

**GENOMICS** INFORMATICS PROTEOMICS METABOLOMICS  
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## A Robust Whole Genome Amplification Method for Agilent Array-based Comparative Genome Hybridization Analysis

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### Abstract

Genomic instability is a classic hallmark of cancer and genetic disorders. The Agilent Oligonucleotide array-based Comparative Genomic Hybridization (aCGH) platform lets you profile DNA copy number variations on a high-throughput and genome-wide scale. We have developed a rapid, low-input whole genome amplification (WGA) method for aCGH analysis that pairs the Sigma GenomePlex WGA Kit with Agilent's established high-performance aCGH microarrays. In addition, aCGH results were validated using the Stratagene Mx3000P<sup>®</sup> QPCR System.<sup>1</sup> Using this powerful system, nanogram quantities of starting genomic DNA can generate robust and precise aCGH data.

Further research highlights can be found in the publication "Oligonucleotide Array CGH Analysis of a Robust Whole Genome Amplification Method" (Brueck, C. *et al.*) from *Biotechniques* (2007) 42(2):230-233.

### Introduction

Array-based Comparative Genomic Hybridization (aCGH) is currently used to determine chromosomal changes in various sample types (Barrett, M.T. *et al.*, 2004). This evolving technology requires DNA inputs of typically 0.5 µg per sample to process one aCGH array (Agilent Application note, 5989-5048EN). However, certain precious samples such as laser capture microdissected (LCM) cells or needle biopsies do not provide enough DNA for labeling and hybridization to an array. Whole genome amplification (WGA) methods are routinely used on genomic DNA for genotyping and sequence analysis when the amount of starting template is extremely low (Kuivaniemi, H.S. *et al.*, 2002; Hosono, S. *et al.*, 2002). Two commonly used WGA strategies are i) primer extension pre-amplification (PEP), a Taq DNA polymerase PCR-based reaction (Zhang, L.X. *et al.*, 1992), and ii) multiple displacement amplification (MDA), an isothermal genome amplification using Phi29 DNA polymerase (Dean, F.B. *et al.*, 2002). In PEP-PCR, due to the usage of completely degenerate primers, shorter products are generated with each cycle. We found that the Phi29 polymerase-based MDA method often produces high noise

<sup>1</sup> Recently, Agilent Technologies acquired Stratagene in an effort to accelerate growth in life sciences through a complementary product portfolio.



levels during genomic microarray analysis with poor quality samples, i.e. degraded or fragmented DNA. Conversely, GenomePlex WGA enables researchers to representatively amplify the genome from samples that have been fragmented to an average size of less than 1 kb. This feature enables aCGH analysis of damaged samples that were previously thought to be unusable due to their high level of DNA degradation. In this study, we applied the GenomePlex WGA to amplify either limited quantities of genomic DNA or fragmented DNA and compared it to the performance of MDA WGA by REPLI-g. Furthermore, we validated the performance of GenomePlex on Agilent aCGH microarrays using the Stratagene Mx3000P QPCR System. Taken together, this application note demonstrates the usage of GenomePlex WGA for generating robust aCGH data which can be effectively validated using the Stratagene Mx3000P QPCR System.

## Materials and methods

### DNA sources

Normal pooled gDNAs were purchased from Promega (P/N G1471 (male) and P/N G1521 (female)). Human colon carcinoma cell line HT29 gDNA was purchased from ATCC (P/N HTB-38D). Frozen breast tumor tissue was obtained from GenomicsCollaborative, Inc (GCI) and gDNA was isolated using the Qiagen DNeasy Blood and Tissue Kit (P/N 69504) per the manufacturer's protocol.

### Genomic DNA fragmentation

Genomic DNAs were sonicated using a sonicator probe (Digital Sonifier 450, Branson Ultrasonics Corp., CA) for 5 sec, 30 sec, 90 sec, or 120 sec. Fragmented gDNAs were then run on a 1.2% agarose gel (E-gel Agarose Gels P/N G5018-01, Invitrogen, CA) to determine the fragment size distributions.

### Whole genome amplification

For REPLI-g MDA, gDNA samples were amplified using the REPLI-g Kit (Qiagen P/N 150043) according to the manufacturer's protocol. The amount of gDNA input into the whole genome amplification reaction was 50 ng. After amplification, excess primers and dNTPs were removed by purifying the amplification product using the QIAprep Spin Miniprep Kit (Qiagen P/N 27106).

For GenomePlex WGA, gDNA samples were amplified using the GenomePlex WGA kit (Sigma P/N WGA2) according to the manufacturer's protocol. The amount of gDNA input into the WGA reactions was 50 ng, except for the titration

experiment where the input amount was as indicated. After amplification, excess primers and dNTPs were removed using Sigma's GenElute PCR Clean-Up Kit (Sigma P/N NA1020).

### DNA labeling and hybridization

Agilent's Genomic DNA Labeling Kit PLUS (Agilent P/N 5188-5309) was used to label the DNA with either Cyanine 3 or Cyanine 5. Following the Agilent Oligonucleotide Array-Based CGH for Genomic DNA Analysis Protocol (version 5.0), 2 µg – 2.5 µg of amplified DNA was used as the input material for each labeling reaction. In the case of un-amplified material, 0.5 µg of un-amplified genomic DNA was used for the labeling reaction. Cyanine 3 and Cyanine 5 labeled samples were mixed and hybridized to Agilent microarrays from a Human Genome CGH Microarray Kit—244A (Agilent P/N G4411B), 105A (Agilent P/N G4412A) or 44B (Agilent P/N G4410B (discontinued); current product P/N G4426B AMADID 14950)—following Agilent's standard aCGH microarray processing recommendations.

### Microarray processing and data analysis

Agilent microarray scanning and image analysis were conducted according to the aCGH protocol above. Microarrays were scanned using an Agilent DNA Microarray Scanner (Agilent P/N G2565BA) with Agilent Scanner Control Software (version 7.0). Agilent's Feature Extraction software (version 9.5) was used to extract data from raw microarray image files in preparation for analysis. Agilent CGH Analytics software (version 3.4) was used to visualize, detect and analyze aberration patterns from aCGH microarray profiles.

### Quantitative PCR methods

To confirm microarray results, exons 2, 3 and 26 within the BRCA2 gene were profiled via SYBR Green quantitative PCR (qPCR). Samples were prepared using Sigma's SYBR Green JumpStart Taq ReadyMix. Data was generated using the Stratagene Mx3000P QPCR System. All reactions were run with equivalent mass to ensure that relative copy number differences could be analyzed via C(t) values.

## Results

### 1. Input DNA titration

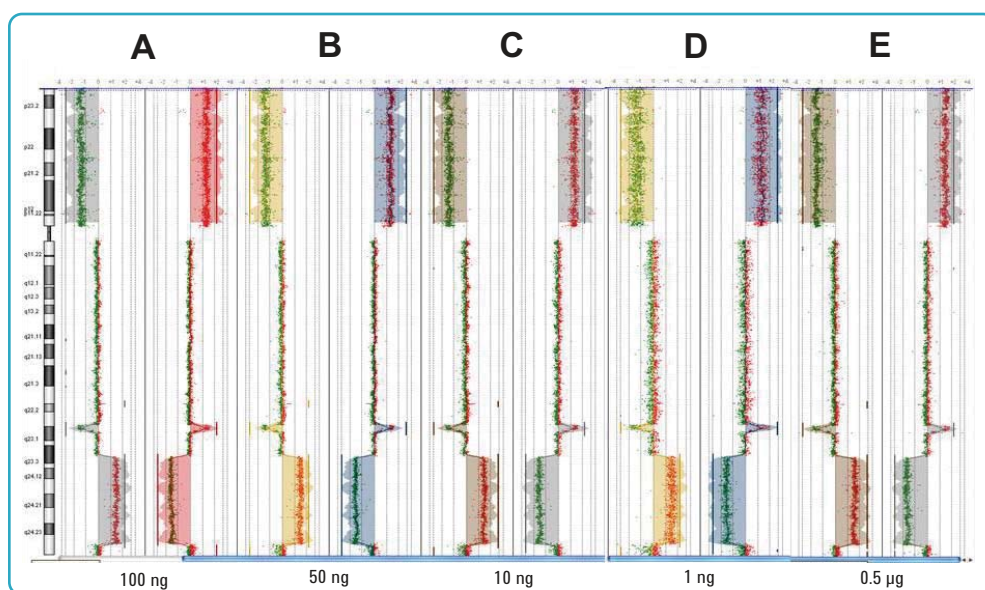
Laser capture microdissected (LCM) cells and needle biopsies often yield limited quantities of genomic DNA (gDNA) making aCGH analysis challenging. Agilent's human genome oligo-based aCGH microarrays were analyzed using GenomePlex WGA and limiting amounts of starting template. One, 10, 50 or

100 ng of normal pooled female gDNA and human colon carcinoma cell line HT29 gDNA were amplified using Sigma's GenomePlex kit. The amplification yields for all inputs were in the range of ~5-10  $\mu$ g. DNA size distributions were between 200 and 5000 bp as measured by 1.2% agarose gel analysis, although the size distribution for WGA product from reactions with 1 ng input was shifted slightly lower (between 200 and 1000 bp) (data not shown).

The human colon carcinoma cell line HT29 amplified DNA was hybridized against the normal pooled female amplified DNA on Agilent Human Genome CGH 2x105K Microarrays (**Figure 1**).

Performance was evaluated using the following metrics:

Derivative Log Ratio Standard Deviation (DLRSD), Signal-To-Noise Ratio (SNR), and Background Noise (BGNoise). These results were comparable to un-amplified gDNA (**Table 1**). While still within the acceptable range, the DLRSD value for the 1 ng input was greater than for other inputs. Chromosomal aberrations could be consistently detected across this wide range of input DNA masses as shown by the low probe-to-probe log ratio noise values. Agilent CGH Analytics software was used to analyze aberration patterns in human colon carcinoma HT29 gDNA amplified across the range of inputs (**Figure 1**). All known aberrations including a deletion across the 8p arm, an amplification of the 8q arm, and a focal deletion



**Figure 1. Agilent aCGH Analytics ideograms illustrating equivalent results when starting with 1 ng to 100 ng of input gDNA.** Varying input gDNAs amplified by GenomePlex were analyzed on Agilent Human Genome CGH 105A microarrays. Dye swap plots are presented side-by-side in each panel (A-E). Polarity +1 (Cy5-HT29/Cy3-Female) is shown on the left and polarity -1 (Cy5-Female/Cy3-HT29) is shown on the right. These aCGH plots compare GenomePlex amplified normal female gDNA vs. human colon carcinoma cell line HT29 gDNA having a known deletion across the 8p arm, an amplification of the 8q arm in the 8q23.3-24.33 region, and a focal deletion in 8q23.1. **A:** amplified from 100 ng input, **B:** amplified from 50 ng input, **C:** amplified from 10 ng input, **D:** amplified from 1 ng input, **E:** un-amplified 0.5  $\mu$ g input.

	<i>DLRSD</i>	<i>SNR-Cy3</i>	<i>SNR-Cy5</i>	<i>BG Noise-Cy3</i>	<i>BG Noise-Cy5</i>
Unamplified (0.5 $\mu$ g input)	0.14 +/- 0.002	155 +/- 1	190 +/-1	2.0 +/-0.2	3.1 +/- 0.16
Amplified (100 ng input)	0.11 +/-0.0002	137 +/-2	192 +/-8	1.9 +/-0.2	2.6 +/- 0.2
Amplified (50 ng input)	0.11 +/-0.002	118 +/-25	190 +/-6	2.4 +/-0.6	2.9 +/- 0.29
Amplified (10 ng input)	0.14 +/-0.003	94 +/-22	202 +/-2	2.4 +/-0.9	2.3 +/- 0.1
Amplified (1 ng input)	0.24 +/-0.012	142 +/-11	221 +/-9	1.7 +/- 0.01	2.2 +/- 0.07

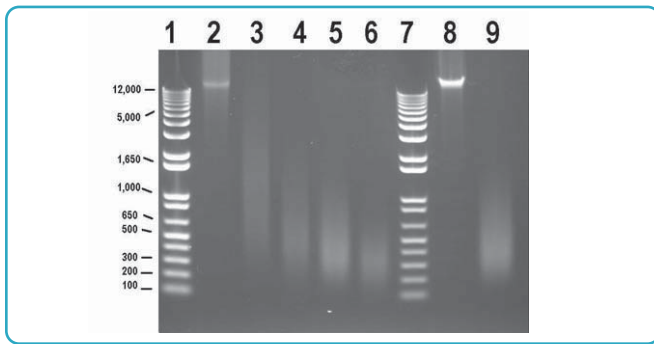
**Table 1. Agilent aCGH Microarray Performance Metrics: DNA Input Titration experiment.** See Appendix for further explanation.

in 8q23.1 were reliably detected in chromosome views for all input levels. These data indicate that amplification integrity is preserved across a wide range of inputs and that GenomePlex amplification from as little as 10 ng of starting genomic DNA can generate consistent aCGH results.

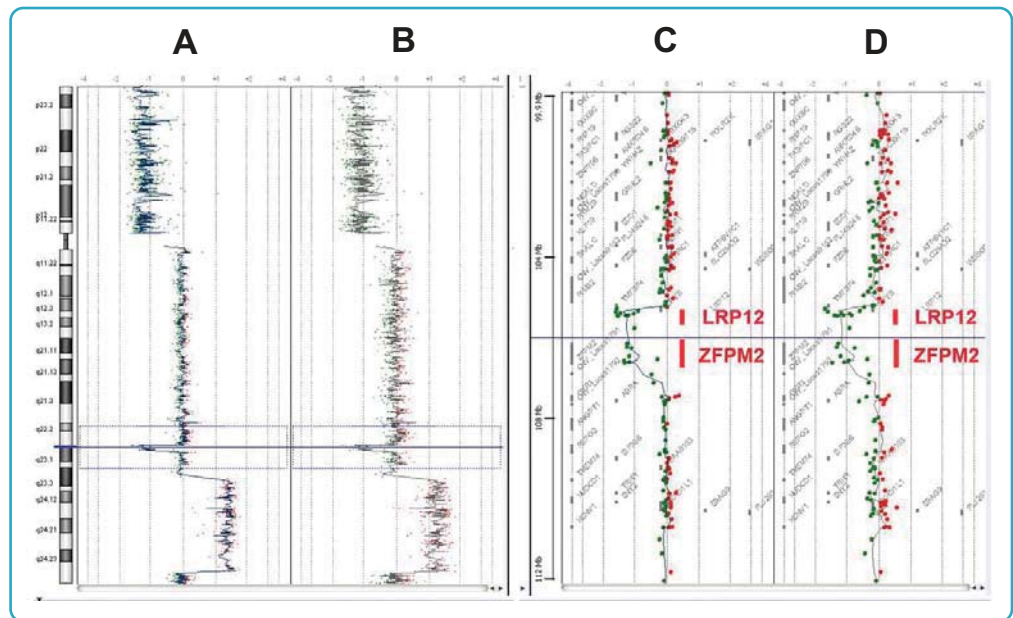
**II. Whole genome amplification of fragmented DNA evaluated by aCGH**

Researchers often only have degraded genomic DNA for aCGH analysis. The purpose of the following experiment was to

determine if amplified fragmented DNAs would yield satisfactory aCGH results using Agilent human genome aCGH microarrays. Intact normal pooled female gDNA and human colon carcinoma cell line HT29 gDNA were sonicated for different lengths of time. Genomic DNA size integrity distributions were determined by agarose gel analysis (**Figure 2**). WGA by GenomePlex was performed using both intact and fragmented HT29 gDNA. Agilent aCGH results are shown in **Figure 3**, revealing all well-known HT29 aberrations on chromosome 8. Furthermore, Agilent microarray performance quality metric



**Figure 2. 1.2% Agarose gel electrophoreses analysis of fragmented gDNA.** DNA fragment size was determined via gel electrophoresis prior to amplification with GenomePlex WGA. Both human female genomic DNA and HT29 genomic DNA yield high molecular weight intact bands (>12,000 bp), whereas the gDNA sonicated intermittently for 5-120 sec yield multiple smaller DNA bands with varying sizes. **Lanes 1 & 7:** Molecular Weight Marker; **Lane 2:** Intact Female gDNA (>12,000 bp); **Lane 3:** Female gDNA sonicated 5 sec (~1,500 bp); **Lane 4:** Female gDNA sonicated 30 sec (~600 bp); **Lane 5:** Female gDNA sonicated 90 sec (~400 bp); **Lane 6:** Female gDNA sonicated 120 sec (~300 bp); **Lane 8:** intact HT29 gDNA (>12,000 bp). **Lane 9:** HT29 gDNA sonicated 90 sec (~400 bp). For aCGH analysis, intact female gDNA (Lane 1) and female gDNA sonicated 90 sec (Lane 5), as well as intact HT29 gDNA (Lane 8) and HT29 gDNA sonicated 90 sec (Lane 9) were used.



**Figure 3. GenomePlex WGA enables amplification of DNA below 1kb with results equivalent to intact gDNA on Agilent human genome aCGH 44B microarrays.** Agilent CGH Analytics chromosome 8 ideograms illustrating data from GenomePlex WGA using both intact and fragmented human colon carcinoma gDNA HT29. GenomePlex WGA product generated from intact HT29 gDNA (**A**) and fragmented HT29 gDNA (**B**) revealed identical known deletions across the 8p arm, amplification along the 8q arm in the 8q23.3-24.23 region, and a focal deletion in 8q23.1 (outlined by dotted blue box). Corresponding zoomed-in gene views (12MB) for data from both intact gDNA (**C**) and fragmented DNA (**D**) focusing on Chr8 q22.2-23.1; distinctly show a ~1.5 MB deletion involving the zinc finger protein ZFP281 and tumor suppressor LRP12 that have been previously reported in BAC array-based analyses (Garnis, C, *et al.* 2004).

values were very similar between intact gDNA and fragmented gDNA (**Table 2**). The results suggest that Agilent microarrays can generate results comparable to those obtained with high molecular weight intact genomic DNA, using amplified sheared gDNA with an average size of at least 400 bp.

### III. Comparison of whole genome amplification methods on fragmented DNA: GenomePlex versus REPLI-g

The high strand displacement, processivity, and requirement of kilobase length template in the REPLI-g method often leads to

unsuccessful amplification of fragmented gDNA samples.

In this experiment, we compared the performance of the MDA-based REPLI-g kit with the WGA GenomePlex kit when using fragmented DNA as input material. Genomic DNA from the human colon carcinoma HT29 cell line was used as a source for intact gDNA, and sonicated gDNA from the HT29 cell line was used as a source for fragmented DNA. 50 ng of starting material input DNA was used for both amplification methods. As shown in **Figure 4** and **Table 3**, MDA by REPLI-g of

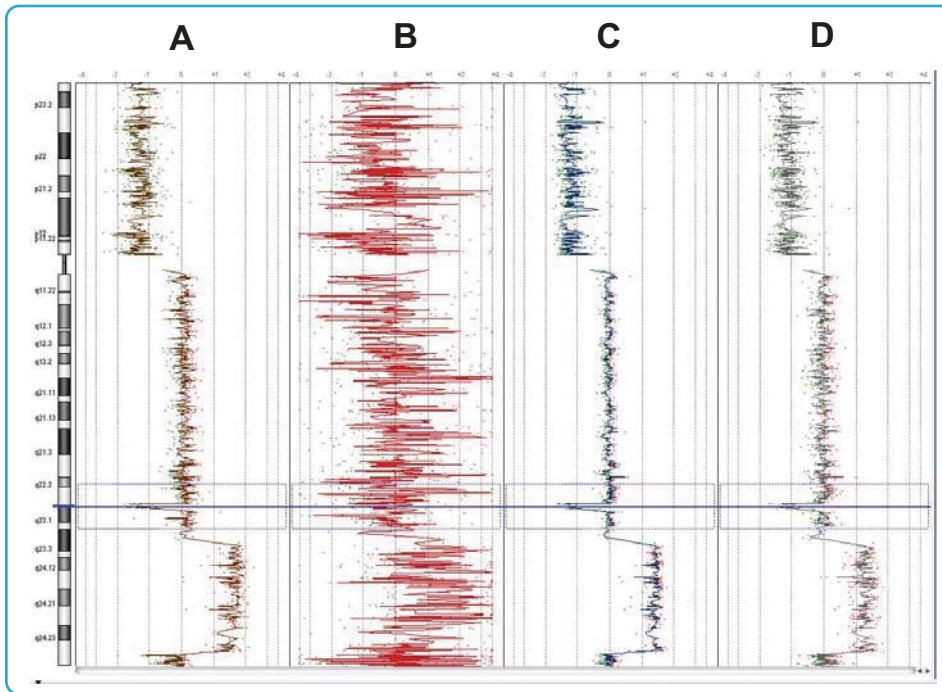
	<i>DLRSD</i>	<i>SNR-Cy3</i>	<i>SNR-Cy5</i>	<i>BG Noise-Cy3</i>	<i>BG Noise-Cy5</i>	<i>Response<sup>1</sup></i>
Intact HT29/Female	0.13 +/- 0.004	68 +/- 3	87 +/- 10	1.8 +/- 0.08	2.5 +/- 0.4	NA
Sonicated (90s) HT29/Female	0.22 +/- 0.012	49 +/- 4	77 +/- 10	2.1 +/- 0.1	2.4 +/- 0.13	NA
Intact Male/Female	0.12 +/- 0.0002	65 +/- 4	96 +/- 4	1.9 +/- 0.14	2.3 +/- 0.2	0.83 +/- 0.02 <sup>#</sup>
Sonicated (90s) Male/Female	0.21 +/- 0.007	53 +/- 5	86 +/- 3	1.9 +/- 0.008	2.0 +/- 0.13	0.73 +/- 0.003

<sup>1</sup>Applies to male versus female comparison only. NA, not applicable. <sup>#</sup>The response is slightly compressed in amplified samples.

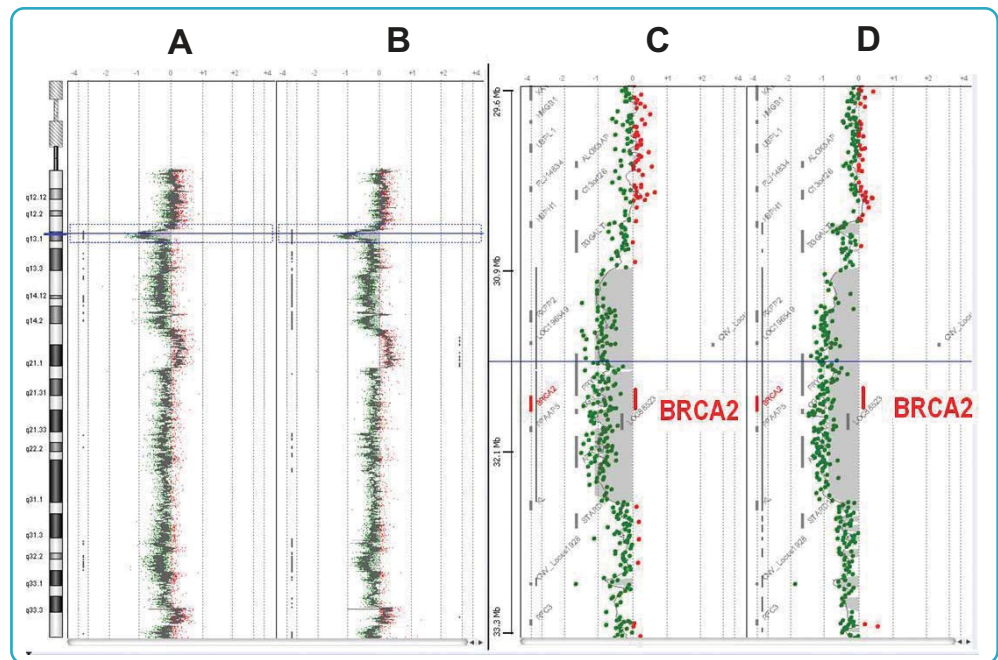
**Table 2. Agilent aCGH Microarray Performance Metrics: DNA Fragmentation Experiment.** See Appendix for further explanation.

<i>HT29/Female</i>	<i>Method</i>	<i>DLRSD</i>	<i>SNR-Cy3</i>	<i>SNR-Cy5</i>	<i>BG Noise-Cy3</i>	<i>BG Noise-Cy5</i>
Intact	GenomePlex	0.13 +/- 0.004	68 +/- 2.5	87 +/- 10.7	1.8 +/- 0.08	2.5 +/- 0.39
	REPLI-g	0.23 +/- 0.0001	70 +/- 10.8	105 +/- 1.04	2.5 +/- 0.35	2.8 +/- 0.06
Sonicated (90s)	GenomePlex	0.22 +/- 0.012	49 +/- 4.0	77 +/- 10.3	2.1 +/- 0.10	2.4 +/- 0.13
	REPLI-g	1.48 +/- 0.014	4 0 +/- 23	13 +/- 1.9	1.7 +/- 0.30	2.0 +/- 0.12

**Table 3. Array Performance Metrics: WGA by GenomePlex and REPLI-g Using Intact and Fragmented DNA.** See Appendix for further explanation.



**Figure 4. Comparison of REPLI-g vs. GenomePlex.** CGH Analytics views of Agilent 44K analyses of human colon carcinoma cell line HT29 DNA hybridized against female gDNA. REPLI-g (A-B) and GenomePlex (C-D) were used for target amplification, respectively. **A:** Intact gDNA HT29/female amplified by REPLI-g. **B:** Fragmented gDNA HT29/female amplified by REPLI-g. **C:** Intact gDNA HT29/female amplified by GenomePlex. **D:** Fragmented DNA HT29/female amplified by GenomePlex. For all the amplification reactions, 50 ng of gDNA was used as starting material.



**Figure 5. Identification of a focal deletion with Agilent human genome aCGH 244K microarrays using GenomePlex amplified DNA.** Array CGH data generated by GenomePlex amplified DNA was compared to that of non-WGA DNA. Scatter plots produced from 244K microarray analysis reveal a focal deletion in the p13.1 region of chromosome 13 (outlined by dotted blue box) in both the amplified (A) and the un-amplified (B) samples. Corresponding zoomed-in gene view (C= amplified, D= un-amplified) which focuses on a 3.7 MB window within 13p13.1 containing the focal deletion. A deletion involving breast cancer gene BRCA2 (red bar) was identified in both the WGA product and un-amplified genomic DNA generated from breast tumor DNA.

fragmented DNA produced very high probe-to-probe log-ratio noise (DLRSD) and as a result failed to identify the known aberrations using Agilent 44K aCGH arrays and CGH Analytics software. In contrast, WGA by GenomePlex detected all the known aberrations in both intact and fragmented HT29 DNA (**Figure 4C-D**). The DLRSD of the fragmented DNA amplified by GenomePlex was about 0.22, an approximately 7-fold lower noise level than that of REPLI-g (1.48). The signal intensities obtained with fragmented DNA amplified by REPLI-g were very low; the Signal-to-Noise ratio of the fragmented DNA amplified by GenomePlex was 40-70, which was 5-10 fold higher than that of REPLI-g (**Table 3**). As expected, results from intact genomic DNAs amplified by both GenomePlex and REPLI-g were comparable (**Figure 4A vs. C**)(**Table 3**) and consistent with data obtained from non-WGA DNA (data not shown). This result indicates that GenomePlex WGA generates robust and precise values on Agilent aCGH microarrays using both intact and fragmented gDNAs.

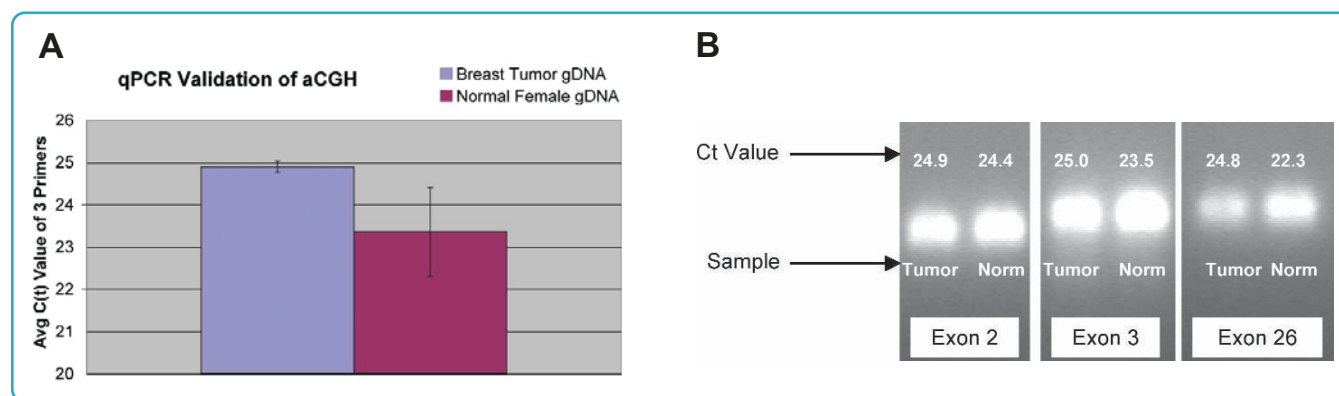
#### IV. Validation of a focal deletion in GenomePlex amplified tumor DNA on Agilent aCGH microarrays using the Stratagene Mx3000P QPCR System

Using the GenomePlex WGA method, we identified a focal deletion in a breast tumor sample on Agilent 244K aCGH microarrays (**Figure 5**). The GenomePlex WGA data was

compared with the un-amplified non-WGA data (**Figure 5**). The data was confirmed by gene-specific qPCR analysis using the Stratagene Mx3000P QPCR System (**Figure 6**). The average of three SYBR Green quantitative PCR primer sets targeting exons 2, 3 and 26 of the BRCA2 gene (Tavtigian, S.V. *et al.*) demonstrated a 1.54 cycle difference between breast tumor gDNA and normal female gDNA (**Figure 6A**). This 2.9 fold ( $2^{1.54}$ ) drop in representation of the BRCA2 gene from the tumor sample compared to the normal sample validates the 13 p13.1 focal heterozygous deletion (**Figure 5C-D**; **Figure 6B**).

#### Conclusion

Agilent's two-color aCGH microarrays can be used successfully with both small quantities of intact DNA (as little as 10 ng) or with fragmented DNA (average size of at least 400 bp) to produce high quality aCGH data. This new amplification method creates an opportunity to use samples from applications such as LCM and needle biopsies, which generally yield less than a microgram of DNA. The GenomePlex whole genome amplification protocol and the Stratagene Mx3000P QPCR System integrate seamlessly into the Agilent aCGH workflow.



**Figure 6. Validation of aCGH breast tumor data using BRCA2 gene-specific quantitative PCR and the Stratagene Mx3000P QPCR System.** **A:** The average of three SYBR Green quantitative PCR primer sets targeting exons 2, 3 and 26 of the BRCA2 gene are shown. Average of Cycle threshold (Ct) values by three primers are shown. Blue bar, breast tumor gDNA. Purple bar, normal female gDNA. **B:** PCR reaction products were analyzed by gel electrophoresis. Tumor: breast tumor PCR DNA. Norm: normal female PCR DNA.

## Appendix

### Array Performance/QC Metrics (CGH Analytics 3.4 software QC metrics)

DLRSD	Derivative Log <sub>2</sub> Ratio Standard Deviation	Standard deviation of the log ratio differences between consecutive probes divided by sqrt(2). This metric calculates probe-to-probe log ratio noise of an array.	Excellent: <0.2 Good: 0.2-0.3 Poor: >0.3
BGNoise	Background Noise	This metric is calculated as the standard deviation of negative control probes after rejecting feature non-uniform outliers, saturated features, and feature population outliers	Excellent: <5 Good: 5-10 Poor: >10
SNR	Signal to Noise Ratio	This metric is calculated as the signal intensity divided by BGNoise.	Excellent: >100 Good: 30-100 Poor: <30
Response <sup>1</sup>	Log Ratio Response	Absolute value of median Log <sub>2</sub> ratio of X-specific probes	Typical good scores: >0.8

<sup>1</sup>For male versus female comparison only.

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