

# Fast, Sensitive Detection of EGF-Induced Receptor Autophosphorylation in Cell Lysates

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## Abstract

We present a rapid, sensitive assay that detects both total and autophosphorylated EGF receptor in A-431 cell lysates. The assay utilizes the novel platform developed by Meso Scale Discovery™ (MSD™) that combines array technologies and electrochemiluminescence detection to achieve ultra-fast, highly sensitive assays in a convenient format. EGFR was solubilized from induced or unstimulated A-431 cells and captured either with an  $\alpha$ -EGFR antibody to the extracellular domain of the receptor or through direct immobilization of lysate constituents via passive adsorption to the surfaces of multi-well plates. Total EGFR was detected with an  $\alpha$ -EGFR antibody to the cytoplasmic domain while autophosphorylated receptor was detected with an  $\alpha$ -phosphotyrosine antibody. In preliminary work, without optimization, 2,000 cell equivalents per well yielded a signal to background ratio of 15 for autophosphorylated receptor. A protocol compatible with HTS is demonstrated.



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## Multi-Array™ Technology

Unified technology platform with instruments, plates and reagents for drug discovery.

Combines the power of microarrays with the sensitivity of electrochemiluminescence.

96-, 384- and 1536 microplate formats.

Multi-Spot™ plates with high density arrays for multiplexing.

Sector HTS™ Instrument: High resolution imaging detection and robotic integration for HTS and large-scale proteomics.

Sector PR™ Instrument: Medium throughput benchtop reader for assay development, cellular and molecular biology, research in therapeutic areas, secondary screening, QC. Assays developed on Sector PR port to Sector HTS.



Sector HTS



Sector PR



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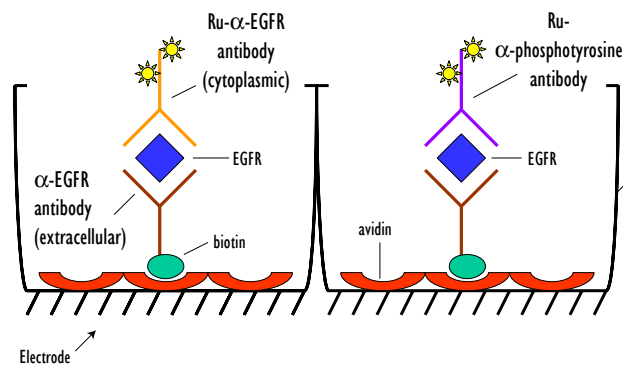
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## Alternative Assay Formats for the Detection of both Total and Autophosphorylated EGF Receptor

- 1) A-431 cells are stimulated with EGF
- 2) Cells are lysed in modified RIPA buffer

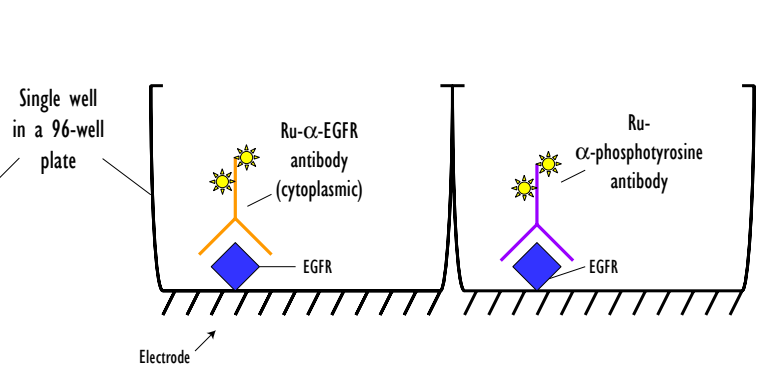
### Sandwich Immunoassay Format

EGFR, solubilized in cell lysates, binds to a biotinylated  $\alpha$ -EGFR antibody immobilized onto an avidin-coated carbon electrode. Total EGF receptor is detected with a ruthenylated (Ru)  $\alpha$ -EGFR antibody to the cytoplasmic domain. Autophosphorylated receptor is detected with a ruthenylated  $\alpha$ -phosphotyrosine antibody.



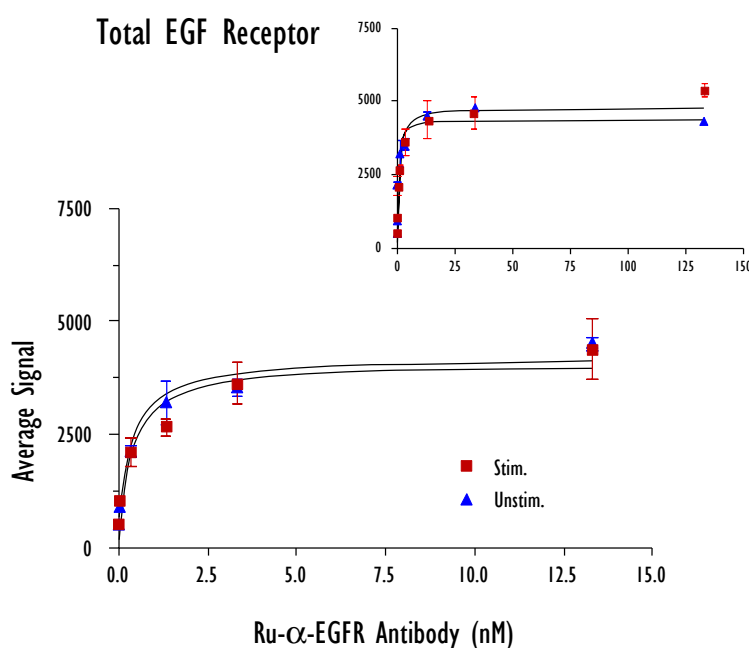
### Direct Immobilization Format

EGFR-containing cell lysates are immobilized directly onto carbon electrodes via passive adsorption. Total EGF receptor is detected with a ruthenylated  $\alpha$ -EGFR antibody to the cytoplasmic domain. Autophosphorylated receptor is detected with a ruthenylated  $\alpha$ -phosphotyrosine antibody.



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Total EGFR Levels are Equivalent in Stimulated and Unstimulated   
A-431 Cells



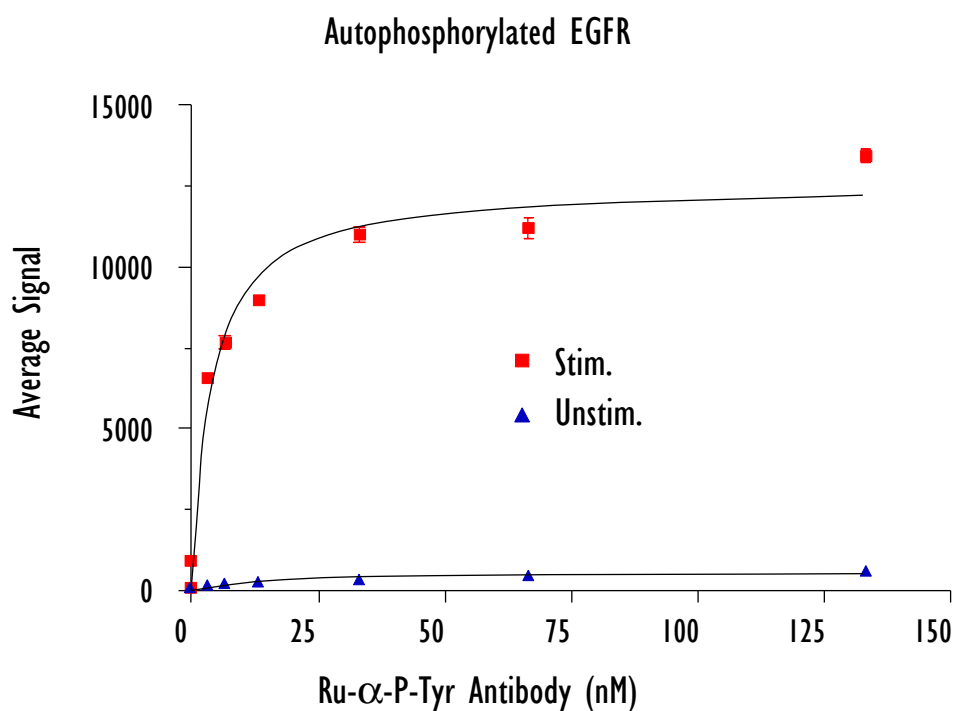
EGFR from A-431 cell lysates was captured and detected via the sandwich immunoassay method. Multi-well plates containing avidin-coated carbon electrodes and immobilized, biotinylated  $\alpha$ -EGFR antibody were prepared. The wells were then challenged with lysate from 20,000 cell equivalents, and subsequently, with varying concentrations of ruthenylated antibody. The addition of each reagent was followed by a 60 min incubation with intermittent agitation and followed by a wash. The average signal obtained is reported for the range of 3pM-13nM (3pM-130nM inset).



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## Rapid, Simple Detection of EGFR Autophosphorylation in A-431 Lysates



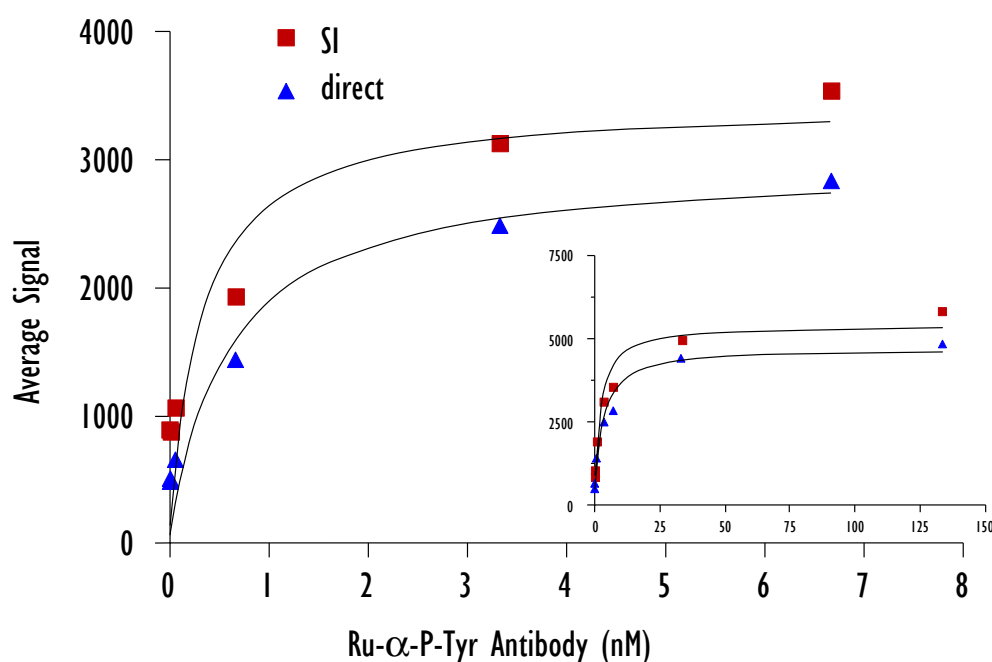
A-431 cell lysates were prepared and used to challenge wells containing immobilized, biotinylated  $\alpha$ -EGFR antibody on avidin-coated carbon electrodes. A ruthenylated  $\alpha$ -phosphotyrosine antibody was used over a range of concentrations in the sandwich immunoassay. The average signal obtained from 20,000 cell equivalents per well is reported. The ratio of the signal from the stimulated to unstimulated cells is 36 at 3nM of  $\alpha$ -phosphotyrosine antibody.



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## Comparison of Alternative Protocols for the Detection of Autophosphorylated EGFR



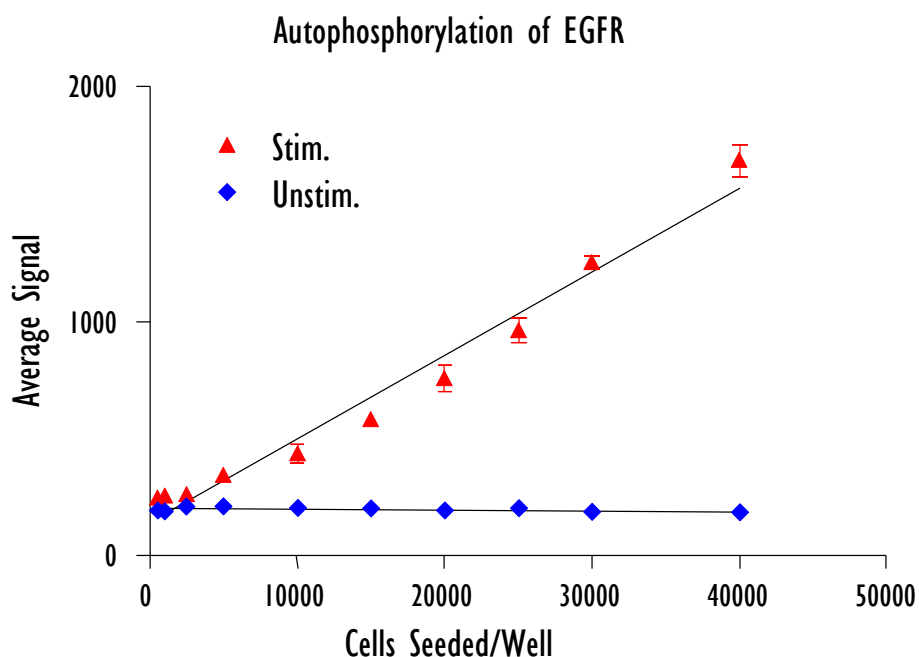
The performance of the sandwich immunoassay (SI) and the direct immobilization (direct) methods were compared as a function of the concentration of ruthenylated  $\alpha$ -phosphotyrosine using only 2,000 cell equivalents per well. The average signal is reported after background correction (the signal from unstimulated cells has been subtracted at each point). The signal/background ratio at 3.3nM antibody is 15 and 5 for the sandwich immunoassay and direct immobilization methods, respectively. The performance difference is largely attributable to differences in background between the two methods.



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## A Simplified Workflow for Detecting Autophosphorylated EGFR is Compatible with HTS



A-431 cells were seeded at various densities in a 96-well plate two days prior to the preparation of cell lysates. Lysates were prepared in situ and directly transferred into wells containing carbon electrodes modified with immobilized, biotinylated  $\alpha$ -EGFR antibody. The sandwich immunoassay method was used with a ruthenylated  $\alpha$ -phosphotyrosine antibody as the reporter. This streamlined protocol facilitates the detection of autophosphorylated receptor from 5,000 or fewer seeded cells per well.



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## Conclusion

We have developed a fast, sensitive assay that uses Multi-Array technology to detect both total and autophosphorylated EGF receptor.

The assay can achieve useful ratios of the signal from stimulated to unstimulated cells even for small numbers of cell equivalents. For example, the assay had a ratio of the signal from stimulated to unstimulated cells of 15 with approximately 2,000 cell equivalents per well in a sandwich immunoassay method.

Two distinct protocols have been developed, offering flexibility in the design and execution of the assay.

A streamlined workflow has been developed that minimizes manipulation of the lysate and increases compatibility with HTS.



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