DNA Quality Control for Oligonucleotide Array CGH (aCGH) with the Agilent 2100 Bioanalyzer

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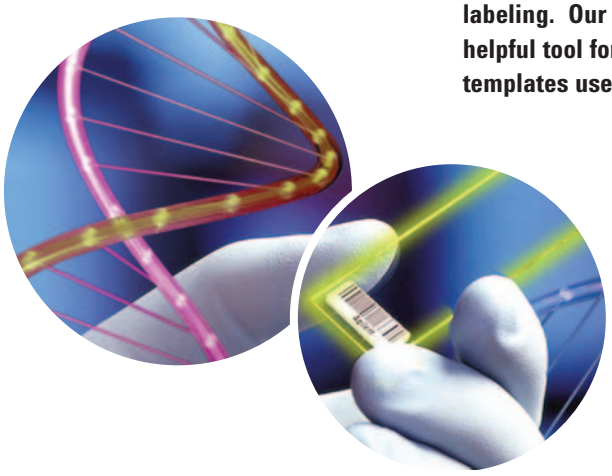
Abstract

Comparative genomic hybridization (CGH) measures copy number variations at multiple loci simultaneously, providing an important tool for studying cancer and developmental disorders, and for developing diagnostic and therapeutic targets. We have recently developed an oligonucleotide microarray platform for microarray-based comparative genomic hybridization (aCGH) analyses that can detect and map copy number alterations in the human genome, including single copy losses, gene specific homozygous deletions, and amplicons of varying sizes.

Robust application of this technology for the study of human disease requires adequate quality control of DNA sample preparation prior to aCGH hybridization. The Agilent 2100 bioanalyzer and associated RNA assays are now established industry standards for measuring the integrity of RNA samples. In addition to RNA sample analyses, the bioanalyzer has on-chip electrophoresis capabilities for DNA analysis. We investigated the use of the bioanalyzer for monitoring critical steps in the workflow of aCGH experiments including whole genome DNA amplification, digestion of template and sample labeling. Our results demonstrate that the bioanalyzer can be a helpful tool for monitoring the quality and quantity of DNA templates used in aCGH experiments.



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Introduction

Comparative genomic hybridization is a technique that allows for the detection of DNA sequence copy number aberrations throughout the genome. We have recently shown that microarrays containing 60mer oligonucleotide probes designed for CGH measurements provide a robust and sensitive platform for detecting chromosomal alterations throughout a genome using high complexity total genomic DNA samples [(1)]. In addition, we have developed protocols using the highly processive DNA polymerase, phi29, to prepare aCGH templates from small samples that yield high quality aCGH measurements, comparable to those derived from unamplified total genomic DNA. The use of high complexity samples, either genomic DNA or template amplified with phi29 DNA polymerase, in conjunction with 60mer oligonucleotide microarrays provides researchers the flexibility to directly study copy number variations in essentially any region of the genome with a single simplified sample preparation.

The Agilent 2100 bioanalyzer has become the industry standard for the quality control of RNA for microarray-based gene expression profiling studies combining sample separation, detection, and analysis all in one step using an automated platform [(2)]. Similar to expression profiling, current protocols for aCGH contain multiple steps for sample preparation and labeling that can benefit from QC procedures to help ensure successful aCGH assays. Specifically we show that the bioanalyzer can be used to monitor phi29 based-amplification of total genomic DNA, restriction enzyme digestion, and Cyanine-3/Cyanine-5 dye labeling.

The current technique of agarose gel analysis for the QC of DNA is limited by its need for relatively large amounts of sample, poor resolution of genomic materials, lack of automation, and exposure to hazardous materials such as ethidium bromide. Furthermore, we demonstrate that agarose gel analysis for the QC of phi29 amplified DNA, fails to provide important information regarding the success of the amplification. Therefore, we are developing Agilent bioanalyzer assays to monitor the quality of DNA samples used in aCGH experiments.

Experimental Design

The oligonucleotide aCGH experiment outlined in this document was designed to examine DNA products from phi29 amplification reactions with varying amounts of input DNA to simulate sub-optimal or failed reactions. Genomic DNA was used as input for the phi29 amplification reactions in amounts ranging from 50ng to 0.5pg. The phi29 amplified samples were digested with restriction enzymes, Cyanine-3 or Cyanine-5 labeled and hybridized to CGH oligonucleotide microarrays. HCT116, a colon cancer cell line derived from a male (XY) patient, was selected for the experimental sample because its genomic aberrations are well characterized in the literature and it retains an intact copy of the X chromosome. Hybridizations with a female (XX) reference allowed us to monitor our ability to detect a single copy deletion of the X chromosome using phi29 amplified DNA from reactions with varying sample input, as well as monitor the ability to detect other known aberrations on the autosomes.

Materials and methods

Bioanalyzer and gel electrophoresis analysis of phi29 amplified DNA
HCT116 (ATCC catalog #CCL-247) cells were grown in culture under conditions recommended by the supplier and genomic DNA was isolated using the DNeasy Tissue kit (Qiagen catalog #69504), as per the manufacturer's recommendations. Normal female (XX) DNA was purchased from Promega (catalog #G1521). A dilution series was prepared for XX and HCT116 genomic DNA consisting of serial 10-fold dilutions in H2O from 50ng/µl to 0.5pg/µl for a total of six samples. Initial sample concentrations were verified using a Nanodrop™ Spectrophotometer. 1µl of each of the diluted samples (50ng, 5ng, 0.5ng, 50pg, 5pg & 0.5pg) was amplified using the RepliG phi29 amplification kit (Qiagen catalog #59045) according to the manufacturer's instructions. A single amplification was done for each of the XX dilution samples and duplicate amplifications were done for the HCT116 dilution samples. Duplicate amplification reactions in the absence of template DNA were performed in which 1µl of DNA was replaced with H_2O. For bioanalyzer analysis, it was necessary to remove the phi29 reaction buffer from the samples. A 5µl aliquot of each of the 50µl amplification reactions was purified using QIAquick columns (Qiagen catalog #28104) and eluted as per the manufacturer's instructions. The purified products were then concentrated under vacuum for 15 minutes at 35ºC to achieve a sample concentration ≥200ng/µl as determined by a Nanodrop™ Spectrophotometer. In preparation for bioanalyzer analysis, samples were heat denatured for 3 minutes in a 100ºC water bath and placed on ice. Immediately following denaturation, an RNA 6000 Nano LabChip (Agilent catalog #5065-4476) was prepared as described in the user manual. 200ng of the purified denatured amplified genomic DNA samples were loaded into each well of the chip along with the Ambion RNA 6000 Ladder (catalog #7152). Samples were analyzed using the mRNA Nano software script, as per the manufacturer's instructions. For comparison, 600ng of the purified amplified material was analyzed by agarose gel electrophoresis on a 0.8% TBE agarose gel run for 1.5 hours at 100 volts.

Bioanalyzer analysis of restriction enzyme digested DNA
The remaining 45µl of unpurified phi29 amplified material from the titration series was digested using 50 Units of AluI (Promega

catalog #R6281) and 50 Units of RsaI (Promega catalog #R6371) in a 100µl volume with 10µl 10X Promega Buffer C. Digestions were carried out for 2 hours at 37°C. The digested samples were purified using QIAprep Spin Miniprep columns (Qiagen catalog #27106) and eluted as per the manufacturer's instructions. The samples were quantitated using a Nanodrop™ Spectrophotometer, and all the samples had similar yields, ~20-30µg, including the no template amplification. 200ng of each sample was analyzed using the Agilent 2100 bioanalyzer with the DNA 7500 LabChip kit and DNA 7500 Software Script as per the manufacturers instructions. For comparison, 600ng of each digested sample was analyzed by agarose gel electrophoresis on a 1.2% TBE agarose gel run for 1.5 hours at 100 volts.

Bioanalyzer analysis of Cyanine-3/Cyanine-5 labeled DNA

The AluI/RsaI digested genomic DNA samples were labeled using the BioPrime Array CGH Labeling kit (Invitrogen catalog #18095-012) according to the manufacturer's protocol, except 10µg of amplified DNA was used in each reaction instead of 4µg. Each XX amplification sample was labeled in duplicate using Cyanine-3-dUTP while the HCT116 cell line amplified DNA samples were labeled with Cyanine-5-dUTP. The two amplification reactions performed in the absence of DNA template were split and labeled with both Cyanine-3 and Cyanine-5. The appropriate experimental (Cyanine-5-HCT116) and reference (Cyanine-3-XX) samples were then combined and the Cyanine-3 and Cyanine-5 no template samples were also combined. The Cyanine-3/Cyanine-5 labeled samples were brought to 500µl with TE (10mM Tris pH 8.0/1mM EDTA) and purified using the Microcon YM-30 columns (Millipore catalog #42410). The 500µl samples were applied to the columns and centrifuged at 8000xg for 10 minutes. The flow through was discarded and an additional 450µl TE was added to the sample on the column and centrifuged at 8000xg for 10 minutes. The column was inverted into a new 1.5ml tube and centrifuged at 8000xg for 1 minute to elute the sample. Eluted samples were brought to a volume of 100µl in H_2O. 1µl of each Cyanine-3/Cyanine-5 labeled sample was analyzed on the bioanalyzer using the DNA 7500 LabChip Kit with the DNA 7500 software as per the manufacturer's instructions.

aCGH Assays

To the 14 purified Cyanine-3/Cyanine-5 labeled samples (7 input DNA concentrations in duplicate) the following hybridization blocking reagents were added: 50µg Cot-1 DNA (Invitrogen #15279-011), 100µg Yeast tRNA (Invitrogen #15401011), and 50µl 10X Control Targets (Agilent catalog #5185-5976). The volume was brought to 250µl with H_2O and 250µl 2X Hybridization Buffer (Agilent catalog #5185-5973) was added. The hybridization mixture was denatured at 100°C for 1.5 minutes in a water bath. Samples were immediately transferred to a 37°C water bath for 30

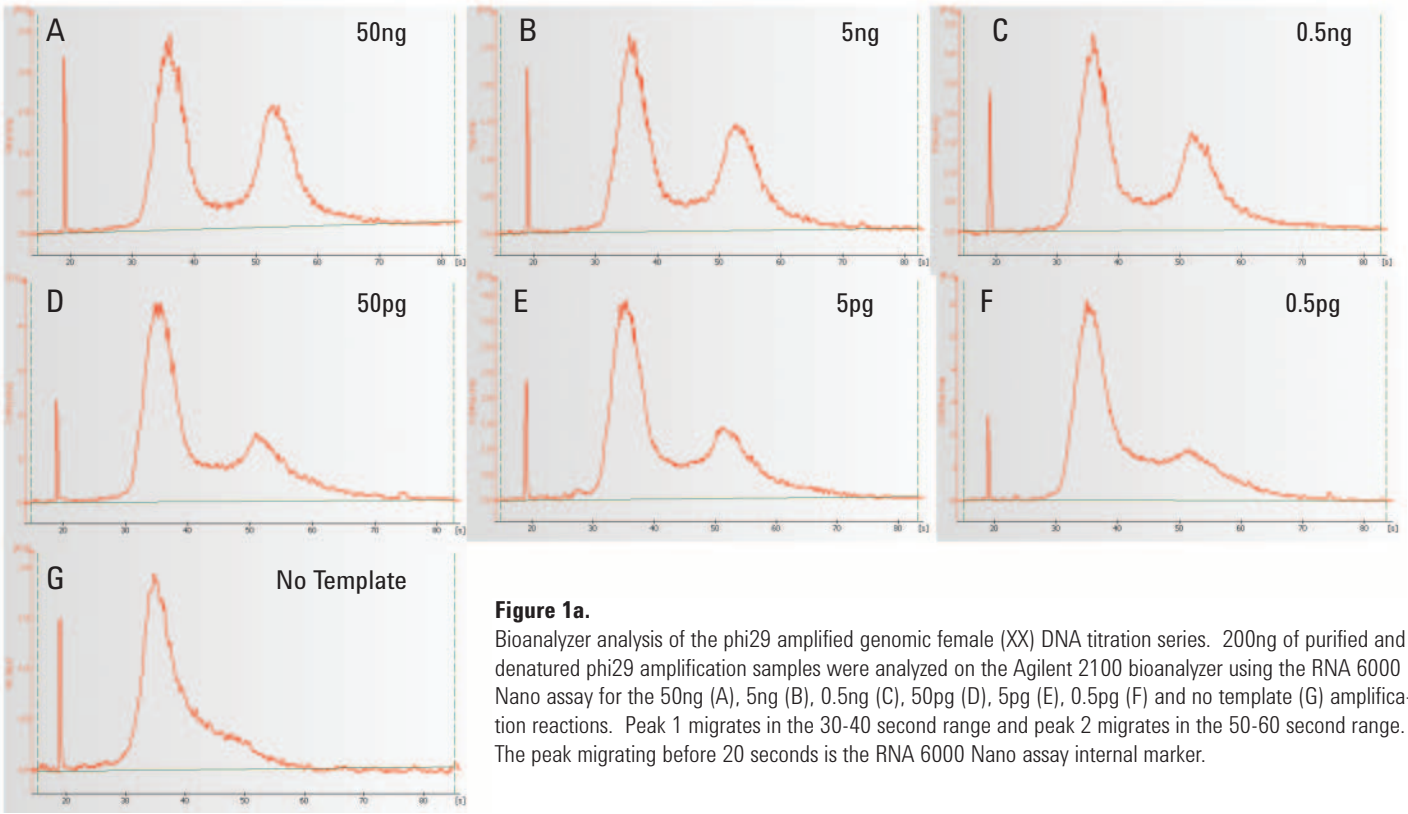

Figure 1a.
Bioanalyzer analysis of the phi29 amplified genomic female (XX) DNA titration series. 200ng of purified and denatured phi29 amplification samples were analyzed on the Agilent 2100 bioanalyzer using the RNA 6000 Nano assay for the 50ng (A), 5ng (B), 0.5ng (C), 50pg (D), 5pg (E), 0.5pg (F) and no template (G) amplification reactions. Peak 1 migrates in the 30-40 second range and peak 2 migrates in the 50-60 second range. The peak migrating before 20 seconds is the RNA 6000 Nano assay internal marker.

minutes to allow pre-annealing of the blocking agents to the labeled sample. Samples were centrifuged for 5 minutes at 16,000xg and immediately applied to Agilent's Human Genome CGH Microarrays (catalog #G4410A) as per the manufacturer's recommendations. Hybridizations were performed at 65°C for 17 hours. The microarrays were disassembled and washed according to Agilent's aCGH hybridization protocol (part # G4410-90010). Microarrays were immediately scanned in the Agilent DNA microarray scanner (catalog #G2565BA) using the default settings. Data was extracted using the Agilent Feature Extraction software 7.5.1 (catalog #G2567AA) using the default settings, except for the following modifications: 1) Background Subtraction, the average of negative control features was used and the spatial detrend option was turned off and 2) Dye normalization, only the linear option was selected.

Results and Discussion

Phi29 amplification analysis

Previous studies have shown that phi29 can be used to prepare templates with good genomic representation from limiting starting materials for aCGH measurements [1,3]. In order to simulate potential failure modes related to phi29 amplification, that we can evaluate on the bioanalyzer, we examined the correlation between aCGH hybridization results and bioanalyzer analysis results from a series of phi29 reactions with decreasing amounts of input DNA. Figure 1a shows the bioanalyzer traces of the XX amplified DNA titration series using the RNA 6000 Nano assay. In the 50ng DNA amplification reaction, there are two distinct peaks in the electropherogram. The first peak, which migrates in the 30-40 second range, is inferred to be nonspecific DNA because it correlates to the peak that is generated from the no DNA template sample. The second peak, which migrates in the 50-60 second range, is believed to be the amplified DNA that is biologically relevant and representative of the starting DNA input. As the amount of input DNA decreases, the first peak increases relative to the second peak. When the amplification was performed in the absence of DNA, only the first peak was observed. Similar results were observed in the bioanalyzer analysis of the duplicate HCT116 amplification titration reactions (data not shown). The RNA 6000 Nano kit was used for this analysis because the DNA LabChip kits were unable to resolve these two populations with the current DNA assay parameters. When these same amplified samples were analyzed using agarose gel electrophoresis, (figure 1b), there was no resolution of the two peaks, only a smear of high molecular weight products. As demonstrated from the analysis of the aCGH results from these samples (see below) the ability to resolve peak one and peak two is important because the ratio of these peaks correlates with the quality of the aCGH results.

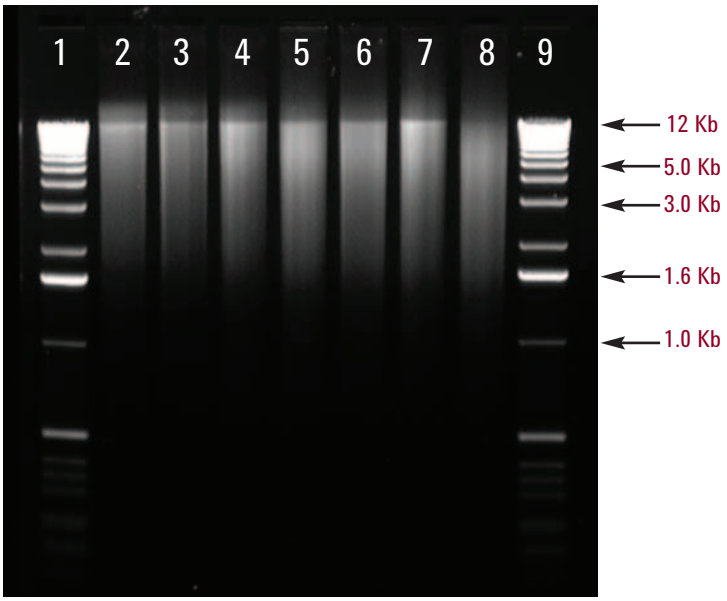

Figure 1b.
Agarose gel analysis of the phi29 amplified genomic female (XX) DNA titration series. 600ng of each sample was electrophoresed on a 0.8% TBE agarose gel and visualized with ethidium bromide staining. Lanes 2-8 contain products from the 50ng, 5ng, 0.5ng, 50pg, 5pg, 0.5pg and no template amplification reactions, respectively. Lanes 1 and 9 contain 1-Kb ladder DNA markers (Invitrogen catalog #15615-016).

DNA restriction enzyme digestion analysis

The digested XX DNA samples were analyzed using the DNA 7500 chip (figure 2a). No differences were observed in the bioanalyzer electropherograms of the titration series samples from 50ng to 5pg. However, in the 0.5pg and no template samples, distinct, but different fragmentation patterns appear. Similar results were observed with the HCT116 samples (data not shown). The appearance of the fragmentation pattern is variable and has been observed either earlier in the titration series, i.e. the 5pg sample, or only in the no template sample. Agarose gel electrophoresis analysis of the restriction enzyme digested samples (figure 2b) shows the same visual results as the bioanalyzer but at a lower resolution.

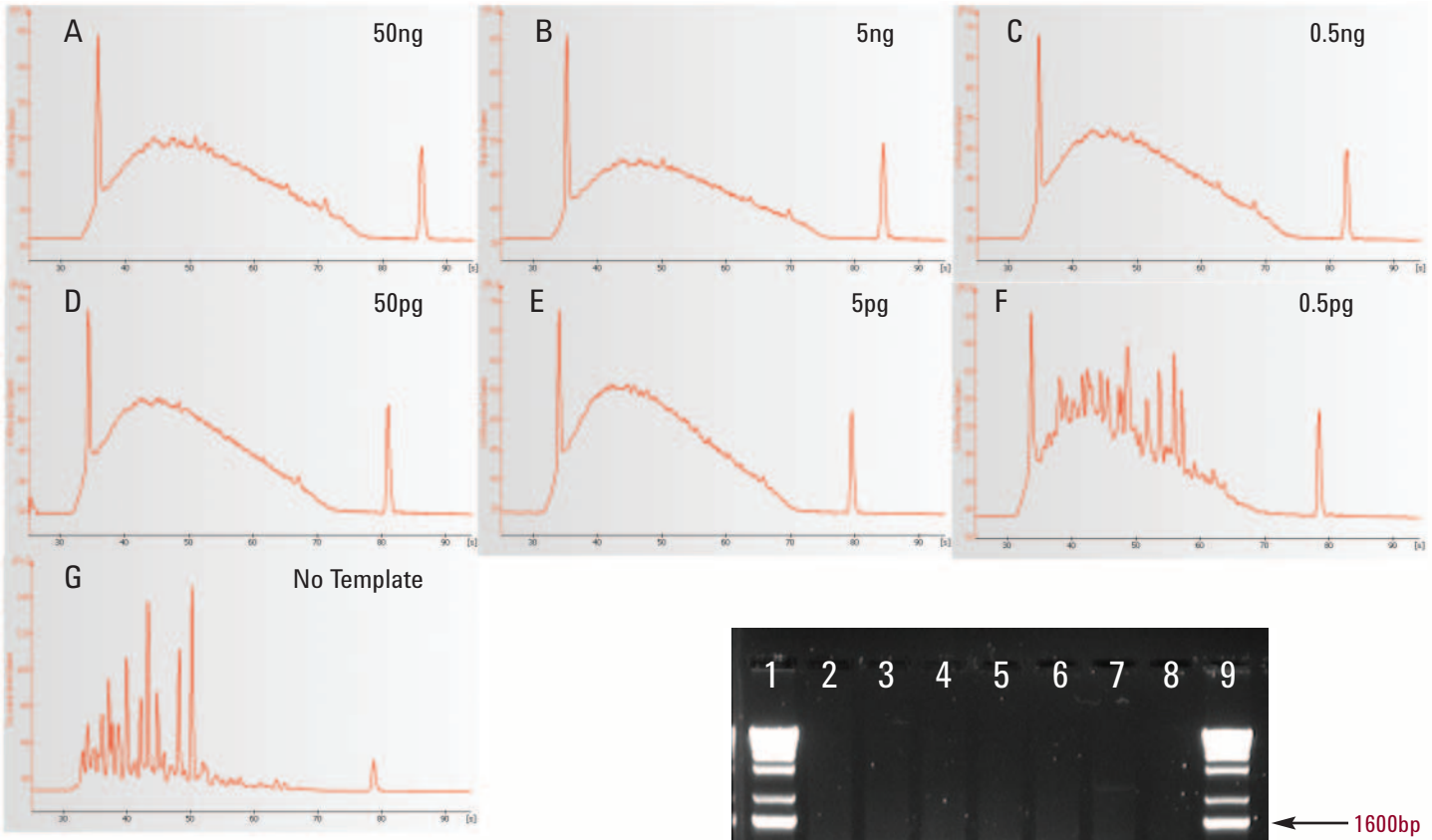

Figure 2a.
Bioanalyzer analysis of restriction enzyme digested phi29 amplified female (XX) DNA samples. 200ng of purified AluI/RsaI digestion samples were analyzed on the Agilent 2100 bioanalyzer using the DNA 7500 assay. The profiles for the 50ng, 5ng, 0.5ng, 50pg, 5pg, 0.5pg and no template reactions are shown in panels A-G, respectively. The peaks migrating at ~35 seconds and ~80 seconds are the DNA 7500 assay lower and upper internal markers, respectively.

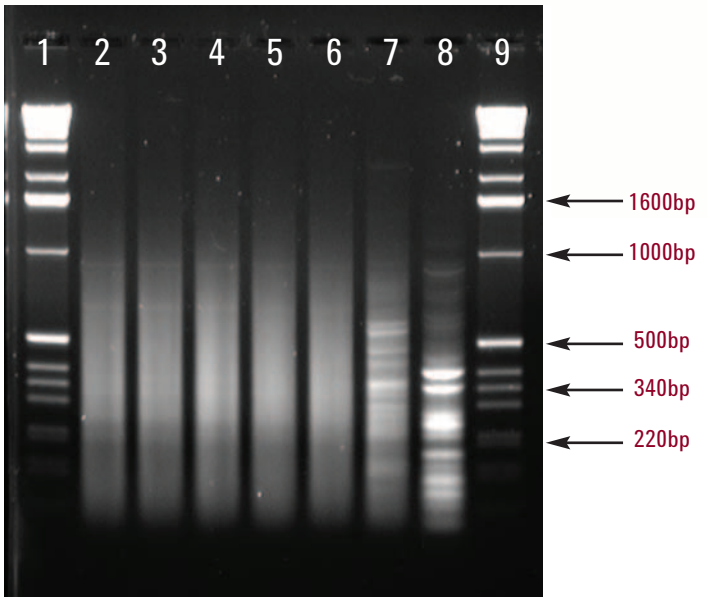

Figure 2b.
Agarose gel analysis of the restriction enzyme digested phi29 amplified female (XX) DNA. 600ng of each sample was electrophoresed on a 1.2% TBE agarose gel and visualized with ethidium bromide staining. Lanes 2-8 contain digested products from the 50ng, 5ng, 0.5ng, 50pg, 5pg, 0.5pg and no template amplification reactions, respectively. Lanes 1 and 9 contain 1Kb ladder DNA markers (Invitrogen catalog #15615-016).

Cyanine-3/Cyanine-5 labeling analysis

The bioanalyzer can also be used to monitor the combined Cyanine-3/Cyanine-5 labeled samples prior to hybridization. Because the bioanalyzer contains a 633 laser that can excite the Cyanine-5 dye but not the Cyanine-3 dye, the fluorescence is a measurement of both the Cyanine-5 dye and the dye in the assay matrix that binds to the DNA in the Cyanine-3/Cyanine-5 labeled samples. The bioanalyzer profiles of the Cyanine-3/Cyanine-5 labeled samples are shown in figure 3. The discrete fragmentation patterns that were observed in the 0.5pg and no template digestion sample profiles (figure 2a) are also present in the profiles from the labeled DNA, but the fragmentation patterns are different. We are currently developing quantitative bioanalyzer metrics to monitor the success of DNA labeling.

Analysis of DNA microarray results

We examined the correlation between three primary measures of the aCGH data quality and the bioanalyzer results. First, we examined the distributions of the log10 red and green background subtracted signals. Microarray data from samples amplified from 50ng, 0.5ng, 0.5pg, and no template starting material is shown in figure 4. Only the biological probes are shown in these plots, all control probes were removed. The 50ng plot shows a relatively tight distribution of signals for the autosome probes (blue) between the HCT116 and XX samples as would be expected from a high quality amplification and hybridization for samples that are both largely diploid (figure 4A). The chromosome X (ChrX) probes, shown in red, are easily separated from the autosome probes as expected, since two vs. one copies of the X chromosome are present in the XX and HCT116 samples, respectively. In the 0.5ng plot, the distribution between the samples widens and the separation between the autosome and ChrX probes diminishes (figure 4B). The 5pg plot shows no correlation between the probe signals for the XX and HCT116 samples and also shows a complete loss of discrimination between the autosome and ChrX probes (figure 4C). For the no template sample, the majority of the feature signals are below 10 counts and are not significantly distinguishable from background.

Second, we examined the impact of the amplification input amount on the ability to detect known genomic aberrations on chromosome 16 of HCT116 by plotting the ratio data along the

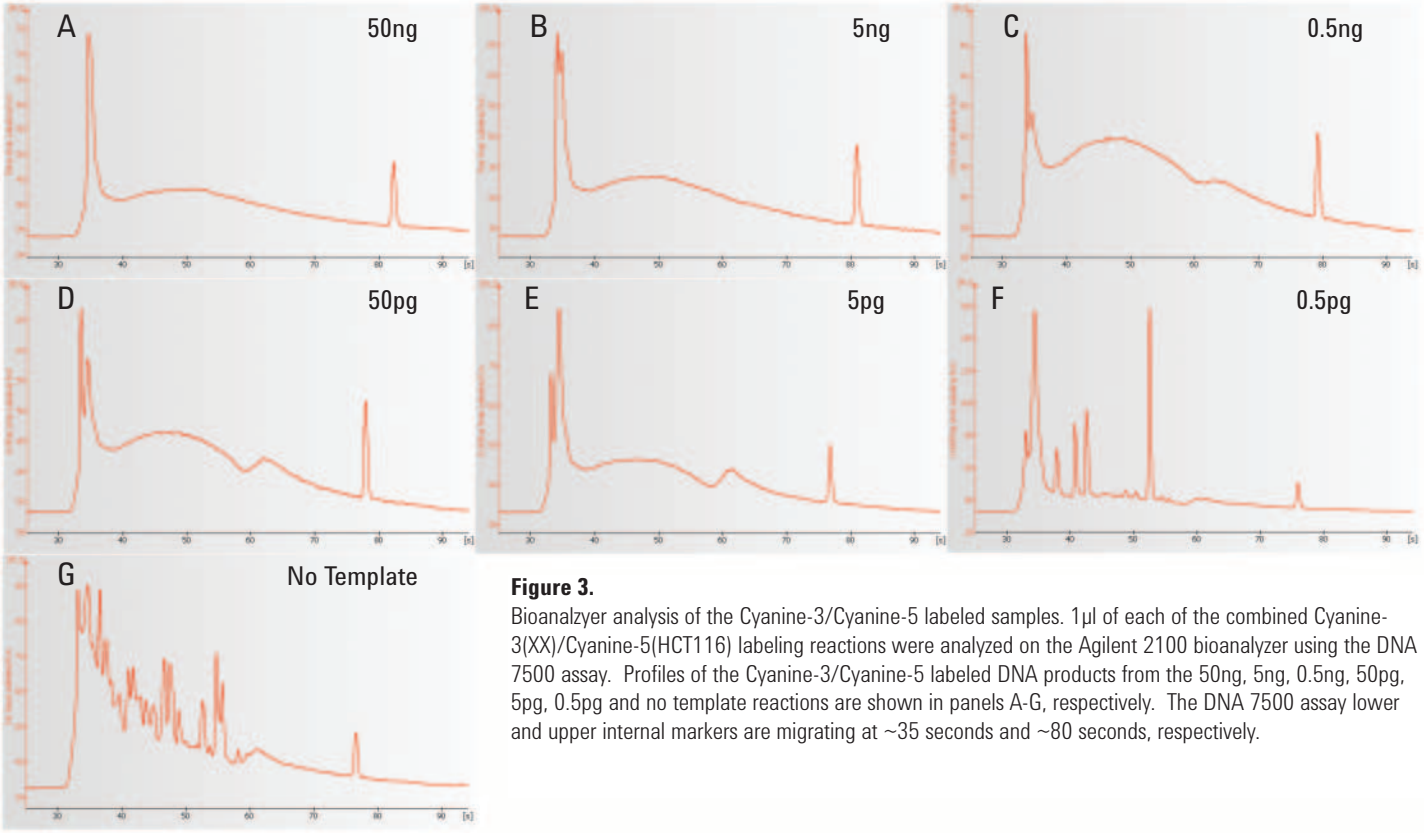

Figure 3.
Bioanalzyer analysis of the Cyanine-3/Cyanine-5 labeled samples. 1µl of each of the combined Cyanine-3(XX)/Cyanine-5(HCT116) labeling reactions were analyzed on the Agilent 2100 bioanalyzer using the DNA 7500 assay. Profiles of the Cyanine-3/Cyanine-5 labeled DNA products from the 50ng, 5ng, 0.5ng, 50pg, 5pg, 0.5pg and no template reactions are shown in panels A-G, respectively. The DNA 7500 assay lower and upper internal markers are migrating at ~35 seconds and ~80 seconds, respectively.

chromosome using Agilent's CGH Analytics software (catalog #G4175AA), (figure 5). The HCT116 cell line contains two homozygous deletions, one in the A2BP1 gene (16p) and one in the WWOX gene (16q), and a region of amplification on the distal portion of the q arm. All three of these aberrations were detectable in the 50ng, 5ng (data not shown) and 0.5ng samples, although the 5ng and 0.5ng samples show significantly more noise in the log ratio values, as is indicated by the greater spread in the diploid regions of chromosome 16. These aberrations were undetectable in hybridizations from the 50pg (data not shown), 5pg and lower DNA input amplifications and the scatter in the log ratio values was greatly increased. Similar results were seen on the other chromosomes with known aberrations (data not shown).

Interestingly, although known copy number aberrations were not detectable from phi29 amplified samples with less than 5ng input DNA, microarray hybridizations from these samples did show a large number of features with signals significantly above background. In the 50ng, 5ng and 0.5ng, amplifications, most of the BGSubsignals fall within the 10-1000 range (figure 4), the expected range for a good aCGH assay performed under current conditions. However, the 50pg (data not shown) and the 0.5pg amplifications have large numbers of probes with BGSubsignals both below 10 and above 1000. A significant increase in the signals from a number of probes, while a larger number of probes show decreased signal, is characteristic of the non-biological DNA which results from amplification reactions with too little input.

Third, as a simple measure of this characteristic low-input signal intensity pattern, we examined the ratio of the median to the mean gBGSubsignals for the XX reference channel for each microarray (figure 6). In a successful amplification, the mean signal intensity is typically 1.1 to 1.4 times the median signal intensity as is observed for the 50ng, 5ng and 0.5ng microarrays. We did observe in this experiment that both the median and mean signals increased with decreasing inputs for the 50ng-0.5ng DNA input range, however this effect was not seen in other experiments. The median/mean ratio decreased dramatically with decreasing input for the 50pg, 5pg and 0.5pg microarrays, consistent with the observation that although many of the feature signals are decreasing with decreasing input DNA, there are a significant number of features that have very high signals. For the no template microarray, both the median and mean signal values

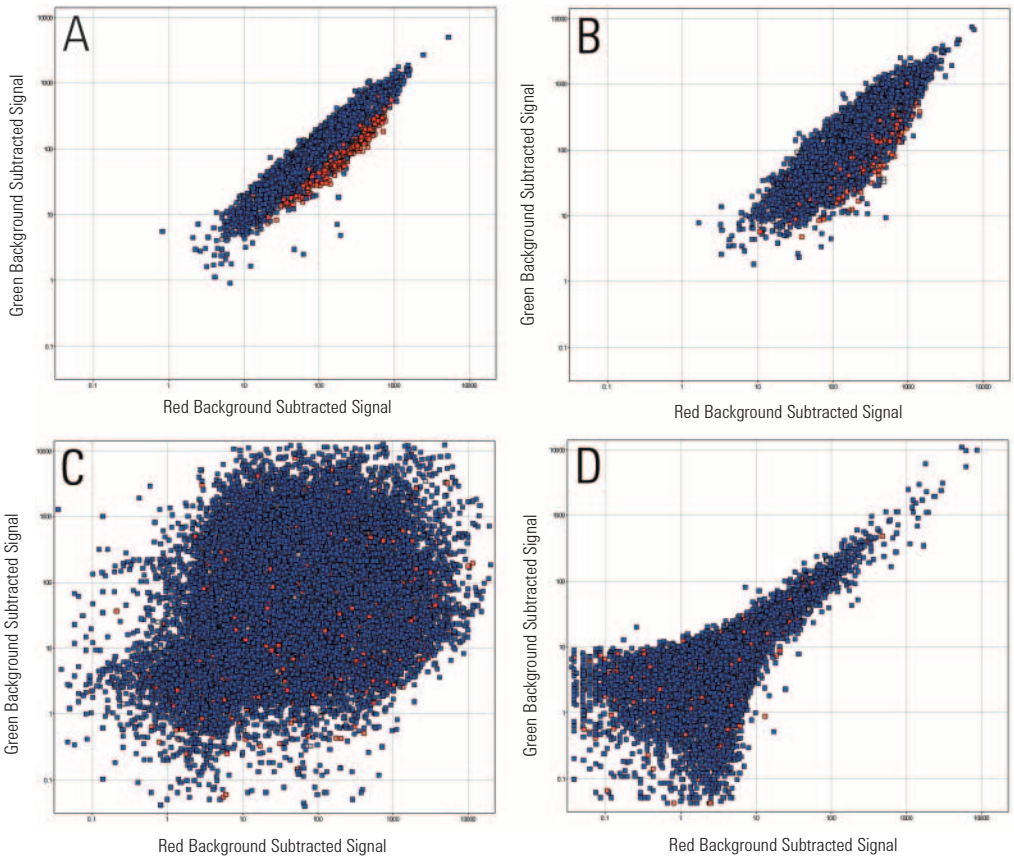

Figure 4.
Cyanine-3 vs. Cyanine-5 signal plots for aCGH assays. The log10 red (Cyanine-5-HCT116) background subtracted signals (rBGSubSignal) are plotted against the log10 green (Cyanine-3-XX) background subtracted signals (gBGSubSignal) for the 50ng (A), 0.5ng (B), 5pg (C) and no template (D) microarrays using Spotfire Decision Site software. The chromosome X (ChrX) probes are shown in red and autosome probes in blue.

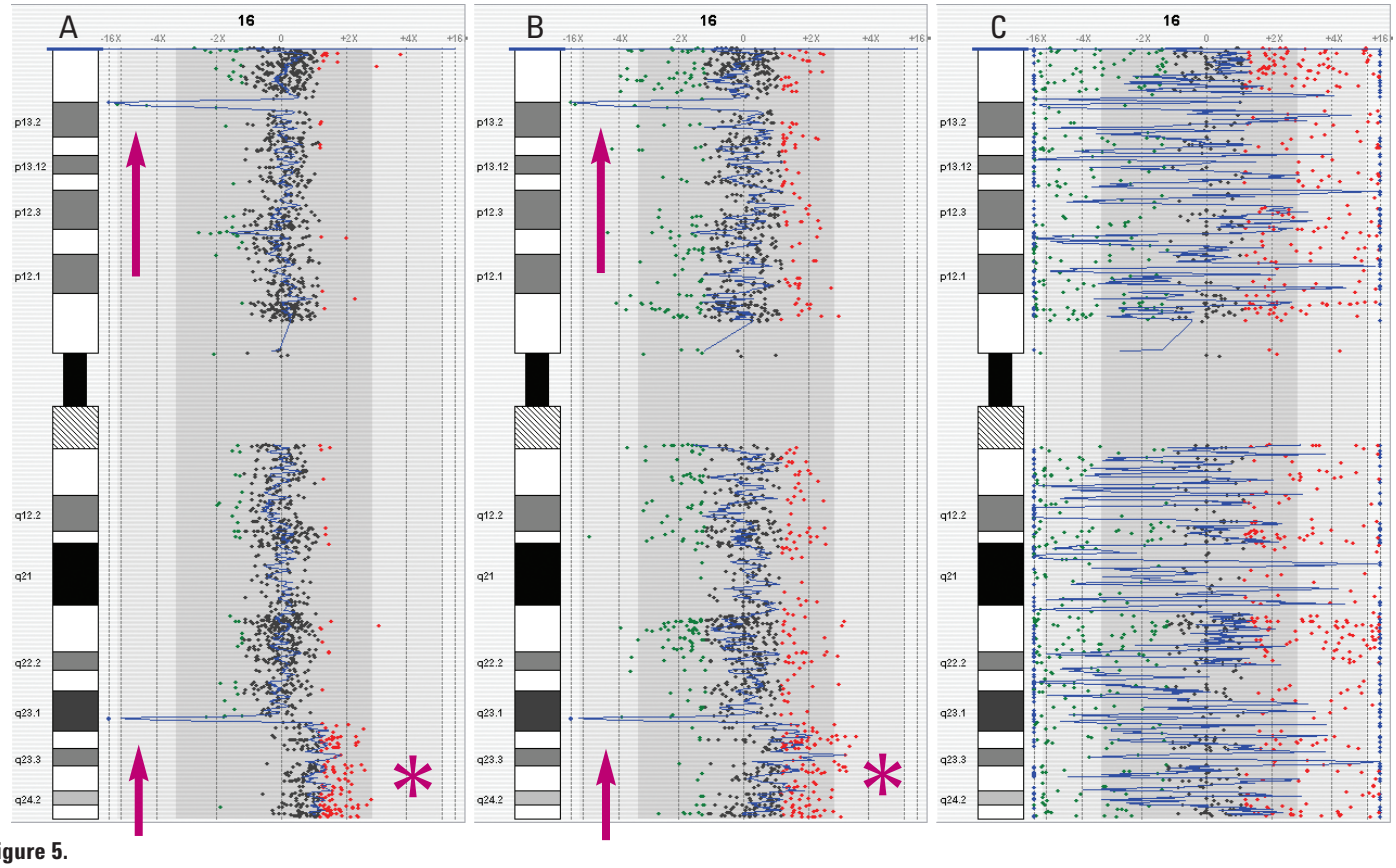

Figure 5.
HCT116/XX chromosome 16 Cyanine-5/Cyanine-3 ratio plots. Agilent's CGH Analytics software was used to plot the Cyanine-5/Cyanine-3 background subtracted signal ratios for all 1647 chromosome 16 probes on the microarray. Data for the 50ng (A), 0.5ng (B) and 5pg (C) representative microarrays are shown. The red points are ratios >1.5 and the green points are ratios <1.5. The blue line represents a 0.5Mb moving average. The two known homozygous deletions (A2BP1-16p) and (WWOX-16q) are delineated with arrows and the distal q arm amplification is denoted with an asterix.

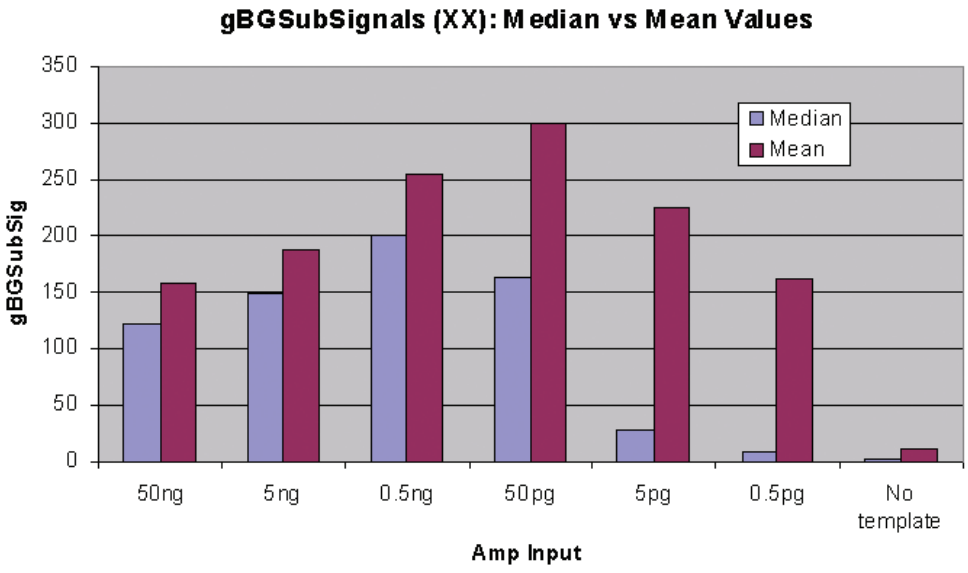

Figure 6.
Graph of median and mean female (XX) background subtracted signals. The background subtracted signal values (Cyanine-3) for the XX samples were plotted as a bar graph. The median/mean signal ratios were very constant for the 50ng, 5ng and 0.5ng samples. However, the ratios were greatly decreased for the 50pg, 5pg and 0.5pg samples. The no template sample had basically no signal.

are very low. The mean and median rBGSubSignals for the HCT116 samples shows the same trend (data not shown). Visual inspection of the raw microarray images (figure 7), yields the same general conclusions regarding the range of feature signal intensities on microarrays hybridized with samples from the different phi29 input DNA amounts. The signal intensities of the features in the 50ng input microarray image are fairly uniform across the microarray in both the Cyanine-3 and Cyanine-5 channels as expected given that HCT116 is largely diploid. When the amplification inputs were lowered, the signal intensities of many features decreased as expected, however the signal intensities of other features increased significantly. There is very little detectable fluorescent signal for the majority of the features on the no template sample microarray.

Further examination of the data revealed little correlation between the identities of the high signal features on the duplicate microarrays for the HCT116 samples from the low input amplification reactions. However, the features with high signals for the XX samples from the low input amounts, which were from the same amplification reaction, were largely correlated on the duplicate microarrays (data not shown). Together these observations suggest that most variation seen with the high signal features arise from differences in the amplification reactions rather than from differences in labeling or hybridization. At this time the specific nature of the hybridizing material generated in the phi29 amplification reactions with low input DNA is unclear.

The results shown above suggest that peak two in the bioanalyzer electropherograms (figure 1) contains phi29 amplified genomic DNA capable of producing high quality aCGH data while peak one represents by-products of the phi29 amplification reaction. In the case of the no input reaction, the labeled peak one products do not hybridize in significant amounts to the microarrays, however, in cases where insufficient DNA is added to the amplification the products can produce high signals for some of the microarray features. It is important to note that high hybridization signals are not necessarily indicative of a good aCGH assay.

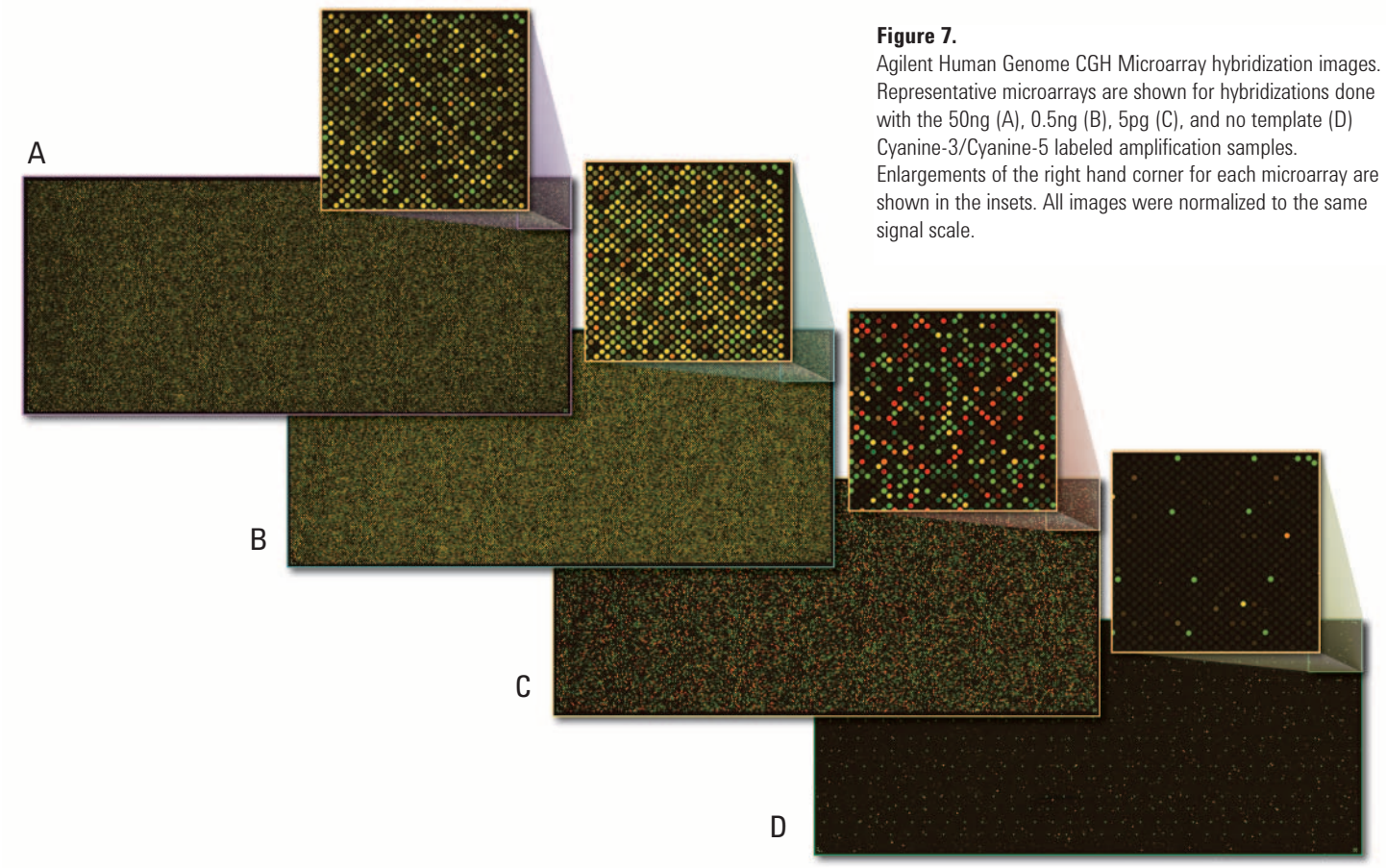

Figure 7.
Agilent Human Genome CGH Microarray hybridization images. Representative microarrays are shown for hybridizations done with the 50ng (A), 0.5ng (B), 5pg (C), and no template (D) Cyanine-3/Cyanine-5 labeled amplification samples. Enlargements of the right hand corner for each microarray are shown in the insets. All images were normalized to the same signal scale.

Conclusions:

Efforts to determine the quality of phi29 amplified DNA preparations have been hampered by the fact that phi29 amplification reactions, with different DNA inputs and even no DNA input, yield the same amount of material when measured by a spectrophotometer or by gel electrophoresis due to the high level of by-products that are produced when optimal template is absent. Here we show that the bioanalyzer is an effective tool for assessing the quality of phi29 amplification reactions because it has the ability to separate the amplification product into two peaks; peak one, the phi29 by-products and peak two, the amplification products believed to represent the specific genomic input DNA.

We are further investigating whether the peak ratios in the bioanalyzer electropherograms for the amplified samples can be correlated to aCGH data to determine what an optimum or acceptable ratio should be. An important point to note is that the no input amplification product produces very little hybridization signal on the microarray. However, material generated in the presence of extremely small amounts of genomic DNA template often causes an increase in peak one in relation to peak two and may produce significant but unpredictable hybridization signals on the microarray for some features. This pattern is quite distinct from the more homogenous level of signals typically observed across features of an microarray that yields high quality aCGH data.

The purpose of the experiment described here was not to determine the optimum DNA input for phi29 amplification reactions, but rather to demonstrate that the bioanalyzer does provide a means for qualitatively and, potentially quantitatively, assessing the quality of phi29 amplification products before downstream use. For this experiment, a titration of DNA input amounts was used as a way to produce and monitor amplifications of varying quality, but there are many other factors that may affect the quality of the phi29 amplification products including: inaccurate input DNA quantitation, degraded input template (i.e. DNA from formalin fixed paraffin embedded samples) in which only a portion of the sample may be acceptable template for phi29 amplification, or inhibiting reagents in the DNA sample. Any of these factors, or others, could result in amplifications that produce large amounts of peak one material compared to peak two material (figure 1) and could lead to poor aCGH experimental results.

We have shown that the bioanalyzer is an effective tool for measuring the quality of phi29 amplification products. We also demonstrated the potential for this technology to allow for more quantitative monitoring of other steps in the aCGH workflow such as restriction enzyme digestion and fluorescent labeling. We are currently working toward developing detailed bioanalyzer based protocols to enable the QC of all steps in the aCGH workflow.

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