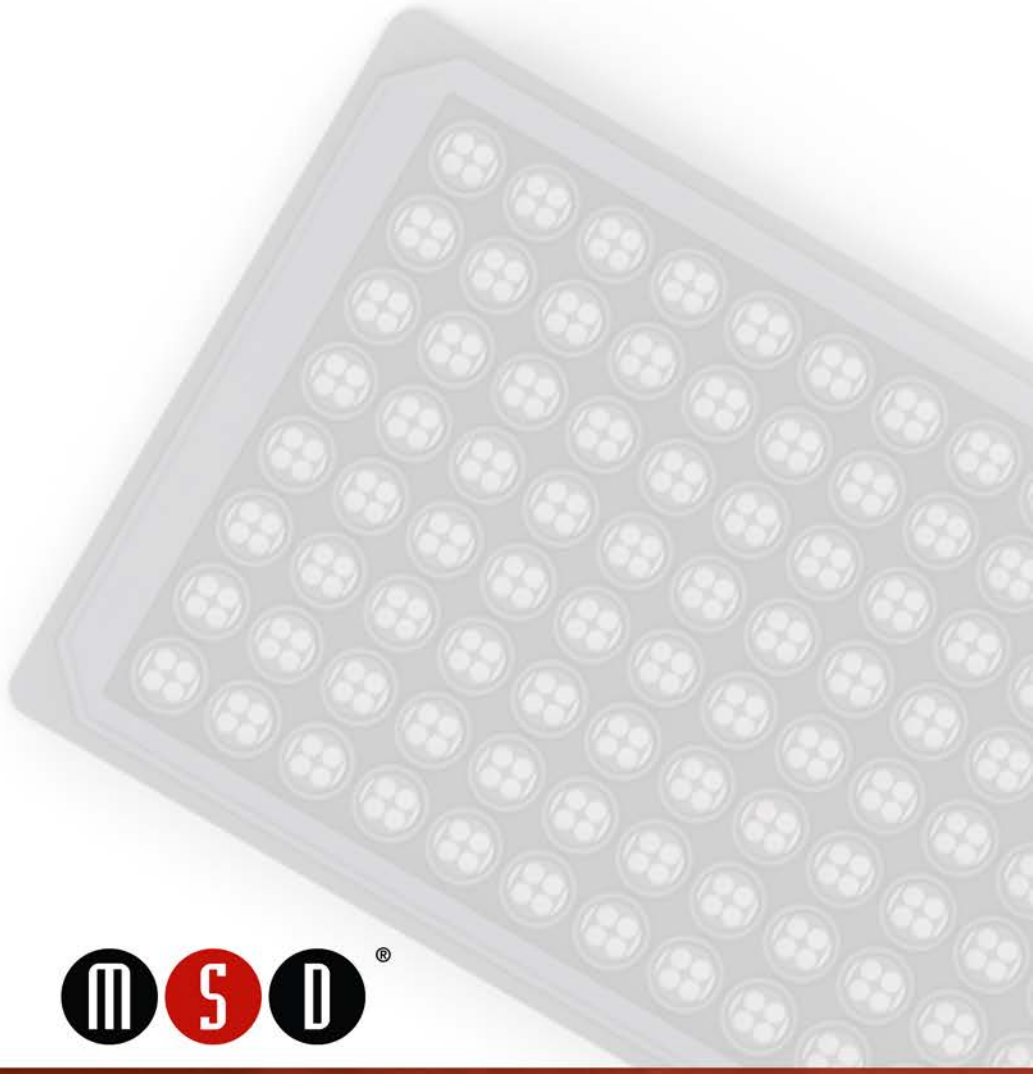
MSD® MULTI-SPOT Assay System

EGFR Family Base Kit

20-Plate Kit

K15106A-3



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MSD Phosphoprotein Assays

EGFR Family

Base Kit

Phospho-EGFR, Phospho-ErbB2, Phospho-IGF-1R

This package insert must be read in its entirety before using this product.

FOR RESEARCH USE ONLY.

NOT FOR USE IN DIAGNOSTIC PROCEDURES.

MESO SCALE DISCOVERY®
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Ordering Information

MSD Customer Service

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MSD Scientific Support

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MSD Advantage

MESO SCALE DISCOVERY'S unique spot patterns are a hallmark of our MULTI-ARRAY® technology, which enables the measurement of biomarkers utilizing the next generation of electrochemiluminescent detection. In an MSD assay, specific capture antibodies for the analytes are coated in arrays in each well of a 96-well carbon electrode plate surface. The detection system uses patented SULFO-TAG™ labels that emit light upon electrochemical stimulation initiated at the electrode surfaces of the MULTI-ARRAY and MULTI-SPOT® plates. The electrical stimulation is decoupled from the output signal, which is light, to generate assays with minimal background. MSD labels can be conveniently conjugated to biological molecules, are stable, and are non-radioactive. Additionally, only labels near the electrode surface are detected, enabling non-washed assays.

One of the advantages of MSD assays is the minimal sample volume required as compared to a traditional ELISA, which is also limited by its inability to measure more than a single analyte. With an MSD assay, up to ten different biomarkers can be analyzed simultaneously using as little as 10-25 µL of sample. These assays have high sensitivity, up to five logs of linear dynamic range, and excellent performance in complex biological matrices. Combined, these advantages enable the measurement of native levels of biomarkers in normal and diseased samples without multiple dilutions. Further, the simple and rapid protocols of MSD assays provide a powerful tool to generate reproducible and reliable results. The MSD product line offers a diverse menu of assay kits for profiling biomarkers, cell signaling pathways, and other applications, as well as a variety of plates and reagents for assay development.

Introduction

Epidermal Growth Factor Receptor (EGFR), Human Epidermal Growth Factor Receptor 2 (Her2/Neu/ErbB2), and Insulin like Growth Factor I Receptor (IGF-1R) are all receptor tyrosine kinases with extracellular ligand binding domains and intracellular tyrosine kinase domains. In normal cells these receptors play key roles in growth and development whereas IGF-1R also plays a critical role in metabolism and determination of overall nutrient state of the organism. All three of these receptors also signal through the PI3K-Akt signaling pathway and play a role in both normal as well as cancer cell development. EGFR and ErbB2 are oncoproteins and are involved in carcinogenesis, tumor growth/progression, and metastasis. Poor patient outcome and constitutive activation of these receptors have been seen in many different forms of cancer.[1] Upon ligand binding EGFR and ErbB2 form homo- and hetero-receptor dimers and autophosphorylate multiple tyrosine residues within the cytoplasmic domain of these receptors.[2] This phosphorylation results in the recruitment of adaptor proteins (such as Ras, Grb2, and Shc), increased complex phosphorylation, and downstream signaling through the PI3K, MAPK, STAT, and phospholipase C signaling pathways.[3] IGF-1R has two extracellular (alpha) domains and two transmembrane (beta) domains with cytoplasmic tails responsible for the downstream signaling mediated by ligand binding.[4,5] Binding induces a conformational change and autophosphorylation of key tyrosine residues in the beta subunits allowing interactions with docking and adaptor proteins (such as Shc, Grb2 and IRS-1).[5] Activation of the receptor and transduction of the intracellular signaling cascades culminates in cell proliferation and anti-apoptotic effects through both PI3K/AKT and Ras/MAPK signaling pathways.[4-6]

Many different cancers (such as breast cancer, prostate cancer, GI cancers, ovarian, neuroblastoma, and others) show increased expression of IGF-1R as well as IGF-1 and the level of expression tracks with tumor progression and cancer stage.[6] IGF-1 and IGF-1R signaling cascades also play a large role in immune system function and development. Autoimmune diseases such as Grave's Disease, rheumatoid arthritis, EAE, and inflammatory bowel disease may involve disruptions in this pathway.[7] EGFR and ErbB2 are also involved in many different types of cancers, such as head, neck, colon, breast, pancreas, and lung cancers.[8] These important intracellular signaling receptors have been the subject of many basic research and drug development programs.

Principle of the Assay

MSD phosphoprotein assays provide a rapid and convenient method for measuring the levels of protein targets within a single, small-volume sample. The assays are available in both singleplex and multiplex formats. In a singleplex assay, an antibody for a specific protein target is coated on one electrode (or "spot") per well. In a multiplex assay, an array of capture antibodies against different targets is patterned on distinct spots in the same well. The EGFR Family is a sandwich immunoassay (Figure 1). MSD provides a plate that has been pre-coated with capture antibodies for total EGFR, total ErbB2, and total IGF-1R on spatially distinct spots. The user adds the sample and a solution containing the detection antibody—anti-phosphotyrosine conjugated with an electrochemiluminescent compound, MSD SULFO-TAG label—over the course of one or more incubation periods. Analytes in the sample bind to the capture antibodies immobilized on the working electrode surface; recruitment of the conjugated detection antibody by bound analytes complete the sandwich. The user adds an MSD read buffer that provides the appropriate chemical environment for electrochemiluminescence and loads the plate into an MSD SECTOR® Imager for analysis. Inside the SECTOR Imager, a voltage applied to the plate electrodes causes the labels bound to the electrode surface to emit light. The instrument measures intensity of emitted light to provide a quantitative measure of phospho-EGFR, phospho-ErbB2, and phospho-IGF-1R present in the sample.

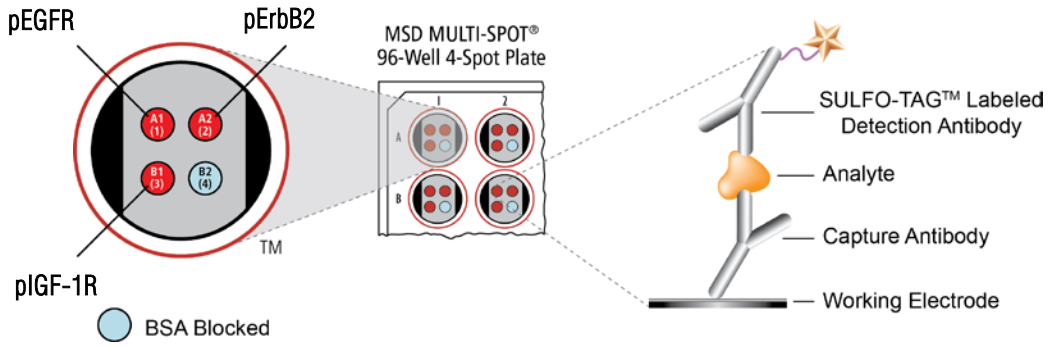

Figure 1. Spot diagram showing placement of analyte capture antibody. The numbering convention for the different spots is maintained in the software visualization tools, on the plate packaging, and in the data files. A unique bar code label on each plate allows complete traceability back to MSD manufacturing records.

Reagents Supplied

Product Description	Storage	Quantity per Kit K15106A-3
MULTI-SPOT 96-Well 4-Spot EGFR Family Plate N45106B-1	2–8°C	20 plates
SULFO-TAG Anti-Phosphotyrosine (PY20) Antibody[1] (50X)	2–8°C	4 vials (375 µL ea)
Read Buffer T (4X) R92TC-3 (50 mL), R92TC-2 (200 mL)	RT	1 bottle (200 mL)

Required Materials and Equipment - not supplied

- Deionized water for diluting Tris Wash Buffer (10X) and Read Buffer T (4X)
- 500 mL bottle for reagent preparation
- 50 mL tubes for reagent preparation
- 15 mL tubes for reagent preparation
- Microcentrifuge tubes for preparing serial dilutions
- Appropriate liquid handling equipment for desired throughput, capable of dispensing 10 to 150 µL into a 96-well microtiter plate
- Plate washing equipment: automated plate washer or multichannel pipette
- Adhesive plate seals
- Microtiter plate shaker

Optional Material — not supplied

- Phosphoprotein Reagent Support Pack (K0000D-3)

Safety

Safe laboratory practices and personal protective equipment such as gloves, safety glasses, and lab coats should be used at all times during the handling of all kit components. All hazardous samples should be handled and disposed of properly, in accordance with local, state, and federal guidelines.

[1] Some SULFO-TAG conjugated detection antibodies may be light-sensitive, so they should be stored in the dark.

Reagent Preparation

Note

The instructions below will prepare the reagents needed as described in the assay protocol. All supplemental reagents (inhibitors, buffers, and blocking reagents) are available for purchase in the MSD Phosphoprotein Reagent Support Pack, or alternatively can be purchased and prepared separately by the end user. Please see the enclosed assay development insert for purchasing and preparation instructions.

Prepare Tris Wash Buffer

Dilute 10X stock of Tris Wash Buffer to 1X as shown below. Tris Wash Buffer (1X) will be used throughout the assay to make additional reagents and wash plates. Approximately 350 mL per plate is required—more if using an automatic plate washer.

For one plate, combine:

- ❑ 35 mL of Tris Wash Buffer (10X)
- ❑ 315 mL deionized water

Excess Tris Wash Buffer may be stored at room temperature in a tightly sealed container for later use.

Prepare Blocking Solution

For one plate, combine:

- ❑ 600 mg Blocker A (dry powder)
- ❑ 20 mL 1X Tris Wash Buffer

Prepare Antibody Dilution Buffer

For one plate, combine:

- ❑ 150 µL 2% Blocker D-M
- ❑ 30 µL 10% Blocker D-R
- ❑ 1 mL blocking solution-A
- ❑ 1.82 mL 1X Tris Wash Buffer

Set aside on ice.

Prepare Complete Lysis Buffer

To 10 mL of Tris Lysis Buffer, add the following supplemental materials to prepare the complete lysis buffer (sufficient for 2-3 plates):

- ❑ 100 µL Protease Inhibitor Solution (100X stock)
- ❑ 100 µL Phosphatase Inhibitor Solution I (100X stock)
- ❑ 100 µL Phosphatase Inhibitor Solution II (100X stock)

The complete lysis buffer should be ice cold before use.

Prepare Detection Antibody Solution

For one plate, combine:

- ❑ 2.94 mL antibody dilution buffer
- ❑ 60 µL 50X SULFO-TAG Anti-Phosphotyrosine (PY20) Antibody (1X final concentration)

Prepare Read Buffer

For one plate, combine:

- ❑ 5.0 mL Read Buffer T (4X)
- ❑ 15 mL deionized water

Diluted read buffer may be stored at room temperature in a tightly sealed container for later use.

Prepare MSD Plate

This plate has been pre-coated with antibodies for the analytes shown in Figure 1. The plate can be used as delivered; no additional preparation (e.g., pre-wetting) is required. The plate has also been exposed to a proprietary stabilizing treatment to ensure the integrity and stability of the immobilized antibodies.

Sample Preparation and Storage

This cell lysis protocol is provided as a reference. Specific cell types or targets may benefit from alternative buffer components or techniques, depending upon the particular research application. Most lysis buffers are compatible with MSD MULTI-SPOT plates, although high concentrations of denaturing detergents (>0.1%) and reducing agents (DTT >1mM) should be avoided. Please contact MSD Scientific Support with any questions regarding lysate preparation options.

All manipulations should be performed on ice. The amount of complete lysis buffer required will vary depending on scale of preparation and type of cells. Larger cells (e.g. NIH3T3, HeLa) should be lysed at concentrations of 1-5 x 10^6 cells per mL of lysis buffer. Smaller cells (e.g. Jurkat) should be lysed at concentrations of 1-5 x 10^7 cells per mL of lysis buffer.

Analysis of proteins in their activated state (i.e. phosphorylated) usually requires stimulation prior to cell lysis. Verification of cell stimulation and sample preparation should be performed prior to using this kit. Phosphate Buffered Saline (PBS) should be ice-cold prior to use.

Suspension Cells

Pellet cells by centrifugation at 500 x g for 3 minutes at 2-8°C. Discard supernatant and wash the pellet once with cold PBS. Pellet the cells again, discard supernatant and resuspend in complete lysis buffer at 1 - 5 x 10^7 cells per mL. Incubate on ice for 30 minutes. A shorter incubation time of 15 minutes may be adequate for many targets. Clear cellular debris from the lysate by centrifugation greater than or equal to 10000 x g, at 2-8°C for 10 minutes. Discard the pellet and determine protein concentration in the lysate using a detergent compatible protein assay such as BCA. Unused lysates should be aliquoted and quickly frozen in a dry ice-ethanol bath and stored at ≤-70°C.

Adherent Cells

All volumes are determined for cells plated in 15 cm dishes. Remove media from the plates and wash cells one time with 5 mL cold PBS. Add 2 mL PBS to the plates and scrape the cells from the surface of the dish and transfer into 15 mL conical tubes. Pellet the cells by centrifugation at 500 x g for 3 minutes at 2-8°C. Discard supernatant and resuspend cells in 0.5 – 2 mL of complete lysis buffer per dish. Incubate on ice for 30 minutes. A shorter incubation time of 15 minutes may be adequate for many targets. Clear cellular debris from the lysate by centrifugation greater than or equal to 10000 x g, at 2-8°C for 10 minutes. Discard the pellet and determine protein concentration in the lysate using a detergent compatible protein assay such as BCA. Unused lysates should be aliquoted and quickly frozen in a dry ice-ethanol bath and stored at ≤-70°C.

Refer to Appendix I for cell lysate preparation protocol modifications that accommodate the use of 96-well culture plates.

Assay Protocol

The following protocol describes the most conservative approach to achieving optimal results with the MULTI-SPOT EGFR Family. The entire assay, including plate analysis on the MSD reader, can be completed in 4.5 hours. Once desired results are achieved, the protocol can be streamlined to eliminate multiple incubations and wash steps. Samples may be prepared for testing in the manner outlined in the Sample Preparation and Storage section.

1. Block Plate and Prepare Samples:
   a. Add 150 µL of blocking solution into each well. Seal the plate with an adhesive plate seal and incubate for 1 hour with vigorous shaking (300–1000 rpm) at room temperature.
   b. Prepare complete lysis buffer just prior to sample dilution.

      Note: Samples, including cell lysates, etc., may be used neat or after dilution.

      - MSD plates are compatible with most sample matrices. Avoid reagents that will denature the capture antibodies (e.g. high concentrations of reducing agents such as DTT should be avoided, and also SDS and other ionic detergents should be 0.1% or less in the sample applied to the well).
      - Depending on the stability of the target in the matrix, additional protease and phosphatase inhibitors may be required in the matrix or diluent.
      - If working with purified protein, only a few nanograms per well will generally provide a strong assay signal. Purified recombinant proteins may exhibit differences in both signal and background as compared to native proteins in cell lysates.
      - Keep diluted samples on ice until use

   c. Prepare positive and negative cell lysates:

      (if purchased separately).

      - Thaw cell lysate samples on ice, and dilute them immediately before use. Keep on ice during all manipulations, and discard all remaining thawed, unused material.
      - Dilute cell lysate in complete lysis buffer to a final concentration of 0.8 µg/µL. This will deliver 20 µg/well lysate in 25 µL.
      - A dilution series may also be prepared if desired.

Notes

Read entire protocol prior to beginning the assay.

Solutions containing MSD Blocker A should be stored at 2-8°C and discarded after 14 days.

Complete lysis buffer should be kept ice-cold during all experimental manipulations.

The sensitivity of MSD immunoassays rivals that of ELISAs and Western blots. The amount of sample required for a given assay will depend on the abundance of the analyte in the matrix and the affinities of the antibodies used.

Samples and standards cannot be serially diluted in the MSD plate. Use microcentrifuge tubes or a separate 96-well polypropylene plate to prepare dilutions.

2. **Wash and Add Samples:** Wash the plate 3 times with 300 µL/well of Tris Wash Buffer. Add 25 µL of samples per well. Seal the plate with an adhesive plate seal and incubate for 2 hours with vigorous shaking (300–1000 rpm) at room temperature.

   Prepare detection antibody solution during this time.

3. **Wash and Add Detection Antibody:** Wash the plate 3 times with 300 µL/well of Tris Wash Buffer. Add 25 µL of detection antibody solution to each well of the MSD plate. Seal the plate with an adhesive plate seal, and incubate for 1 hour with vigorous shaking (300–1000 rpm) at room temperature.

   Prepare 1X Read Buffer T during this time.

4. **Wash and Read:** Wash the plate 3 times with 300 µL/well of Tris Wash Buffer. Add 150 µL of 1X Read Buffer T to each well of the MSD plate.

   Analyze the plate on the SECTOR Imager:

   a. Double click on DISCOVERY WORKBENCH® icon on computer desktop (if not already open).
   b. Click the SECTOR Imager icon in upper left corner of screen (if not already open to plate reading screen).
   c. From the pull down menu select "Read From Barcode."
   d. If only reading one plate check "Return Plate to Input Stack." Then check "Read Plates(s)" checkbox and enter 1.
   e. If reading multiple plates, check the "Read Plate(s)" checkbox and enter number of plates to be read in the text field. For example, if five plates need to be read, type in "5."
   f. Click the "Run" button. The "Run Options" window will be displayed
   g. If the data from each microplate is to be exported as individual files, select "Separate Files" in the "Export" area of the "Run Options" window. Select "Appended File" if all data from the entire stack run is to be exported to one file. Select "Default" in the "Export Format" area. Check the box to export default data file.
   h. If desired, make selections to export a custom data file.
   i. Browse and select the location to export data files.
   j. Click OK to initiate the run.
   k. Data will be automatically saved in the software database. Text versions of the requested data files will be exported to the designated folder.

Notes

Shaking a 96-well MSD MULTI-ARRAY or MULTI-SPOT plate during an incubation step will typically accelerate capture at the working electrode.
The lysate sample incubation time provided is optimized for the use of MSD cell lysates. Samples from other sources may require a longer incubation.

Excess diluted read buffer may be kept in a tightly sealed container at room temperature for later use.

Bubbles introduced during the read buffer addition will interfere with imaging of the plate and produce unreliable data.

Plate should be imaged within 5 minutes following the addition of read buffer. Due to the varying nature of each research application, assay stability should be investigated prior to allowing plates to sit with read buffer for extended periods.

An all-inclusive indelible copy of the data and associated instrument information will be saved on the internal database, regardless of data file export selection. Additional copies of the data can be exported in any layout at a later time using this database. Consult the instrument user manual for more information.

Analysis of Results

The percent phosphoprotein in a sample can be calculated using independent MSD phosphoprotein and total protein singleplex assays or MSD phospho-/total multiplex phosphoprotein assays.

INDEPENDENT ASSAY FORMAT: Anti-Total Singleplex and Anti-Phospho-Singleplex Assays
% Phosphoprotein = (Phospho-signal / Total signal) x 100

MULTIPLEX ASSAY FORMAT: Anti-Total and Anti-Phospho-Assay in the same well
% Phosphoprotein = ((2 x Phospho-signal) / (Phospho-signal + Total signal)) x 100

Note:

1. The above calculation assumes that the capture antibodies on the anti-phospho and anti-total spots have very similar binding affinities.
2. The numerator in the equation contains a distribution factor of 2 based on the assumption that the phosphorylated isoform of the protein binds with a similar affinity to the phospho-specific and total capture antibodies. Given equivalent binding of the phosphorylated isoform to both capture antibodies, half of the phosphorylated species will be captured by the phospho-specific and the other half will be captured by the phosphorylation-independent (total) antibody. Therefore, the phospho-specific signal can be referred to as 2X of the phospho spot.
3. The denominator is "phospho + total" because this represents the total of all the analyte captured on both of the spots.
4. If the % phosphorylation is > 100%, then the distribution factor in the numerator may be adjusted to less than 2X such that the % phosphorylation with the control lysates is 100%.

Example:

Phosphoprotein Assay							
Lysates (µg)	Positive Control Lysate			Negative Control Lysate			P/N
	Average Signal	StdDev	%CV	Average Signal	StdDev	%CV	
0	245	4	1.4	242	6	26.0	
5.0	19235	2342	12.2	461	3	0.6	42

Total Protein Assay							
Lysates (µg)	Positive Control Lysate			Negative Control Lysate			P/N
	Average Signal	StdDev	%CV	Average Signal	StdDev	%CV	
0	561	18	3.2	569	19	3.4	
5.0	7304	1227	16.8	14530	585	4.0	0.5

% Phosphoprotein = [(2 x Phospho signal) / (Phospho signal + Total signal)] x 100

Therefore, % phosphoprotein with 5 µg of positive lysate will be:

[(2 x 19235) / (19235 + 7304)] x 100 = 144% phosphorylation

In this case, the constant in the numerator may be adjusted using the control lysates as follows:

[(1.38 x 19235) / (19235 + 7304)] x 100 = 100% phosphorylation

1.38 should be used as the numerator for further calculations in the same experiment.

Typical data

Representative results for the MULTI-SPOT EGFR Family are illustrated below. The signal and ratio values provided below are example data; individual results may vary depending upon the samples tested.

Results with SKOV3 cells: Control Lysates for phospho-EGFR and phospho-ErbB2

Serum deprived SKOV3 cells were treated with Compound 56 and AG825 (1 μM each, 2.5 hours) (negative) or pretreated with sodium vanadate (1 mM, 4 hours) and stimulated with EGF (100 ng/mL, 10 minutes) (positive). Whole cell lysates were added to MSD MULTI-SPOT 4-Spot plates coated with anti-total EGFR, anti-total ErbB2, and anti-total IGF-1R antibodies on three of the four spatially distinct electrodes within a well. Phosphorylated EGFR, ErbB2, and IGF-1R were detected with an anti-phosphotyrosine antibody conjugated with MSD SULFO-TAG reagent. Western blot analyses of each lysate type were performed with phospho-EGFR, phospho-ErbB2, and phospho-IGF-1R antibodies and are shown below for comparison.

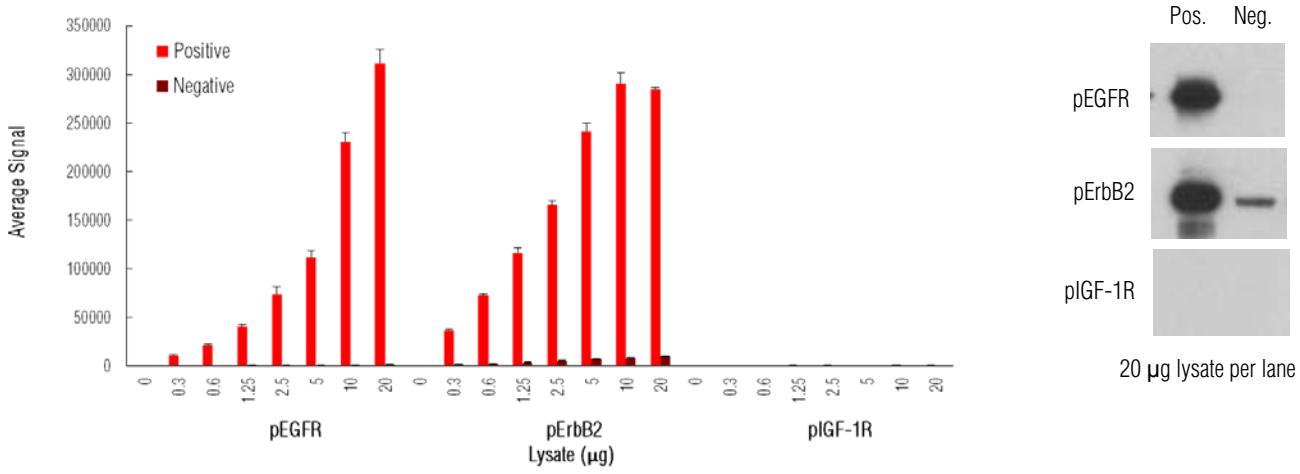

Figure 2: *Sample data generated with MULTI-SPOT EGFR Family. Increased signals for pEGFR and pErbB2 were observed with only the positive cell lysate. Signals for phospho-IGF-1R remained low throughout the titration of positive lysate. Signals for negative lysate were also low throughout the titration for all assays.*

Lysate Titration

Data for positive and negative SKOV3 cell lysates using the MULTI-SPOT EGFR Family are presented below.

	Lysate (µg)	Positive			Negative			P/N
		Average Signal	StdDev	%CV	Average Signal	StdDev	%CV	
pEGFR	0	81	26	32.1	74	13	17.2	
	0.31	10782	779	7.2	141	27	19.4	76
	0.63	21653	981	4.5	195	9	4.6	111
	1.3	40238	2499	6.2	239	13	5.6	169
	2.5	74001	7543	10.2	295	16	5.4	251
	5.0	111613	6754	6.1	348	34	9.7	321
	10	230708	8906	3.9	706	63	8.9	327
	20	311189	14667	4.7	1166	183	15.7	267
pErbB2	0	139	29	21.2	123	17	13.9	
	0.31	36591	1301	3.6	1152	52	4.5	32
	0.63	72742	1514	2.1	2117	46	2.1	34
	1.3	116381	5075	4.4	3448	130	3.8	34
	2.5	165277	5082	3.1	4818	413	8.6	34
	5.0	241421	8847	3.7	7184	231	3.2	34
	10	290486	11243	3.9	8151	255	3.1	36
	20	284703	2170	0.8	9648	110	1.1	30
pIGF-1R	0	93	8	8.2	103	16	15.7	
	0.31	503	60	11.9	131	21	15.8	3.8
	0.63	614	24	3.9	154	6	3.8	4.0
	1.3	784	18	2.3	167	5	3.0	4.7
	2.5	963	81	8.5	167	12	7.3	5.8
	5.0	592	36	6.1	133	40	29.8	4.4
	10	1102	29	2.6	181	4	2.2	6.1
	20	813	53	6.5	193	17	8.8	4.2

Results with A431 cells: Control Lysates for phospho-IGF-1R

Serum deprived A431 cells (negative) were pretreated with sodium vanadate (1 mM, 4 hours) and stimulated with IGF-1 (100 nM, 10 minutes) (positive). Whole cell lysates were added to MSD MULTI-SPOT 4-Spot plates coated with anti-total EGFR, anti-total ErbB2, and anti-total IGF-1R antibodies on three of the four spatially distinct electrodes per well. Phosphorylated EGFR, ErbB2, and IGF-1R were detected with anti-phosphotyrosine antibody conjugated with MSD SULFO-TAG reagent. Western blot analyses of each lysate type were performed with phospho-EGFR, phospho-ErbB2, and phospho-IGF-1R antibodies and are shown below for comparison.

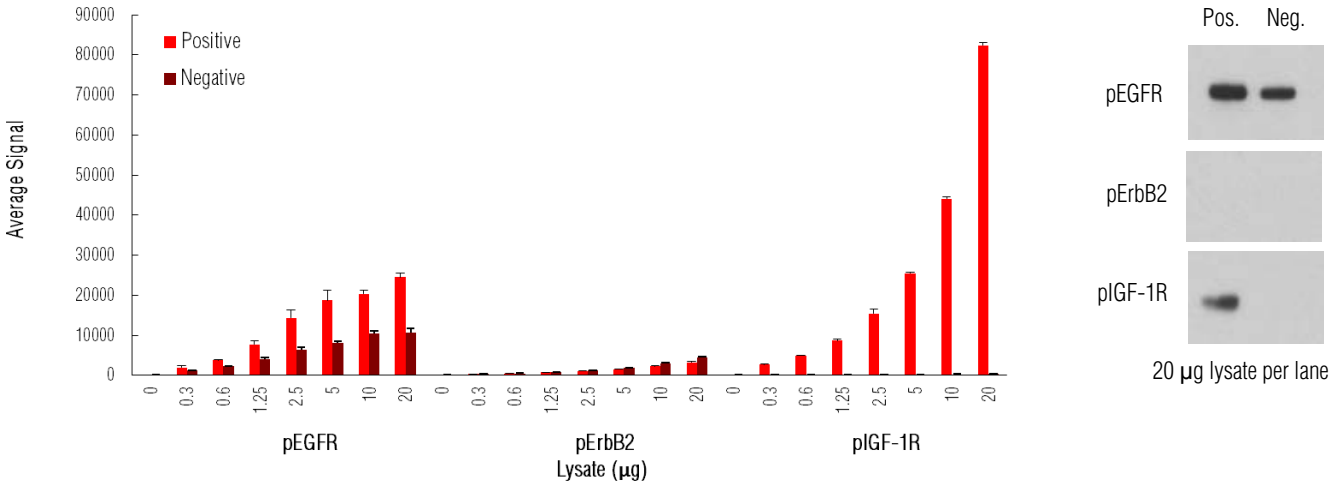

Figure 3: *Sample data generated with MULTI-SPOT EGFR Family. Increased signals for pEGFR and pIGF-1R were observed with the positive cell lysate. Signals for phospho-ErbB2 were relatively low.*

Lysate Titration

Data for positive and negative A431 cell lysates using the MULTI-SPOT EGFR Family are presented below.

	Lysate (µg)	Positive			Negative			P/N
		Average Signal	StdDev	%CV	Average Signal	StdDev	%CV	
pEGFR	0	72	7	10.0	70	15	20.9	
	0.31	1933	406	21.0	1174	35	3.0	1.6
	0.63	3720	129	3.5	2289	80	3.5	1.6
	1.3	7733	907	11.7	3972	462	11.6	1.9
	2.5	14358	2046	14.2	6346	672	10.6	2.3
	5.0	18697	2466	13.2	8113	471	5.8	2.3
	10	20289	1016	5.0	10537	627	5.9	1.9
	20	24540	1068	4.4	10558	1230	11.6	2.3
pErbB2	0	110	13	12.1	111	1	1.0	
	0.31	297	23	7.7	312	19	6.1	0.9
	0.63	480	15	3.1	483	29	5.9	1.0
	1.3	729	22	3.0	740	17	2.3	1.0
	2.5	1027	54	5.2	1177	29	2.4	0.9
	5.0	1500	95	6.4	1807	20	1.1	0.8
	10	2254	96	4.3	2970	122	4.1	0.8
	20	3228	226	7.0	4506	231	5.1	0.7
pIGF-1R	0	109	7	6.3	93	5	5.4	
	0.31	2682	178	6.6	152	12	7.7	18
	0.63	4975	82	1.6	165	4	2.6	30
	1.3	8623	373	4.3	196	10	4.8	44
	2.5	15432	1163	7.5	228	7	3.1	68
	5.0	25444	297	1.2	222	22	9.7	115
	10	44107	563	1.3	267	11	4.1	165
	20	82362	649	0.8	299	21	7.0	276

Assay Components

The capture and detection antibodies used in this assay are listed below. They cross-react with human whole cell lysates.

	Source Species	
Analyte	MSD Capture Antibody	MSD Detection Antibody
Phospho-EGFR	Mouse Monoclonal	Mouse Monoclonal
Phospho-ErbB2	Mouse Monoclonal	Mouse Monoclonal
Phospho-IGF-1R	Mouse Monoclonal	Mouse Monoclonal

Limitations of the Procedure

The following points should be noted with the MULTI-SPOT EGFR Family to maximize assay sensitivity and performance.

- A no-wash assay format may be employed, however lower sensitivity may be observed.
- All buffers containing phosphate should be avoided when detecting phosphoproteins.
- Due to the unstable nature of phosphoproteins, cell lysates should be thawed immediately prior to use, and any remaining thawed material should be subsequently discarded.

References

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Appendix

96-well Culture Plate Modifications

Successful adaptation to a 96-well culture format is cell type and target-dependent. The number of cells to be plated per well should be determined for each cell type. General recommended plating concentrations for adherent cells range from 1 x 10^4 – 5 x 10^4 cells per well and approximately 2 x 10^6 cells per mL (50 - 75 µL per well) for suspension cells. These numbers are provided as a guide, and the optimal concentrations will vary depending upon cell line used.

Suspension Cells

For flat bottom plates, experiments should be designed such that the final volume per well is 50 – 75 µL. Perform cell lysis using a 4X complete lysis buffer concentrate, supplemented with protease and phosphatase inhibitors at 4X concentrations. Add 4X complete lysis buffer directly to cells in the growth medium for a final 1X concentration in the well.

Note: With some effort, a 10X complete lysis buffer can also be prepared.

(For conical microwell plates, perform lysis by pelleting the cells, removing most of the growth medium, and adding a constant amount of 1X complete lysis buffer).

Adherent Cells

Plate cells on biologically treated tissue culture ware (such as BD BioCoat™ Cellware (Becton, Dickinson and Company, Franklin Lakes, NJ) to reduce variability due to cells lost as growth medium is removed. Treat cells as desired. Gently aspirate growth medium from microwell plate. A PBS wash step is not required and can introduce variability. Add 50-100 µL 1X complete lysis buffer per well.

Cell lysis time should be determined by the end user. Some targets are immediately available for detection. Other targets may require an incubation step at room temperature, 45°C, or on ice with gentle agitation.

Carefully pipet cell lysate onto prepared capture plate, and proceed with assay protocol.

It is important to transfer a constant volume and avoid pipetting too vigorously, as the introduction of air bubbles may result. (Targets can be captured from a volume greater than 25 µL).

Summary Protocol

MSD 96-well MULTI-SPOT EGFR Family Assay Kit

MSD provides this summary protocol for your convenience. Please read the entire detailed protocol prior to performing the MULTI-SPOT EGFR Family Assay.

Step 1 : Block Plate and Prepare Samples

Add 150 µL/well of blocking solution.
Incubate at room temperature with vigorous shaking (300-1000 rpm) for 1 hour.
Prepare complete lysis buffer just prior to sample dilution.
Prepare positive and negative cell lysates and keep on ice until use.

Step 2 : Wash and Add Sample

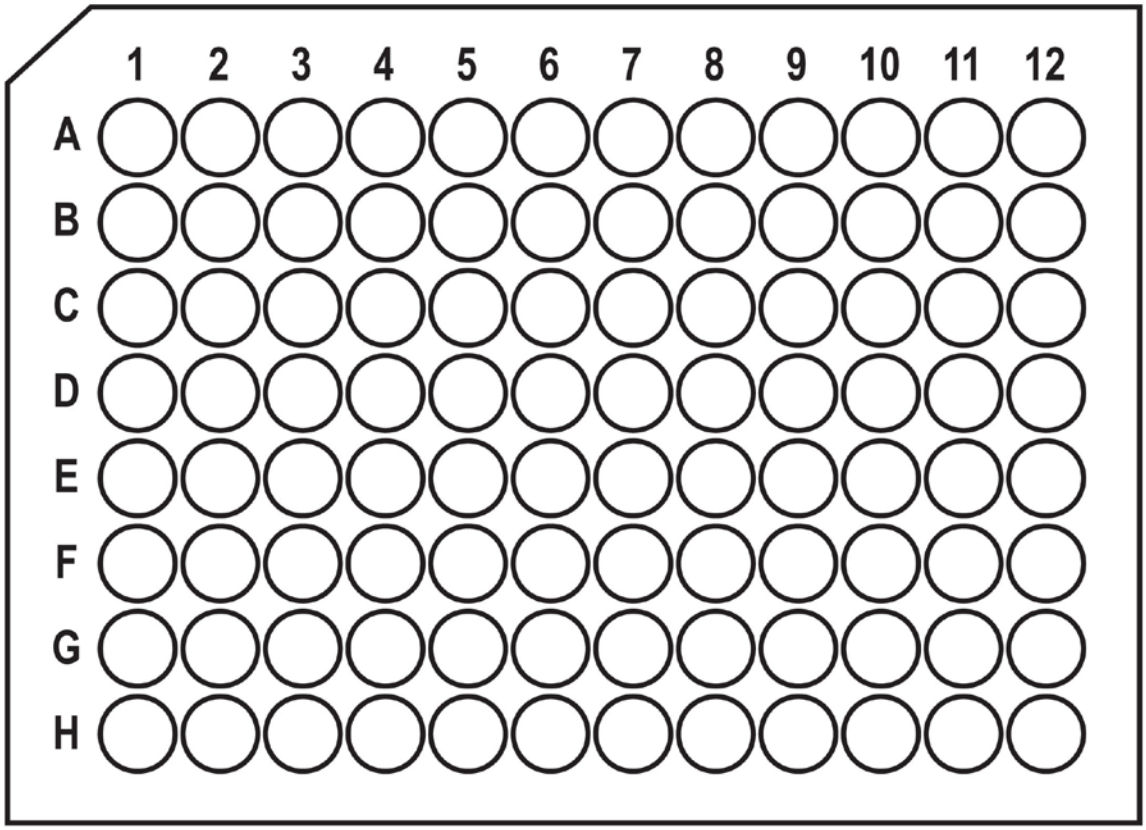
Wash the plate 3 times with 300 µL/well of Tris Wash Buffer.
Dispense 25 µL/well samples.
Incubate at room temperature with vigorous shaking (300-1000 rpm) for 2 hours.

Step 3 : Wash and Add Detection Antibody Solution

Wash the plate 3 times with 300 µL/well of Tris Wash Buffer.
Dispense 25 µL/well of 1X detection antibody solution.
Incubate at room temperature with vigorous shaking (300-1000 rpm) for 1 hour.

Step 4 : Wash and Read Plate

Wash the plate 3 times with 300 µL/well of Tris Wash Buffer.
Dispense 150 µL/well of 1X Read Buffer T.
Analyze plate on SECTOR Imager within 5 minutes of read buffer addition.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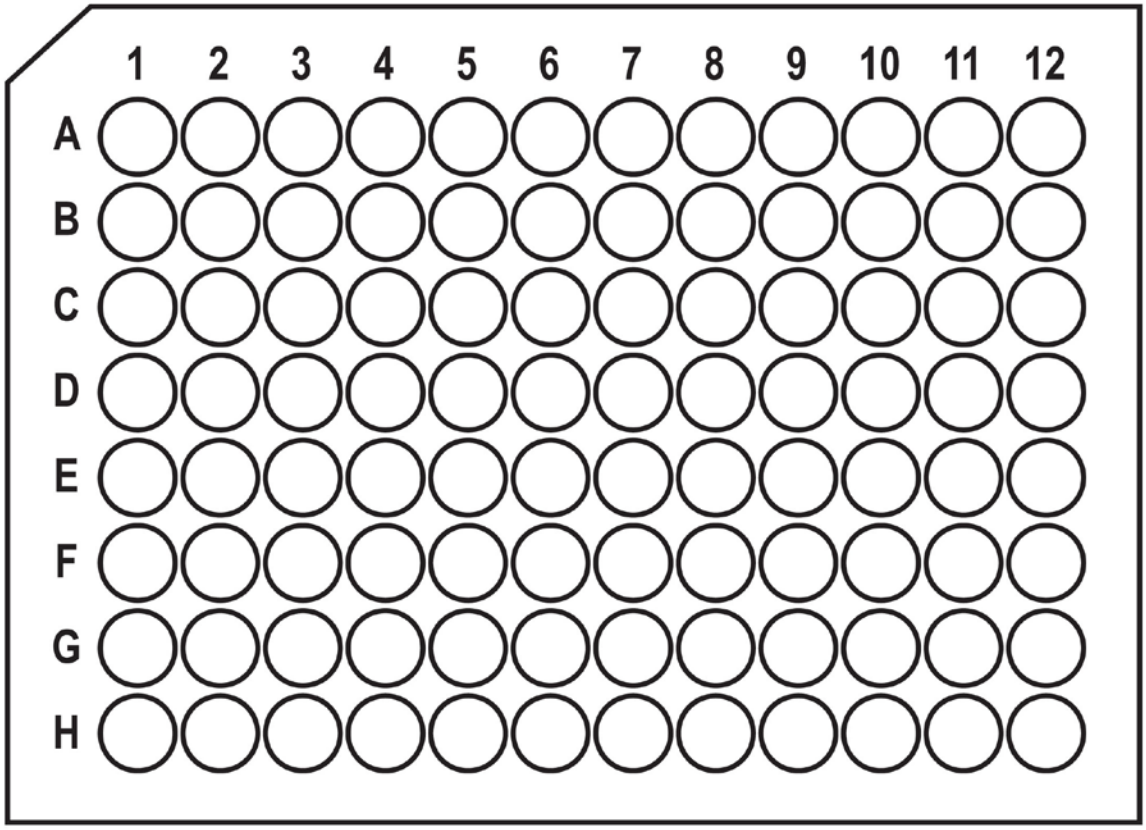
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