Methods 59 (2013) 183–186

Contents lists available at SciVerse ScienceDirect

Methods

journal homepage: www.elsevier.com/locate/ymeth

Review Article

Droplet Digital™ PCR quantitation of HER2 expression in FFPE breast cancer samples

Nicholas J. Heredia [a], Phillip Belgrader [a], Shenglong Wang [a], Ryan Koehler [a], Jack Regan [a], Angela M. Cosman [b], Serge Saxonov [a], Benjamin Hindson [a], Stephanie C. Tanner [b], Alexandra S. Brown [b], George Karlin-Neumann [a,*]

[a] Digital Biology Center, Bio-Rad Laboratories Inc., 7068 Koll Center Pkwy, Ste. 401, Pleasanton, CA 94566, USA
[b] University of Mississippi Medical Center, Department of Pathology, 2500 N. State St., Jackson, MS 39216, USA

ARTICLE INFO

Article history:
Available online 2 October 2012

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 HER2 is of clinical relevance in breast cancer due to its prognostic value correlating elevated expression with worsening clinical outcome. At the same time, HER2 assessment is also of importance because successful anti-tumor treatment with Herceptin® is strongly correlated with HER2 overexpression in the tumor (approximately 30% of all breast tumors overexpress HER2). In a comprehensive national study, Wolff et al. [1] state that "Approximately 20% of current HER2 testing may be inaccurate" which underscores the importance of developing more accurate methods to determine HER2 status. Droplet Digital™ PCR (ddPCR™) has the potential to improve upon HER2 measurements due to its ability to quantitate DNA and RNA targets with high precision and accuracy. Here we present a study which investigates whether ddPCR can be used to assess HER2 transcript levels in formalin-fixed paraffin embedded (FFPE) human breast tumors and whether these ddPCR measurements agree with prior assessments of these same samples by pathologists using immunohistochemistry (IHC) and in some cases fluorescence in situ hybridization (FISH). We also determined the copy number of *HER2* in these samples as compared to the CEP17 reference gene. Results: Clinical FFPE samples were successfully studied using ddPCR and compared to results from standard FISH and IHC methodology. 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.

1. Introduction

The HER2 gene expression level is of clinical significance in correlating with patient prognosis in breast cancer [1]. Higher levels are correlated with poorer prognosis yet successful treatment of breast cancer with the drug Herceptin® is positively correlated with HER2 overexpression, and thus its accurate measurement is important for proper diagnosis and treatment [2]. IHC and FISH are the standard methods for evaluating tumor status and patient prognosis but are subject to errors in classification. qPCR has also been used to quantitate nucleic acids, however a recent study has shown that for copy number alterations in *HER2*, digital PCR is much more sensitive in resolving copy number changes compared to qPCR [3]. In this study we employed Droplet Digital PCR technology (ddPCR) to reliably quantitate HER2 expression levels in clinical FFPE samples and compare them, where possible, with IHC and FISH performed on the same sample. The copy number of the *HER2* gene was also assessed via ddPCR since gene amplifications are also common in breast cancer tumors.

2. Material and methods

RNA from both fresh-frozen and FFPE samples was examined in this study. Fresh-frozen RNA from Her2+ breast tissue was obtained from Origene Inc. RNA from FFPE clinical breast tumor samples was provided by the University of Mississippi, Department of Pathology. Purified total RNA from normal breast tissue was from Ambion. cDNA was generated in a bulk reaction prior to droplet formation using the High Capacity cDNA Reverse Transcription Kit from Applied Biosystems (4368814). HER2 RNA expression levels from all RNA sources were determined with 2 different HER2 TaqMan® probe hydrolysis assays and each was duplexed with and normalized to both glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and elongation factor 2 (EEF2) expression levels using the Bio-Rad QX100 ddPCR platform.

* Corresponding author.
E-mail address: george_karlin-neumann@bio-rad.com (G. Karlin-Neumann).

1046-2023/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.ymeth.2012.09.012

Table 1
TaqMan assays and clinical samples used.

Gene expression assays	Part number
EEF2	ABI, Hs00157330_m1
GAPDH	ABI, Hs02758991_g
ERBB2 (HER2) best coverage	ABI, Hs01001580_m1
ERBB2 (HER2) 3′ coverage	ABI, Hs99999005_mH
Copy number assays	
erbB2	ABI, Hs02803918_cn
CEP17	In-house custom assay
Samples used in this study	
Origene	CR562124
Origene	CR560536
Origene	CR561507
Origene	CR560258
50 Clinical breast tumor FFPE samples	University of Mississippi
Ambion human breast total RNA	AM6952

Briefly, the Droplet Digital PCR workflow begins by partitioning the TaqMan™ reaction mix containing sample cDNA or DNA into aqueous droplets in oil via the QX100 Droplet Generator; after transfer of droplets to a 96-well PCR plate, a 2-step thermocycling protocol (95 °C × 10 min; 40 cycles of [94 °C × 30 s, 60 °C × 60 s (ramp rate set to 2 °C/s)], 98 °C × 10 min) is carried out in a conventional thermal cycler, such as the Bio-Rad C1000; and the PCR plate is then transferred to the QX100 Droplet Reader (a droplet flow cytometer) for automatic reading of samples in all wells. A detailed description of the ddPCR method and workflow can be found in Hindson et al., 2011 [4]. Bio-Rad QX100 reagents and consumables were used for the experiments including droplet generator oil (186–3005), DG8 cartridges and gaskets (186–3006), droplet reader oil (186–3004), and ddPCR supermix for probes (186–3010). *HER2* DNA copy number was also assessed in parallel ddPCR reactions for the clinical samples obtained from the University of Mississippi Medical Center Department of Pathology. TaqMan® assays were used at a final concentration of 1×(final concentrations: 900 nM each primer, 250 nM each probe) in all ddPCR reactions and are listed in Table 1. The CEP17 assay is an in-house-designed custom assay for comparing FISH and ddPCR results. The assay resides near the chr17 centromere on the q-arm. The sequence for CEP17 custom assay is: forward primer 5′-GCTGATGATCATAAAGCCACAGGTA-3′; reverse primer 5′-TGGTGCTCAGGCAGTGC-3′; and probe 5′VIC-TGCTGCAATAGGCGG-MGB-3′.

3. Results

The first series of experiments were designed to qualify commercially available assays for gene expression of the HER2 target and GAPDH and EEF2 reference genes. cDNA was made from purified RNA of fresh-frozen samples and tested in ddPCR. Fig. 1 demonstrates that the HER2 and GAPDH commercial assays run in duplex give a linear response to input cDNA amount across at least two orders of magnitude. This is also reflected in the consistency of the normalized ratios (maroon circles) of the 2 transcript concentrations across the range of input cDNA amounts. Similar results were obtained with HER2 and EEF2 (data not shown). Fig. 2 illustrates the ability of digital PCR to quantify HER2 gene expression levels and simultaneously normalize these values to EEF2 reference gene expression in cDNA from various fresh-frozen samples. Although additional reference genes could be used for normalization, such as RPL37, and the combined use of multiple reference genes could potentially normalize the data more effectively, here we are reporting on this pilot study using only a single reference gene at a time for normalization.

We next tested the performance of the HER2 and reference gene assays and the compatibility of the ddPCR system for quantifying more challenging RNA from FFPE samples, which is typically more degraded from processing. We tested 50 FFPE clinical breast tumor samples for HER2 gene expression normalized to GAPDH or EEF2, and for *HER2* genomic DNA copy number normalized to the CEP17 reference locus. Fig. 3 shows a subset of these samples with HER2 gene expression levels normalized to two independent

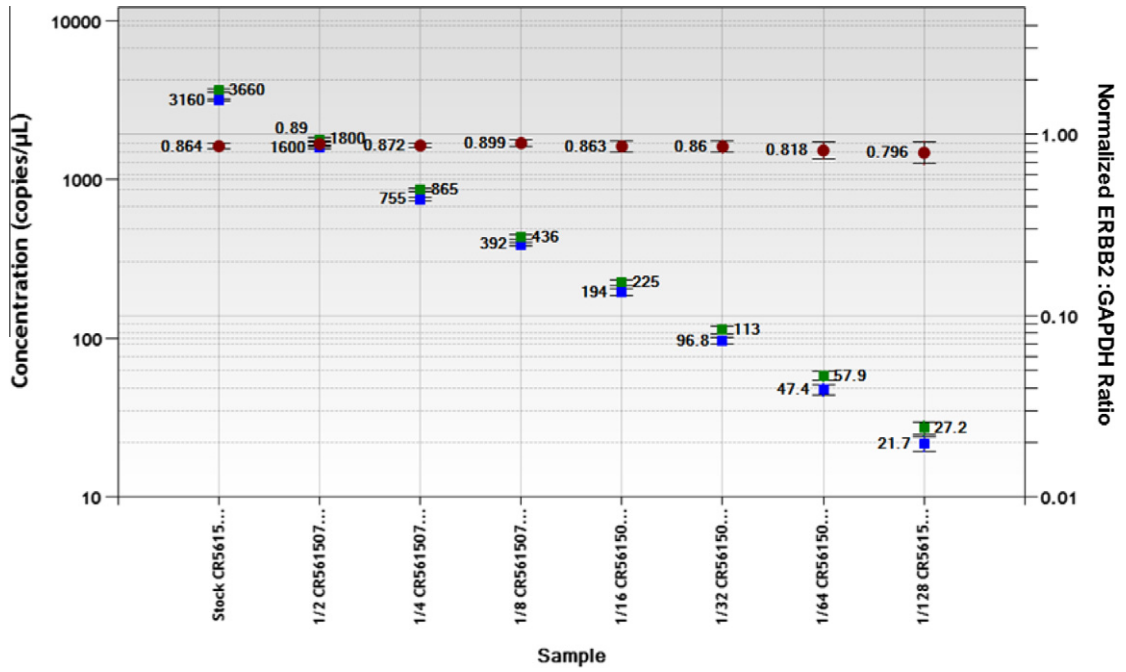

Fig. 1. Linearity of HER2 transcript concentration measurement by ddPCR. A twofold dilution series of cDNA derived from Origene® fresh-frozen sample CR561507 assessed by ddPCR with duplexed assays for HER2 (ERBB2 Best Coverage, FAM-labeled; blue squares) and GAPDH (VIC-labeled; green squares). The *x*-axis represents the dilution series. The normalized ratio of HER2 to GAPDH is represented by the maroon circles. The error bars associated with each point represent the 95% confidence interval.

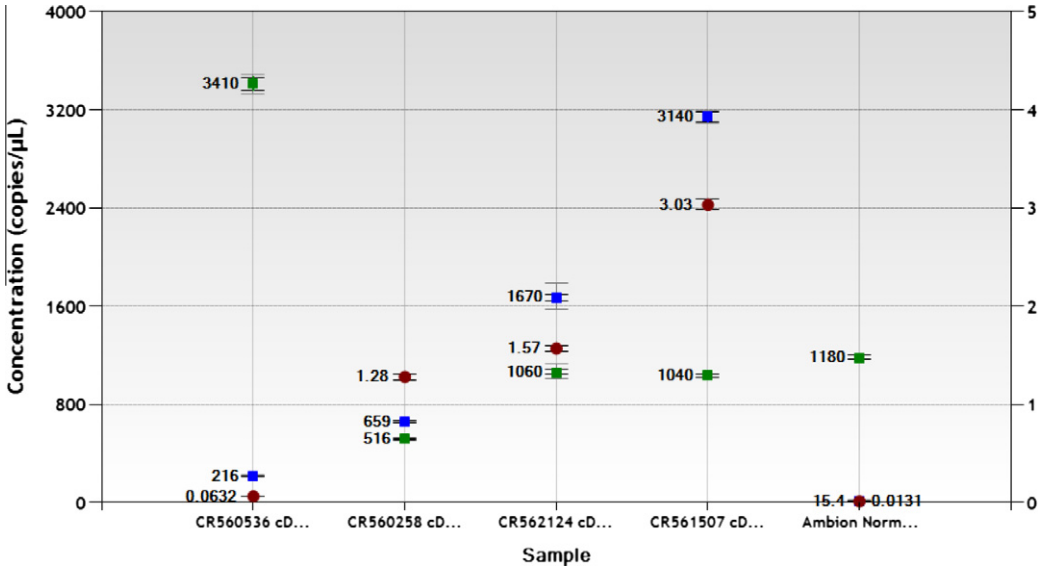

Fig. 2. ddPCR detects HER2 and EEF2 transcript levels in weakly and strongly Her2+ fresh-frozen samples. Five cDNA samples run in duplex ddPCR reaction with HER2 assay (ERBB2 Best coverage, FAM labeled; blue squares) and EEF2 (VIC labeled; green squares). Four of the samples were derived from RNA of fresh-frozen adenocarcinoma breast tissue from Origene (CR560536, CR560258, CR562124, and CR561507) and one sample was from Ambion RNA from normal breast tissue for comparison. The normalized ratio of HER2 to EEF2 are represented with maroon circles. The error bars associated with each point represent the 95% confidence interval.

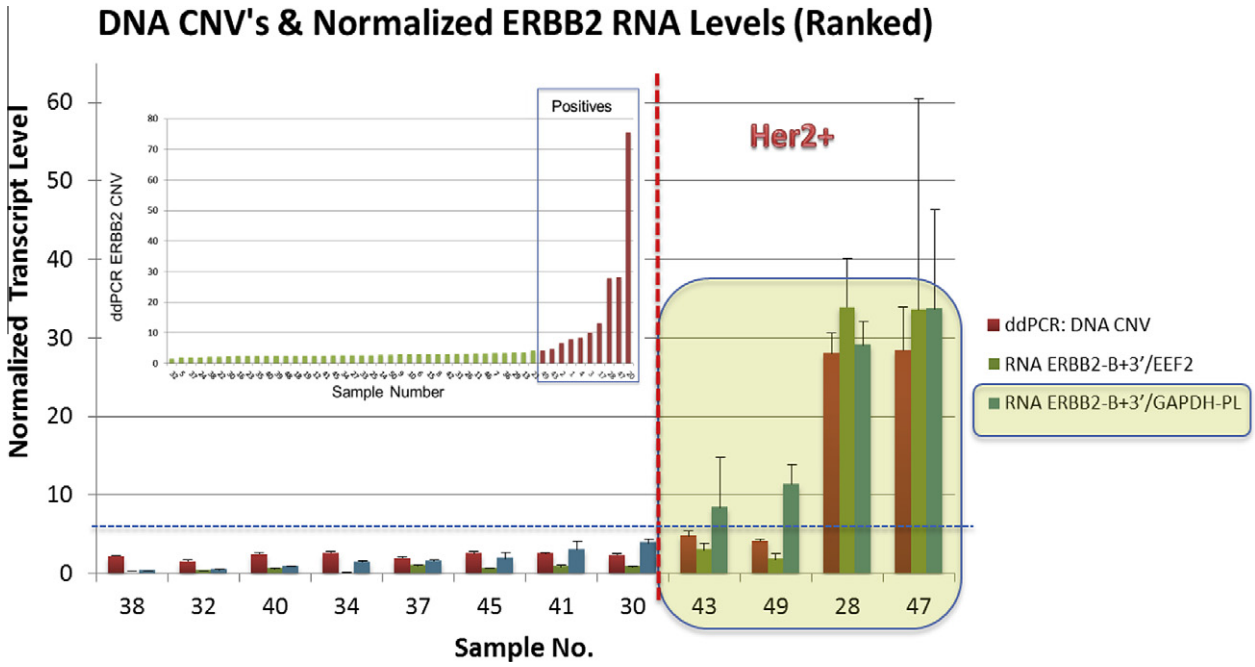

Fig. 3. Normalized HER2 gene expression and CNA (CNV) values for clinical FFPE samples. The larger plot is for a subset of twelve samples assessed for HER2 gene expression and CNA's via ddPCR. The normalized HER2 expression values denoted by "RNA ERBB2-B + 3′/EEF2" and "RNA ERBB2-B + 3′/GAPDH-PL" are each an average of two different HER2 TaqMan™ assays, Hs01001580_m1 and Hs99999005_mH, each run in duplex with one of the 2 reference assays, either EEF2 (olive green bars) or GAPDH (blue bars). Genomic DNA copy number alterations of *HER2* were normalized to CEP17 (ddPCR:DNA CNV, red bars). Samples judged to be Her2− or Her2+ are delineated as lying to the left or to the right of the red dotted line, respectively, and the positive HER2 samples are outlined in blue. In the inset plot, fifty clinical breast tumor samples (including the subset of 12) were also assessed via ddPCR for genomic DNA copy number alterations of *HER2* normalized to CEP17 assay. The genomic DNA copy number ddPCR results are rank-ordered from lowest to highest, left to right, with corresponding pathology results determined via IHC and/or FISH denoted as Her2− (olive green) and Her2+(red).

reference genes, GAPDH or EEF2, for comparison. The main graph in Fig. 3 shows samples rank-ordered by HER2 gene expression levels after normalization against GAPDH. Samples to the right of the largest discontinuity in HER2 expression levels, marked by the vertical red line, were judged to be Her2+. When compared to the samples known to be Her2+ by IHC and/or FISH, all those appearing together at the extreme right of the graph were confirmed to be Her2+ by the pathology results (Table 2). Rank-ordering samples by *HER2* DNA copy number normalized to CEP17 also segregated all Her2+ samples (based on IHC & FISH) at the right side of the smaller inset graph where the highest copy number samples are located (Fig. 3, upper left). Table 2 presents a comparison of ddPCR

Table 2
Comparison of rank-ordering by ddPCR measurements with clinical pathology assessments in breast cancer samples.

		Clinical pathology		ddPCR: CNV's 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*

results for a subset of the clinical samples seen in Fig. 3 to the pathology analysis using either IHC, FISH, or both. The green portion of Table 2 represents the samples that are considered negative for HER2 (ErbB2) overexpression and the red portion delineates HER2 positive samples.

4. Discussion

The HER2 studies presented here demonstrate the power of digital PCR in quantifying gene expression levels and copy number alterations in different samples and sample types, including degraded RNA and DNA from FFPE samples. The FFPE clinical sample data show that normalizing HER2 gene expression with two independent reference gene expression assays provides a means of segregating Her2− from Her2+ samples. A similar grouping of Her2− and Her2+ samples was achieved by using the normalized HER2 copy number values. Staging and analysis of tumor samples by standard pathology methods requires the expertise of a seasoned pathologist to make the determination of which samples are positive and which are negative, and grade them on a scale. This classification is prone to significant variation and error between labs [1]. Droplet Digital PCR could potentially offer a quantitative method for determination of Her2 status that is less sensitive to the judgment of the practitioner and to the lab in which it is performed.

5. Conclusions

ddPCR gives absolute quantitation of nucleic acids in samples with varying extents of degradation and can be duplexed with reference assays for determination of either normalized transcript concentrations or DNA copy number. The clinical samples tested by ddPCR for HER2 gene expression levels resulted in excellent correlation with IHC and FISH results. Droplet Digital PCR offers a unique approach for discrimination of HER2+ and HER2− samples due to its absolute quantitative nature without the need for calibration curves. Droplet Digital PCR may enable more reliable predictors of tumor status and patient prognosis by using absolute quantitation paired with more sophisticated reference normalizations with multiple reference assays.

6. Disclaimer

The author and contributing authors who are designated as affiliated with the Bio-Rad Digitial Biology Center are current or former employees of Bio-Rad Laboratories Inc. and have interests in Bio-Rad Laboratories, Inc.

References

[1] A.C. Wolff, E.H. Hammond, J.N. Schwartz, et al., J. Clin. Oncol. 26 (1) (2007) 118–145.
[2] S. Lee, W. Yang, K. Lan, et al., Cancer Res. 62 (2002) 5703–5710.
[3] A.S. Whale, J.F. Huggett, S. Cowen, et al., Nucleic Acids Res. 40 (11) (2012) e82.
[4] B.J. Hindson, K.D. Ness, D.A. Masquelier, et al., Anal. Chem. 83 (22) (2011) 8604–8610.