A Homogeneous Multiplex Immunoassay for RTK Phosphorylation State that is Adaptable to HTS

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INTRODUCTION

Receptor Tyrosine Kinases (RTKs) are widely expressed transmembrane receptors for growth factors and other extracellular signaling molecules. Binding of a specific ligand leads to phosphorylation at specific sites in the cytoplasmic tail that triggers further signal transduction processes. RTKs play critical roles in the regulation of developmental processes, including cell survival, proliferation, and motility. In addition, when unregulated, they are involved in cancer formation. For these reasons, RTKs have become popular therapeutic targets for drug development.

The VersaMAP™ Magnetic Human RTK Multiplex Kits are Luminex ® bead-based multiplex immunoassays for the analysis of RTK phosphorylation. Due to the homogeneous assay format and small sample volume required, these assays can be readily adapted for high-throughput screening with minimal manual intervention from cell treatment to data collection. To demonstrate this, several different cell lines were grown in 96-well plates, treated with various stimulants or inhibitors, and lysed in the same plate. Diluted lysates were transferred to the assay plate for analysis of RTK phosphorylation using the Human RTK VersaMAP Magnetic Kit. Using these assays, relative phosphorylation states were successfully determined for the various cell types and treatments.

MATERIALS AND METHODS

CELL CULTURE AND REAGENTS

A431 human epidermoid carcinoma cells, MDA-MB-453 human breast cancer cells, and CCD1070SK human foreskin fibroblast cells were originally from American Type Culture Collection (Manassas, VA). The cells were maintained in the recommended media. All biochemical reagents were from Tocris, an R&D Systems Company (Minneapolis, MN), including the kinase inhibitors HDS 029 (Catalog # 2646), SU 6668 (Catalog # 3335), and GTP 14564 (Catalog # 2086), and the Insulin Receptor/EGF R activator Demethylasterriquinone (DAQ) B1 (Catalog # 1819). All recombinant proteins were from R&D Systems, including recombinant human EGF (Catalog # 236-EG), NRG1-β1/HRG1-β1 (Catalog # 396-HB), and PDGF-BB (Catalog # 220-BB).

Phosphorylation was measured using either the Human RTK VersaMAP Magnetic Multiplex System Kit A (Catalog Number # VMAPMAGA) or the Human RTK VersaMAP Magnetic Multiplex System Kit B (Catalog Number # VMAPMAGB). Data were collected using the Luminex MAGPIX® analyzer.

PHOSPHORYLATION AND INHIBITION OF EGF R

A431 cells were grown in a 96-well, high binding tissue culture plate to 95% confluency. For inhibitor experiments, the cells were incubated with 220 nM HDS 029 for 1 hour prior to stimulation. Cells were then stimulated for 5 minutes with 500 µM DAQ B1 to induce EGF R phosphorylation. The cells were then lysed and assayed.

In addition, A431 cells were grown in a 96-well, high binding tissue culture plate to 95% confluency. For inhibitor experiments, the cells were incubated with 110 nM HDS 029 for 1 hour prior to stimulation. The cells were then stimulated for 5 minutes with 100 ng/mL recombinant human EGF to induce tyrosine EGF R phosphorylation. The cells were then lysed and assayed.

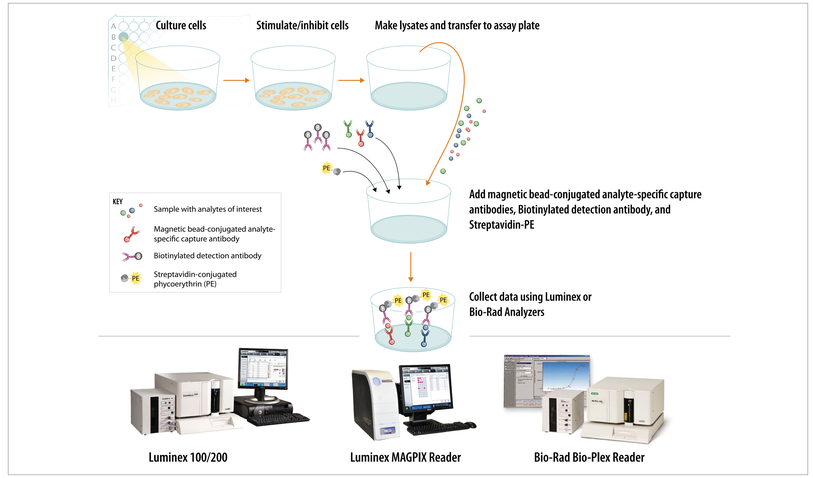
PHOSPHORYLATION AND INHIBITION OF ErbB3

MDA-MB 453 cells were grown in a 96-well, high binding tissue culture plate to 95% confluency. For inhibitor experiments, the cells were incubated with 220 nM HDS 029 for 1 hour prior to stimulation. Cells were stimulated for 5 minutes with 100 ng/mL recombinant human NRG1-β1/HRG1-β1 to induce ErbB3 phosphorylation. The cells were then lysed and assayed.

PHOSPHORYLATION AND INHIBITION OF PDGF Rβ

CCD1070SK cells were grown in a 96-well, high binding tissue culture plate to 95% confluency. For inhibitor experiments, the cells were incubated with 5 µM GTP 14564 or 0.3 µM SU 6668 for 1 hour prior to stimulation. The cells were then stimulated for 5 minutes with 100 ng/mL recombinant human PDGF-BB to induce PDGF Rβ phosphorylation. The cells were then lysed and assayed.

HOMOGENEOUS MULTIPLEX ASSAY



CELL TYPE	RTK	SENSITIVITY (ng lysate/well)	VersaMAP MAGNETIC KIT
Hek293-EphA5	MSP R	<1	VersaMAP Magnetic **Human RTK Kit A**
	TrkA	10	
	Flk-2/Flt-3	1	
	EphA4	254	
	EphA5	1	
	EphA6	41	
	EphA7	1	
	EphA8	508	
	EphB1	508	
	EphB2	1015	
	EphB3	652	
	EphB4	254	
	EphB6	5	
	HGF R	10	
HEK293-EPHA1	Ret	1	
	EphA1	674	
	EphA2	1428	
MDA-MB 453	ErbB2	713	
	ErbB3	45	
A431	EGF R	65	
C6-SCF R	SCF R	2416	
HepG2	ErbB4	3	
	M-CSF R	3	
	IGF-I R	178	
	Insulin R	2	VersaMAP Magnetic **Human RTK Kit B**
EOL-1	FGF R3	24	
	Tie-1	24	
	PDGF Ra	12	
	ROR-1	<1	
	Mer	<1	
	TrkC	1	
	MuSK	2	
	DDR1	6	
	DDR2	24	
	PDGF Rb	24	
	FGF R1	24	
	TrkB	<1	
	CCK4	1	
	Dtk	49	
	ROR2	<1	
	LTK	<1	
	ALK	<1	
A172	Tie-2	5	
	Axl	12	
	VEGF R3	5	
HEK293 FGF4	FGF R4	166	
*In Vitro**	VEGF R1	4	
**Measured in an in vitro binding assay with recombinant human VEGF*	VEGF R2	10	

TABLE 1. Sensitivity of VersaMAP Magnetic Human RTK Multiplex assays. Cells were stimulated either by treating with specific ligands or with pervanadate. Cell lysates were serially diluted and run in both VersaMAP Magnetic RTK A and B Multiplex assays as 24-plexes. Sensitivity was defined as the minimum total lysate protein per well required to produce a median fluorescence intensity (MFI) of at least 2 times the baseline signal. For VersaMAP RTK Panel A, the sensitivity is typically less than 2500 ng (2.5 µg) of lysate per well; for VersaMAP RTK panel B, the sensitivity is typically less than 200 ng (0.2 µg) of lysate per well.

RESULTS

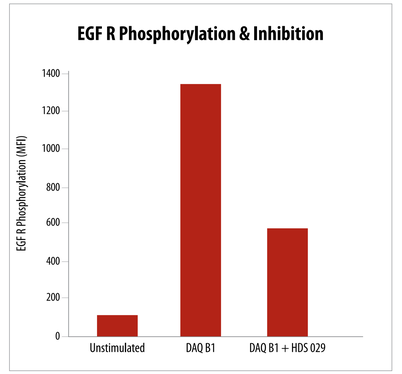
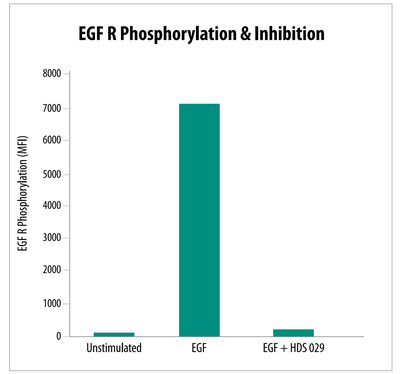
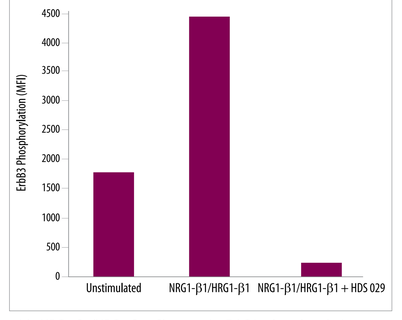
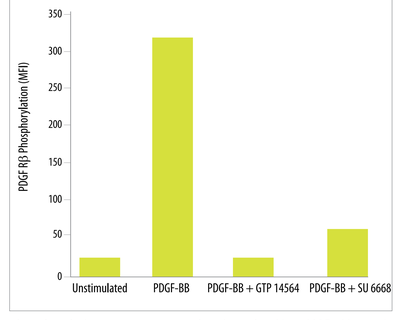


FIGURE 1. A431 cells were unstimulated, stimulated with the Insulin Receptor/EGF R activator DAQ B1, or pretreated with the ErbB inhibitor HDS 029 followed by stimulation with DAQ B1. Changes in EGF R phosphorylation were measured using the VersaMAP Magnetic Custom Human RTK Multiplex Kit A.**FIGURE 2.** A431 cells were unstimulated, stimulated with EGF, or pretreated with the ErbB inhibitor HDS 029 before stimulation with EGF. Changes in EGF R phosphorylation were measured using the VersaMAP Magnetic Custom Human RTK Multiplex Kit A.**FIGURE 3.** MDA-MB 453 human breast cancer cells were unstimulated, stimulated with NRG1-β1/HRG1-β1, or pretreated with the ErbB inhibitor HDS 029, followed by stimulation with NRG1-β1/HRG1-β1. Changes in ErbB3 phosphorylation were measured using the VersaMAP Magnetic Custom Human RTK Multiplex Kit A. **FIGURE 4.** CCD1070SK human foreskin fibroblast cells were unstimulated, stimulated with PDGF-BB, pretreated with the Class III RTK inhibitor GTP 14564 or the PDGF R/VEGF R/FGF R inhibitor SU 6668, followed by PDGF-BB stimulation. Changes in PDGF Rβ phosphorylation were measured using the VersaMAP Magnetic Custom Human RTK Multiplex Kit B.







CONCLUSION

- The assay described is a homogeneous multiplex immunoassay that allows cells to be grown in 96-well plates, treated, lysed, and tested in a continuous process that requires no wash steps.
- This assay offers a small sample size requirement, with typically < 2.5 µg and as little as 1 ng of total cellular protein in a sample volume of 25 µL.
- When used as a screening tool, this assay can allow for the rapid screening and selection of RTK inhibitors for further analyses.
- VersaMAP Magnetic Multiplex Kits are available as complete ready-to-use kits that allow the user to choose as many as 24 RTKs for primary screening, or as smaller panels for targeted follow-up analysis.

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