Technical Advance

Subtracted, Unique-Sequence, *In Situ* Hybridization *Experimental and Diagnostic Applications*

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Nonrandom chromosomal aberrations, particularly in cancer, identify pathogenic biological pathways and, in some cases, have clinical relevance as diagnostic or prognostic markers. Fluorescence and colorimetric *in situ* hybridization methods facilitate identification of numerical and structural chromosome abnormalities. We report the development of robust, unique-sequence *in situ* hybridization probes that have several novel features: 1) they are constructed from multimegabase contigs of yeast artificial chromosome (YAC) clones; 2) they are in the form of adapter-ligated, short-fragment, DNA libraries that may be amplified by polymerase chain reaction; and 3) they have had repetitive sequences (eg, *Alu* and LINE elements) quantitatively removed by subtractive hybridization. These subtracted probes are labeled conveniently, and the fluorescence or colorimetric detection signals are extremely bright. Moreover, they constitute a stable resource that may be amplified through at least four rounds of polymerase chain reaction without diminishing signal intensity. We demonstrate applications of subtracted probes for the *MYC* and *EWS* oncogene regions, including 1) characterization of a novel *EWS*-region translocation in Ewing's sarcoma, 2) identification of chromosomal translocations in paraffin sections, and 3) identification of chromosomal translocations by conventional bright-field microscopy. (*Am J Pathol* 1998, 153:1401–1409)

tological, mesenchymal, neural, and epithelial neoplasms, and, in some cases, the specific oncogene and tumor suppressor gene targets have been characterized.² One major goal in studying cancer chromosome and gene aberrations is to elucidate biological pathways responsible for neoplastic transformation. Indeed, many such pathways have been identified through characterization of particular cancer chromosome aberrations.^{1,3} Another goal, in cancer chromosome evaluations, is identification and validation of novel diagnostic and prognostic markers.^{4,5} Cytogenetic markers have potential utility as histological adjuncts, and methods that delineate diagnostic chromosomal aberrations, or associated molecular changes, are increasingly important in experimental pathology.^{6–8}

In situ hybridization (ISH) is a robust analytic method that enables evaluation of numerical and structural chromosome aberrations in both fresh and archival tumor specimens^{9–13} and has extended the capabilities of conventional tumor karyotyping.^{14–17} Particular advantages include applicability to interphase cell populations and the ability to demonstrate, unambiguously, chromosome rearrangements that target specific loci of interest.^{12,18–20} Several technical innovations have permitted the application of ISH in genome-wide screening studies. Comparative genomic hybridization (CGH),²¹ combinatorial multicolor ISH,²² and multicolor spectral karyotyping,²³ all provide a broad perspective, complementary to that afforded by chromosome banding methods.

Our general aim, in the present studies, was development of novel unique-sequence genomic probes suitable for both fluorescence and colorimetric ISH. The specific aims were to create libraries of DNA fragments that could be labeled by polymerase chain reaction (PCR), random priming, or nick translation, that could be hybridized in the absence of competitor DNAs, and that would constitute a virtually permanent resource of ISH probe DNA. These aims have been accomplished by 1) assembling

Alteration of chromosome structure is a key tumorigenic mechanism in most human cancers.¹ Recurrent large-scale chromosomal abnormalities, particularly translocations and deletions, have been identified in many hema-

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Figure 1. CEPH mega-YAC clones flanking the EWS and MYC regions. Clone insert sizes are those reported by CEPH. Gaps between the contigs are estimates based on published mapping data.

contigs of yeast artificial chromosome (YAC) clones that cover multimegabase genomic regions and yield bright ISH signals, 2) developing subtraction protocols that enable production of repeat-free libraries of adapter-ligated DNA fragments from YAC and other large insert clones, and 3) demonstrating the stability of the subtracted DNA library after multiple rounds of amplification by PCR. Diagnostic and experimental applications are described for subtracted, unique-sequence probe sets that detect rearrangements in the *MYC* gene region of chromosome 8 and the *EWS* gene region of chromosome 22.

Materials and Methods

Selection of YACs for MYC and EWS Translocation Detection

The *MYC* gene maps to chromosome subband 8q24.1 and is involved in three well characterized translocations in Burkitt lymphomas.^{24,25} The *EWS* gene maps to chromosome band 22q12 and is involved in at least eight different translocations in soft-tissue tumors.²⁶⁻³⁴ YAC clones centromeric and telomeric to these genes were selected based on published maps³⁵⁻³⁷ that were cross-referenced with physical mapping data from the Whitehead Institute Center for Genome Research and CEPH-Génethon websites (<http://www-genome.wi.mit.edu> and <http://www.cephb.fr>, respectively). YAC clones were selected based on appropriate map location and absence of features suggesting chimerism. Potential chimerism was determined using both sequence-tagged site (STS) and *Alu*-PCR data from the Whitehead Institute and CEPH. All YAC clones were obtained from Research Genetics (Huntsville, AL), and YAC DNAs were isolated

as described previously.¹³ Chimerism was evaluated formally by fluorescence ISH (FISH) against normal male lymphocyte metaphase and interphase preparations,³⁸ and chimeric clones were excluded from the final contigs. Figure 1 depicts the YAC clones that comprise the centromeric and telomeric contigs flanking *MYC* and *EWS*.

Subtractive Hybridization

Genomic subtractive hybridization removes sequences from a tracer DNA population by hybridizing with a molar excess of driver DNA.³⁹ The driver DNA is chemically modified such that it may be selectively removed from solution along with driver-tracer hybrid molecules. *MYC*- and *EWS*-region YAC contigs (Figure 1) were repeatedly hybridized with a 40-fold excess of biotinylated YACs containing abundant repetitive, eg, *Alu* and LINE element, sequences. Consequently, repetitive sequences present in the *EWS*- and *MYC*-region contigs were quantitatively removed.

Tracer Preparation

Tracer was prepared by combining 2 μ g of each clone from a particular contig. These pooled DNAs were then sonicated to 0.1 to 8 kb and size-fractionated on 1.5% agarose gels. The 0.4- to 2-kb fractions were cut from the gels, purified using QIAquick gel extraction kits (Qiagen, Santa Clarita, CA), blunt ended, and ligated to the T-1/T-2 adapter. The T-1/T-2 adapter was constructed by annealing polyacrylamide gel electrophoresis (PAGE)-purified oligos 5'CTGAGCGGAATTCGTGAGACC (T-1) and 5'(PO₄)GGTCTCACGAATTCGCTCA GTT (T-2).⁴⁰ Adapter-ligated fragments were then PCR amplified, in

multiple 25- μ L reactions, using the T-1 sequence as primer. Amplified fragments were purified using QIAquick PCR purification kits (Qiagen) and then eluted in 1 mmol/L Tris/Cl, pH 8.0. PCR reactions here and elsewhere, unless otherwise indicated, were done in 25- μ L volumes using KlenTaq reaction buffer (Clontech, Palo Alto, CA), 0.2 mmol/L dNTPs, 1.2 μ mol/L PAGE-purified primer, and 0.1 U/ μ L KlenTaq DNA polymerase (Clontech). PCR cycling conditions were 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 3 minutes for 25 to 30 cycles, followed by 72°C for 9 minutes.

Driver Preparation

Biotinylated driver DNA was prepared from three nonchimeric chromosome 21 YAC clones (746-b-10, 745-c-11, 615-c-9) that are known to be rich in repetitive sequences.⁴¹ YAC DNA was isolated, as described,⁴² from a 500-ml culture consisting of all three clones. Nucleic acid thus isolated was precipitated twice in 6.5% polyethylene glycol/0.8 mol/L sodium chloride, washed in 70% ethanol, and dissolved in distilled water. Twenty micrograms of this DNA was sonicated and size fractionated as described above. Fragments ranging in size from 0.4 to 2 kb were gel purified, blunt ended, and ligated to a D-40/D-41 adapter constructed by annealing PAGE-purified oligos 5'-AATTCTTGCGCCTTAAACCAAC (D-40) and 5'-(PO₄)GTTGGTTTAAGGCGCAAG (D-41). PCR was performed using 5'-(biotin)AATTCTTGCGCCTTAAACCAAC (D-40B) as primer. A second round of PCR was performed using the first-round product as the template. In the second round of PCR, the concentration of D-40B was increased to 6 μ mol/L, the dNTP concentration was increased to 0.4 mmol/L, and the number of cycles was reduced to 20. Several hundred micrograms of biotinylated YAC driver were generated in multiple 25- μ L reactions. The biotinylated PCR products were purified using QIAquick PCR purification kits (Qiagen), precipitated in ethanol, and dissolved at 1.5 μ g/ μ L in EE buffer (10 mmol/L Na *N*-[2hydroxyethyl]piperazine-*N'*-[3-propane-sulfonic acid], 1 mmol/L EDTA, pH 8.0).

Subtraction

Subtraction was performed by mixing 250 ng of tracer DNA with 10 μ g of biotinylated driver DNA, 2 μ g of T-1, 5 μ g of D-41, and 20 μ g of yeast tRNA as carrier. This mixture was denatured at 99°C for 1 minute, lyophilized, redissolved in 5 μ L of EE buffer/1 mol/L NaCl, and then incubated at 65°C for 24 to 48 hours. Biotinylated molecules (including tracer-driver hybrids) were removed using avidin-polystyrene beads as described.⁴³ Remaining unbiotinylated tracer fragments were precipitated in ethanol before proceeding with the next round of subtraction. Each of three rounds of subtraction was performed exactly as described above. After the third round, remaining tracer fragments were amplified by PCR using the T-1 sequence as primer.

Dual-Color ISH

Probe Preparation

The subtracted contigs telomeric to the *EWS* and *MYC* loci (*EWS*.T and *MYC*.T, respectively, Figure 1) were labeled with biotin using the BioPrime random octamer priming kit (Gibco BRL/Life Technologies, Gaithersburg, MD). The subtracted contigs centromeric to the *EWS* and *MYC* loci (*EWS*.C and *MYC*.C, respectively, Figure 1) were labeled with fluorescein isothiocyanate (FITC), also by random octamer priming. The final nucleotide concentrations for FITC labeling were 0.2 mmol/L dCTP, 0.2 mmol/L dGTP, 0.2 mmol/L dATP, 0.1 mmol/L dTTP, and 0.1 mmol/L FITC-12-dUTP (NEN, Boston, MA). Residual primers and unincorporated nucleotides were removed by S-200HR spin-column chromatography (Pharmacia, Uppsala, Sweden). The purified products were precipitated in ethanol and dissolved in a solution containing 50% formamide, 10% dextran sulfate, and 2X SSC (0.3 mol/L sodium chloride, 0.03 mol/L sodium citrate, pH 7.0).

Slide Preparation

Formalin-fixed, paraffin-embedded, 4- μ m tissue sections were applied to silanized slides, baked at 65°C for 16 hours, and stored at room temperature. Slides were processed for ISH using Oncor Tissue kits (Oncor, Gaithersburg, MD) according to the manufacturer's specifications with minor variations. Briefly, after deparaffination in xylene and dehydration in 100% ethanol, all tissue sections were incubated in 30% pretreatment solution for 15 minutes, followed by 15 to 40 minutes of protease treatment. Digestion times were optimized on a case-by-case basis. Slides were denatured in a solution containing 70% formamide, 2X SSC, pH 7.0, at 75°C for 8 minutes. Slides were dehydrated in ice-cold 70%, 85%, and 95% ethanol and then air dried. Cytogenetic preparations were processed as described previously.¹³

Hybridization and Detection

Aliquots of labeled DNA were diluted to a concentration of 100 to 200 ng/ μ L in hybridization solution (50% formamide, 10% dextran sulfate, 2X SSC), denatured at 75°C for 5 minutes, placed immediately onto denatured slides, and covered with a glass coverslip that was sealed with rubber cement. The slides were then placed in a humidified chamber at 37°C for 12 to 16 hours. Slides were washed in 0.5X SSC at 72°C for 5 minutes and then in PN buffer (0.1 mol/L sodium phosphate, pH 8.0, 0.1% Nonidet P-40) at room temperature. Biotinylated probes were detected with 5 μ g/ml Texas Red-avidin DCS (Vector Laboratories, Burlingame, CA). FITC-labeled probes were visualized directly and, in some cases, amplified using FluorAmp kits (Oncor) according to the manufacturer's specifications. FluorAmp consists of mouse anti-FITC followed by FITC-conjugated goat anti-mouse. For colorimetric detection, sequential peroxidase reactions were performed using horseradish peroxidase (HRP)-conjugated goat anti-FITC (Zymed Laboratories, South

Figure 2. Dot blot of subtracted and unsubtracted EWS.C and EWS.T tracer DNAs, probed with Cot-1 and total yeast genomic DNA. Amounts of 300, 100, 30, 10, and 1 ng (left to right) of yeast genomic DNA (AB1380) and each tracer DNA were spotted on the membrane. Both Cot-1 (A) and total yeast genomic DNA (B) hybridize to unsubtracted DNAs but not to their subtracted counterparts, demonstrating removal of both human repetitive sequences and yeast sequences after three rounds of subtractive hybridization.

San Francisco, CA) with the diaminobenzidine (DAB) substrate kit (Zymed), followed by HRP-conjugated streptavidin (Zymed) with the VIP substrate kit (Vector).⁴⁴ Cells were counterstained with Gill's hematoxylin (Vector) and mounted in Permount (Sigma-Aldrich Corp., St. Louis, MO).

Dot Blotting

Total genomic DNA from *Saccharomyces cerevisiae* strain AB1380 (a negative control) as well as subtracted and unsubtracted tracer DNA (EWS.C and EWS.T) were evaluated. Amounts of 300, 100, 30, 10, and 1 ng of each DNA were denatured and spotted onto a positively charged nylon membrane, which was then baked. The membrane was probed successively with radiolabeled human Cot-1 DNA (Gibco BRL/Life Technologies) and total yeast genomic DNA (strain AB1380).

Results

Subtraction of Human and Yeast Repetitive Sequences

Dot blots of subtracted and unsubtracted tracer DNAs were evaluated for the presence of human repetitive sequences using human Cot-1 (repetitive-sequence-enriched) DNA as probe. This experiment demonstrated complete subtraction of human repetitive sequences from the tracer DNAs (Figure 2A). Reprobing with total yeast genomic DNA demonstrated substantial removal of yeast sequences (Figure 2B). All subtracted tracer DNA libraries were then evaluated as

FISH probes, in the absence of Cot-1 competitor DNA or preannealing. FISH signals localized exclusively to the expected chromosome regions, and signal intensities were identical to those obtained using unsubtracted DNAs (the latter preannealed and hybridized in the presence of excess Cot-1 DNA to suppress nonspecific background staining). Potential PCR-related biases were evaluated by subjecting the subtracted probe pools to four successive rounds of PCR amplification. Probe aliquots were labeled after each round of amplification and were hybridized against two different slides. No diminution in probe signal intensity was seen after four rounds of amplification (Figure 3, A and B). This experiment was validated by repeating the four rounds of PCR amplification using another aliquot of subtracted probe. Again, there was no fall-off in FISH signal intensity after the fourth round of PCR. The Gibco BioPrime random octamer labeling approach permitted substantial amplification of the template DNA during the labeling process.⁴⁵ Typical yields, starting with 200 ng of subtracted tracer, were 5–10 μ g (25–50 fold amplification) of labeled DNA after a 5-hour, 50- μ l random priming reaction. PCR incorporation, using the T-1 adapter primers with biotin- or digoxigenin-conjugated nucleotides, was an equally effective alternative to random priming (J.M. Davison and J.A. Fletcher, unpublished observations).

Application 1: Evaluation of EWS-Region Rearrangements

The subtracted EWS-region ISH probe set was evaluated against 1) a primary cutaneous Ewing's sarcoma, 2) a

Figure 3. Dual-color FISH with EWS and MYC probe sets. Probes on the centromeric and telomeric sides of the genes are detected using FITC (green) and Texas Red, respectively. **A:** Unsubtracted EWS.C/EWS.T probes hybridized to a primary Ewing's sarcoma in the presence of Cot-1 competitor DNA. Split red and green signals indicate EWS-region rearrangement of chromosome 22. **B:** Fourth-generation subtracted EWS.C/EWS.T probes hybridized without Cot-1 to the same primary Ewing's sarcoma as in **A**. FISH signals localize exclusively to the expected regions and signal intensities are undiminished after the four rounds of PCR amplification. **C:** FISH with subtracted EWS.C/EWS.T probes in 4- μ m section of paraffin-embedded primary cutaneous Ewing's sarcoma. Split signals indicate EWS-region rearrangement. **D:** FISH with subtracted EWS.C/EWS.T probes in 4- μ m section of paraffin-embedded clear-cell sarcoma. Split signals indicate EWS-region rearrangement. **E:** FISH with subtracted EWS.C/EWS.T probes in a primary Ewing's sarcoma. One EWS.T probe is translocated to chromosome band 1q42 (arrow), localizing a potential novel EWS translocation partner. **F:** FISH with subtracted MYC.C/MYC.T probes in interphase Burkitt lymphoma cells from a pleural effusion. The split probes establish a MYC-region rearrangement. **G:** FISH with subtracted MYC.C/MYC.T probes in a metaphase cell from the same pleural effusion shown in **F**. One MYC.T probe is translocated to chromosome 14 (arrow), consistent with an immunoglobulin heavy chain gene rearrangement. **H:** Colorimetric detection of MYC.C (DAB; brown) and MYC.T (VIP; purple) in interphase (upper panel) and metaphase (lower panel) cells from the same Burkitt lymphoma shown in **F** and **G**. The MYC.T translocation, to chromosome 14, is indicated by an arrow (lower panel).

clear-cell sarcoma, and 3) a tibial Ewing's sarcoma that lacked, cytogenetically, a typical t(11;22). The cutaneous Ewing's sarcoma, as reported previously,⁴⁶ was an axillary lesion in a 19-year-old woman. The clear-cell sarcoma was a right knee mass in a 39-year-old woman. The histological differential diagnosis, for the knee mass, included both clear-cell sarcoma (melanoma of soft parts) and metastatic cutaneous melanoma. Greater than 65% of clear-cell sarcomas contain a t(12;22)(q13;q12), resulting in fusion of *EWS* and *ATF-1*,²⁷ whereas this translocation has not been reported in cutaneous malignant melanoma. FISH analysis of 4- μ m paraffin sections revealed splitting of one *EWS.C/EWS.T* probe pair, consistent with *EWS*-region rearrangement, in both the cutaneous Ewing's sarcoma (Figure 3C) and putative clear-cell sarcoma (Figure 3D). The tibial Ewing's sarcoma, diagnosed in a 17-year-old boy, had a cytogenetically aberrant chromosome 22 homolog, whereas chromosomes 2, 7, 11, 17, and 21 (chromosomes containing ETS family genes involved in known Ewing's sarcoma *EWS* fusions) were unremarkable by banding analysis. *EWS.C/EWS.T* FISH evaluation revealed a reciprocal translocation, t(1;22)(q42;q12), of the *EWS* region (Figure 3E), and reverse transcriptase PCR was negative for *EWS-FLI1* or *EWS-ERG* fusion transcripts (C.-J. Chen and J.A. Fletcher, unpublished data). These data support a unique *EWS* translocation, potentially involving a novel ETS family locus on chromosome band 1q42.

Application 2: Evaluation of MYC-Region Rearrangement

The subtracted *MYC*-region ISH probe was evaluated against a malignant pleural effusion in which cytological evaluation, but not immunophenotype, was classical for Burkitt's lymphoma. The pleural fluid was from a 10-year-old boy with a 2-month history of anorexia and abdominal pain and with radiological evidence of mesenteric/mediastinal adenopathy and bilateral pleural effusions. Cytological evaluation revealed a homogeneous population of small lymphoid cells with bluish vacuolated cytoplasm and round nongrooved nuclei, whereas flow immunophenotyping was notable for the absence of surface immunoglobulin. Cytogenetic banding studies were inconclusive because the chromosome morphology was poor. FISH analysis, using the subtracted *MYC.C/MYC.T* probe set, demonstrated *MYC*-region rearrangement in metaphase (Figure 3F) and interphase cells (Figure 3G). *MYC*-region rearrangement was also demonstrated convincingly by colorimetric detection (Figure 3H).

Discussion

The general aim in these studies was development of novel approaches that expand the role of *in situ* hybridization in diagnostic and research applications. ISH probes derived from pericentromeric α -satellite repeat sequences have been used extensively to evaluate numerical chromosome aberrations. Many molecular cyto-

genetic research and diagnostic questions, however, are best addressed using unique sequence probes to particular chromosome regions. Large-insert clones, eg, BAC, PAC, P1, and YAC clones, are suitable for molecular cytogenetic applications, including those using paraffin-embedded material. The fluorescence signals obtained using large-insert clones are often as bright as those obtained with α -satellite probes, particularly when the human genomic inserts are rich in unique sequences. As we demonstrate, these large probes are also suitable for bright-field studies, using either peroxidase/alkaline phosphatase (B.-L. Hsi, unpublished observations) or sequential peroxidase detection strategies.

In addition to the potential for excellent ISH signal intensity, a major advantage of mega-YACs is the wealth of physical mapping data accessible via the internet at websites such as those of the Whitehead Institute and CEPH (see Materials and Methods). These data facilitate selection of mega-YAC clones in a region of interest. We tested multiple mega-YACs in the *EWS* and *MYC* regions and selected clones with superior ISH signals for inclusion in the final contigs (Figure 1). The probes reported here are YAC contigs spanning 1.5 to 5.0 megabases. This contig size was associated with excellent ISH signal intensity without sacrificing compactness of the signals.

We also report adaptation of subtractive hybridization to the synthesis of ISH probes. Subtractive hybridization has been used primarily for analysis of differences between two DNA populations, whether these be expressed (cDNA) or genomic sequences.⁴⁷⁻⁵¹ Subtraction enables enrichment of the population of interest, or tracer DNA, for sequences absent or substantially underrepresented in the driver. We used subtraction to remove repetitive sequences, while preserving unique sequences, in the mega-YAC contigs. The physical basis of subtraction lies in the kinetics of DNA annealing in solution.³⁹ Due to the presence of molar excesses of biotinylated driver DNA, denatured tracer repetitive sequences are far more likely to anneal to their counterparts in the driver population rather than to each other. Biotinylated molecules, including driver-tracer hybrids, are subsequently removed from solution using a matrix of avidin-conjugated beads. Our protocol, based on the subtraction methods developed by Straus et al,^{52,53} was designed to maximize both quantitative removal of repetitive sequences and retention of sequence complexity in the tracer. A related method, in which biotinylated Cot-1 DNA is used to subtract human repeats from chromosome microdissection probes,⁵⁴ was reported recently by Craig et al.⁵⁵ Our method differs in that yeast sequences are included in the driver DNA library. Hence, both yeast and repetitive human sequences are removed efficiently from the tracer (probe) libraries.

The subtraction approach described herein accomplishes two objectives. The first is repetitive-sequence removal, and the second is creation of a library of DNA fragments that can be manipulated readily for large-scale DNA preparation and labeling. Repetitive sequences in ISH probes are generally competed through preannealing with total genomic or repeat-sequence-enriched DNA.^{11,56} Preannealing is effective, but this step adds

both expense and time to the ISH protocol. In addition, some labeled repetitive sequences will likely come in contact with the slide. It is fundamentally desirable to remove these sequences from the probe altogether, thus eliminating a potential source of nonspecific fluorescence.

We demonstrate that subtracted mega-YAC probes may be amplified through at least four rounds of PCR without diminishing the ISH signal intensity. Each 25-cycle round of PCR amplifies the starting material 500- to 1000-fold. Additional amplification is then achieved using random octamer priming⁴⁵ or PCR to incorporate labeled nucleotides. Hence, subtraction converts 250 ng of adapter-ligated tracer DNA into a virtually permanent resource that can serve to generate kilogram amounts of repeat-sequence-depleted probe for *in situ* hybridization. This approach is particularly advantageous in the case of CEPH mega-YACs, which are known to be unstable in the strain of yeast in which they were cloned.^{57,58} CEPH mega-YACs often develop large deletions due to recombination and selection events. By converting contigs of mega-YAC clones into adapter-ligated libraries, we have created stable and readily amplifiable probes.

There are several well established techniques for production of large-insert ISH probes. These include *Alu*-PCR and DOP-PCR, which are rapid and universally applicable methods for amplification of YAC or bacterial artificial chromosome human inserts.⁵⁹⁻⁶¹ *Alu*-PCR is performed using *Alu*-sequence primers. An advantage in this approach is that *Alu*-containing, ie, human, sequences are amplified. However, the amplified sequences are limited primarily to those situated between closely neighboring, and appropriately oriented, *Alu* repeats, thus biasing the final pool of amplified DNA fragments. DOP-PCR is performed using degenerate oligonucleotide primers. This method, although nonselective for human *versus* bacterial or yeast sequences, may enable a more complex and representative amplification of the human insert than does *Alu*-PCR. Both *Alu*-PCR and DOP-PCR are effective and straightforward methods requiring substantially less up-front effort than subtraction. However, neither approach is designed to generate probes depleted of repetitive sequences. In our experience, *Alu*-PCR and DOP-PCR YAC probes are generally associated with a lower signal-to-noise ratio, despite Cot-1 preannealing, than subtracted probes. DOP-PCR chromosome painting probes, on the other hand, have been extremely successful.^{54,62}

The *EWS* and *MYC* probe sets described herein are particularly effective in a screening mode. *EWS* translocations are found in Ewing's sarcoma, clear-cell sarcoma, desmoplastic small-round-cell tumor, extraskeletal myxoid chondrosarcoma, and (infrequently) myxoid liposarcoma. At least nine different partner genes participate in the translocation-related *EWS* fusion oncogenes in these tumors.²⁶⁻³⁴ The *EWS* ISH screening approach allows efficient evaluation of an *EWS* rearrangement in any of the aforementioned tumors. In cases with abnormal ISH patterns, the specific fusion oncogene can then be established, using appropriate oligonucleotide primers, by reverse transcription PCR. As we have demon-

strated, this screening approach also enables localization of previously uncharacterized translocation partners (Figure 3E). *MYC* translocations are also particularly amenable to ISH detection. Translocation breakpoints, in HIV-associated and endemic Burkitt lymphomas, are often 100 to 300 kb upstream of *MYC*,⁶³⁻⁶⁵ whereas translocation breakpoints in most sporadic Burkitt lymphomas involve *MYC* exon 1 or intron 1.^{64,65} However, 10% to 20% of sporadic Burkitt lymphomas contain rearrangements of κ or λ light chain loci, and these cases typically have translocation breakpoints 200 to 300 kb downstream of *MYC*.⁶⁶ We designed a *MYC* ISH probe set with a 500-kb gap on either side of the gene (Figure 1) such that virtually all *MYC* translocations, whether upstream, intragenic, or downstream, are detected.

In summary, we report a new method for constructing and synthesizing DNA probes from multimegabase YAC contigs. This method enables creation of adapter-ligated DNA libraries that are free of repetitive sequences. We demonstrate that subtracted unique-sequence probes are detected readily using standard fluorescence and colorimetric reagents. The probes are labeled conveniently and are hybridized without competitor DNA or preannealing, thus simplifying the *in situ* hybridization protocol.

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References

1. Rabbitts TH: Chromosomal translocations in human cancer. *Nature* 1994, 372:143-149
2. Mitelman F, Mertens F, Johansson B: A breakpoint map of recurrent chromosomal rearrangements in human neoplasia. *Nature Genet* 1997, 15:417-474
3. Look AT: Oncogenic transcription factors in the human acute leukemias. *Science* 1997, 278:1059-1064
4. Delattre O, Zucman J, Melot T, Garau XS, Zucker JM, Lenoir GM, Ambros PF, Sheer D, Turc-Carel C, Triche TJ, Aurias A, Thomas G: The Ewing family of tumors: a subgroup of small-round-cell tumors defined by specific chimeric transcripts. *N Engl J Med* 1994, 331:294-299
5. Sandberg AA, Turc-Carel C, Gemmill RM: Chromosomes in solid tumors and beyond. *Cancer Res* 1988, 48:1049-1059
6. Sreekantaiah C, Ladanyi M, Rodriguez E, Chaganti RS: Chromosomal aberrations in soft tissue tumors: relevance to diagnosis, classification, and molecular mechanisms. *Am J Pathol* 1994, 144:1121-1134
7. Speel EJ, Ramaekers FC, Hopman AH: Cytochemical detection systems for *in situ* hybridization and the combination with immunocytochemistry: Who is still afraid of red, green and blue? *Histochem J* 1995, 27:833-858
8. Hopman AH, Claessen S, Speel EJ: Multi-colour brightfield *in situ* hybridisation on tissue sections. *Histochem Cell Biol* 1997, 108:291-298
9. Hopman AH, Poddighe PJ, Smeets AW, Moesker O, Beck JL, Vooijs GP, Ramaekers FC: Detection of numerical chromosome aberrations

- in bladder cancer by *in situ* hybridization. *Am J Pathol* 1989, 135: 1105-1117
10. Cremer T, Lichter P, Borden J, Ward DC, Manuelidis L: Detection of chromosome aberrations in metaphase and interphase tumor cells by *in situ* hybridization using chromosome-specific library probes. *Hum Genet* 1988, 80:235-246
11. Pinkel D, Landegent J, Collins C, Fuscoe J, Segraves R, Lucas J, Gray J: Fluorescence *in situ* hybridization with human chromosome-specific libraries: detection of trisomy 21 and translocations of chromosome 4. *Proc Natl Acad Sci USA* 1988, 85:9138-9142
12. Tkachuk DC, Westbrook CA, Andreeff M, Donlon TA, Cleary ML, Suryanarayan K, Homge M, Redner A, Gray J, Pinkel D: Detection of bcr-abl fusion in chronic myelogenous leukemia by *in situ* hybridization. *Science* 1990, 250:559-562
13. Xiao S, Renshaw AA, Cibas ES, Hudson TJ, Fletcher JA: Novel fluorescence *in situ* hybridization approaches in solid tumors: characterization of frozen specimens, touch preparations, and cytologic preparations. *Am J Pathol* 1995, 147:896-904
14. Poddighe PJ, Moesker O, Smeets D, Awwad BH, Ramaekers FC, Hopman AH: Interphase cytogenetics of hematological cancer: comparison of classical karyotyping and *in situ* hybridization using a panel of eleven chromosome specific DNA probes. *Cancer Res* 1991, 51:1959-1967
15. Gray JW, Pinkel D: Molecular cytogenetics in human cancer diagnosis. *Cancer* 1992, 69:1536-1542
16. Wolman SR: Fluorescence *in situ* hybridization: a new tool for the pathologist. *Hum Pathol* 1994, 25:586-590
17. Tiainen M, Hopman A, Moesker O, Ramaekers F, Wessman M, Laasonen A, Pyrhonen S, Tammilehto L, Mattson K, Knuutila S: Interphase cytogenetics on paraffin sections of malignant pleural mesothelioma: a comparison to conventional karyotyping and flow cytometric studies. *Cancer Genet Cytogenet* 1992, 62:171-179
18. Manuelidis L, Langer-Safer PR, Ward DC: High-resolution mapping of satellite DNA using biotin-labeled DNA probes. *J Cell Biol* 1982, 95:619-625
19. Pinkel D, Straume T, Gray JW: Cytogenetic analysis using quantitative, high-sensitivity, fluorescence hybridization. *Proc Natl Acad Sci USA* 1986, 83:2934-2938
20. Lichter P, Tang CJ, Call K, Hermanson G, Evans GA, Housman D, Ward DC: High-resolution mapping of human chromosome 11 by *in situ* hybridization with cosmid clones. *Science* 1990, 247:64-69
21. Kallioniemi A, Kallioniemi OP, Sudar D, Rutovitz D, Gray JW, Waldman F, Pinkel D: Comparative genomic hybridization for molecular cytogenetic analysis of solid tumors. *Science* 1992, 258:818-821
22. Speicher MR, Gwyn Ballard S, Ward DC: Karyotyping human chromosomes by combinatorial multi-fluor FISH. *Nature Genet* 1996, 12: 368-375
23. Schrock E, du Manoir S, Veldman T, Schoell B, Wienberg J, Ferguson-Smith MA, Ning Y, Ledbetter DH, Bar-Am I, Soenksen D, Garini Y, Ried T: Multicolor spectral karyotyping of human chromosomes. *Science* 1996, 273:494-497
24. Taub R, Kirsch I, Morton C, Lenoir G, Swan D, Tronick S, Aaronson S, Leder P: Translocation of the c-myc gene into the immunoglobulin heavy chain locus in human Burkitt lymphoma and murine plasmacytoma cells. *Proc Natl Acad Sci USA* 1982, 79:7837-7841
25. Davis M, Malcolm S, Rabbitts TH: Chromosome translocation can occur on either side of the c-myc oncogene in Burkitt lymphoma cells. *Nature* 1984, 308:286-288
26. Delattre O, Zucman J, Plougastel B, Desmaze C, Melot T, Peter M, Kovar H, Joubert I, de Jong P, Rouleau G, Aurias A, Thomas G: Gene fusion with an ETS DNA-binding domain caused by chromosome translocation in human tumours. *Nature* 1992, 359:162-165
27. Zucman J, Delattre O, Desmaze C, Epstein AL, Stenman G, Speleman F, Fletcher CD, Aurias A, Thomas G: EWS and ATF-1 gene fusion induced by t(12;22) translocation in malignant melanoma of soft parts. *Nature Genet* 1993, 4:341-345
28. Sorensen PH, Lessnick SL, Lopez-Terrada D, Liu XF, Triche TJ, Denny CT: A second Ewing's sarcoma translocation, t(21;22), fuses the EWS gene to another ETS-family transcription factor, ERG. *Nature Genet* 1994, 6:146-151
29. Ladanyi M, Gerald W: Fusion of the EWS and WT1 genes in the desmoplastic small round cell tumor. *Cancer Res* 1994, 54:2837-2840
30. Jeon IS, Davis JN, Braun BS, Sublett JE, Roussel MF, Denny CT, Shapiro DN: A variant Ewing's sarcoma translocation (7;22) fuses the EWS gene to the ETS gene ETV1. *Oncogene* 1995, 10:1229-1234
31. Gill S, McManus AP, Crew AJ, Benjamin H, Sheer D, Gusterson BA, Pinkerton CR, Patel K, Cooper CS, Shipley JM: Fusion of the EWS gene to a DNA segment from 9q22-31 in a human myxoid chondrosarcoma. *Genes Chromosomes & Cancer* 1995, 12:307-310
32. Clark J, Benjamin H, Gill S, Sidhar S, Goodwin G, Crew J, Gusterson BA, Shipley J, Cooper CS: Fusion of the EWS gene to CHN, a member of the steroid/thyroid receptor gene superfamily, in a human myxoid chondrosarcoma. *Oncogene* 1996, 12:229-235
33. Panagopoulos I, Hoglund M, Mertens F, Mandahl N, Mitelman F, Aman P: Fusion of the EWS and CHOP genes in myxoid liposarcoma. *Oncogene* 1996, 12:489-494
34. Peter M, Couturier J, Pacquement H, Michon J, Thomas G, Magdelenat H, Delattre O: A new member of the ETS family fused to EWS in Ewing tumors. *Oncogene* 1997, 14:1159-1164
35. Hudson TJ, Stein LD, Gerety SS, Ma J, Castle AB, Silva J, Slonim DK, Baptista R, Kruglyak L, Xu SH, Hu S, Colbert AME, Rosenberg C, Reeve-Daly MP, Rozen S, Hui L, Wu X, Vestergaard C, Wilson KM, Bae JS, Maitra S, Gaiatsas S, Evans CA, DeAngelis MM, Ingalls KA, Nahf RW, Horton LT, Anderson MO, Collymore AJ, Ye W, Kouyoumjian V, Zemsteva IS, Tarn J, Devine R, Courtney DF, Renaud MT, Nguyen H, O'Connor TJ, Fizames C, Faure S, Gyapay G, Dib C, Morissette J, Orlin JB, Birren BW, Goodman N, Weissenbach J, Hawkins TL, Foote S, Page DC, Lander ES: An STS-based map of the human genome. *Science* 1995, 270:1945-1954
36. Collins JE, Cole CG, Smink LJ, Garrett CL, Leversha MA, Soderlund CA, Maslen GL, Everett LA, Rice KM, Coffey AJ, Gregory SG, Gwilliam R, Dunham A, Davies AF, Hassock S, Todd CM, Lehrach H, Hulsebos TJM, Weissenbach J, Morrow B, Kucherlapati RS, Wadley R, Scambler PJ, Kim UJ, Simon MI, Peyrard M, Xie YG, Carter NP, Durbin R, Dumanski JP, Bentley DR, Dunham I: A high-density YAC contig map of human chromosome 22. *Nature* 1995, 377:367-379
37. Kim UJ, Shizuya H, Kang HL, Choi SS, Garrett CL, Smink LJ, Birren BW, Korenberg JR, Dunham I, Simon MI: A bacterial artificial chromosome-based framework contig map of human chromosome 22q. *Proc Natl Acad Sci USA* 1996, 93:6297-6301
38. Huang SF, Xiao S, Renshaw AA, Loughlin KR, Hudson TJ, Fletcher JA: Fluorescence *in situ* hybridization evaluation of chromosome deletion patterns in prostate cancer. *Am J Pathol* 1996, 149:1565-1573
39. Milner JJ, Cecchini E, Dominy PJ: A kinetic model for subtractive hybridization. *Nucleic Acids Res* 1995, 23:176-187
40. Lovett M: Direct selection of cDNAs using genomic contigs. *Current Protocols in Human Genetics*. Edited by Dracopoli NC, Haines JL, Korf BR, Moir D, Morton CC, Seidman CE, Seidman JC, Smith DR: New York, John Wiley and Sons, 1994, pp 6.3.1-6.3.15
41. Xu H, Wei H, Tassone F, Graw S, Gardiner K, Weissman SM: A search for genes from the dark band regions of human chromosome 21. *Genomics* 1995, 27:1-8
42. Silverman GA: Purification of YAC-containing total yeast DNA. *YAC Protocols*. Edited by Markie D. Totawa, NJ, Humana Press, 1995, pp 65-68
43. Straus D, Ausubel FM: Genomic subtraction for cloning DNA corresponding to deletion mutations. *Proc Natl Acad Sci USA* 1990, 87: 1889-1893
44. Lanciego JL, Goede PH, Witter MP, Wouterlood FG: Use of peroxidase substrate Vector VIP for multiple staining in light microscopy. *J Neurosci Methods* 1997, 74:1-7
45. Mackey J, Darfler M, Nisson P, Rashtchian A: Use of random primer extension for concurrent amplification and nonradioactive labeling of nucleic acids. *Anal Biochem* 1993, 212:428-435
46. Hasegawa SL, Davison JM, Rutten A, Fletcher JA, Fletcher CD: Primary cutaneous Ewing's sarcoma: immunophenotypic and molecular cytogenetic evaluation of five cases. *Am J Surg Pathol* 1998, 22:310-318
47. Nedivi E, Hevroni D, Naot D, Israeli D, Citri Y: Numerous candidate plasticity-related genes revealed by differential cDNA cloning. *Nature* 1993, 363:718-722
48. Mor O, Messinger Y, Rotman G, Bar-Am I, Ravia Y, Eddy RL, Shows TB, Park JG, Gazdar AF, Shiloh Y: Novel DNA sequences at chromosome 10q26 are amplified in human gastric carcinoma cell lines: molecular cloning by competitive DNA reassociation. *Nucleic Acids Res* 1991, 19:117-123
49. Nussbaum RL, Lesko JG, Lewis RA, Ledbetter SA, Ledbetter DH: Iso-

- lation of anonymous DNA sequences from within a submicroscopic X chromosomal deletion in a patient with choroideremia, deafness, and mental retardation. *Proc Natl Acad Sci USA* 1987, 84:6521–6525
50. Kunkel LM, Monaco AP, Middlesworth W, Ochs HD, Latt SA: Specific cloning of DNA fragments absent from the DNA of a male patient with an X chromosome deletion. *Proc Natl Acad Sci USA* 1985, 82:4778–4782
51. Lamar EE, Palmer E: Y-encoded, species-specific DNA in mice: evidence that the Y chromosome exists in two polymorphic forms in inbred strains. *Cell* 1984, 37:171–177
52. Rosenberg M, Przybylska M, Straus D: "RFLP subtraction": a method for making libraries of polymorphic markers. *Proc Natl Acad Sci USA* 1994, 91:6113–6117
53. Reijo R, Lee TY, Salo P, Alagappan R, Brown LG, Rosenberg M, Rozen S, Jaffe T, Straus D, Hovatta O, de la Chapelle A, Silber S, Page DC: Diverse spermatogenic defects in humans caused by Y chromosome deletions encompassing a novel RNA-binding protein gene. *Nature Genet* 1995, 10:383–393
54. Guan XY, Meltzer PS, Trent JM: Rapid generation of whole chromosome painting probes (WCPs) by chromosome microdissection. *Genomics* 1994, 22:101–107
55. Craig JM, Kraus J, Cremer T: Removal of repetitive sequences from FISH probes using PCR-assisted affinity chromatography. *Hum Genet* 1997, 100:472–476
56. Lichter P, Cremer T, Borden J, Manuelidis L, Ward DC: Delineation of individual human chromosomes in metaphase and interphase cells by in situ suppression hybridization using recombinant DNA libraries. *Hum Genet* 1988, 80:224–234
57. Kohno K, Oshiro T, Kishine H, Wada M, Takeda H, Ihara N, Imamoto F, Kano Y, Schlessinger D: Construction and characterization of a rad51rad52 double mutant as a host for YAC libraries. *Gene* 1997, 188:175–181
58. Le Y, Dobson MJ: Stabilization of yeast artificial chromosome clones in a rad54-3 recombination-deficient host strain. *Nucleic Acids Res* 1997, 25:1248–1253
59. Lengauer C, Green ED, Cremer T: Fluorescence in situ hybridization of YAC clones after Alu-PCR amplification. *Genomics* 1992, 13:826–828
60. Lengauer C, Riethman HC, Speicher MR, Taniwaki M, Konecki D, Green ED, Becher R, Olson MV, Cremer T: Metaphase and interphase cytogenetics with *Alu*-PCR-amplified yeast artificial chromosome clones containing the *BCR* gene and the protooncogenes *c-raf-1*, *c-fms*, and *c-erbB-2*. *Cancer Res* 1992, 52:2590–2596
61. Bohlander SK, Espinosa R III, Fernald AA, Rowley JD, Le Beau MM, Diaz MO, Espinosa R: Sequence-independent amplification and labeling of yeast artificial chromosomes for fluorescence in situ hybridization. *Cytogenet Cell Genet* 1994, 65:108–110
62. Guan XY, Trent JM, Meltzer PS: Generation of band-specific painting probes from a single microdissected chromosome. *Hum Mol Genet* 1993, 2:1117–1121
63. Joos S, Haluska FG, Falk MH, Henglein B, Hameister H, Croce CM, Bornkamm GW: Mapping chromosomal breakpoints of Burkitt's t(8;14) translocations far upstream of *c-myc*. *Cancer Res* 1992, 52:6547–6552
64. Veronese ML, Ohta M, Finan J, Nowell PC, Croce CM: Detection of *myc* translocations in lymphoma cells by fluorescence in situ hybridization with yeast artificial chromosomes. *Blood* 1995, 85:2132–2138
65. Pelicci PG, Knowles DM II, Magrath I, Dalla-Favera R, Knowles DM: Chromosomal breakpoints and structural alterations of the *c-myc* locus differ in endemic and sporadic forms of Burkitt lymphoma. *Proc Natl Acad Sci USA* 1986, 83:2984–2988
66. Zeidler R, Joos S, Delecluse HJ, Klobeck G, Vuillaume M, Lenoir GM, Bornkamm GW, Lipp M: Breakpoints of Burkitt's lymphoma t(8;22) translocations map within a distance of 300 kb downstream of *MYC*. *Genes Chromosomes & Cancer* 1994, 9:282–287