Evaluation of Whole Genome Amplification Protocols for Array and Oligonucleotide CGH

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Abstract: Genome-based technologies such as genomic arrays and next generation sequencing are poised to make significant contributions to clinical oncology. However, translation of these technologies to the clinic will require that they produce high-quality reproducible data from small archived tumor specimens and biopsies. Herein, we report on a systematic and comprehensive microarray analysis comparing multiple whole genome amplification methods using a variety of difficult clinical specimens, including formalin-fixed and paraffin-embedded tissues. Quantitative analysis and clustering suggest that Sigma's whole genome amplification protocol performed best on all specimens and, moreover, worked well with a formalin-fixed, paraffin-embedded biopsy.

Key Words: whole genome amplification, aCGH, oCGH

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Recurrent structural alterations of the genome such as amplifications, deletions, and translocations are common to many malignancies.[1–5] Array comparative genomic hybridization (aCGH) is a powerful and sensitive tool for the identification of copy number aberrations (CNAs). Identification of diagnostic, predictive, and prognostic biomarkers based on CNAs is an important goal of research aimed at improving cancer management. The importance of such biomarkers is best illustrated by the power of HER2 amplification in predicting response to Herceptin and the BCR-ABL fusion for imanitib mesylate in chronic myeloid leukemia. That so few validated biomarkers exist for cancer speaks of the difficulty of developing such markers. In theory, aCGH is an ideal tool for discovery of candidate genome-based biomarkers; however, its requirement of $\sim 1\,\mu g$ of good quality tumor DNA limits its actual utility.[3,6]

aCGH, using DNA obtained from frozen and formalin-fixed, paraffin-embedded (FFPE), archived radical prostatectomy tissue blocks, is successful when sufficient tumor tissue is available.[3,7,8] Prostate cancer is heterogeneous, necessitating macro and microdissection to isolate tumor cells of the same grade and to separate out interspersed normal and stromal tissue, with the end result being very limited quantities of DNA for analysis.[9] In addition, FFPE specimens typically yield degraded DNA.[3,10] Application of aCGH to biomarker discovery and its extension to a clinical screening tool will require high-quality data using limited DNA obtained from FFPE and small tumor specimens such as those from fine needle aspirates and biopsies. At present, these applications demand a reliable, high fidelity amplification scheme to produce sufficient DNA for aCGH.

Efforts have been made to design and optimize DNA amplification methodologies.[11–14] Various polymerase chain reaction (PCR) strategies, including degenerate oligonucleotide primer PCR, have demonstrated efficacy.[11,14] A slightly improved thermal cycling protocol involving random fragmentation and PCR amplification using ligated adapters (eg, Sigma's Whole Genome Amplification kit, WGA) has been developed and is commercially available.[15,16] Multiple displacement amplification (MDA), which uses the bacteriophage Phi29 DNA Polymerase and random, exonuclease-resistant primers on long DNA templates (eg, Qiagen's RepliG kit, RG) or short, circularized DNA templates (Restriction and Circularization-Aided Rolling Circle Amplification, RCA-RCA), provide high throughput, isothermal amplification systems.[13,17–19] Although some groups have sought to optimize amplification of degraded DNA products from FFPE and fresh-frozen samples,[17,19] a thorough comprehensive study comparing the different amplification protocols on a variety of difficult clinical specimens is lacking. Given the limitations of DNA quality from these archival sources, and also the variety of available amplification systems, we sought to identify the most reliable, reproducible amplification technology that will be suitable for use across a wide spectrum of clinical specimens, using our optimized DNA extraction protocols. In addition, a common aCGH labeling method (random priming, RP) was modified for the purpose of whole genome amplification and also evaluated in this study. Genomic DNA extracted from a FFPE specimen, fresh-frozen tissues, an immortalized cell line, and commercially available DNA isolated from blood was amplified with RG, RCA-RCA, WGA, and RP

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protocols. Fidelity of the different amplification technologies was assessed using aCGH to confirm concordance of genome profiles after amplification. To investigate possible clinical utility, DNA from an FFPE prostate biopsy was amplified, using the amplification protocol that performed the best in this study, and evaluated on a higher resolution oligonucleotide CGH platform.

MATERIALS AND METHODS

Samples and DNA Extraction

DU145 cells were obtained from American Type Culture Collection (Manassas, VA) and grown in Minimum Essential Medium (MEM Eagle) with 2 mM of L-glutamine and Earle's BSS adjusted to contain 1.5 g/L of sodium bicarbonate, 0.1 mM of nonessential amino acids, 1 mM of sodium pyruvate, and 10% fetal bovine serum. Cells were trypsinized and pelleted from 2 confluent 75 cm^2 flasks. Genomic DNA was extracted using the Promega (Madison, WI) Wizard Kit, according to the manufacturer's protocol for tissue culture cells.

The radical prostatectomy and the biopsy cases were obtained from the UCSF Tissue Core. For the frozen cases (PR374, PR659), ten 10-μm sections were cut from the fresh-frozen blocks. A pathologist marked areas of greater than 80% tumor on hematoxylin and eosin (H&E) guide slides. Macrodissection was performed using a scalpel. The Promega Wizard kit was used to extract DNA, according to the manufacturer's instructions for tissue. The DNA was subjected to 2 rounds of phenol:chloroform:isoamyl alcohol (25:24:1) extractions using Eppendorf (Westbury, NY) PhaseLock Gel tubes, followed by an ethanol-sodium acetate precipitation.

Ten 10-μm slices were cut from the FFPE block (41P). Tumor regions (> 75%) on the H&E guide slides were outlined with the help of a pathologist. Macrodissection was performed with a scalpel. The Gentra (Minneapolis, MN) Puregene DNA Isolation kit was used to extract the DNA. The manufacturer's protocol was followed, except that the final elution volume was 30 μL. Phenol chloroform extraction and ethanol precipitation was performed as described above.

The biopsy specimen (B1) from a routine fine needle biopsy was embedded in an FFPE block. An H&E guide slide was used to macrodissect tumor tissue (> 90%) from 10 slides of 10-μm thickness. The tissue was digested using proteinase K for 48 hours at 56°C. Genomic DNA was isolated using the Qiagen (Valencia, CA) QIAamp DNA Micro purification kit using the manufacturer's protocol for biopsy specimens. This was followed by an ethanol precipitation as described above.

Amplification

RepliG

One hundred nanograms of template DNA was amplified using Qiagen's RepliG Mini kit, according to the manufacturer's instructions. After denaturation and neutralization of the template, the MDA principle forms the basis of the protocol.[13] The amplification products were purified using Qiamp micro columns following Qiagen's Qiamp DNA Micro protocol, and eluted in 25 to 30 μL Tris-EDTA (TE) buffer.

WGA

Sigma's (St Louis, MO) GenomePlex Whole Genome Amplification kit uses a proprietary technology based on random fragmentation of genomic DNA and conversion of the resulting small molecules to PCR-amplifiable library molecules flanked by universal priming sites. The library molecules are then subsequently used in PCR amplification. One hundred nanograms of template DNA was amplified using the GenomePlex kit according to the manufacturer's instructions. The amplified DNA was cleaned up using Qiagen's Qiaquick PCR purification kit and the DNA eluted in 25 to 30 μL TE buffer.

Random Priming

One hundred nanograms of genomic DNA was heat-denatured at 99°C for 10 minutes in a 21 μL reaction mix, followed by incubation at 4°C for 15 minutes to allow random, octamer primers to anneal to the template. dNTPs (25 nmol) were then added to the mix along with 20 units of Klenow. This solution was then incubated overnight at 37°C to allow for amplification. All reagents (Klenow, primers, dNTPs) were from Invitrogen's (Carlsbad, CA) Bioprime DNA labeling kit. The products were then subjected to the Qiagen Qiaquick PCR purification kit.

Restriction and Circularization-aided Rolling Circle Amplification, RCA-RCA

The originally described RCA-RCA protocol was adopted,[19] with minor modification. Briefly, genomic DNA was digested with 0.5 μL Nla-III (New England Biolabs, Beverly, MA) at 37°C or 2 hours in 10 μL of 1X T4 DNA ligase buffer (New England Biolabs). Each sample was heated at 65°C for 20 minutes to inactivate Nla-III. The fragmented DNAs were circularized with 1000 units of T4 DNA ligase (New England Biolabs) in a volume of 15 μL at room temperature for 2 hours. After inactivation of ligase at 65°C for 10 minutes, linear DNAs were eliminated with 6 units of Lamda Exonuclease (New England Biolabs) and 6 units of Exonuclease I (New England Biolabs) in a volume of 25 μL at 37°C for 1 hour. The circularized DNAs were then purified using a QIAquick PCR Purification Kit (Qiagen) and eluted in 35 μL of water. Four microliters of circular DNA was mixed with 200 ng hexamers (Sigma) and 0.5 μL binding buffer [400 mM Tris-HCl (pH 8.0), 160 mM KCl]. The DNA was amplified using 3 units of Phi29 DNA polymerase (New England Biolabs), complemented with 2 μL of 10x Phi29 DNA polymerase buffer, 0.2 μL of 100x BSA, 3.2 μL of 2.5 mM dNTP (Applied Biosystems, Foster City, CA), 1 μL of 20% DMSO (Sigma), and 20 ng T4 gene32 (Amersham Biosciences, Piscataway, NJ) in a volume of 20 μL at 30°C for 20 hours. The Phi29

DNA polymerase was inactivated at 70°C for 15 minutes, and the amplification product was digested with 3.5 μL of Nla-III at 37°C for 2 hours in a volume of 80 μL.

aCGH

aCGH was run with 1 μL of DNA to compare the individual amplification systems. The commercial male DNA from blood served as a reference in all experiments. The human version 3.1 BAC arrays were purchased from the UCSF Array Core. Each array consists of 2460 BAC clones spotted in triplicate onto chromium slides, with an average resolution of 1.4 Mb. The hybridization protocol, processing of the arrays, and data analysis were according to laboratory-established protocols.[7]

Oligonucleotide Comparative Genomic Hybridization

Agilent's human oligonucleotide comparative genomic hybridization (oCGH) arrays with a resolution of ~9 kb, achieved with 244,000 probes covering the entire genome, were used to evaluate the robustness of the biopsy amplification. Five hundred nanograms of sample and reference DNA were digested with AluI and RsaI and the products labeled with cy3 and cy5, respectively. The labeled DNA was then hybridized to the array and scanned using the Agilent array scanner. The Agilent protocol was followed during sample preparation, hybridization, and the scans. Feature Extraction 9.1 and CGH Analytics 3.4 were used with manufacturer recommended settings to analyze the array data.

Statistical Analysis

We derived several copy number based measures, both descriptive and quantitative, to evaluate the comparative performance of the technologies. In particular, we quantified the signal to noise ratio of the individual hybridizations and compared them between the amplification protocols. We also evaluated the bias of various amplification strategies by using a novel profile subtraction approach to detect small differences in hybridization profiles. We then looked at the agreement between amplified and nonamplified samples in making gain and loss calls. More detailed description of the methods is presented in the Results section of this manuscript and described in the aCGH package in Bioconductor/R.[20,21]

RESULTS

The different amplification protocols were evaluated on a spectrum of specimens, including commercially available normal female genomic DNA extracted from blood, DNA extracted from an immortalized human prostate cancer cell line (DU145), 3 radical prostatectomy samples, 2 of which had been preserved as fresh-frozen material (PR374 and PR659) and 1 as an FFPE block (41P), and also 1 FFPE biopsy. To maintain consistency across each amplification system, commercial male genomic DNA from blood was used as the reference DNA during hybridization in CGH, for each of the specimens. One hundred nanograms of test and reference genomic DNA were individually amplified with each of the 4 amplification protocols.

The genomic copy number profile for every specimen from each amplification protocol was compared with the aCGH results obtained with corresponding nonamplified sample and reference DNA, and also the results from the other amplification platforms. As an internal control, the loss of the Y chromosome and gain of the X chromosome were confirmed when the female commercial DNA from blood was hybridized against the male reference DNA for all of the amplification protocols. All 4 amplification protocols gave consistent aCGH results for female-male blood DNA hybridizations when compared by visual inspection with the corresponding aCGH result for nonamplified DNA (data not shown).

The different amplification technologies were then evaluated on genomic DNA extracted from the DU145 prostate cancer cell line. aCGH with amplified DU145 DNA, hybridized against amplified commercial, genomic male DNA, showed similar chromosome losses and gains as the aCGH run with nonamplified genomic material (Fig. 1). The WGA and RG protocols, in particular, seem to produce consistent, unbiased amplifications, yielding similar copy number profiles, which in turn are comparable with known, preestablished genomic changes in DU145.[22,23]

To analyze the fidelity of the amplification technologies on clinically relevant material, 100 ng of DNA was amplified from fresh-frozen radical prostatectomy samples (PR374, PR659) and an FFPE radical prostatectomy sample (41P). The aCGH profile with each amplified material was compared with the aCGH copy number profiles obtained with nonamplified DNA. Figure 2 shows the distribution of genomic losses and gains found on chromosome 10 for the FFPE sample, as an example to compare the performance of the protocols in closer detail.

In addition to a visual assessment of the reproducibility of the aberrations across the amplification protocols in every sample, we also performed an unsupervised cluster of the aCGH $\log_2$ratio values of all of the samples. This helped to assess whether amplification bias was introduced by any of the amplification protocols as demonstrated by recurrent gains and/or losses of particular genomic loci across all of the samples. The clustering was found to occur by sample identity rather than any particular amplification protocol, thereby confirming that these protocols did not result in over or underrepresentation of any specific genomic loci on a regular basis (Fig. 3).

Several other statistical copy number based measures were used to compare the performance of the amplification technologies. Smoothed values for each amplification aCGH were computed by using circular binary segmentation[24] with default parameters to translate experimental intensity measurements into regions of equal copy number. This was implemented using the

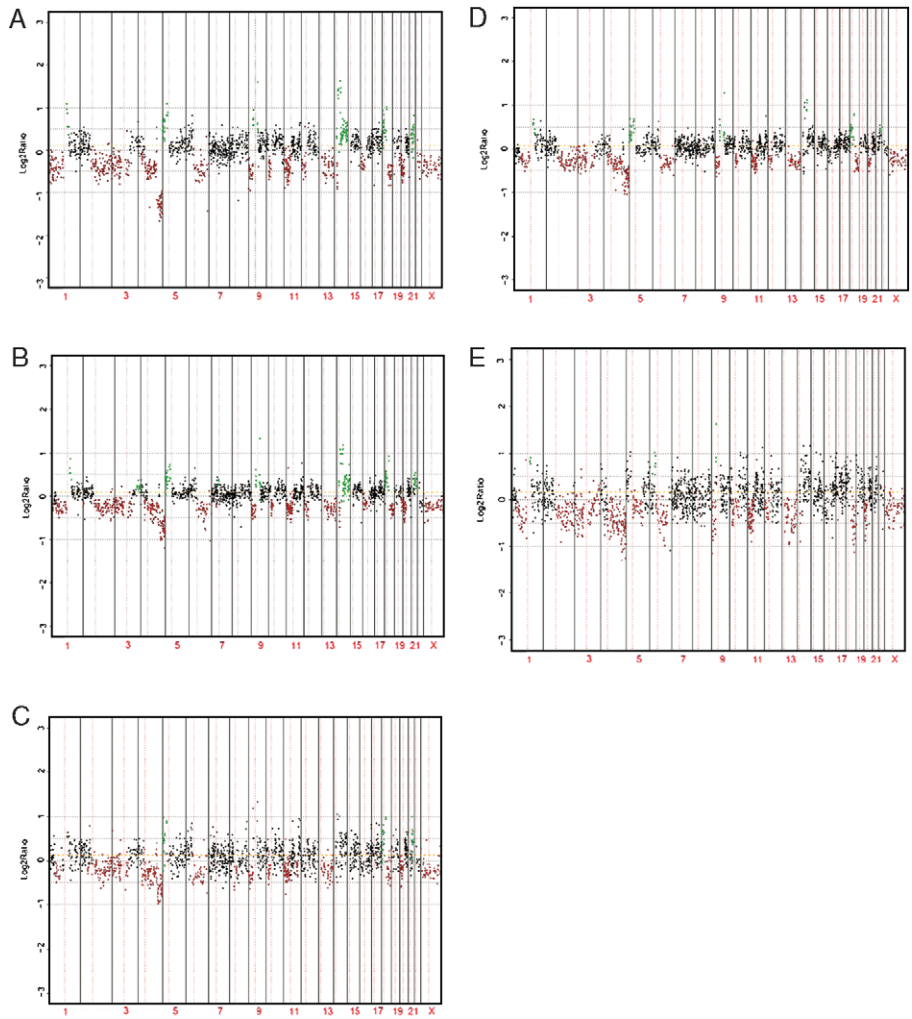

FIGURE 1. DU145 aCGH. A–E, The genomic profile with nonamplified DNA, followed by the 4 amplification protocols evaluated in this study—WGA, RP, RG, and RCA-RCA, respectively. Each is a plot of $\log_2$ratios versus genomic position. Red denotes a loss and green represents a gain of genomic material.

DNAcopy R/Bioconductor package, and the median of each segment was assigned to the clones in that segment. Experimental variation was then estimated for each sample by calculating the median absolute deviation (MAD) of the difference between the observed and smoothed values (Fig. 4A). Higher MAD scores correspond to lower measurement precision. Figure 4A shows that the WGA method most closely corresponds to the MAD score of the respective nonamplified sample, thereby demonstrating that Sigma's WGA introduces the least experimental variability. The median MAD value of Sigma's WGA across the samples tested was the lowest, at 0.14, among the 4 protocols. The cluster nodes in Figure 3 further confirm that the Sigma WGA amplification protocol produces the most concordant CNA profile when compared with the nonamplified sample.

It is difficult to confirm the conservation of aberrations in an amplified tumor sample as the genetic profile and aberration locations of the primary tumor are not known a priori. Thus, identifying false positives and false negatives requires other measures to evaluate the performance of the amplification techniques. Instead one can assess amplification fidelity by focusing on measures that reflect signal to noise ratios in other ways. Figure 4 shows box plots and the corresponding Table 1 represents the spread of these values across amplification protocols for the different measures. For example, we can quantify the amount of observed overlap between probes at different copy number levels. This can be approximated by taking each pair of neighboring segments, with segments defined by the circular binary segmentation breakpoints, and calculating the median distance between the medians of the neighboring segments (Fig. 4B).

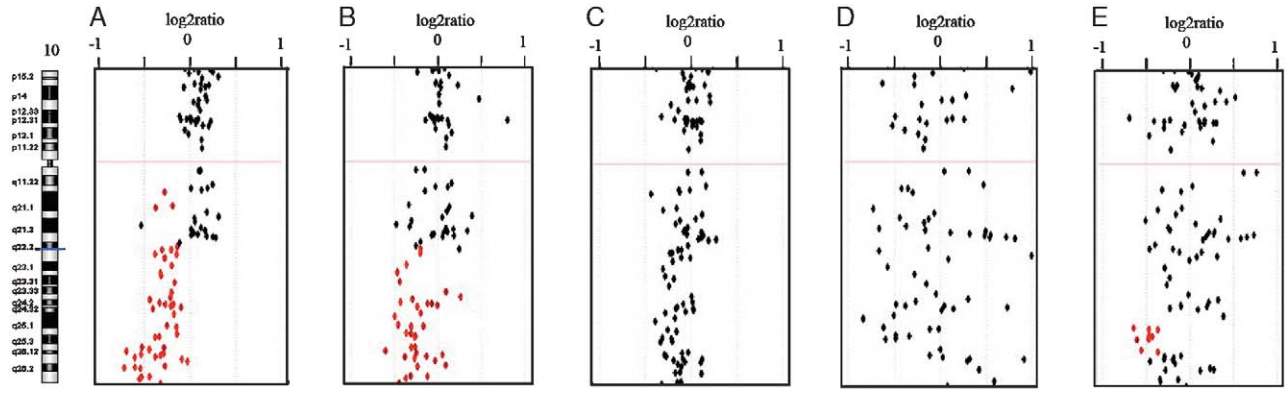

FIGURE 2. Chromosome 10 copy number changes in FFPE sample, 41P. A–E, The chromosome profile with nonamplified DNA, followed by the 4 amplification protocols evaluated in this study—WGA, RP, RG, and RCA-RCA, respectively. Log_2ratios are plotted for the probes on chromosome 10. Red denotes a loss of genomic material in the tumor sample.

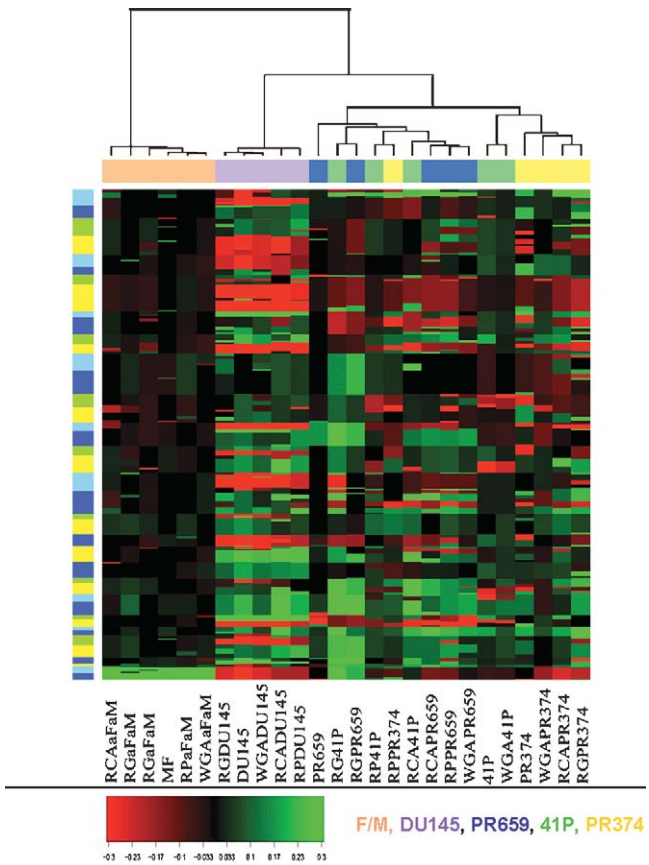

FIGURE 3. Heatmap generated by unsupervised clustering of the smoothed aCGH log_2ratios for all of the samples. The color spectrum shows the color legend for the heatmap. Each sample is also color coded as noted below the heatmap. Note that none of the amplification techniques clustered together, suggesting that there were no artificial copy number changes being consistently introduced by these amplification techniques.

The larger the distance the between segments, the higher the signal. For robustness, only segments with at least 10 admissible probes were used. However, median distance in neighboring segments (Fig. 4B), on its own, is not very informative as it suggests RCA, RG, and RP do better than the control group even though MAD values (Fig. 4A) showed these produced noisy data. To assess signal to noise ratios, the distance between the medians of neighboring segments was normalized by the MAD for a given sample (Fig. 4C). Large distances indicate

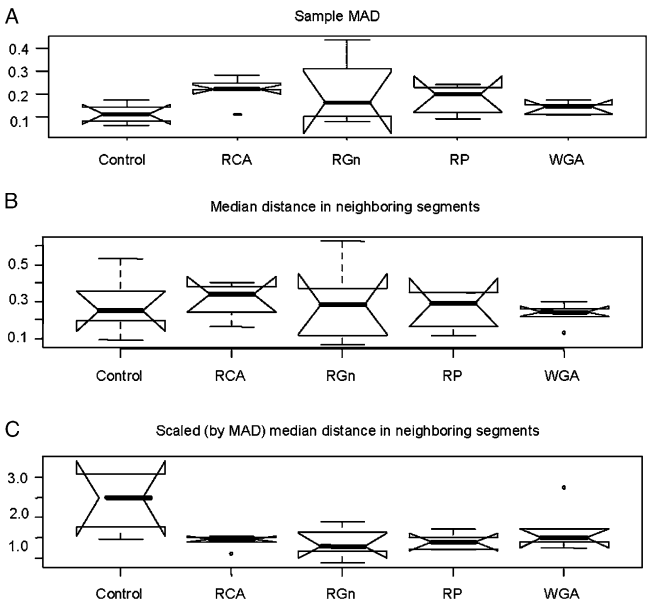

FIGURE 4. Box plot with panels (A–C) showing the distribution of the sample MAD values, median distance in neighboring segments, and median distance in neighboring segments scaled by MAD, respectively. The median is represented by the thick solid line, whereas the bottom and top of each box represent the 25th and 75th percentile, respectively. The whiskers extend to 1.5 times the interquartile range from the box. Outlier values are indicated with circles.

TABLE 1. A Comparison of the Performance of the Different Amplification Protocols to Their Respective Control Array Experiment Performed With Nonamplified Test and Reference DNA

Specimen	Amplification Method	MAD	OverlapSegPropMedian	OverlapSegDist	OverlapSegDistNorm
F/M	None	0.0631	0.5598	0.0928	1.4708
F/M	WGA	0.1073	0.4630	0.1340	1.2483
F/M	RP	0.0918	0.5669	0.1127	1.2277
F/M	RGn	0.1007	0.4231	0.1198	1.1892
F/M	RCA	0.1102	0.4063	0.1613	1.4642
DU145	None	0.1725	0.0839	0.5297	3.0703
DU145	WGA	0.1108	0.1605	0.3012	2.7191
DU145	RP	0.1996	0.2734	0.3471	1.7390
DU145	RGn	0.1201	0.2684	0.2279	1.8982
DU145	RCA	0.2484	0.3333	0.3752	1.5105
PR374	None	0.1115	0.2471	0.1969	1.7651
PR374	WGA	0.1733	0.4412	0.2586	1.4923
PR374	RP	0.2421	0.4722	0.2935	1.2125
PR374	RGn	0.2057	0.2727	0.3414	1.6594
PR374	RCA	0.2207	0.5125	0.2453	1.1112
PR659	None	0.0813	0.2591	0.2489	3.0631
PR659	WGA	0.1550	0.4081	0.2165	1.3972
PR659	RP	0.2287	0.4036	0.3487	1.5248
PR659	RGn	0.3111	0.3433	0.3705	1.1910
PR659	RCA	0.2200	0.3174	0.3389	1.5403
41P	None	0.1415	0.0921	0.3510	2.4798
41P	WGA	0.1440	0.4078	0.2469	1.7143
41P	RP	0.1178	0.4399	0.1642	1.3944
41P	RGn	0.4375	0.1338	0.6214	1.4204
41P	RCA	0.2830	0.4156	0.3989	1.4097

Values corresponding to every array's respective sample MAD, median proportion overlapping clone in neighboring segments (OverlapSegPropMedian), median distance in neighboring segments (OverlapSegDist), scaled (by MAD) median distance in neighboring segments (OverlapSegDistNorm), are reported.

DU145 indicates the cell line DNA; F/M, sex mismatched DNA samples from blood; PR374 and PR659, frozen samples; 41P, FFPE radical prostatectomy case.

desirable higher signal to noise ratios. Accordingly, the signal to noise is highest for the control group, as expected. Overall, by observing Figures 4A–C, we conclude that WGA does best among the amplification methods. Additional measures were calculated only for the cell line sample (Table 2) because they are not as robust for low CNAs. Prostate cancer tumors are inherently heterogeneous and exhibit low copy number changes. To analyze the agreement between the amplified and nonamplified samples, the proportion of agreement for calling gains, losses, and no alterations and the Pearson correlation coefficient were calculated for all of the amplification protocols. Sigma's WGA protocol generally exhibited the best agreement with nonamplified sample for all 3 measurements. Proportion of overlap for calling gains, losses, or no alterations was higher for longer segments than for shorter ones in the case of Sigma's WGA.

The BAC aCGH arrays used in this study offer a resolution of approximately 1.4 Mb. Although comprehensive for tracking genomic copy number changes, CGH technology can now offer kilobase resolution with the advent of oligonucleotide arrays (oCGH). As a final test, with clinical diagnostics in mind, we amplified 100 ng of DNA extracted from a fine needle prostate biopsy using the Sigma WGA protocol and analyzed 500 ng of the amplified product on the Agilent oCGH 244 K platform. A very high degree of concordance was obtained (Fig. 5) when compared with the oCGH result for the nonamplified biopsy DNA.

TABLE 2. Results for Metrics Comparing Nonamplified and Amplified Samples

Amplification Method	Pearson Correlation	Proportion of Gain	Proportion of Loss	Proportion of No Alteration	Proportion of Overlap Gain < 30 Mb/≥ 30 Mb	Proportion of Overlap Loss < 30 Mb/≥ 30 Mb	Proportion of Overlap No Alterration < 30 Mb/≥ 30 Mb
RCA	0.8249	0.0438	0.8275	0.9155	0.1129/0.0000	0.4954/0.4522	0.7161/0.53491
RG	0.9089	0.5125	0.8387	0.9965	0.4827/0.4630	0.8185/0.7650	0.7680/0.7572
RP	0.8426	0.3750	0.8261	0.9474	0.2641/0.4259	0.7516/0.7048	0.4697/0.5461
WGA	0.9653	0.9375	0.9579	0.9698	0.5811/0.9907	0.8464/0.8899	0.8323/0.8628

All results are for the DU145 cell line sample.

The following data are presented: the Pearson correlation (based on smoothed values obtained from CBS), the proportion of agreement in calling gains, losses or no alterations, and the proportion of overlap based on segment length for regions of copy number gain, loss, or no alteration.

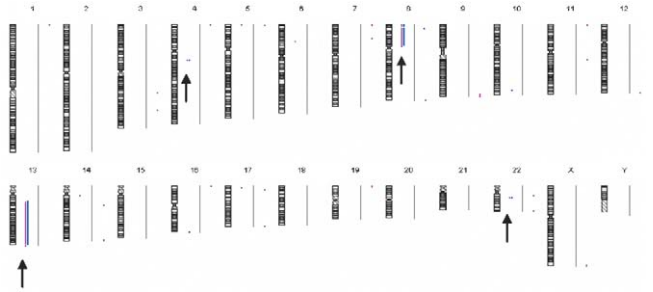

FIGURE 5. CNAs in nonamplified and Sigma WGA amplified biopsy (B1) on Agilent oCGH. The aberrations of the nonamplified biopsy are seen in pink, whereas the aberrations of the amplified biopsy are seen in blue. Copy number gains are seen as colored vertical bars to the right of the vertical bar representations of every chromosome, and copy number losses are to the left of the bars. Arrows denote aberrations in common between the nonamplified and amplified biopsy samples.

DISCUSSION

Genomic profiles, defined by copy number changes, have been identified in prostate cancer specimens and could be useful early markers to help assess and predict the risk of recurrence and metastasis.[4,25,26] aCGH, in particular, has been a useful tool in the identification of such biomarkers as it can expeditiously and quantitatively identify and locate genomic amplifications and losses. Our laboratory has shown that aCGH is a robust system that can provide consistent results from fragmented, relatively poor quality DNA obtained from archived FFPE prostate tissue.[3,4,7,27] The importance of the DNA extraction protocol cannot be stressed enough. The aforementioned protocols have been extensively optimized and evaluated in our laboratory from tissues around the world and proven effective for aCGH and oCGH[3] (and data not shown). The importance of such archival material cannot be understated when we consider the value of long clinical follow-up for a potentially slow growing, indolent disease, such as prostate cancer. The wealth of information from the study of such samples has proven invaluable to the discovery and identification of prostate biomarkers with prediction potential for recurrence and severity.[4,8,25] Furthermore, all patients with prostate cancer are first identified by prostate biopsy, thus affording the opportunity to segregate patients with clinically relevant disease from those who may not require intervention. Therefore, to translate genomic biomarkers to the clinic, it is important to tackle the bottleneck of limited DNA sample to make detailed, genome wide analysis possible on biopsy material.

An ideal amplification protocol yields an accurate, complete, and reproducible copy of a DNA template from limited starting material. It should be versatile and robust, thereby functional, irrespective of the DNA source (blood, cell line, frozen tissue, or the more challenging FFPE tissue), and provide exponential amplification. The resulting amplified products should be free of any contamination that will impede usability, in various down-stream processes, on multiple high throughput platforms. In our hands, Sigma's WGA kit provided the most robust, highly reliable, and versatile amplification system across our variety of DNAs. Although the other systems were also easy to use and provided ample amplification, they were limited in their fidelity, versatility, or unbiased amplification across the samples studied here. Sigma's WGA kit involves a straightforward procedure and has a relatively minor requirement for high precision thermal cycling reactions, which will greatly help maintain amplification kinetics in suboptimal conditions, in diagnostic laboratories, etc.

FFPE specimens provide a major hurdle in clinical research. This valuable repository of archived samples is moderately to highly degraded and thus often difficult to amplify. Lage et al[13] have demonstrated that MDA (eg, RG) amplification proficiency rapidly diminishes as the molecular weight of the template decreases, thus limiting its use in FFPE derived specimens. Wang et al[19] showed that some of the MDA limitations are eliminated by using RCA-RCA on FFPE samples. Conversely, Bredel et al[17] demonstrated comparable amplification efficiencies in DNA extracted from pairs of FFPE and fresh-frozen samples, by aCGH, using a random priming protocol and the Phi29 DNA polymerase. Superiority of linker adapted PCR (the basis of what became the Sigma WGA protocol) is however proven over degenerate oligonucleotide primer PCR-based methods[15] and was recommended as the method of choice for difficult and limited samples using FISH based CGH. Similarly, Little et al[28] showed that Sigma's WGA protocol was equally robust on matched frozen and FFPE samples. In our hands, the Sigma WGA system maintained the highest fidelity in FFPE-derived samples when the input DNA quantity was 100 ng, even though the manufacturer suggests that one can use as little as 5 ng starting material (data not shown). The Sigma WGA protocol also yielded sufficient, high-quality amplified DNA from biopsy material, which gave comparable CNA profiles on the 244 K, high-resolution oCGH platform as compared with nonamplified DNA. It should be noted that both the FFPE radical prostatectomy and the biopsy samples were archived approximately 6 years ago. Further work is warranted on high-resolution array platforms to ensure that narrow discreet regions are being well represented by Sigma's WGA protocol.

Sequence bias from any amplification method may arise from a variety of factors including priming efficiency, template accessibility, GC content, and proximity to telomeres and centromeres. Isothermal protocols were developed to minimize the drawbacks introduced by thermo cycling-based protocols. MDA, the underlying principle of the Qiagen RG kit, which was assessed as part of this study, is one such option. Similarly, RCA-RCA uses isothermal amplification preceded by a DNA-fragment circularization step that enables degraded FFPE samples to be amplified via an MDA-like reaction. The isothermal amplification protocols are reported to give better coverage and adequate average DNA size

compared with other amplification methods. However, prior studies have raised doubts that PCR-based whole genome amplification methods may generate nonspecific amplification artifacts, give incomplete coverage of loci, and generate DNA less than 1-kb long, which cannot be used in many down-stream applications.[12] These studies, however, did not simultaneously compare the performance of these amplification protocols across a spectrum of difficult clinical samples, and neither did they perform detailed product quality assessments or evaluate down-stream application usability. We, on the other hand, observed that the Sigma WGA kit had the most consistent and robust performance across the various sample types we examined, and compared the best with nonamplified material (ie, without the introduction of any extraneous gains or losses). Using Sigma's WGA protocol, we have successfully shown for the first time both the amplification of DNA from an FFPE prostate biopsy and its genomic profiling on a high-resolution oCGH platform.

It should be mentioned that caution should be exercised before applying any whole genome amplification method to FFPE specimens of older age, as the higher the DNA fragmentation the more constrained becomes the amplification. Methods that are tolerant toward sample degradation, such as Sigma's WGA and RCA-RCA, have advantages for amplification of FFPE specimens over methods such as Qiagen's RG that require intact material. Further, it should be mentioned here that the labeling step for aCGH could be a source for the introduction of copy number changes, but the protocol used in all of these experiments was random priming, which is well established and is proven to not introduce any aberrations by selectively labeling particular regions of the genome.[29] Also, hybridization of amplified sample and reference is proven to give better results than hybridizations where only the test sample is amplified and the reference DNA is not.[30] We similarly observed the introduction of random CNAs when aCGH was performed with only the sample being amplified, but these CNAs promptly disappeared when the aCGH was repeated with amplified reference DNA (data not shown). Therefore, for all experiments in this study, the test sample and reference DNAs were amplified. The performance of the arrays is not a variable in these experiments as the reproducibility of replicate experiments is excellent for this platform. For example, the average standard deviation of $\log_2$ratios for a cell line on the UCSF BAC arrays is 0.08.[31]

In summary, small amounts of template DNA can be efficiently and reliably reproduced from a variety of tissue sources. The amplification of valuable, limited clinical material will further expand opportunities for longitudinal and translational studies. Sigma's WGA is a valuable tool that can effectively be integrated into CGH technology for the identification of tumor-specific genomic changes and, thus, the identification and development of biomarkers for diagnosis and, ultimately, clinical intervention.

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