Copy number variation in breast cancer translational research

The QuantStudio™ 3D Digital PCR System as a cost-effective and sensitive alternative for HER2 gene amplification assessment

“Initial cost analysis and testing suggests that the QuantStudio™ 3D Digital PCR System may provide an affordable frontline screening method with future utility in the clinic.”

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Researcher profile
Bruno Ping is a member of the Molecular Diagnostics Department at Royal Surrey County Hospital (Guildford, UK), one of the leading hospitals and cancer centers in South East England. The laboratory capabilities range from diagnostics to translational research in oncology. One of his personal interests lies in breast cancer research, especially looking at copy number variation as a major contributor to sample stratification.

Application
HER2 (human epidermal growth factor 2, also known as ERBB2) is a proto-oncogene and driver of cellular proliferation in cancer cells under specific circumstances. There are three mechanisms in which the proto-oncogene contributes to tumorigenesis: (1) chromosomal rearrangement resulting in a hyperactive gene fusion, (2) point mutations or deletions in the coding sequence leading to a hyperactive protein, and (3) gene amplification resulting in protein overexpression.

HER2 gene amplification has been recognized for many years as an indicator of poor prognosis in early breast cancer and a critical factor in selecting the best treatment options (e.g., HER2 protein–targeted therapy with trastuzumab [Herceptin®]) for early- and advanced-stage cancers [1–3].

Currently, fluorescence in situ hybridization (FISH) and silver in situ hybridization (SISH) are routine methodologies used to assess prognosis and drug eligibility. However, disadvantages with these methods include lengthy and laborious processes for staining and scoring slides, specialized training required for performing the techniques, high overall cost, and subjective interpretation of results in some challenging cases [3].
Royal Surrey County Hospital was interested in exploring the use of digital PCR (dPCR) as an alternative to SISH for HER2 testing for technical and cost improvements. Digital PCR enables absolute quantitation of a target DNA molecule through partitioning a sample into many individual reactions. Some of these reactions contain a target molecule (and thus amplify) and some do not (do not amplify and are called as negative). Following PCR amplification, the fraction of negative reactions is used to generate an absolute quantity of target molecules in a sample, without reference to standards. As such, this technology has the potential to reduce subjectivity and improve standardization compared to the SISH methods currently used to assess HER2 gene amplification.

**Results**
A predesigned TaqMan® Copy Number Assay for ERBB2 (HER2 gene) [Life Technologies Cat. No. 4400291, Assay ID Hs00817646_cn, Entrez Gene ID 2064] and TaqMan® RNase P Copy Number Reference Assay [Life Technologies Cat. No. 4403326] was used to assess HER2 status across 48 formalin-fixed, paraffin-embedded (FFPE) samples. DNA from the samples was amplified using the GeneAmp® PCR System 9700 and read on the QuantStudio™ 3D Digital PCR System. The ratio of HER2 to RNase P indicates the number of copies of HER2 per haploid genome. HER2 amplification status was assessed using American Society of Clinical Oncology (ASCO) and the College of American Pathologists (CAP) guidelines (Table 1). At this time, there are no accepted criteria for assessment by dPCR. Therefore, the in situ hybridization (ISH) cutoff guidelines were applied to the dPCR results. Based on these criteria, 14 samples showed HER2 amplification, 31 samples had values suggesting they did not have amplification, and 3 samples had a ratio that was considered equivocal (undetermined) (Figure 1).

<table>
<thead>
<tr>
<th></th>
<th>HER2 positive</th>
<th>HER2 equivocal*</th>
<th>HER2 negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>ISH (ratio HER2:C17)†</td>
<td>&gt;2.2</td>
<td>1.8–2.2</td>
<td>&lt;1.8</td>
</tr>
<tr>
<td>IHC (score based on staining intensity)</td>
<td>3+</td>
<td>2+</td>
<td>0.1+</td>
</tr>
<tr>
<td>dPCR (ratio HER2:RNase P)</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

*Equivocal results require additional testing using either the same test or a different test.

† C17 is the reference sequence, which targets the centromeric region of chromosome 17.

To validate the dPCR results, data generated from the same samples previously analyzed by SISH were compared. The SISH results confirmed the results obtained by dPCR and suggested that dPCR on the QuantStudio™ 3D system potentially offers finer resolution when compared to the SISH results (Figure 1).

Although this is still considered early exploratory research, the team proposed a possible testing scheme that would incorporate dPCR on the QuantStudio™ 3D Digital PCR System (Figure 2). The streamlined workflow and data precision makes it practical to implement dPCR as a frontline screening technology, reserving the more costly approaches such as SISH for second-line or confirmatory testing. Even when the threshold cutoff window is expanded from the 2007 ASCO guideline cutoffs of 1.8–2.2 to a more stringent 1.1–2.2 for dPCR, the number of samples needing to be assessed by ISH technology would fall from 48 to only 14—that is, only those considered equivocal in an initial dPCR screen would have to be subjected to SISH. This would provide the group substantial savings in time and resources.

**Conclusions**
Digital PCR on the QuantStudio™ 3D Digital PCR System provides a sensitive, robust, and cost-effective method to assess HER2 gene amplification in breast cancer samples. A working model is proposed by the Royal Surrey County Hospital team that will be further tested in a long-term parallel study. Perhaps most exciting to the team, initial cost analysis incorporating dPCR as a frontline screen shows savings of over 75% compared to SISH, suggesting that dPCR is an affordable alternative with future utility in the clinic.
Figure 1. Analysis of samples. Forty-eight FFPE samples were analyzed for HER2:RNase P copy ratios as determined on the QuantStudio™ 3D Digital PCR System. The ratios were subsequently converted to diploid copy number. Samples that were equivocal by 2007 ASCO guidelines are highlighted in yellow. For confirmation, each of the samples was also analyzed by SISH. The HER2 amplification status is shown as not amplified (N, green) or amplified (P, red). Note that the SISH results suggest the range of samples with equivocal status (orange outline on graph) could be expanded to reflect the increased sensitivity of dPCR. *HER2:RNase P ratios were calculated in Excel® software.

Figure 2. Working model proposed to assess HER2 gene amplification using dPCR. This workflow recommends the use of 2 reference genes to calculate the HER2 amplification state. The choice of the best reference genes will depend on the biology of the tissue and tumor.
References

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