

## Abstract

**Introduction:** The interaction of phosphoinositide 3-kinase (PI3K) with phosphorylated HER3 is a key mechanism by which the PI3K pathway can be activated in solid tumors. Assays capable of detecting the HER3-PI3K protein complex in formalin-fixed, paraffin-embedded (FFPE) tumor samples hold promise for better understanding activation of this pathway and for guiding patient response to therapies that directly, or indirectly, target PI3K.

**Experimental procedures:** Measurements of the HER3-PI3K complex and other markers of HER3 activation, such as receptor phosphorylation and formation of HER2/HER3 heterodimers were made using a new fluorescence-based, proximity assay, capable of quantitatively measuring protein-protein interactions in FFPE samples. The VeraTag™ assay utilizes fluorescently tagged antibodies targeted against proteins of interest and measures complexes of proteins in close association by relying on a singlet oxygen-mediated cleavage of the tags, which is distance-dependent. Measurements were made on a series of FFPE cell line controls generated in-house and data was verified by performing co-immunoprecipitation experiments followed by Western blot detection (Co-IP Western) on lysates prepared from the same cells. In addition, a range of breast and ovarian tumors were examined.

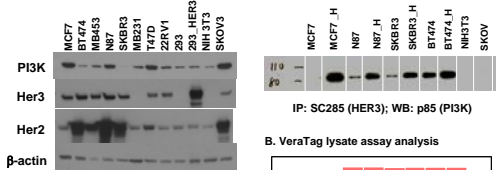
**Results:** Levels of the HER3-PI3K complex were measured in FFPE sections of the breast and ovarian cancer cell lines MCF-7, T47D, MB231, SKBR3 and SKOV3 under conditions of serum starvation or treatment with the HER3 ligand, heregulin. An antibody against the intracellular domain of HER3 and an antibody targeting the p85 sub unit of PI3K were used in the assay, and as high as a 10-fold increase in level of the HER3-PI3K complex could be observed as a result of ligand-induced HER3 activation. Co-IP Western experiments from the same series of cell lines were in qualitative agreement with results obtained from the VeraTag assay. In addition, measurements of the HER3-PI3K complex were made in FFPE breast and ovarian tumors as part of an ongoing landscaping study. The level of HER3-PI3K complex was highly correlated to other markers of HER3 activation such as receptor-phosphorylation. Efforts to verify these observations utilizing Co-IP Western experiments from matched frozen tissue is currently underway. Finally, we have examined the capability of the VeraTag assay to measure the disruption in HER3-PI3K signaling caused by the HER2 monoclonal antibody, 2C4, an antibody that inhibits HER2 receptor dimerization. In the MCF7 cell line treated with the heregulin ligand the expected, decrease in the level of HER2/HER3 heterodimer, HER3 phosphorylation, HER3-PI3K complex formation and AKT phosphorylation was observed in the drug-treated cells compared to untreated controls.

**Conclusion:** A new fluorescence-based proximity assay, capable of detecting levels of the HER3-PI3K complex in FFPE tumor samples has been developed. The performance of the assay has been demonstrated in FFPE cell line controls and verified using conventional Co-IP Western experiments. Data has been generated in a series of breast and ovarian tumors.

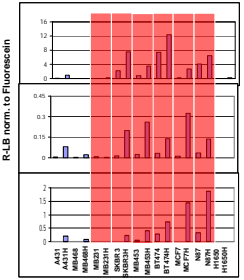
## Control Cell Line Characterization

A series of cell lines expressing a broad range of HER2, HER3 and PI3K were treated with the HER3 ligand heregulin and characterized by immunoblot and VeraTag cell lysate assays

A. Western and Co-IP Western analysis



B. VeraTag lysate assay analysis

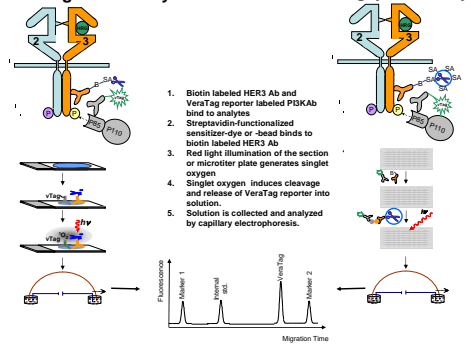


Cell line	HER2	HER3	PI3KCA Mutation*
MCF7	35,000	35,000	E545K
T47D	60,000	30,000	H1047R
MB453	300,000	35,000	H1047R
SKOV3	500,000	500	
SKBR3	1,000,000	27,000	Nona
BT474	1,100,000	35,000	K111N

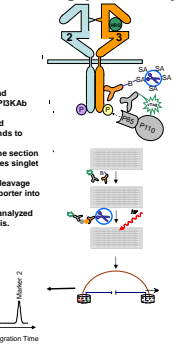
\* Stemke-Hale et al, Cancer Res, 68, 6084-91 (2008)

## Methods

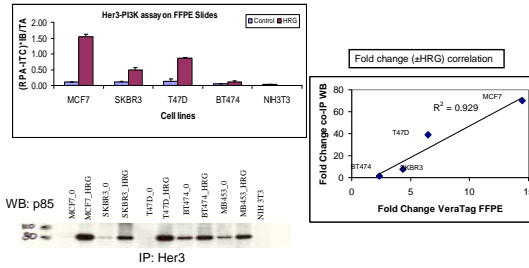
### VeraTag FFPE assay



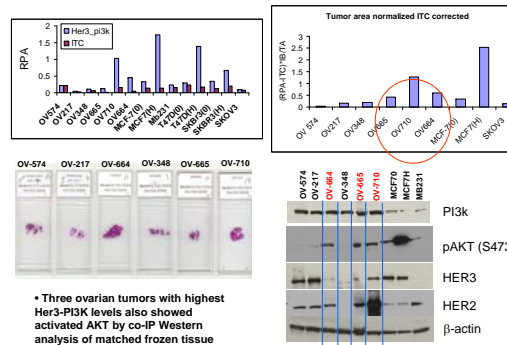
### VeraTag lysate assay



## Cross Validation with Co-IP Western



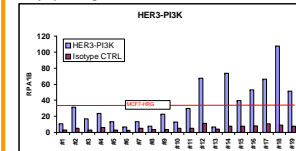
## HER3-PI3K Measurement in Ovarian Tumors



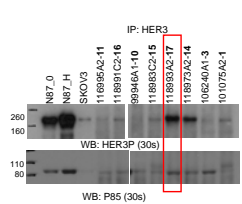
• Three ovarian tumors with highest Her3-PI3K levels also showed activated AKT by co-IP Western analysis of matched frozen tissue

## HER3-PI3K Measurement in Breast Tumors

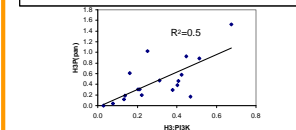
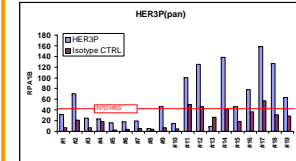
A: 19 breast tumors were analyzed by VeraTag FFPE assays profiling HER3-PI3K activation



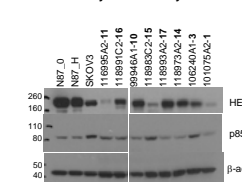
B: Subset of tumor lysates were analyzed by co-IP Western



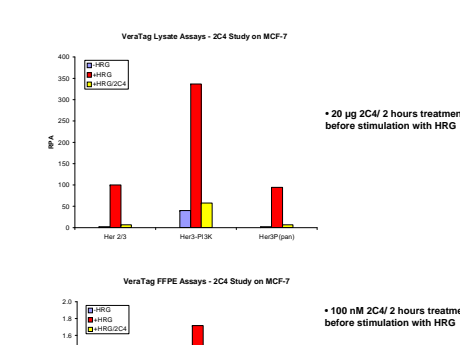
Good agreement between VeraTag lysate and FFPE assays. 2C4 effectively inhibits HER2 heterodimerization and HER3 pathway activation



C: Western analysis of tumor lysates



## 2C4 Disruption of HER3-PI3K Signaling



• 20 µg 2C4 2 hours treatment before stimulation with hHRG

• 100 nM 2C4 2 hours treatment before stimulation with hHRG

## Summary

• Both cell lysate and FFPE HER3-PI3K assays were developed and optimized in cell lines using the VeraTag technology

• Significant fold change in the level of HER3-PI3K complex was measured in FFPE control cell lines upon ligand stimulation. Data from these assays were cross-validated using co-immunoprecipitation and Western blotting

• HER3-PI3K measurements were made in breast and ovarian FFPE tumors

• In a subset of ovarian tumors the HER3-PI3K level in FFPE sections correlated with measurements of AKT activation performed on tumor lysates prepared from the same tumor

• In breast tumors VeraTag FFPE assays measuring HER3-PI3K and HER3 phosphorylation showed correlation