

## Abstract

**Background:** Ligand-bound and phosphorylated ErbB/HER heterodimers are the proposed potent signaling forms of this receptor family, and quantitative measurements of these active receptors may be predictive of response to targeted drugs. Using VeraTag™ technology, we developed and characterized quantitative assays in FFPE tumor cell lines measuring EGF-dependent increases in activated HER receptors. We utilized these assays to determine the prevalence and distribution of activated HER1, HER2, and HER1-HER2 heterodimer receptors in breast and head/neck FFPE tumors.

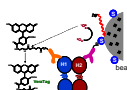
**Materials and Methods:** Assays for activated HER1 and HER2 receptors in FFPE and cell lysate formats were developed using VeraTag technology, which requires the proximity of an antibody pair for light-dependent release of a fluorescently labeled tag, followed by capillary electrophoresis-based quantitation.

**Results:** We identified a panel of cell lines that differentially express a range of EGF-dependent HER1-HER2 heterodimers using the lysate format of the VeraTag proximity assay. This cell line panel was used to develop FFPE assays that measure a >10-fold range of EGF-dependent HER1-HER2 heterodimer signal. An activated HER2 VeraTag FFPE assay and two activated HER1 VeraTag FFPE assays were also developed, all of which detect up to 100-fold ranges of EGF-dependent phosphotyrosine signal in cell lines. All VeraTag FFPE assays were cross-validated by co-immunoprecipitation. These assays, in addition to our HER1 and HER2 (HERmark™) assays, were implemented to profile 43 HER2-positive FFPE breast tumors. 63% of the samples displayed phosphorylated HER2 at levels that were significantly greater than the corresponding isotype control, across a >20-fold dynamic range of signal. These results were cross-validated by co-immunoprecipitation and western blotting in matched fresh-frozen samples. HER1-HER2 heterodimers were detected in ~10% of HER2-positive tumors, and these also expressed HER1. Additionally, we surveyed 29 head and neck tumors, most of which expressed HER1. Phosphorylated HER1 and HER1-HER2 heterodimers were detected in a subset of the HER1-positive tumors. Further, these VeraTag lysate and FFPE assays were used to characterize the effects of 2C4, erlotinib, and lapatinib on basal and ligand-dependent dimerization of HER1 and HER2 receptors in cell lines.

**Conclusions:** Quantitative VeraTag FFPE assays for HER1, HER2, phosphorylated HER1, phosphorylated HER2 and HER1-HER2 heterodimers were used to profile breast and head/neck tumors. Activated receptors were detected in a subset of tumors that expressed HER1 or HER2, supporting the hypothesis that FFPE assays measuring HER phosphorylation and dimerization status may have utility in patient selection for HER targeted therapies.

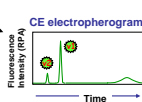
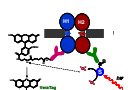
## Methods

## VeraTag lysate assay



- Ab-biotin and Ab-VeraTag reporter bind to analytes.
- Streptavidin-functionalized sensitizer dye binds to Ab-biotin.
- 670 nm light causes singlet oxygen release.
- Singlet oxygen induces cleavage and release of VeraTag reporter into solution.
- Solution is collected and analyzed by capillary electrophoresis.

## VeraTag FFPE assay

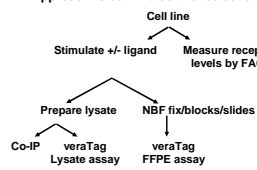


## Results: VeraTag Lysate Assays

## 1. Identification and characterization of HER1-HER2 control cells

Cell line controls spanning a range of HER1-HER2 heterodimer expression were selected by VeraTag lysate assays and confirmed by co-immunoprecipitation. HER1-HER2 heterodimers were induced upon stimulation with EGF, while total HER1 and HER2 protein expression levels were unchanged.

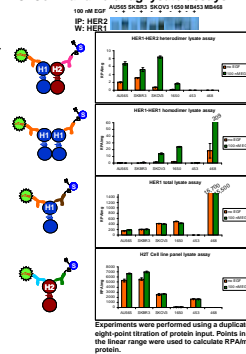
## A. Approach to cell line control selection



## B. Receptor levels measured by FACS

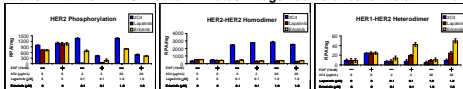
Cell line	HER1	HER2
AU565	204,560	1,447,688
SKBR3	143,599	1,402,832
SKOV3	387,771	657,000
1650	158,872	53,810
MB453	5,316	292,984
MB468	3,389,807	1,209

## C. Co-IP and veraTag lysate assays

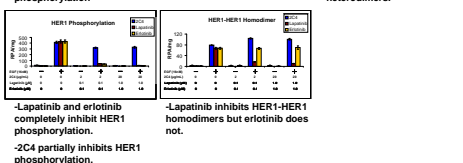


## 2. 2C4, lapatinib, erlotinib differentially modulate HER1 and HER2

• SKOV3 cells were serum-starved overnight then exposed 2hrs to either 2C4, lapatinib, or erlotinib at two different doses, followed by stimulation or mock-stimulation with 16 nM EGF for ten minutes. Drugs had no effect on total HER levels.



• Lapatinib completely inhibits HER2 phosphorylation.  
• 2C4 and erlotinib partially inhibit EGF-dependent HER2 phosphorylation.  
• 2C4 significantly increases HER2-HER2 homodimer measurements.  
• 2C4 inhibits EGF-dependent HER1-HER2 heterodimers.  
• Erlotinib, inactivates EGF-dependent, stabilizes EGF-dependent, inhibits HER1-HER2 heterodimers.

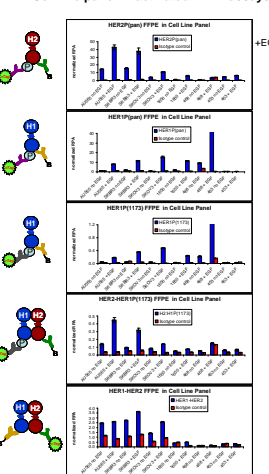


## Results: VeraTag FFPE Assays

## 3. Activated HER1, HER2 and HER1-HER2 in FFPE cell lines

Cell line controls identified by VeraTag lysate assays were used for development of FFPE assays measuring activated HER1, HER2 and HER1-HER2 heterodimers. Assay signals in FFPE track with levels expected from lysate assays. FFPE assays were cross-validated by co-immunoprecipitation.

## A. Cell line panel in activated FFPE assays

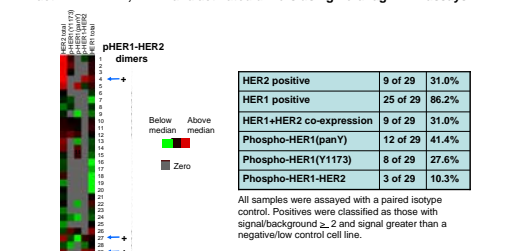


## B. Cell line panel in Co-IP/Westerns



## 4. A subset of HER1 receptors are activated in SCCNH tumors

29 squamous cell carcinoma tumors of the head/neck were surveyed for activated HER1, HER2 and activated dimers using VeraTag FFPE assays.

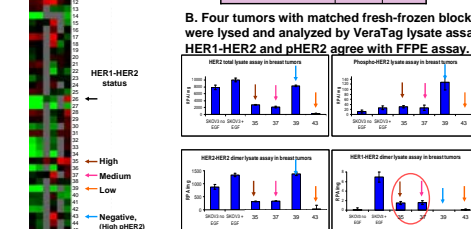


All samples were assayed with a paired isotope control. Positives were classified as those with signal/background  $\geq 2$  and signal greater than a negative/low/control cell line.

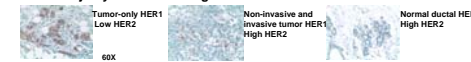
## 5. A subset of HER2 receptors are activated in breast tumors

A. Distribution of analyses in the HER1-HER2 axis in 48 breast tumors that were preselected for high HER2 using VeraTag FFPE assays.

HER2 positive	43 of 48	89.6%
Phospho-HER2	27 of 48	56.3%
HER1 positive	13 of 48	27.0%
HER1+HER2	12 of 48	25.0%
HER1-HER2	3 of 48	6.3%
Phospho-HER1-HER2	14 of 48	29.2%



## C. The majority of HER1 IHC signal localizes to tumor cells



## Conclusions

- VeraTag lysate assays revealed inhibitory and stabilizing effects of 2C4, lapatinib, and erlotinib on the HER1-HER2 axis in SKOV3 cells.
  - 2C4 inhibited EGF-dependent HER1-HER2 heterodimers and phospho-HER1 and -HER2, and stabilized HER2-HER2 homodimers.
  - Lapatinib inhibited phospho-HER1 and -HER2 and HER1-HER1 homodimers, but did not affect HER1-HER2 heterodimers.
  - Erlotinib completely inhibited phospho-HER1, partially inhibited phospho-HER2, stabilized EGF-dependent HER1-HER2 heterodimers, but does not affect HER1-HER1 heterodimers.
- We profiled 48 HER2-positive FFPE breast tumors for analyses in the HER1-HER2 axis:
  - 90% of tumors were HER2-positive; 50% were positive for phospho-HER2
  - Approximately 25% of the HER2-positive tumors also expressed HER1.
  - HER1-HER2 heterodimers were detected in 15-60% of this HER1-expressing subset, depending on detection method, and agreed with 6/30 = 20% HER1 IHC in tumor cells only.
- We profiled nearly 30 FFPE head/neck tumors (SCCHN) for analyses in the HER1-HER2 axis.
  - 90% of tumors expressed HER1; 32-45% were positive for phospho-HER1, depending on detection method.
  - 17% of tumors expressed high levels of HER2; 14% expressed elevated levels.
  - HER1-HER2 heterodimers were detected in 33% of tumors that co-expressed HER1 and HER2.
- VeraTag lysate assays can be used as a tool for understanding the effects of HER-family inhibitors in the preclinical setting, while the combination of our current CLIA-validated FFPE total and HER2 homodimer assays (HERmark™) with our new VeraTag-activated FFPE assays may provide powerful tools to more accurately predict clinical response to both HER1 and HER2 targeted therapies in tumor biopsy tissue.