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An improved method for generating BAC DNA suitable for FISH

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Abstract. Fluorescence in situ hybridization (FISH) is commonly used to identify chromosomal aberrations such as translocations, deletions, duplications, gene fusions, and aneuploidies. It relies on the hybridization of fluorescently labeled DNA probes onto denatured metaphase chromosomes or interphase nuclei. These probes are often generated from DNA sequences cloned within bacterial artificial chromosomes (BACs). Growing these BACs in adequate amounts for FISH can be demanding. We describe FISH performed with bacteriophage Phi29 DNA polymerase amplified BAC DNA. Generating this material required significantly smaller cultures and less time than standard methods. The FISH results obtained were comparable with those obtained from standard BAC DNA. We believe this method of BAC DNA generation is useful for the entire FISH community as it improves considerably on prior methods.
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Fluorescence in situ hybridization (FISH) is a well established tool that is used in both basic research and in clinical diagnostics. In this technique, fluorescently labeled DNA probes hybridize to denatured metaphase chromosomes or interphase nuclei on a slide. The slide is then washed, counterstained, and analyzed by fluorescent microscopy. There are several different types of FISH probes including unique sequence probes, whole chromosome painting probes, repetitive probes, gene fusion probes and break-apart probes (Price, 1993; Wolff et al., 2007). Commonly, FISH probes are generated from DNA sequences cloned into bacterial artificial chromosomes (BACs). Libraries of BACs are commercially available but growing these clones in sufficient quantities for FISH is time consuming and laborious. BACs are plasmids present in bacteria at a low copy number (1–2 copies per cell). Unlike high copy number cloning systems (i.e. cosmids), the genomic inserts in BACs are more stable, but significantly larger cultures are necessary to generate the same amount of material (Shizuya and Kouros-Mehr, 2001). Here we describe FISH performed with whole genome amplification products of BAC DNA using bacteriophage Phi29 DNA polymerase. Phi29 DNA polymerase amplifies DNA through an isothermal strand displacement reaction and can generate micrograms of material from a few nanograms of template (Lovmar and Syvänen, 2006). The FISH results obtained with Phi29 DNA polymerase amplified BAC DNA and the results obtained from standard BAC DNA were compared.

Material and methods

BAC DNA clones, locus specific for the oncogenes *ERBB2* and *SEPT9*, were grown in 3 ml cultures overnight and purified with the Perfectprep Plasmid Mini kit (Eppendorf, Germany). Purification of BAC DNA involved a two step process. First, plasmid DNA was released from cells and separated from proteins, chromosomal DNA and large RNA through alkaline lysis. Next, the plasmid DNA was purified from small RNA, salts, and weakly bound substances through a column purification. After purification, BAC DNA was Phi29 DNA polymerase amplified using the GenomiPhi DNA Amplification kit (GE

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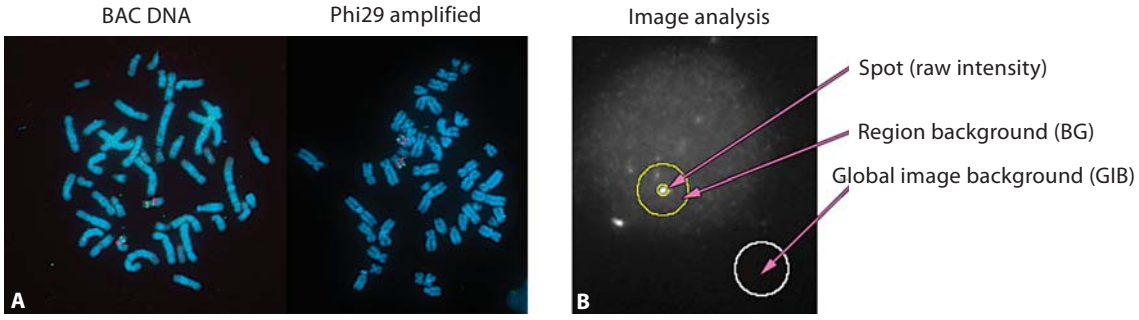

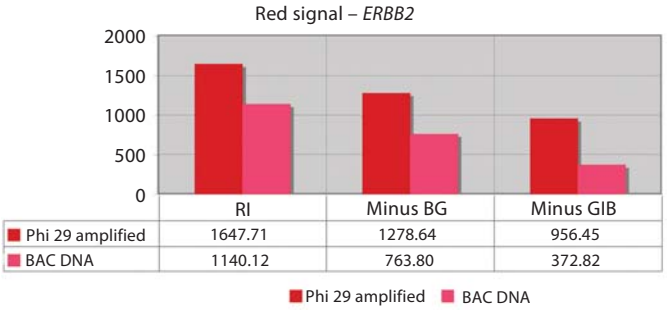

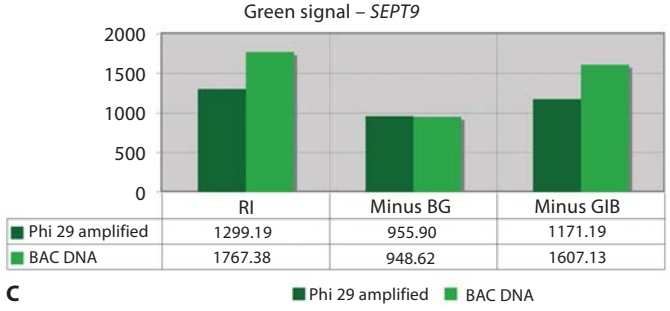

Fig. 1. Hybridization of locus specific probes and analysis of intensities of BAC DNA compared to Phi29 amplified probes. (**A**) Images of metaphase chromosomes of *SEPT9* (green) and *ERBB2* (red) locus specific probes. Both probes label chromosome 17 (17q25 and 17q12, respectively). Left panel BAC DNA, right panel Phi29 amplified material. (**B**) Identification of regions for pixel intensity measurements. Spot (Raw Intensity, RI) represents the gene locus of interest; Region Background (BG) represents the background within the nucleus; global image background (GIB) represents the background within the hybridization slide. (**C**) Average of pixel intensities measured for the three regions identified as in **B** for the *SEPT9* and the *ERBB2* locus.

Healthcare, UK). Amplifications were performed as described in the kit's manual with two key differences. The DNA was denatured with NaOH instead of heat to reduce excess nicking and a modified purification procedure was used to increase the recovery of high molecular weight DNA and to reduce recovery of random hexamers present in the GenomiPhi sample buffer. Briefly 10 ng of BAC DNA was combined with 5 μl of a 0.4 M NaOH, 0.4 mM EDTA solution and the volume increased to 10 μl with water. The mixture was incubated at room temperature for 5 min, 1.5 μl of 3 M acetic acid and 1 μl of glycogen were added and then the sample was mixed well. Subsequently, 100 μl of cold isopropanol was added, the sample precipitated and spun at 20,000 *g* for 10 min. Following centrifugation, the supernatant was not removed and 1 ml of 70% ethanol added to wash the pellet. After a second centrifugation at 20,000 *g* for 10 min, the supernatant was removed and the pellet allowed to air dry. The pellet was resuspended in 10 μl of GenomiPhi sample buffer. The sample was combined with 9 μl of GenomiPhi reaction buffer and 1 μl of Phi29 DNA polymerase and incubated at 30°C for 16 h and then 65°C for 10 min. 70 μl of water and 10 μl of a 1.5 M NaOAc, 200 mM EDTA solution were added and then the pellet was precipitated using 233.3 μl of 100% ethanol and a 15 min centrifugation at 20,817 *g*. The supernatant was removed and the pellet washed with 1 ml of 70% ethanol and a 5 min centrifugation at 20,000 *g*. It was resuspended in 52 μl of TE; 2 μl of this was used to quantify the DNA with a Nanodrop ND-1000 spectrophotometer. The Phi29 amplifications yielded *SEPT9* DNA at a concentration of 107 ng/μl and *ERBB2* DNA at a concentration of 114 ng/μl.

500 ng each of *ERBB2* and *SEPT9* Phi29 amplified DNA were labeled by nick translation. The reactions were carried out in 100 μl total volume (Tris HCl 0.05 M, $MgCl_2$ 5 mM, BSA 0.05 mg/ml, β-mercaptoethanol 0.01 M, dATP-dCTP-dGTP 0.05 mM, dTTP 0.01 mM, DNase I 0.02 ng/μl, DNA polymerase I 10 U) at 16°C for 30 min. *ERBB2* DNA was labeled with Spectrum Orange dNTP (1.6 nmol, Abbott Molecular, USA) and *SEPT9* DNA was labeled with Spectrum Green dNTP (1.6 nmol, Abbott Molecular). 5 μl of nick translated DNA was run on a 1.5% agarose gel to verify DNA size (0.5–1.6 kb). 10 μl of *ERBB2* Spectrum Orange labeled probe was combined with 10 μl of *SEPT9* Spectrum Green labeled probe and precipitated overnight (2.5 vol ethanol abs., 1/10 vol 3 M NaOAc, 10 μg CotI DNA). The DNA pellet was resuspended in 3 μl of 70% deionized formamide and 3 μl of master mix (50% Dextran sulfate, 4× SSC). After probe denaturation (5 min at 86°C) and reannealing (30 min at 37°C), probes were hybridized to pretreated (5 min in 10 mg/ml pepsin) denatured slides (1 min and 30 s at 86°C) at 37°C in a humidified chamber overnight. Metaphase chromosomes were derived from normal B lymphocytes as previously described (http://www.riedlab.nci.nih.gov/protocols.asp). Control reference probes of unamplified *ERBB2* and *SEPT9* BAC DNA were labeled and hybridized under the same conditions. After hybridization, slides were washed three times in 50% formamide and 2× SSC at 45°C and subsequently in 1× SSC at 45°C. Slides were stained with DAPI and mounted with antifade (phenylene diamine).

Results and discussion

Metaphase cells were imaged with an Olympus BX61 microscope with an UPlanSApo 100× N.A. 1.4 objective, an Hg arc lamp for excitation and narrow band filters for all fluorescent emission and equipped with a Cooke SensicamQE camera with IPLab for image acquisition (Fig. 1A). Images of metaphase chromosomes and interphase cells for each slide were acquired for the Spectrum Orange and Spectrum Green dyes (intensities of 20 to 36 spots were analyzed for each locus specific signal). Exposure time was set to 800 and 1,200 ms for the green and red channels, respectively. Exposure time was determined on the first acquisition and kept unchanged for the subsequent image acquisitions (for both Phi29 polymerase amplified and unamplified BAC DNA). After image acquisition, areas for pixel intensity determination were selected as follows: spot area (Raw Intensity, RI) was selected as the locus identified from the DNA probe and a circle was drawn around the brightest pixel intensities; region background area (BG) was selected as 'concentrical' to the spot area and includes the background of the cell nucleus; and global image background (GIB) was selected in a region of the hybridization slide where no cells were present (Fig. 1B). Raw intensities were measured within the three selected areas with image processing analysis tools available through ImageJ (http://rsb.info.nih.gov/ij/). Pixel intensities were averaged for all the spots measured in the Spectrum Orange and Spectrum Green channels (Fig. 1C, RI columns). Region background and global image background were subtracted from raw measurements (RI – BG and RI – GIB) to obtain locus specific intensities (Fig. 1C, Minus BG and Minus GIB, respectively). *ERBB2* and *SEPT9* average raw intensities for Phi29 amplified BAC DNA and unamplified BAC DNA are within the same range, 1,647 a.i. vs. 1,140 a.i. (arbitrary units = intensity directly proportional to number of photons) and 1,299 a.i. vs. 1,767 a.i. respectively for the Spectrum Orange and Spectrum Green signals. After BG and GIB subtraction, the signal intensities remain within comparable ranges (Fig. 1C). Our results indicate that the quality of hybridization and the intensities of locus specific signals generated by hybridizing BAC DNA and Phi29 amplified DNA are similar.

Since Phi29 amplified BAC DNA probes are reliable for FISH, there is no longer a need to grow large cultures to obtain sufficient quantities for the technique. We have found 3 ml cultures grown overnight and purified with Eppendorf's Perfectprep Plasmid Mini kit sufficient. Also subsequent to this work, we have begun using the GenomiphiV2 kit for our applications with similar results. This kit requires only 2 h incubation at 30°C.

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