Detection of Activated EGFR by the Association of Signaling Proteins: Stat3, Grb2 and Shc

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❶ Abstract

The association of signaling and adapter proteins to receptor tyrosine kinases is a critical initial step in the relay of extracellular cues into the nucleus. One example is the epidermal growth factor receptor (EGFR) that upon activation by ligand binding recruits a number of Src-homology 2 (SH2) and phosphotyrosine binding domain (PTB) containing proteins including Stat3, Grb2 and Shc. We have developed novel assays that quantify the activation of EGFR through the binding of SH2 domain proteins to specific phosphorylated tyrosine residues. They utilize activated receptor molecules or individual phosphopeptides representing SH2 docking sites immobilized on MSD MULTI-ARRAY™ or MULTI-SPOT™ plates. These molecules are challenged with proteins labeled with an electrochemiluminescent label. The assays can measure binding interactions with dissociation constants that range from low picomolar to low micromolar.

❷ MSD MULTI-ARRAY™ and MULTI-SPOT™ Plates

Instrument Features

- Highly sensitive
- SECTOR Imager 6000 designed for high-throughput screening (HTS)
- SECTOR PR 100 Reader ideal for assay development
- Custom optics
- High-speed motion control systems
- Electrochemiluminescence (ECL) detection

SECTOR™ Imager 6000

SECTOR™ PR 100 Reader

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❷ MSD MULTI-ARRAY™ and MULTI-SPOT™ Plates (cont.)

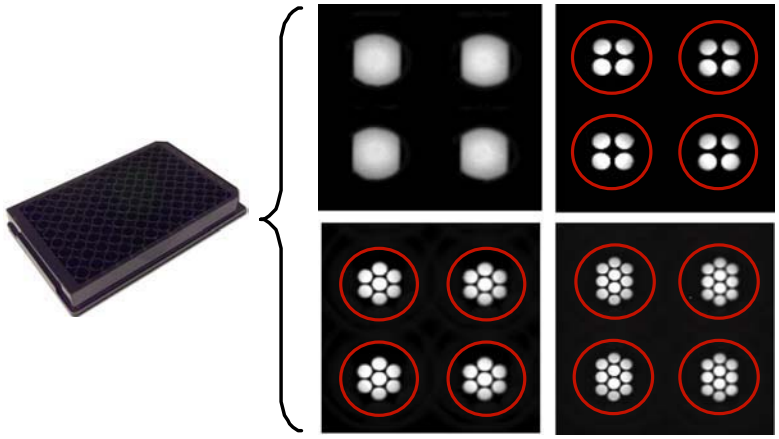

Plate Features

- Disposable Plates
- Carbon Electrodes with high binding capacity
- Suitable electrochemistry for ECL
- Biocompatible: direct immobilization of avidin, IgG, membrane fragments, intact cells, etc.
- Functional Assays: simple binding reactions, GPCRs, enzyme cascades, post-translational modifications, etc.

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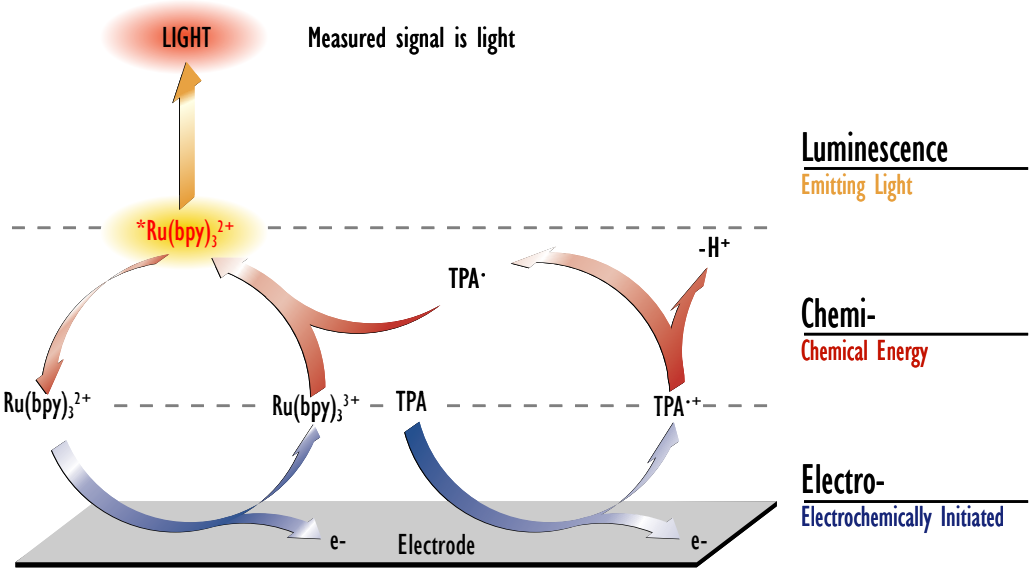
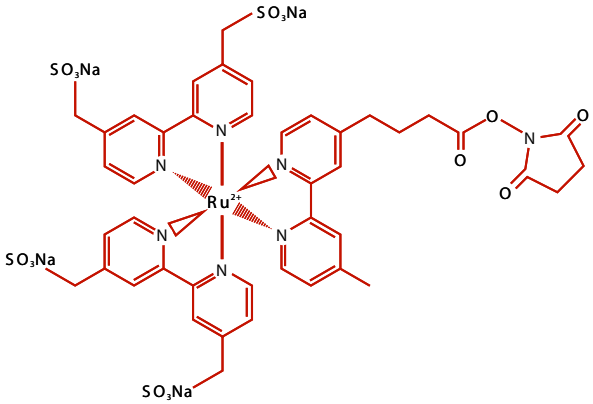
❸ Electrochemiluminescence (ECL)



Ruthenium (II) tris-bipyridine-(4-methylsulfone) NHS ester (MSD SULFO-TAG™ label)



- Selective
- Convenient chemistry
- Robust, stable
- Few interferences

- Size, MW: ~1200 daltons
- Stability: Years
- Solubility: Aqueous, DMSO
- Functionality: Hydrophilic
- Specificity: High

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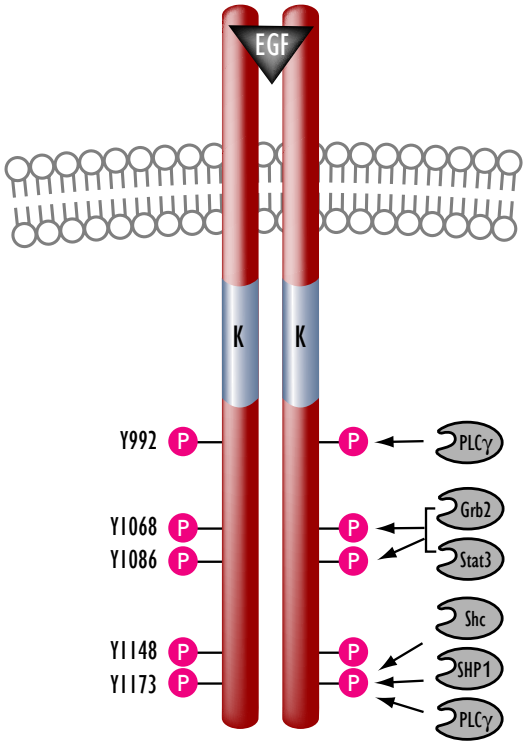
4 Association of SH2 Domain Proteins to EGFR



992	DADEYLIPQQ
1068	PVPEYINQSV
1086	QNPVYHNQPL
1148	DNPDYQQDFF
1173	ENAEYLRVAP

Binding of adapter proteins to phosphorylated receptor tyrosine residues are critical to a number of important signaling pathways. Binding or "docking" is achieved by Src-homology-2 (SH2) or phosphotyrosine binding (PTB) domains that recognize phosphotyrosine residues located within distinct amino acid contexts. The specificity of these interactions allows for the development of screening strategies for inhibitors of particular protein/protein interactions.

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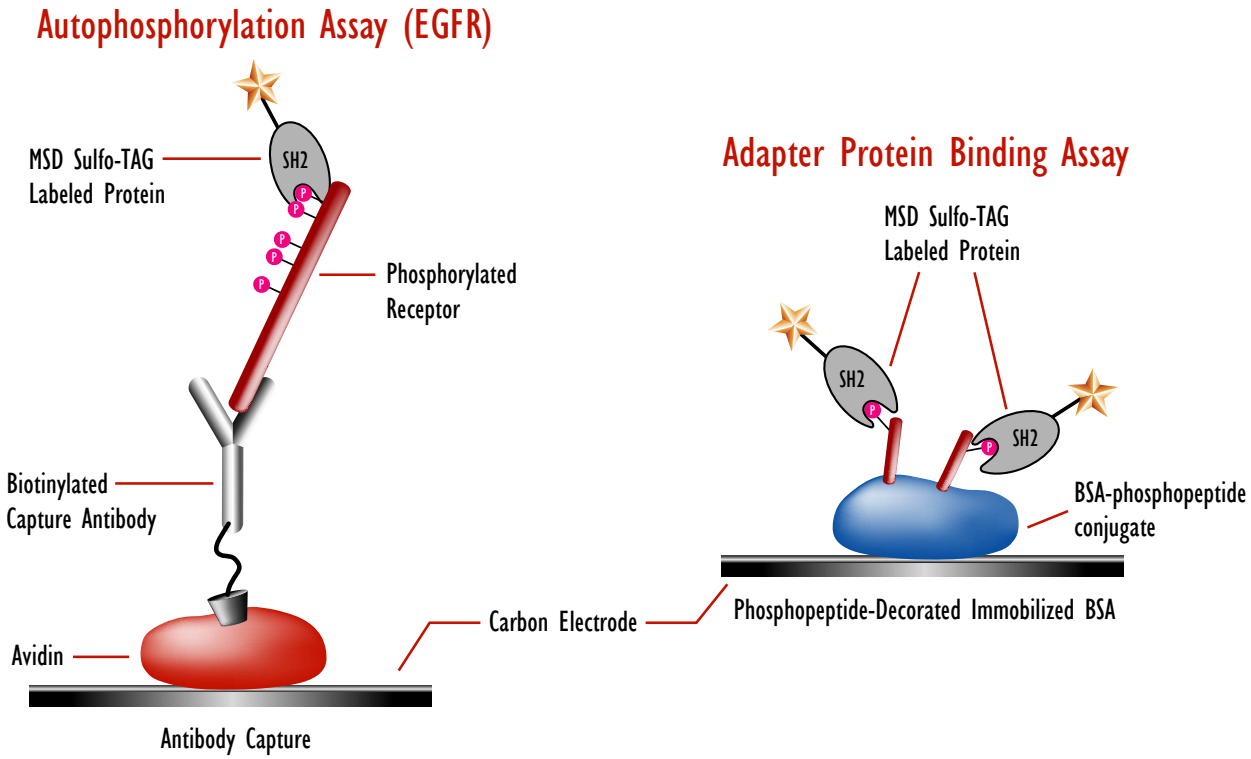
5 Assay Formats



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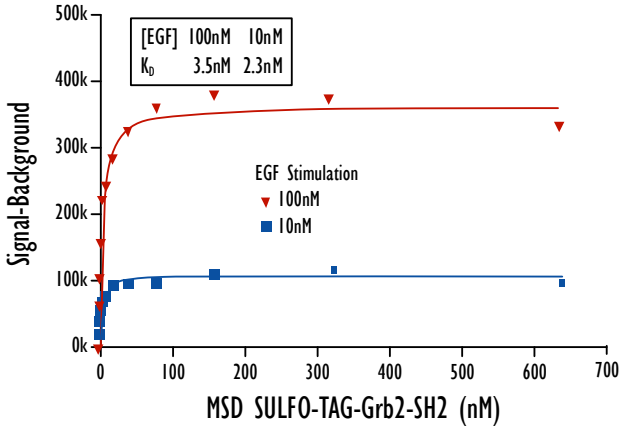
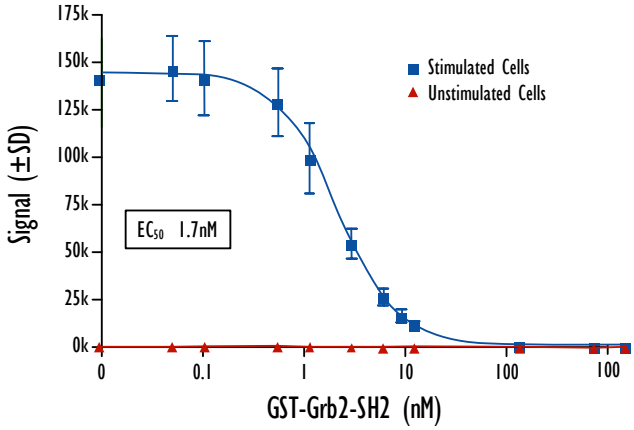
6 Detection of Activated EGFR by the Association of Grb2





EGF-treated cells were lysed *in situ* in multi-well plates. Portions of the lysate were transferred to avidin coated MULTI-ARRAY plates containing a biotinylated EGFR capture antibody. Left panel: MSD SULFO-TAG-labeled recombinant GST-Grb2 SH2 domain protein was then added to the wells at the concentrations indicated. Right panel: Competition with unlabeled GST-Grb2 SH2 protein (MSD SULFO-TAG-labeled species at 3nM).

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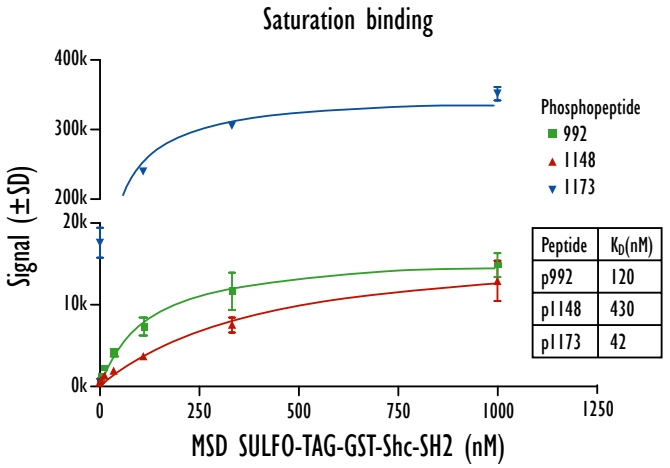
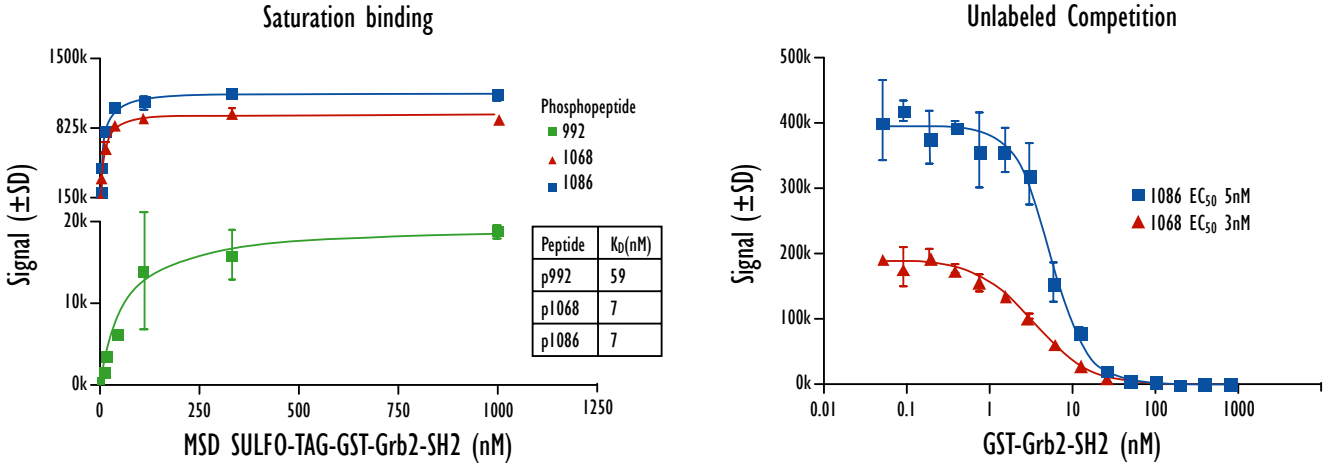
7 Binding of the Shc SH2 Domain to EGFR Phosphopeptides



BSA with coupled phosphopeptides was deposited onto the surface of a MULTI-ARRAY plate. MSD SULFO-TAG-labeled recombinant GST-Shc SH2 domain protein was then added to the wells at the concentrations indicated. The plate was incubated at room temperature for 4 hours, washed and MSD Read Buffer added. It was then imaged on the SECTOR Imager 6000. The results are consistent with molecular genetic evidence that indicates the Shc SH2 domain preferentially binds to pY1173.

8 Binding of the Grb2 SH2 Domain to EGFR Phosphopeptides



BSA with coupled phosphopeptides was deposited onto the surface of a MULTI-ARRAY 384-well plate. Left panel: MSD SULFO-TAG-labeled recombinant GST-Grb2 SH2 domain protein was then added to the wells at the concentrations indicated. The plate was incubated at room temperature for 4 hours, washed and MSD Read Buffer added. It was then imaged on the SECTOR Imager 6000. Right panel: Competition with unlabeled GST-Grb2 SH2 protein (MSD SULFO-TAG-GST-Grb2-SH2 species at 6 nM). The results are consistent with those in the literature that have shown that Grb2 binds to both pY1068 and pY1086 using molecular genetic methods.

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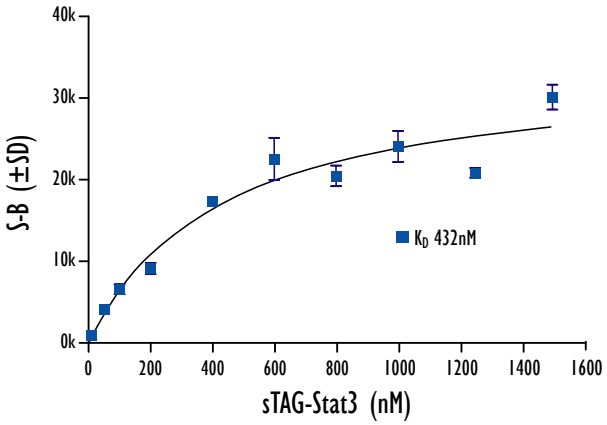
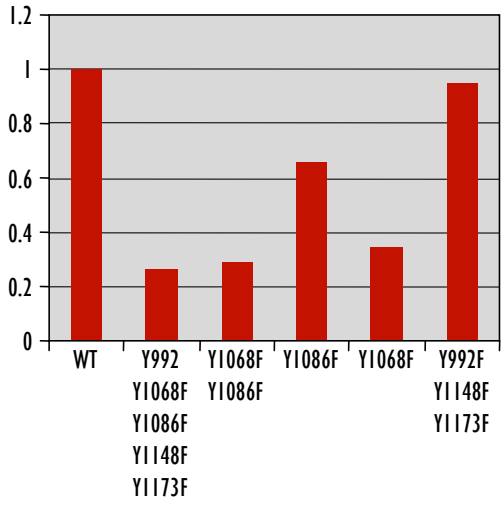
9 Binding of Stat3 to Stat3-Derived Phosphopeptide



BSA-coupled phosphopeptides were deposited onto the surface of a 384-well MULTI-ARRAY plate. MSD SULFO-TAG-labeled recombinant, dimer-incompetent Stat3 protein was then added to the wells at the concentrations indicated. The plate was incubated at room temperature for 4 h, washed and MSD Read Buffer added. It was then imaged on the SECTOR Imager 6000 reader.

10 Binding of Stat3 to EGFR



EGFR from cell lysates expressing the indicated mutant was captured onto the surface of a 384-well MULTI-ARRAY plate with a biotinylated EGFR antibody. MSD SULFO-TAG-labeled recombinant dimer-incompetent Stat3 protein was then added to the wells (200nM final concentration). The plate was incubated at room temperature for 1 h, washed and MSD Read Buffer added. It was then imaged on the SECTOR Imager 6000. Binding relative to wildtype receptor is shown.

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⓫ Conclusions

Cell based assays have been developed that report the phosphorylation status of EGFR through "docking" of specific adapter proteins.

The use of signaling or adapter proteins allows one to monitor phosphorylation events associated with a particular signal transduction pathway.

Isolated phosphopeptides can be used to quantify the binding affinity of SH2 domain proteins to specific receptor residues.

The assays can be employed in discovery efforts to identify specific interactions between phosphopeptides and signaling or adapter molecules.

These assays have been developed to facilitate HTS efforts to identify novel small molecules that inhibit specific SH2/phosphopeptide interactions relevant to individual signaling pathways.

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