

Simultaneous Quantification of Total vs Phospho Signaling Molecules - Dissect the AKT Pathway with ProcartaPlex Dual Reporter Assays

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INTRODUCTION

The serine-threonine kinase Akt (also known as protein kinase B) is a central convergence node in a broadly influential signaling network. Akt activation serves as a master switch of these cellular signaling pathways, generating a multitude of intracellular responses through a plethora of downstream targets and interacting partners (Figure 1). Because of its pivotal role in cell signaling and the consequences of its misregulation in diseases ranging from cancer and diabetes to neurodegeneration, Akt is one of the most actively studied kinases in both basic research and drug development. Here we introduce our newest Invitrogen™ ProcartaPlex™ (PPX) product for analyzing the Akt signaling pathway and its downstream targets.

With the introduction of the new Luminex™ xMAP™ INTELLIFLEX System (Figure 2) an additional laser and a second reporter channel open up the ability to simultaneously acquire data for two parameters per analyte. This new instrument feature enables the simultaneous quantitation of phosphorylated and total signaling proteins on the same Luminex bead.

Akt Signaling Pathway: The Master Regulator in Human Disease

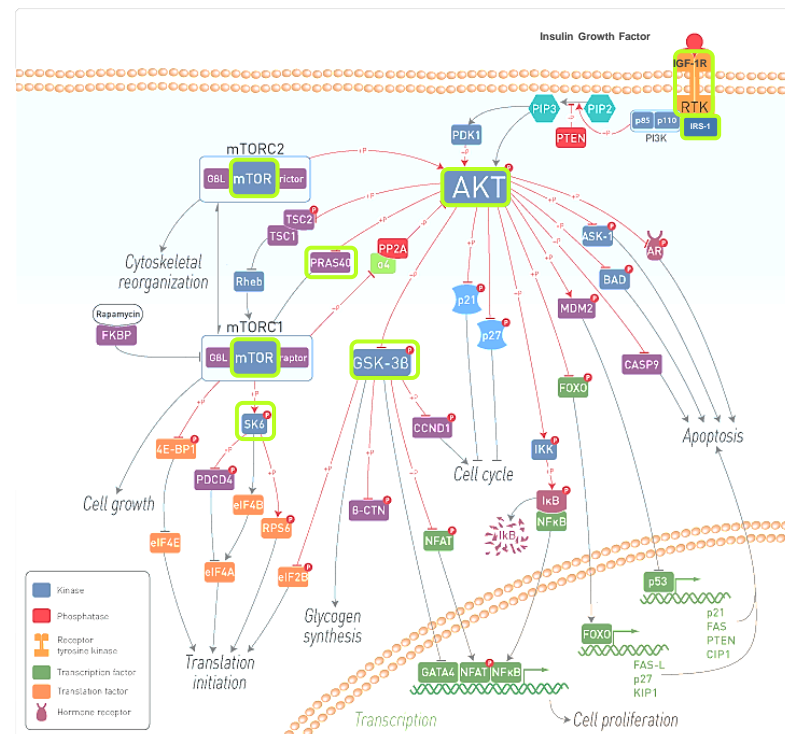
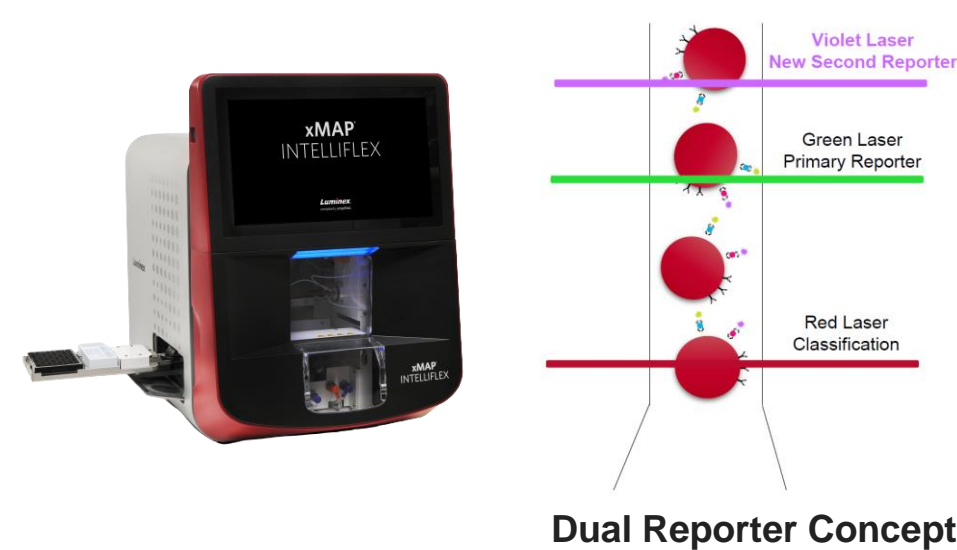


Figure 1: The Akt signaling pathway
The Akt signaling pathway is activated via growth factors such as insulin or Insulin Growth Factor (IGF) promoting phosphorylation of corresponding receptor tyrosine kinases (RTKs) like IGF-1R and propagating further signal transduction through phosphorylation of downstream targets such as IRS-1, Akt, GSK-3β, p70S6K, mTOR or CREB to regulate cell processes that are associated with tumor development, including regulators of apoptosis, gene transcription, cell cycle progression, and cellular metabolism. Key regulators of the pathway which are included in the ProcartaPlex Human Akt Pathway Dual Reporter Panel 2x8-plex are framed in green.

The Luminex xMAP INTELLIFLEX System

Figure 2: INTELLIFLEX DR-SE System.
The most advanced and versatile multiplexing platform

This system enables data acquisition for two parameters per bead simultaneously by introducing an additional laser and a second reporter channel.



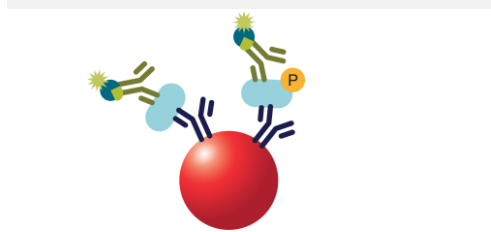
In addition to the traditional xMAP single green reporter channel (Single Reporter), the xMAP INTELLIFLEX DR-SE instrument is designed with a second reporter channel in the violet spectrum (Dual Reporter).

The system is compatible with all Invitrogen ProcartaPlex multiplex immunoassays and QuantiGene Plex multiplex gene expression assays.

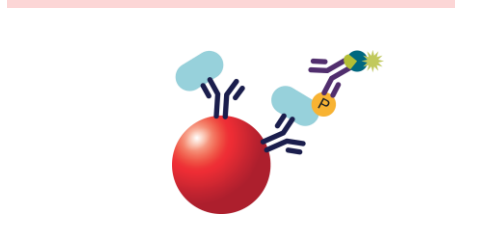
Human Akt Pathway Dual Reporter Panel 2x8-plex – ASSAY DESIGN

Luminex beads are coupled with target specific pan-capture antibodies which bind the signaling proteins independent of their phosphorylation state. Panels for separate multiplex analysis of total or phospho signaling proteins are established with either non phospho-specific or phospho-specific detection antibodies both using PE as the dye for reporter channel 1, so suitable for all Luminex instruments. For the Dual Reporter Panel, the phospho-specific detectors use a new violet dye for reporter channel 2 on the INTELLIFLEX DR-SE combined with the pan-detector read in the PE reporter channel 1 to simultaneously quantify total and phosphorylated protein in the Human Akt Pathway Dual Reporter Panel 2x8-plex.

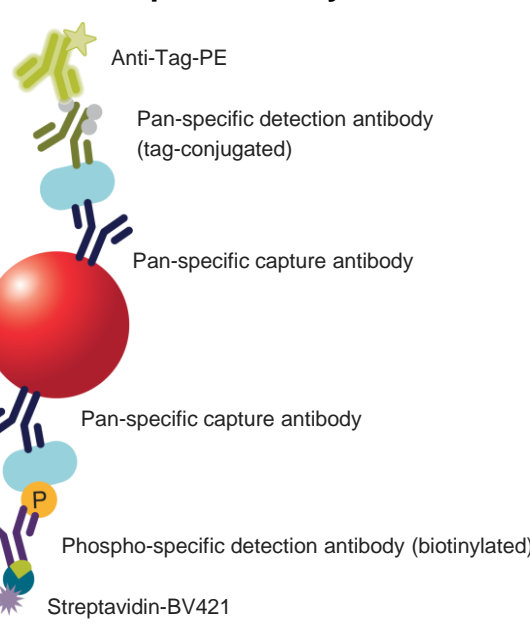
PPX Total Assay:
detector not phospho-specific



PPX Phospho Assay:
detector phospho-specific



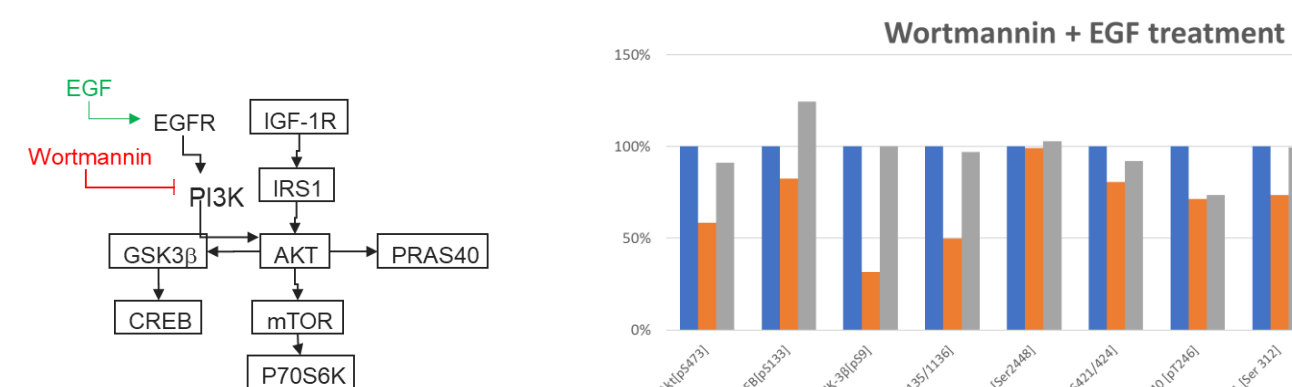
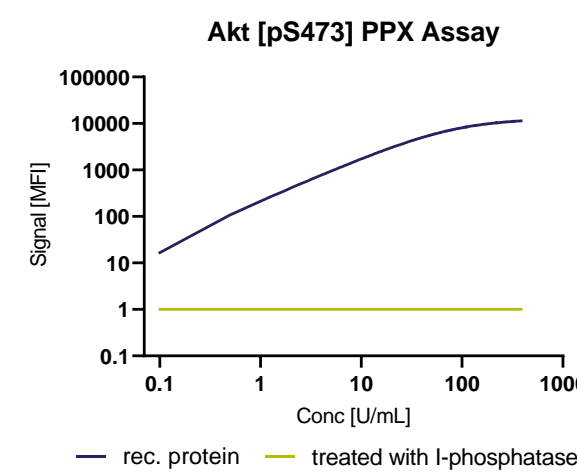
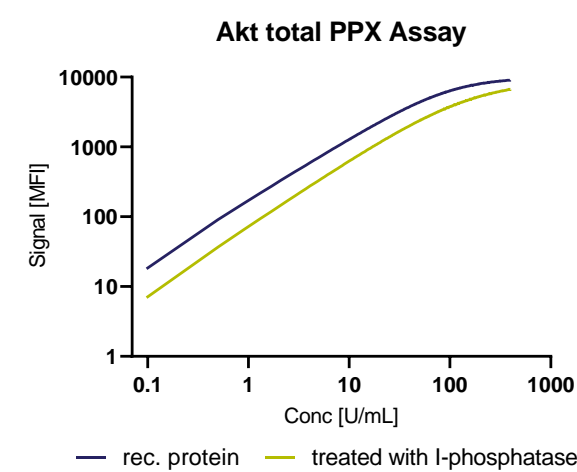
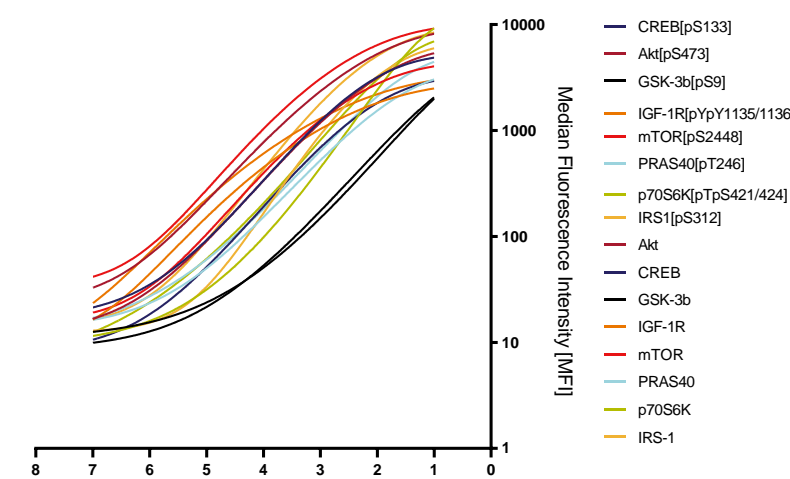
Dual Reporter Assay



ProcartaPlex Human Akt Pathway Total Panel 8-plex			
Cat No.: EPX080-97000-901			
Akt	CREB	GSK-3β	IGF-1R
mTOR	PRAS40	IRS-1	p70S6K
ProcartaPlex Human Akt Pathway Phospho Panel 8-plex			
Cat No.: EPX080-97100-PHO			
Akt[pS473]	CREB[pS133]	GSK-3β[pS9]	IGF-1R[pYpY1135/1136]
mTOR[pS2448]	PRAS40[pT246]	IRS-1[pS312]	p70S6K[pTpS421/424]
ProcartaPlex Human Akt Pathway Dual Reporter Panel 2x8-plex			
Cat No.: EPX080-97200-DR			
Akt	CREB	GSK-3β	IGF-1R
Akt[pS473]	CREB[pS133]	GSK-3β[pS9]	IGF-1R[pYpY1135/1136]
mTOR	PRAS40	IRS-1	p70S6K
mTOR[pS2448]	PRAS40[pT246]	IRS-1[pS312]	p70S6K[pTpS421/424]

Assay Characteristics – CALIBRATION and SPECIFICITY

Standard curves with phosphorylated proteins enable relative quantification (U/ml) for all signaling proteins (total and phospho). These recombinant proteins were also used to confirm phospho-specificity. We either used posttranslationally modified active proteins and dephosphorylated those with lambda phosphatase, which resulted in loss or reduction of signal in the p-assay whereas the signal of the dephosphorylated protein remained unchanged in the total assay (data shown for Akt) or verified phospho-specificity by analyzing our phospho-standard proteins before and after phosphorylation (data not shown)

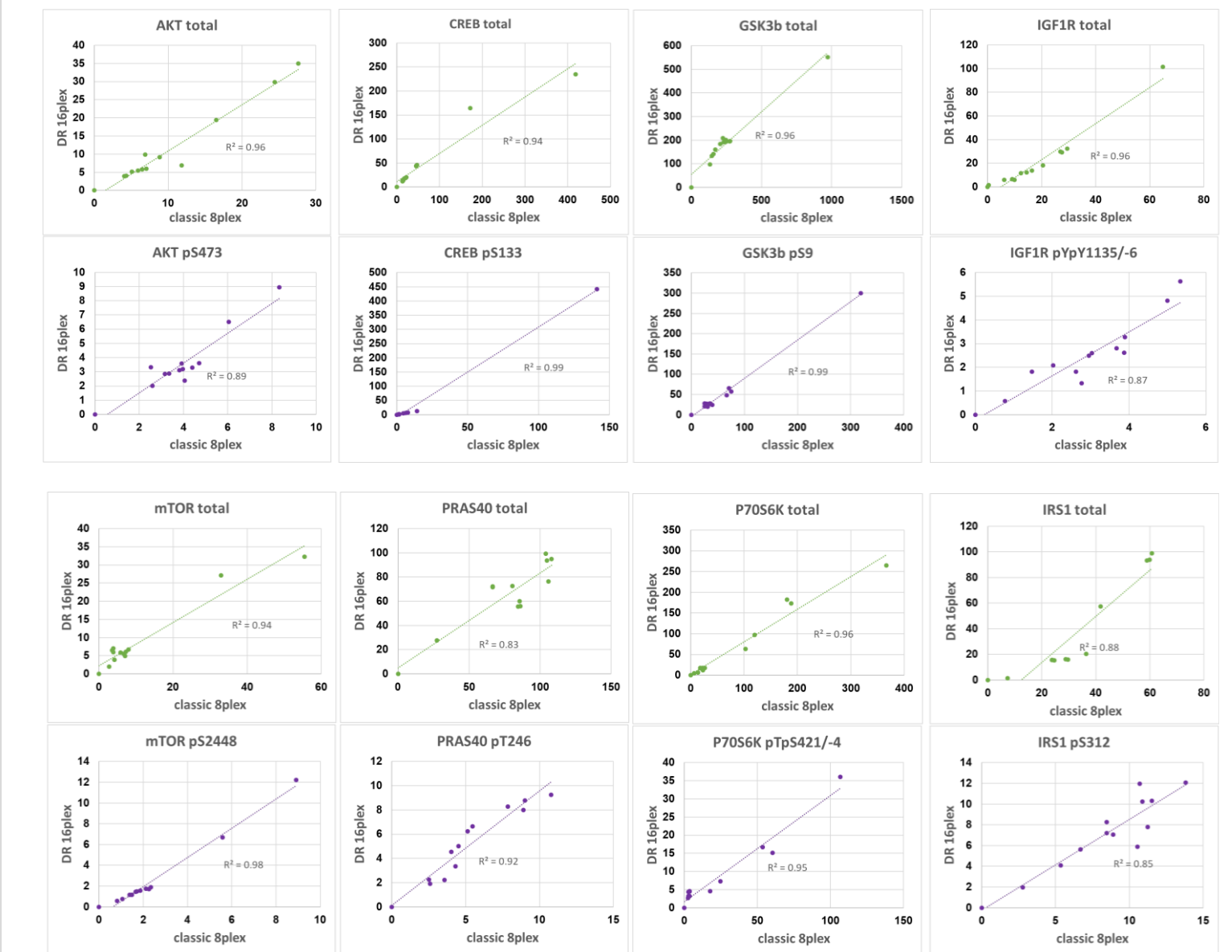


U87MG cells, a cell-line with mutation of PTEN, thus expressing high AKT levels, were treated with Wortmannin and Wortmannin + EGF.

Phospho-target levels were normalized to total protein levels and are shown relative to untreated levels. A reduction due to Wortmannin treatment and recovery of levels after EGF treatment could be shown.

CORRELATION of separate panels to Dual Reporter Panel

The conventional separate ProcartaPlex 8-plex panels for either total or phosphorylated signaling proteins were run side-by-side with the new Dual Reporter 2x8-plex panel to confirm consistency and scalability.



CONCLUSIONS

The new Invitrogen ProcartaPlex Human Akt Pathway Dual Reporter Panel 2x8-plex provides a unique tool to analyze all relevant key molecules of the Akt signaling pathway quantitatively and simultaneously. We leveraged the dual reporter functionality of the xMAP INTELLIFLEX DR-SE System to enable the quantification of the total protein and the phosphorylated portion thereof in the same well and on the same Luminex bead.

TRADEMARKS/LICENSING

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