

Method for Manufacturing Whole-Genome Microarrays by Rolling Circle Amplification

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Comparative genomic hybridization (CGH) to metaphase chromosomes is a method for genome-wide detection of chromosomal aberrations in DNA samples. Recent advances in microarray technology have improved CGH by replacing metaphase chromosomes with a collection of mapped genomic clones placed on glass slides. However, it is quite expensive and labor-intensive to prepare DNA from the genomic clones for use in constructing genomic microarrays. Here we used strand-displacement rolling circle amplification (RCA) to manufacture whole-genome microarrays by using a collection of about 4,500 mapped RPCI-II BAC clones that cover the human genome at approximately a 1-Mb resolution. These genomic microarrays detected all major chromosomal aberrations in cancer cells lines and in cell lines with aneuploidy. In this article, we discuss the advantages of using RCA for the manufacturing of large genomic microarrays. © 2004 Wiley-Liss, Inc.

Comparative genomic hybridization (CGH) to metaphase chromosome targets (or chromosome CGH) has contributed to the current understanding of genomic alterations associated with certain diseases, particularly cancer (Kallioniemi et al., 1992; reviewed in Lichter et al., 2000). Although chromosome CGH offers a whole-genome screening capability for chromosomal abnormalities, two major limitations restrict its usefulness as a comprehensive screening tool. CGH to metaphase chromosomes provides only limited resolution in the identification of deletions and gains, which at best is on the order of 3–10 Mb (Kallioniemi et al., 1992; Parente et al., 1997; Lichter et al., 2000). In addition, chromosome CGH cannot easily be automated because of the need to identify individual chromosomes. Recent advances in microarray technology allow the circumvention of these limitations by replacing metaphase chromosomes with a collection of mapped genomic clones placed on glass slides (Solinas-Toldo et al., 1997; Pinkel et al., 1998; Snijders et al., 2001; reviewed in Beheshti et al., 2002). This new platform for genomewide analysis of chromosomal imbalances is generally referred to as microarray CGH or genomic microarrays.

Early experiments with genomic microarrays were limited by the lack of mapped clones. Therefore, investigators used small collections of mapped cosmids, yeast artificial chromosome (YAC) clones, and bacterial artificial chromosome (BAC) clones. To allow genome-wide analysis at high resolution by use of genomic microarrays, we mapped a col-

lection of about 4,500 mapped BAC clones that covers the human genome at an approximately 1-Mb resolution (Cheung et al., 1999, 2001; Morley et al., 2001). The clones were anchored to STS markers from the GeneBridge 4 radiation hybrid map. Each clone was mapped by filter hybridization and verified by polymerase chain reaction (PCR) to contain the STS marker. Chromosomal locations of some of the clones were verified by fluorescence in situ hybridization (Kirsch et al., 2000). The mapped clones were further characterized by *Hind*III fingerprinting and end-sequencing. The clones were anchored to the current human genome sequence assemblies by aligning the end sequences of the BAC clones to the DNA sequences at the UCSC Genome Browser. Information about the mapped BAC clones is available on our Web-based database GenMapDB, <http://genomics.med.upenn.edu/genmapdb>, and on the UCSC Genome Browser (<http://genome.ucsc.edu> on the GenMapDB clone track). Glycerol stocks of

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all the clones are available through various repositories (<http://genomics.med.upenn.edu/genmapdb>).

Despite the availability of mapped clones, the manufacturing of genomic microarrays is still challenging because of the difficulty of preparing adequate amounts of DNA from genomic clones. Initially, for microarray CGH studies, DNA from genomic clones was extracted by use of a large-scale alkaline lysis method followed by ion-exchange chromatography (Solinas-Toldo et al., 1997; Pinkel et al., 1998). When expanded to thousands of clones, this approach becomes expensive and time-consuming. Unfortunately, small-scale DNA preparations do not produce sufficient amounts of genomic DNA to manufacture genomic microarrays. In addition, *E. coli* DNA is a very common contaminant of DNA preparations from large insert clones when small-scale silica- or filtration-based DNA preparation miniprep kits are used (10%–25% of total purified DNA; Foreman and Davis, 2000). The presence of *E. coli* DNA compromises the use of degenerate oligonucleotide-primed PCR approaches for subsequent amplifications of genomic clone DNA samples (Fiegler et al., 2003; Smirnov and Cheung, unpublished observations).

Strand-displacement rolling circle amplification (RCA) is an amplification method that utilizes an oligonucleotide primer annealed to a circular template (Walter and Strunk, 1994; Fire and Xu, 1995; Lizardi et al., 1998; Dean et al., 2001; Nelson et al., 2002). RCA amplifies circular DNA templates selectively and exponentially even in the presence of contaminating bacterial genomic DNA (Lizardi et al., 1998; Dean et al., 2001; Nelson et al., 2002). A modified version of RCA, known as multiply primed RCA, utilizes random primers and Phi29 DNA polymerase to achieve thousandfold preferential amplification of circular DNA templates (Dean et al., 2001). Phi29 DNA polymerase is highly stable and has a very low error rate of 1 in 10^6 – 10^7 , in contrast to native Taq DNA polymerase, which has an error rate of 3 in 10^4 (Eckert and Kunkel, 1991; Esteban et al., 1993). This DNA polymerase is also capable of performing strand-displacement DNA synthesis for more than 70 kb without dissociating from the template (Blanco et al., 1989). Here, we describe an alternative approach for manufacturing large genomic microarrays based on multiply primed RCA.

To test the applicability of RCA for manufacturing genomic microarrays, we used it to amplify 4,308 mapped RPCI-11 BAC clones that cover the

human genome at about a 1-Mb resolution (Cheung et al., 1999, 2001; Morley et al., 2001). For initial BAC template preparation, individual BAC colonies were grown overnight and subjected to partial purification by the alkaline lysis method. One-microliter aliquots of partially purified BACs (out of a total of 50 μ l recovered from a 1-ml overnight culture) were amplified by use of the TempliPhi RCA protocol by incubation overnight at 30°C. Amplified DNA was spotted onto the glass slides without further processing, essentially as described previously (Cheung et al., 1999; Watts et al., 2002). The entire protocol was executed in 96-well plates. The partially purified BAC suspension can be stored at -70°C and used for RCA after prolonged storage (Smirnov et al., unpublished observations). The detailed protocol is available online at <http://genomics.med.upenn.edu/rcaarray/>.

To test whether the microarrays produced by RCA amplification can be used for detecting copy number changes, we performed a series of hybridization experiments with genomic DNA samples that have known chromosome CGH and microarray CGH profiles. The test samples were labeled with Cy3 fluorescent tags and cohybridized with Cy5-labeled control samples. The differential hybridization intensities of each clone on the array were used as an indication of copy number changes. Because the samples hybridized to the microarrays were labeled with two dyes, Cy3 and Cy5, that have different incorporation efficiencies, it is necessary to normalize signal intensities. A method that is used frequently in microarray data analysis is to normalize all the Cy3 and Cy5 intensities to the value of 1. However, this global normalization method is not appropriate for many CGH experiments, especially those with tumor samples that have high percentages of chromosomal aberrations (Wessendorf et al., 2002). Normalization based on regions known to have normal chromosome profiles is more precise but requires prior knowledge of the copy number changes in that sample.

Here, we used the rank invariant normalization method, which allows us to perform normalization without prior knowledge of the degree of chromosomal aberrations in the samples (Tseng et al., 2001). In this method, normalization is based on clones whose rankings by intensity signal in the two channels are the most similar. These clones most likely represent regions with normal chromosomal profiles. After normalization, the *t* statistic for each clone was used to measure the differences between the Cy3: Cy5 ratios from the test sample:

reference sample hybridizations and those from the reference sample:reference sample hybridizations. The t -statistic values were plotted for the clones along the chromosomes; the clones with high absolute t -score values are likely to represent areas in the genome where copy number changes occur (Moore et al., 1997; Yu et al., 1997; Kirchoff et al., 1998; Goeze et al., 2002). To test the performance of RCA-amplified genomic microarrays, we performed several array CGH hybridization experiments with a DNA sample from an individual with X-chromosome aneuploidy and two samples from cancer cell lines. All experiments were done with four replicates.

First, we tested genomic DNA samples from a cell line (GM11226) of an individual with X-chromosome aneuploidy (48,XXXX). Figure 1A shows that the majority of clones with high t -statistic values mapped to the X chromosome. Clones mapped to chromosome 16 had the next highest average t scores (Fig. 1A). Concentration of the clones with high t scores in the region of chromosome arm 16p is consistent with the known interchromosomal duplication of a gene-rich cluster between chromosome arms 16p and Xq (Fig. 1A; Eichler et al., 1996). The dynamic range of RCA genomic microarrays appears to be slightly lower than that in other genomic microarrays. This is possibly a result of incomplete suppression of the repetitive sequences during hybridization and increased hybridization complexity because of unbiased amplification of genomic DNA by RCA.

Next, we hybridized genomic DNA from peripheral blood acute promyelocytic leukemia cell line HL60 onto microarrays. This cell line was recently characterized by chromosome and microarray CGH (Wessendorf et al., 2002). We identified several copy number aberrations in the HL60 genome that agree well with previous HL60 CGH profiles (Fig. 1A). We observed amplifications of the regions on chromosome bands 2p16 and 8q24. We also observed deletions in the regions of 5q, 8p22, 17p13–17p12, and 17q and along the whole length of the X chromosome (Fig. 1A). Many of the amplified regions contain known oncogenes, such as *MYC* (on chromosome 8), *REL*, and *BCL11* (on chromosome 2), whereas the regions with losses contain known tumor-suppressor genes, such as *TP53* (on chromosome 17) and *FEZ1* (on chromosome 8; Joos et al., 1992; Fisher et al., 1997; Martin-Subero et al., 2002; Pollack et al., 2002; Toyooka et al., 2002; Wessendorf et al., 2002).

Finally, we tested genomic DNA samples isolated from mammary gland epithelium ductal carcinoma cell line ZR75-30. Genomic DNA was recently profiled both by chromosome CGH (Kallioniemi et al., 1994; <http://amba.charite.de/~ksch/cghdatabase/index.htm>) and by microarray CGH (Pollack et al., 2002). Our data agree with those generated by other groups. We detected amplifications in chromosome bands 5p13, 8q24, 14q32, and 17q21 (Fig. 1A). We also observed losses in the region of 1pter and in multiple regions along chromosomes 2 and 18 (Fig. 1A). Both the leukemia HL60 and the carcinoma ZR75-30 cancer cell lines share losses within chromosome band 8p22 and gains within chromosome band 8q24 (Fig. 1B). This latter band is the location of *MYC*, which is involved in regulation of cellular proliferation. These observations are consistent with observed recurrent copy number changes found in a variety of human cancer cells (Forozan et al., 2000; Crawley and Furge, 2002; Gebhart et al., 2002; Pollack et al., 2002; Wreesmann et al., 2002).

Genomic microarray is a useful tool for high-resolution chromosomal analysis for copy number changes in a variety of biological samples, including human tumors. Coupled with methodologies such as genomic mismatch scanning, genomic microarrays can also be used for gene mapping (Cheung et al., 1998). We believe that the RCA method offers an alternate approach for the manufacture of genomic microarrays. The phi29 DNA polymerase-based protocol is rapid because it does not require preliminary extensive purification of BAC DNA. It is very well suited for automation because all the steps can be accomplished in a 96-well format. Amplification reactions that utilize phi29 DNA polymerase do not require thermocyclers because they proceed at a constant temperature of 30°C. Recent comprehensive studies demonstrated that, when compared to other whole-genome amplification techniques, such as degenerate oligonucleotide-primed PCR (DOP-PCR) and primer extension preamplification (PEP), phi29 DNA polymerase-based amplification appears to have the lowest bias toward amplification of a particular genomic sequence (Faruji et al., 2001; Dean et al., 2002; Detter et al., 2002). In addition, RCA preferentially amplifies circular templates, minimizing the impact of contaminating bacterial genomic DNA (Lizardi et al., 1998; Dean et al., 2001; Nelson et al., 2002). With genomic microarrays made using the RCA approach, we were able to detect all major copy number changes

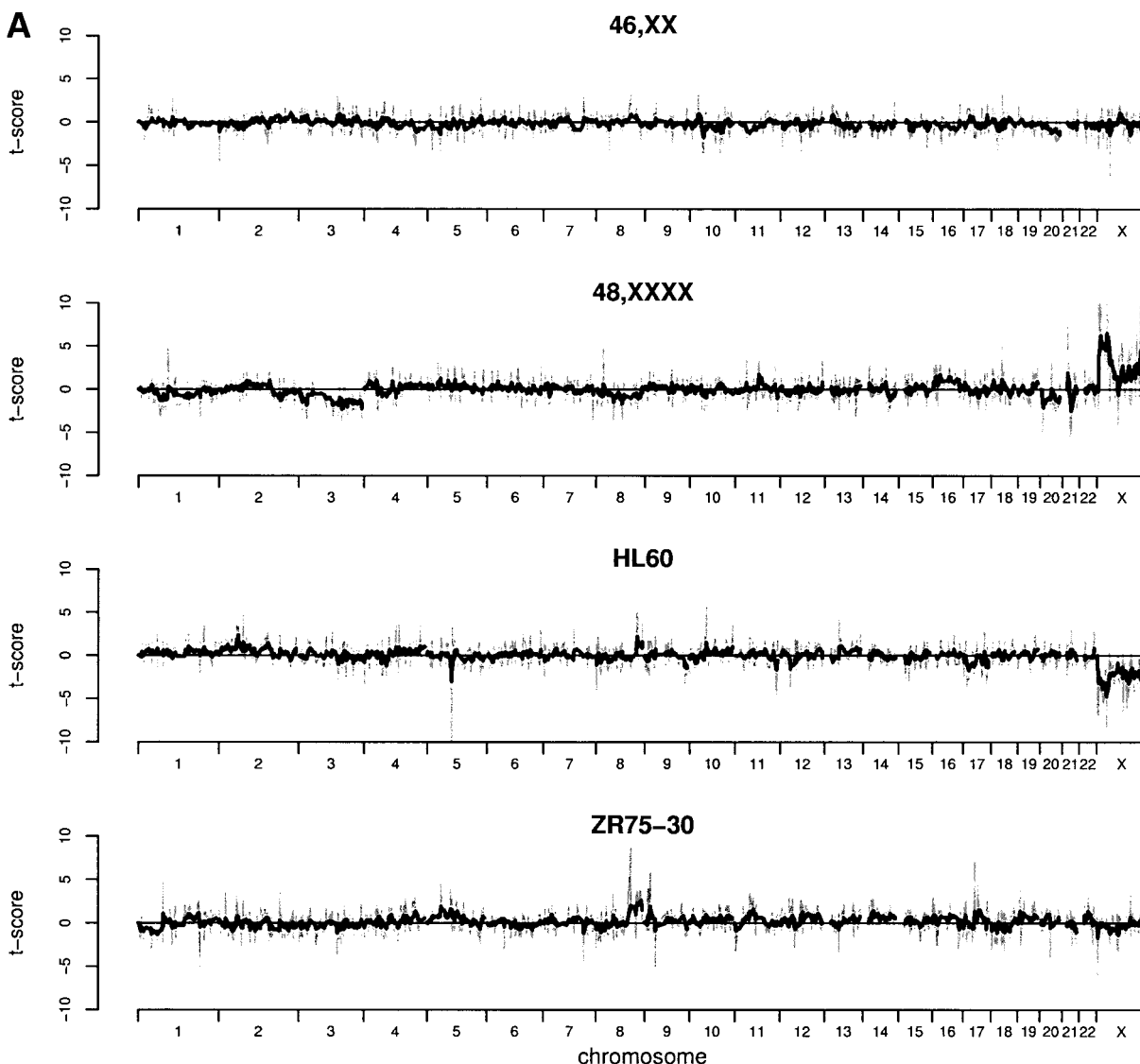


Figure 1. Results of microarray CGH experiments: *t* scores for individual clones are represented by gray bars. Exponentially weighted moving average of the *t* scores are plotted as black lines. (Detailed results from each hybridization experiment are available online at <http://genomics.med.upenn.edu/rcaarray/>.) (A) Genomewide view of DNA copy number alterations in a cell line from a normal female (46,XX) labeled with Cy3 and Cy5 and cohybridized onto a microarray containing DNA from 4,308 human BAC clones; [48,XXXX]: Cy3-labeled genomic DNA from a cell line with X chromosome aneuploidy (48,XXXX) compared with a Cy5-labeled 46,XY DNA sample. The presence of extra X chromosomes was detected; [HL60]: Cy3-labeled genomic DNA sample from peripheral blood acute promyelocytic leukemia cell line HL60, compared with a Cy5-labeled 46,XX DNA sample. Amplifications of the regions on chromosome bands 2p16 and 8q24 and deletions in the regions of chromosome bands 5q, 8p22, 17p13–17p12, and 17q, and the X chromosome were observed; [ZR75-30]: Cy3-labeled genomic DNA sample from a mammary gland epithelium ductal carcinoma ZR75-30, compared with a Cy5-labeled 46,XX DNA sample. Amplifications in chromosome bands 5p13, 8q24, 14q32, and 17q21 and losses in the regions of chromosomes 1, 2, 8, and 18 were observed. (B) Enlarged view of DNA copy number alterations across chromosome 8 in promyelocytic leukemia cell line HL60 and epithelium ductal carcinoma cell line ZR75-30. Both cancer cell lines share losses within chromosome band 8p22 and gains within chromosome band 8q24.

detected by chromosome CGH and genomic microarrays prepared by other techniques. Based on these observations, RCA amplification of genomic clones offers advantages over other amplification methods for manufacturing genomic microarrays. Ongoing efforts to improve RCA amplification protocols coupled with improvement of detection and

analysis protocols can make RCA a method of choice for manufacturing large genomic microarrays.

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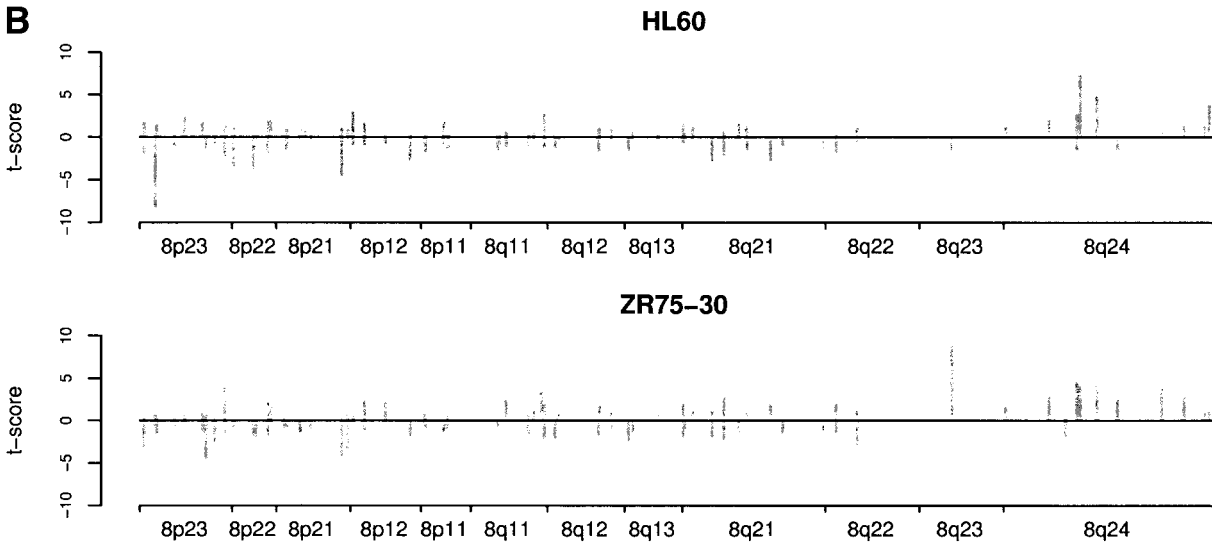


Figure 1. (Continued)

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