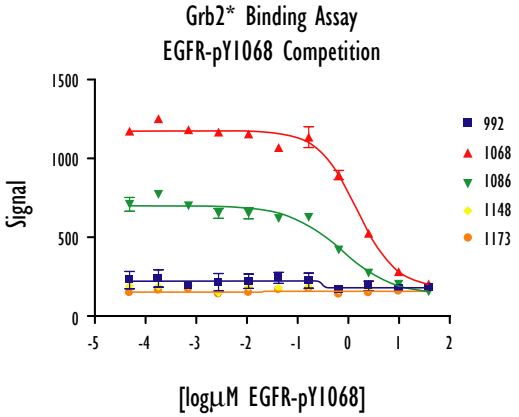
## Multiplex Assay to Assess SH2 Domain/Phosphopeptide Associations

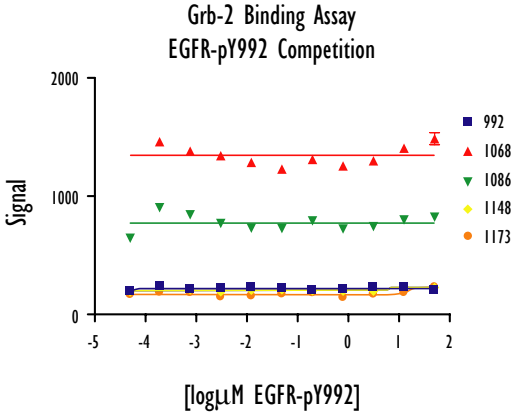
Conjugated phosphopeptides are named for the amino acid position of the EGFR tyrosine (Y) they contain.




Phosphospeptides corresponding to segments of EGFR were synthesized in the phosphorylated state, coupled to BSA and spotted on Multi-Array 7-spot plates. Subsequently, ruthenylated Grb2, a "docking" protein, alone or along with "free"pY1068 phosphopeptide in solution was added to the well, allowed to bind, and visualized in the Sector HTS Reader.




*Grb2 does not bind pY992

### Multi-Spot plates permit simultaneous quantification of preferential binding affinity among multiple targets

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## Conclusions

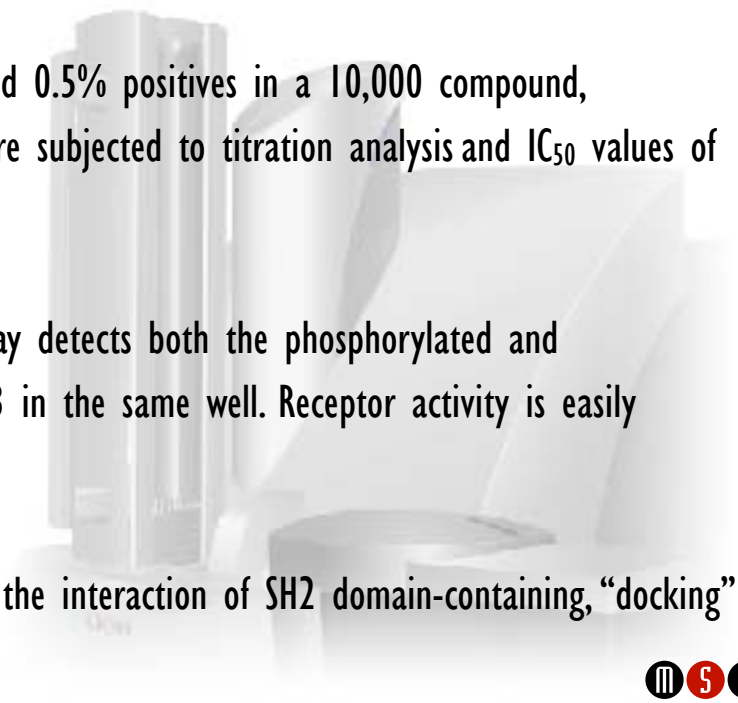
1. A variety of approaches may be used to immobilize membrane fragments on the Multi-Spot plates. EGF-TAG binding to EGFR-containing A431 membranes occurred using membranes that were immobilized directly, bound to native lectins, or bound via biotinylated lectins bound to avidin.

2. Whole cells immobilized on Multi-Array plates retain receptor binding characteristics and specificity. The assay was a simple, non-washed format that is complete in 1.5h with as little as 1,000 cells per well in 96-well MSD proprietary coated plates.

3. A high-throughput homogeneous assay yielded 0.5% positives in a 10,000 compound, representative screen. Re-confirmed "hits" were subjected to titration analysis and $IC_{50}$ values of 500 nM to 5 μM were observed.

4. The multiplex EGFR autophosphorylation assay detects both the phosphorylated and nonphosphorylated receptor at tyrosine 1173 in the same well. Receptor activity is easily controlled and quantified.

5. The multiplex format can be used to assess the interaction of SH2 domain-containing, "docking" proteins and immobilized phosphopeptides.





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