# Droplet Digital™ PCR Detection of HER2 Expression in FFPE Breast Tissue Samples

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## Abstract

The human epidermal growth factor receptor 2 (HER2, also known as ERBB2) gene is involved in signal transduction for cell growth and differentiation. It is a cell surface receptor tyrosine kinase and a proto-oncogene. Overexpression of ERBB2 is of clinical relevance in breast cancer due to its prognostic value in correlating elevated expression with worsening clinical outcome. ERBB2 assessment is also of importance because successful anti-tumor treatment with Herceptin is strongly correlated with ERBB2 overexpression in the tumor (approximately 30% of all breast tumors overexpress ERBB2). In a comprehensive national study by Wolff et al. (JCO 2007), "Approximately 20% of current ERBB2 testing may be inaccurate," which underscores the importance of developing more accurate methods to determine ERBB2 status. Droplet Digital™ PCR (ddPCR™) has the potential to improve upon ERBB2 measurements because of its ability to quantitate DNA and RNA targets with high precision and accuracy. Here we present a study which investigates whether Droplet Digital PCR can be used to assess expression levels of HER2 transcripts in FFPE human breast tissues samples that were concurrently assessed by pathologists using FISH and IHC. We also determined copy number of ERRB2 compared with reference genes.
**Results:** Clinical FFPE samples were studied using ddPCR and standard FISH and IHC methodologies. The results demonstrate that ddPCR can rank order the samples in complete agreement with the current standard methods and that ddPCR has the added benefit of providing quantitative results, rather than relying on the expert skill of a seasoned pathologist for determination.

## Methods

ddPCR was performed using the Bio-Rad QX100™ Droplet Digital™ PCR system, ddPCR supermix, and Bio-Rad standard reagents for droplet generation and reading. Assays were purchased from Applied Biosystems (ABI) at 20x concentration (see list below) and cDNA was generated with MultiScribe high capacity kit (without RNase inhibitor). Samples were either from Origene fresh-frozen RNA, Ambion, or 50 clinical FFPE human breast tumor samples from the University of Mississippi, which had IHC and FISH analyses performed, of which a subset of 12 was assessed by ddPCR for gene expression levels.

TaqMan assays used in this study include:

### Gene Expression Assays

- Hs00157330_m1 (EEF2)
- Hs02758991_g1 (GAPDH)
- Hs01001595_m1 (ERBB2 5')
- Hs01001580_m1 (ERBB2 best coverage)
- Hs99999005_mH (ERBB2 3')
- Hu GAPDH PL

### Copy Number Assays

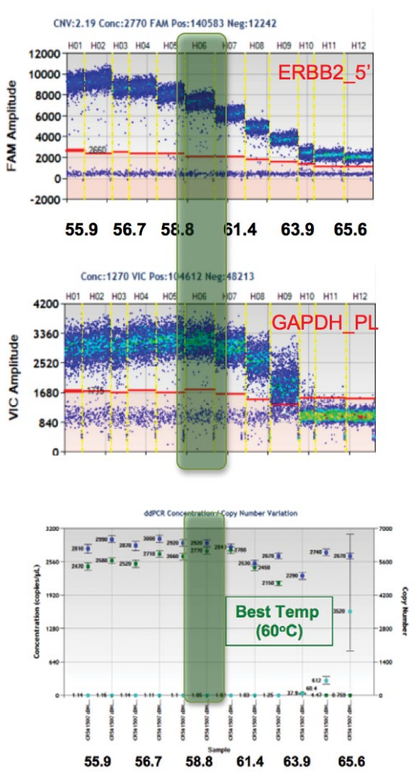
- Hs02803918_cn (ERBB2)
- CEP17

Samples used in this study include:

- Origene CR562124
- Origene CR560536
- Origene CR561507
- Origene CR560258
- Ambion normal breast RNA
- 50 FFPE derived breast tissue sections from the University of Mississippi Medical Center Department of Pathology

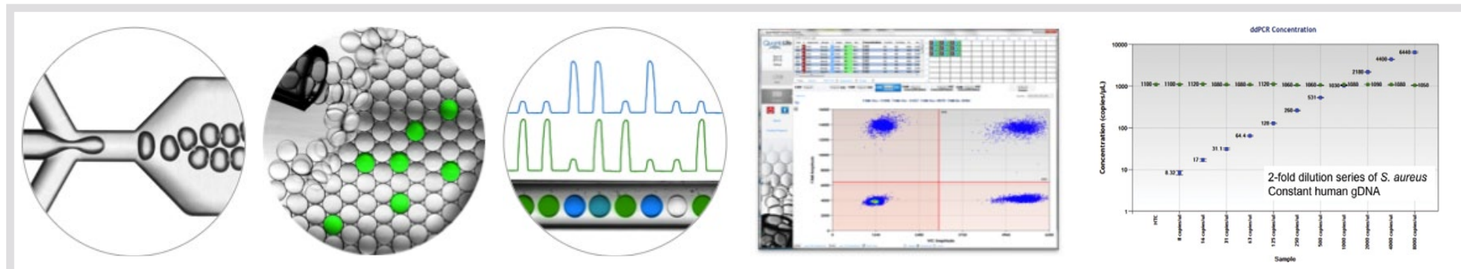
## Thermal Gradient Optimization

**Example of thermal gradient optimization for duplex assay: ERBB2/GAPDH.** In this case Applied Biosystems gene expression assays were used (Hs01001595_m1 ERBB2 and Hu GAPDH PL). The optimum temperature for both assays in duplex was 60°C.




- Strong Her2+ FF breast tumor RNA
- ERBB2_5' (64nt)
- GAPDH_pl (122nt)

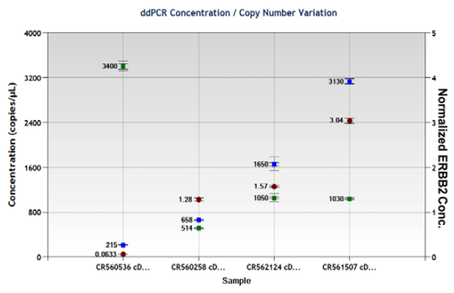
## Generate Cycle Read Analyze Evaluate



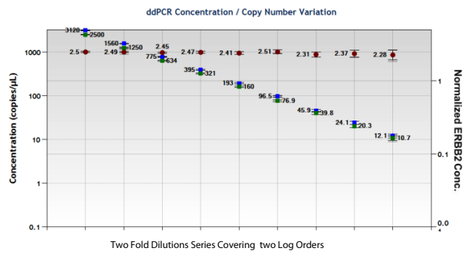

## Gene Expression

**Example of determination of ERRB2 transcript levels relative to EEF2 reference gene.** A "best coverage" ERBB2 assay was tested with four fresh-frozen breast tumor RNA samples (Origene). FAM (ERBB2) concentration, VIC (EEF2) concentration, and normalized ERBB2 concentrations are shown in panel A (blue, green, and maroon, respectively). Panel B is a twofold dilution series of sample CR561507, which demonstrates linearity of the assay spanning two log orders. Panel C represents examples of threshold settings used in determining the concentrations of the transcripts from the dilution series in B. Panel D is an example of normal breast tissue compared with Her2+ tissues (fresh frozen).

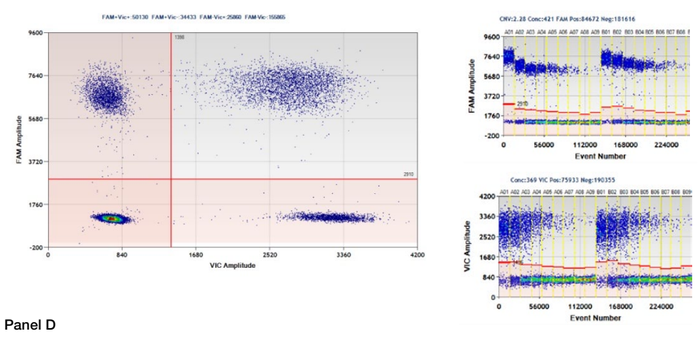
Panel A



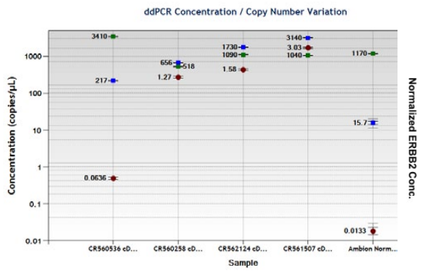

Panel B




Panel C




Panel D




**Comparison of ERBB2 expression levels in four Origene fresh-frozen samples and Ambion normal breast cDNA with "best coverage" assay normalized to GAPDH.**

## Summary Comparison of FAM and VIC Concentrations in Single and Duplex Reactions

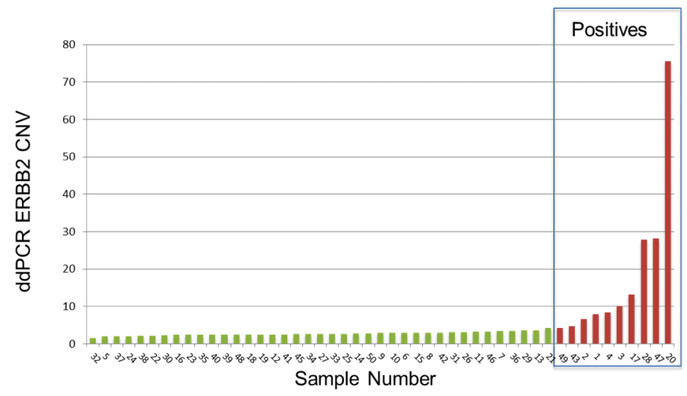
**Example of selection of best reference assays to pair with target assays.** Three different ERBB2 gene expression assays were run in single and duplex reactions with four different gene expression reference assays. Ensure that the assays are well matched in terms of thermal profiles and assay compatibility if the assays are run in duplex; however, most off-the-shelf assays work well in duplex.

| | Hs03044964 RPL37 | Hs00157330 _m1 EEF2 | Hs01922876 _u1 GAPDH | Hu GAPDH PL | Splx ERBB2 |
|---|---|---|---|---|---|
| Hs99999005 _mH ERBB2 | F 2460 V 634 | F 2480 V 809 | F 2360 V 2780 | F 2410 V 2930 | F 2550 |
| Hs01001580 _M1 ERBB2 | F 2580 V 671 | F 2660 V 794 | F 2650 V 2860 | F 2650 V 2870 | F 2750 |
| Hs01001595 _m1 ERBB2 | F 2920 V 516 | F 2970 V 748 | F 2940 V 2760 | F 3470 V 2920 | F 3120 |
| Splx refs | V 813 | V 846 | V 2980 | V 2940 | |

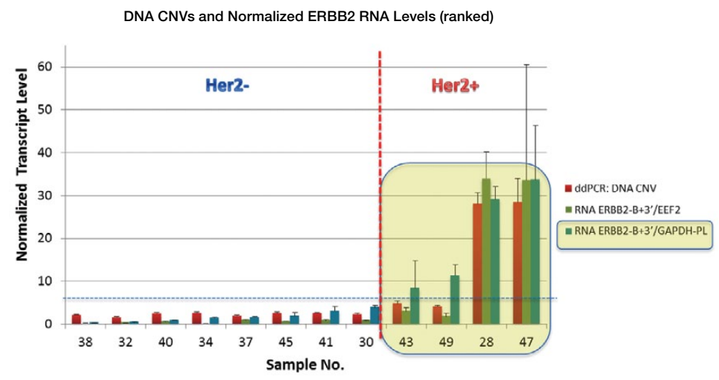
- Most duplexes do not interfere, except for RPL37 (reduced positive/negative separation)

## ERBB2 Copy Number Alterations in Breast Cancer Tissues

**Example of genomic copy number determinations.** Commercial (ABI) ERRB2 and CEP17 TaqMan assays were tested with tissue and tumor FFPE samples. Droplet Digital PCR allows determination of the genomic copy number of each gene and correction for sample degradation. Samples highlighted in red were tumors that pathology had determined as Her2+.




**Example of quantification of RNA transcript levels compared with DNA copy number.** Commercial (ABI) ERRB2, GAPDH-PL, and EEF2 gene expression TaqMan assays were tested with breast tumor FFPE samples. Droplet Digital PCR was used to determine the amounts of transcripts in a subset of 12 samples (from the genomic copy number study above) that were normalized to commonly expressed genes GAPDH and EEF2.




**Example of comparison of IHC, FISH, genomic copy number, and transcript level of clinical samples.** The table below demonstrates the capability of ddPCR to determine copies in genomic DNA and ERBB2 cDNA transcript levels when normalized to a reference transcript of either EEF2 or GAPDH. ddPCR results are in excellent agreement with pathology results, and have the added benefit of being quantitative.

| | | Clinical pathology | | ddPCR: CNVs or Normalized RNA Levels | | |
|---|---|---|---|---|---|---|
| Sample # | Year specimen taken | HER2 IHC Score | FISH HER2/CEP-17 ratio | DNA Her2/CEP17 | RNA ERBB2-B+3'/EEF2 | RNA ERBB2-B+3'/GAPDH |
| 38 | 2009 | 1+ | 0.8 | 2.2 | 0.22 | *0.27* |
| 32 | 2010 | 1+ | 1.2 | 1.56 | 0.29 | *0.46* |
| 40 | 2008 | 2+ | negative | 2.46 | 0.54 | *0.88* |
| 34 | 2009 | 1+ | not done | 2.61 | 0.17 | *1.42* |
| 37 | 2008 | 0 | not done | 1.96 | 0.98 | *1.54* |
| 45 | 2006 | negative | not done | 2.55 | 0.63 | *1.89* |
| 41 | 2008 | 1+ (resection) | negative | 2.53 | 0.85 | *3.01* |
| 30 | 2009 | 1+ | not done | 2.37 | 0.86 | *3.87* |
| 43 | 2007 | 2+ | positive | 4.76 | 2.97 | *8.31* |
| 49 | 2006 | 3+ | not done | 4.15 | 1.79 | *11.3* |
| 28 | 2010 | 3+ | not done | 28 | 33.9 | *29.1* |
| 47 | 2006 | 3+ | not done | 28.4 | 33.6 | *33.7* |

## Conclusions

- Droplet Digital PCR is a new and powerful tool to further elucidate clinically relevant materials at genomic and transcriptional levels
- ddPCR provides absolute quantitation of nucleic acids in samples with varying extents of degradation and can be paired with reference assays for DNA copy number assessments or normalized transcript concentrations
- In the example of ERBB2, ddPCR results are well correlated with IHC and FISH results
- Better discrimination of ERBB2+ and ERBB2– samples and better predictors may be enabled by more sophisticated reference normalizations using multiple reference assays