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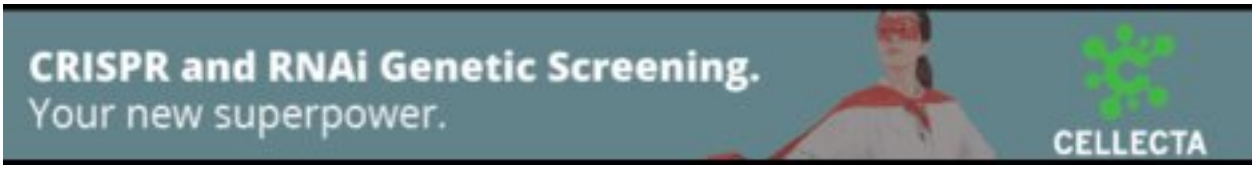

LETTER

Genome Mapping by Fluorescent Fingerprinting

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The construction of sequence-ready maps of overlapping genomic clones is central to large-scale genome sequencing. We have implemented a method for fluorescent fingerprinting of bacterial clones to assemble contig maps. The method utilizes three spectrally distinct fluorescently tagged dideoxy ATPs to specifically label the *Hin*dIII termini in *Hin*dIII and *Sau*3AI restriction digests of clones that are multiplexed prior to electrophoresis and data collection. There is excellent reproducibility of raw data, improved resolution of large fragments, and concordance between the results obtained using this and the equivalent radioactive protocol. This method also allows detection of smaller overlaps between clones when compared to the analysis of restriction digests on nondenaturing agarose gels.

Central to large-scale genomic sequencing is the construction of sequence-ready maps containing ordered sets of overlapping clones. To make the best choice of clones for the minimally overlapping set (or tiling path), the optimal strategy is to assemble a deep contig using a method that provides accurate information on the extent of each clone and its neighbors. Restriction digest fingerprinting can be used to process large numbers of bacterial clones rapidly and economically. Restriction patterns are visualized by polyacrylamide or agarose gel electrophoresis and can be scanned and analyzed to identify statistically significant overlaps. This information is then used to assemble contigs either automatically or following visual inspection of the raw data.

The approach was first developed to assemble contigs of cosmids or bacteriophage λ clones of the *Caenorhabditis elegans* (Coulson et al. 1986) and *Saccharomyces cerevisiae* (Olson et al. 1986; Riles et al. 1993) genomes, respectively. In the *C. elegans* project, purified cosmid DNA was digested with *Hin*dIII, end labeled, and recut with *Sau*3AI. The sizes of the products were then determined following electrophoresis on sequencing gels to obtain maximum resolution. The technique of generating overlapping fragment sets has also been used as the basis for other mapping projects with the aim of producing sequence-ready maps (Carrano et al. 1989; Heding et al. 1992; Taylor et al. 1996). As larger projects are undertaken, there is a need to streamline robust methods by incorporating increased levels of automation, safety, and convenience so that they may be scaled up. With this aim in mind we have implemented a fluorescent fingerprinting technique based on the methods used in the *C. elegans* project by Coulson et al. (1986), with the modifications of Taylor et al. (1996) and Tang et al. (1994).

We have developed a one-reaction technique where purified bacterial clone DNA is digested with *Hin*dIII and *Sau*3AI, with the *Hin*dIII ends being specifically labeled with one of three fluorescent dye-labeled dideoxynucleotides in preparation for multiplexing and electrophoresis on a polyacrylamide gel. Fingerprint data are collected using a model 377 automated DNA sequencer (Perkin Elmer Applied Biosystems), analyzed, and visually checked before entry into the database.

RESULTS

The following developments were required to implement nonisotopic fingerprinting on a large scale. First, the ''microprep'' procedure to prepare clone DNAs in a 96-well format was modified as follows: The supernatants from the alkaline lysis step were passed through a filter-bottomed microtiter plate before isopropanol precipitation, and the samples were resuspended in T0.1E containing RNase and stored overnight at 4°C. This protocol resulted in an increased yield and, hence, signal intensity of the fingerprints, and also increased the pass rate from ~80%–90% to >95% using cosmids, fosmids, P1 or bacterial artificial chromosome [PAC (Iaonnou et al. 1994) or BAC (Shizuya et al. 1992), respectively] clones. We defined pass rate as the pro-

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portion of clones producing fingerprints of sufficient quality to warrant data entry and analysis, expressed as a percentage of the total number of clones selected for DNA preparation and fingerprinting. The most common cause of fingerprint failure was poor quality or low-yield DNA preparations (data not shown).

Second, we substituted the previous marker set (a *Sau*3AI digest of λ DNA) with a *Bsa*JI digest of λ DNA, which produced a more even distribution of fragments across the complete size range required. The optimum temperature for this enzyme was also compatible with the conditions used for end labeling by *Taq* FS polymerase, so that both steps could be carried out in a single reaction.

Third, the new fluorescently labeled dideoxy adenosine triphosphate (ddATP) derivatives were synthesized with the fluorophore dyes TET or NED attached (kindly provided by P.E. Applied Biosystems). As these and the previously available HEX ddATP are all fluoresceine derivatives (see Fig. 1), the mobility difference of identical fragments labeled with different fluorophores was identical (data not shown), thus eliminating the need for correction during analysis. The availability of the three different fluorescently tagged ddATP molecules allowed us to multiplex three clone fingerprints plus the marker digest in each lane of the gel (Fig. 2a).

All data were collected using ABI 377 Prism Collection software (v. 1.1), and the unprocessed gel images were downloaded onto a UNIX workstation from which the data file could be imported into *Image 3* (for more details concerning the installation and documentation of the Image software, see http://www.sanger.ac.uk/Software/Image) for further editing (Fig. 2b) before transfer to fingerprinted contigs (FPC) (Soderlund et al. 1997) for analysis (Sulston et al. 1988; Fig. 2c). This eliminated all of the gel processing, autoradiography, and scanning required for radioactive fingerprinting. Collection of the data in real time also improved the resolution of the larger fragments. This, when coupled with incorporation of the standard marker set in each lane, allowed us to determine the relative mobility of each fragment in the 30-bp to 2.8-kb size range detected by the marker digest with greater accuracy than was previously possible. This improvement is especially valuable for analysis of the more complex fingerprint patterns of clones with larger inserts (data not shown).

Some variation in the intensity of labeling across the size range of fingerprint bands was noted. The intensity of these bands proved to be consistent between the fingerprints of overlapping clones. We

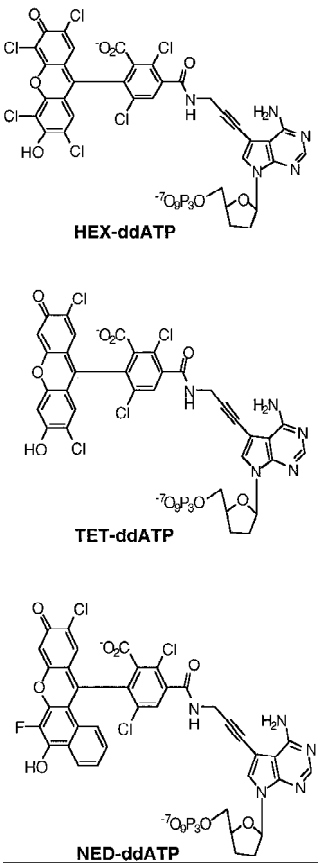

Figure 1 A representation of the chemical structures of HEX, TET, and NED, the three identically sized fluorophores used to produce fluorescent fingerprints.

believe that the observed band-specific variation is caused by the local context (sequence and/or conformation) at the ends of the DNA fragments being labeled by the Thermosequenase.

Unlike the single enzyme agarose-based fingerprinting methods (M. Marra, pers. comm.), we were not able to detect band multiplicity. The identification of bands of identical size and migration rate corresponding to a relative increase in signal strength of fingerprint bands is of less significance as the *Hin*dIII–*Sau*3AI fingerprints do not represent all of the cloned insert (see later).

The same PAC clone was fingerprinted 10 times with each of the three dyes and compared. All fingerprints were highly reproducible. The migration of specific fragments in independent fingerprints of the same or different clones was found to be the same within the tolerances set. In all cases examined, the variation of migration of bands in 30 independent fingerprints was ±0.25% (maximum observed; i.e., ±11/4500 intervals), which is less than the tolerance used in the analysis.

Next we fingerprinted the 49 PAC clones of a

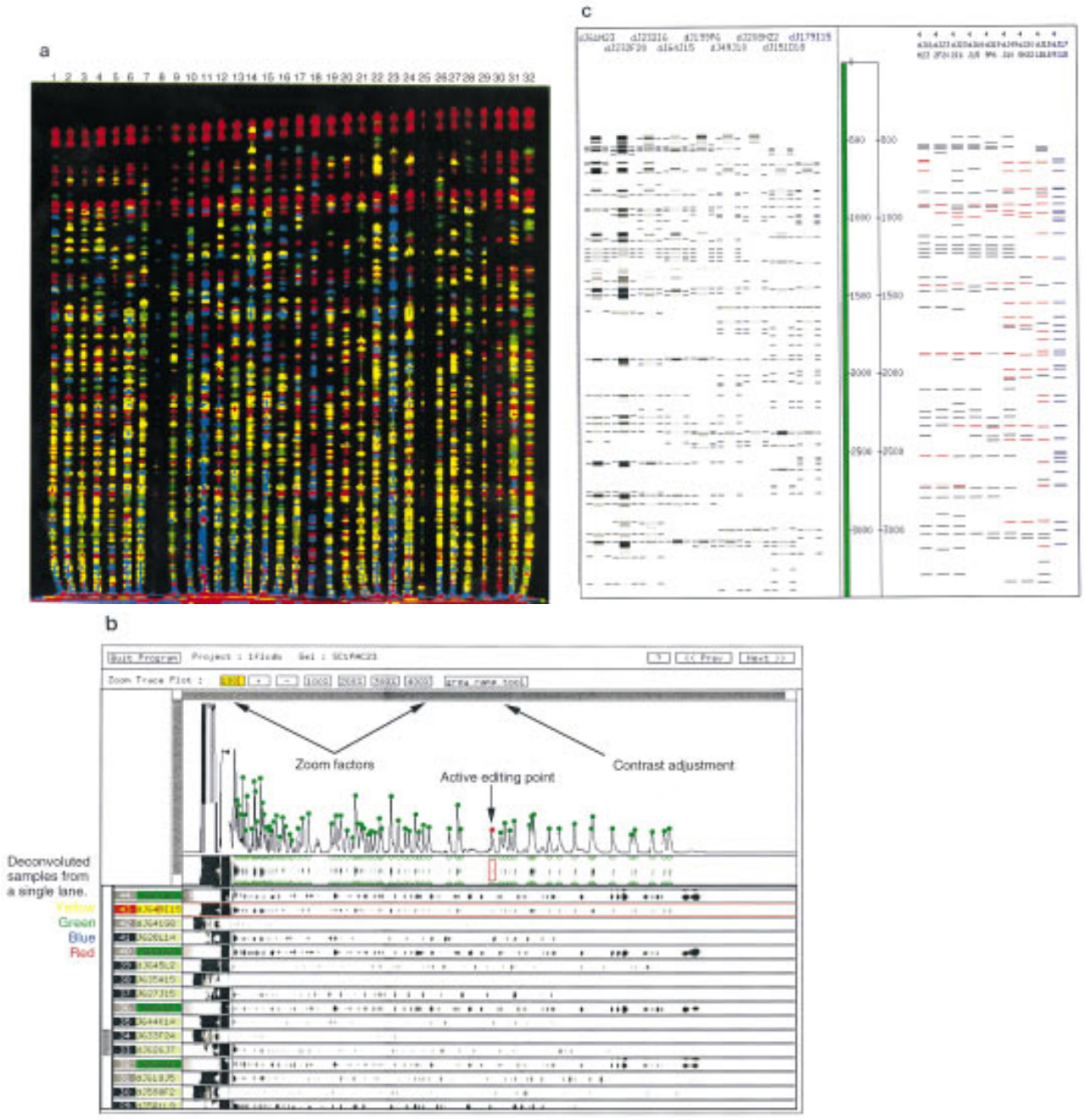

Figure 2 Fluorescent fingerprint electrophoresis data, entry, and analysis. (*a*) The gel image of a fluorescent fingerprint gel (ABI Prism GeneScan Analysis 2.0.2). The 32-lane gel contains three clones in each well, giving a present capacity of 96 clones per run. We are updating our collection of software to double the number of lanes per gel. (*b*) Editing of fingerprint bands using *Image3.* (*c*) Digitized images of gel traces and corresponding fingerprints in FPC.

1400-kb contig by both radioactive and fluorescent methods and compared another nonisotopic method in which digests of purified clone DNA are separated on SYBR green-stained agarose gels and the data are collected using a Fluorimager (Molecular Dynamics). Analysis of the data and contig assembly was performed independently. The assembly results of each of the four different methods were compared to the overlaps defined by sequencing of the 14 clones of a minimum set (Fig. 3a). Vertical arrows in Figure 3b represent the overlaps that were not found between clones in each of the

four methods. Figure 3c represents the contigs as constructed by the fluorescent fingerprinting method. The vertical arrows coincide with those in Figure 3b, denoting overlaps that were not detected by fingerprinting alone. There was excellent agreement between the contigs assembled using either the radioactive or fluorescent *Hin*dIII–*Sau*3AI data. Minor variations were observed where small overlaps between clones fell just outside the probability cutoff used. (These overlaps were originally detected by end-probe hybridization and later confirmed by the sequence.)

There was also good agreement between the *Hin*dIII–*Sau*3AI results and those of the *Hin*dIII and *Eco*RI fingerprints resolved on agarose gels, but matches in the latter protocols required a higher statistical probability of overlap, that is, smaller overlaps were not detected. This can be attributed in part to the fact that each agarose fingerprint pattern is divided into 2000 intervals along a lane as opposed to the 3400 and 4500 intervals of the radioactive and fluorescent fingerprints, respectively. A smaller number of intervals implies that there is a greater probability that bands will occur randomly in the same bin and contribute to background matches. However, it should be pointed out that the two approaches are both useful in different ways. The *Hin*dIII–*Sau*3AI method can detect smaller overlaps, but samples 256/4096 bp (14%) of the insert DNA of a clone and provides no information on the remainder of the sequence. In contrast, the use of complete *Eco*RI, *Hin*dIII, or similar digests resolved on agarose gels, although achieving lower resolution, does display bands representing all of the DNA of the cloned insert. The latter method is therefore more appropriate for verifying the integrity of the sequence compared to all genomic clones covering the region. It also confers the ability to identify possible deletions or other rearrangements that result in de-

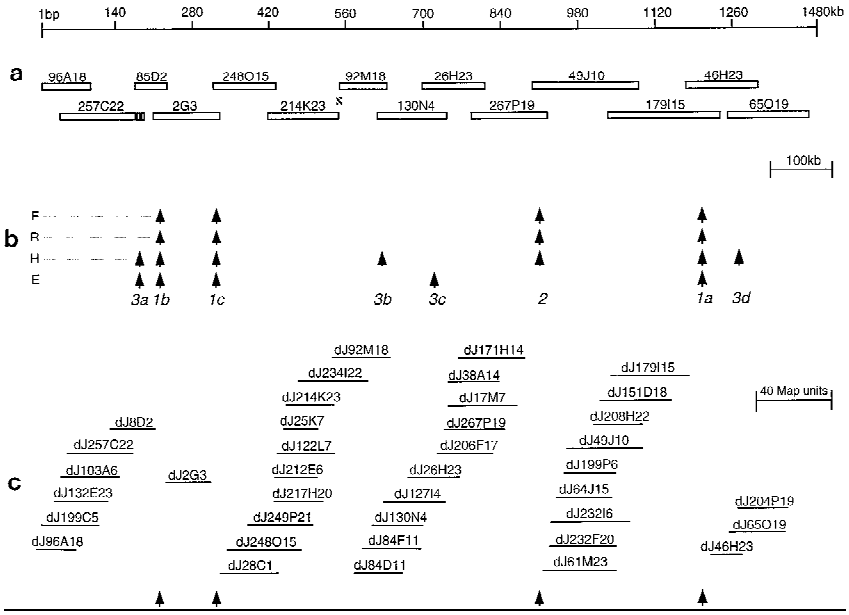

Figure 3 Comparative fingerprinting data of 49 clones covering a 1400-kb interval sequence that was determined from 14 clones comprising a minimum tiling path and a PCR product between 214K23 and 92M18 (hatched). (*a*) Finished sequence data were used to define the overlaps between clones and absolute length of each of the clones (except 257C22, where data were incomplete). (*b*) Vertical arrows denote overlaps that were not detected by each method. (F) Fluorescence, (R) radioactive; (H) *Hin*dIII, and (E) *Eco*RI. As expected, there is variation based on recognition sites between the overlaps detected using *Hin*dIII (*3b* and *3d*) and those established with *Eco*RI (*3c*). Details of the analysis of undetected overlaps are as follows: (Overlap *1a,* 62 kb) Nine *Hin*dIII and 11 *Eco*RI fragments are present in this overlap on the basis of DNA sequence. Statistical probability on the basis of these bands shared between 179I15 and 46H23 is 1×10^{-4} (-4) fluorescence, -1 radioactive, -4 *Hin*dIII, and -5 *Eco*RI (whose probability cutoffs for all matches are -4, -5, -7, and -7, respectively). (Overlap *1b,* 25 kb) Clone 2G3 is unattached. Three *Hin*dIII or six *Eco*RI fragments shared with clone 85D2 are insufficient to score the overlap. (Overlap *1c,* 11 kb) Clone 2G3 is unattached. Four *Hin*dIII or three *Eco*RI fragments shared with clone 248O15 are insufficient to detect the overlap. (Overlap *2,* 24 kb) Overlap between 267P19 and 49J10 was not found by any of the *Hin*dIII methods. Six *Hin*dIII fragments are not sufficient to detect overlap. Arrows *3a–3d* denote where only the agarose methods failed to find overlap. (Overlap *3a*) Overlap between 257C22 and 85D2 could not be determined because of incomplete overlap data and therefore was not analyzed. (Overlap *3b,* 18 kb) Four *Hin*dIII fragments shared between 130N4 and 92M18 are not sufficient to detect overlap. (Overlap *3c,* 44 kb) Six *Eco*RI fragments shared between 130N4 and 267P19 are not sufficient to detect overlap. (Overlap *3d,* 55 kb) 16 *Hin*dIII fragments between 46H23 and 65O19 should have been significant. The lack of overlap may be attributed to the migration of similar-sized fragments in the same location of the agarose gel and therefore not be representative of the cloned insert. (*c*) Although all but 1 of the 49 clones (2G3) fell into contigs, only 4 of the contigs that lie within the sequence interval are represented. Arrows denote where the overlaps between clones were not found.

tectable size alterations in individual restriction fragments.

Where all four methods failed to detect overlaps between adjacent clones (Fig. 3b, overlaps 1a–1c), the overlapping bands were only contained within two clones. This problem would be resolved with greater contig depth. Details of analysis of overlaps that were not detected by individual methods are given in the legend to Figure 3.

In a more extensive comparison of fluorescent and radioactive methods, 96 and 785 clones were analyzed independently on chromosomes X and 1, respectively, using both fingerprinting methods. The initial pass rate of the fluorescent fingerprinting was markedly higher for the chromosome 1 analysis (87%), as compared to the radioactive method (65%). When repeat fingerprints were included in the data sets for both projects fluorescent fingerprinting yielded a marginally higher overall pass rate of 96% as opposed to 90% radioactively. Closer inspection of the analyzed fingerprint data revealed the same number of contigs had been generated that contained the same clones in identical order (data not shown). We are currently using the fluorescent fingerprinting technique in our chromosome mapping projects on chromosomes 1, 6, 20, and X. To date, we have fluorescently fingerprinted a total of 12,500 clones (generating 1216 contigs), which provide an estimated 156 Mb in contigs of sequence-ready map.

DISCUSSION

The advent of large-insert bacterial clone libraries of PACs and BACs for construction of high-resolution maps has facilitated sequencing and gene identification in chromosomal regions. Initial identification of the clones is achieved by using hybridization- or PCR-based markers to screen the available libraries. The degree of coverage of the region obtained following screening depends on the initial marker density and distribution. In regions of high marker density (when multiple markers are found in each bacterial clone) the existence of overlaps between clones may be identified on the basis of shared marker content. However, as a marker represents one point in the genomic DNA, no measure of the extent of overlap is obtained, and in most regions of the human genome at present the marker density is too low to achieve closure of all gaps by marker content alone. Fingerprinting can detect overlaps where there is no marker available and gives an indication of the extent of each overlap.

Alternative methods to define overlaps between bacterial clones include the hybridization of end probes to clone arrays (Evans and Lewis 1989), high-resolution fluorescence in situ hybridization (FISH) using DNA fibers (Wang et al. 1996), and matching of end sequences to complete insert sequences (Kupfer et al. 1995; Roach et al. 1995). End-probe hybridizations do not define the extent of overlap, and the approach is not easily scaled up. The same limitation is true of whole cosmid-to-cosmid hybridizations (Xie et al. 1993), and all hybridization-based methods employing undefined sequences can yield false-positive signals because of cross-hybridization of repeat motifs. The end-sequencing strategy provides precise information on extent of overlap but relies on extensive prior investment in sequencing all the clones in the library and also does not permit assembly of contigs prior to sequencing of the inserts. Fiber–FISH, using bacterial clones as probes and yeast artificial chromosomes (YACs) as a target, provides good estimates of both the extent of overlaps and sizes of gaps between clones but requires the existence of a well-defined YAC map across the region of interest and has yet to be scaled up.

Previous approaches to fluorescent-based fingerprinting include ligation of fluorochrome-labeled oligonucleotide adaptors to *Eco*RI digests of cosmids that were resolved on agarose gels (Lamerdin and Carrano 1993); this is now superseded by the VISTRA–green-stained agarose gel fingerprints (M. Marra, pers. comm.) as used here. Our approach has extended this to multiplex *Hin*dIII–*Sau*3AI fingerprints of three clones (plus markers) using three novel dye–ddA terminators, thus enabling automatic data collection of 96 clones, increasing to 192 clones with 64 lane upgrades, for a 4.5-hr run and a steady-state throughput of 400 clones per person per week including analysis.

METHODS

Bacterial Clone DNA Preparation

Our microprep method is based on the alkaline lysis procedure of Birnboim and Doly (1979) and produces ~200 ng of PAC, BAC, fosmid, and cosmid DNA. Bacterial cultures (500 µl) in 2× TY containing the appropriate antibiotic, were grown in 1-ml deep-well microtiter plates (Beckman), with shaking at 300 rpm at 37°C for 18 hr. A portion of the culture (250 µl) was then transferred using a 50- to 250-multichannel pipette (Finnpipette) to a ''U''-bottom microtiter plate (Greiner), and the cells were pelleted by centrifugation at 1000*g* for 4 min (PAC and BAC clones) or 2 min (cosmids, fosmids). The supernatant was discarded and the pellet resuspended by gently vortexing the pellet. Twenty-five microliters of solution I (50 mM glucose, 25 mM Tris, 1 mM EDTA, stored

at 4°C) was added to the wells using a 5- to 50-multichannel pipette (Finnpipette) and mixed by carefully tapping the side of the plate. Twenty-five microliters of solution II (0.2 M NaOH, 1% SDS, made fresh) was added and mixed as before, and left at room temperature for 5 min until the solution cleared. Twenty-five microliters of cold solution III (3.5 M K acetate, stored at 4°C) was added and mixed as described previously, and left at room temperature for 5 min. The total well volume was transferred to a 2 µM filter-bottomed plate (Millipore), which had been taped in place on a U-bottom microtiter plate (Greiner) containing 100 µl of isopropanol, and spun at 2500 rpm for 2 min at 20°C. After the filter plate was removed, the microtiter plate was left at room temperature for 30 min before spinning at 3200 rpm for 20 min at 20°C. The supernatant was removed, and the precipitated DNA was dried briefly before washing with 100 µl of 70% ethanol. After spinning at 3200 rpm the supernatant was removed, and the pellet was dried to ensure no ethanol remained and resuspended in 5 µl of T0.1E (pH 7.4) with RNase (10 mg/ml). Samples were stored at −20°C.

Fluorescent Marker

Three microliters of λ DNA (500 ng/µl NEB) was digested with 3.0 µl of *Bsa*JI (2.5 U/µl) and labeled with 2.0 µl of ROX ddC (5.08 µM) (PE-ABI, cat. no. 402117) using 2.0 µl *Taq* FS (8 U/µl) in the presence of 5.0 µl of NEB2 buffer and 35.0 µl of T0.1E (pH7.4) in a 500-µl microcentrifuge tube for 1 hr at 60°C. The DNA was recovered by the addition of 50.0 µl of 0.3 M Na acetate and 200 µl of 96% ethanol. The contents of the tube were mixed briefly by vortexing, left at room temperature (in the dark) for 15 min and then at −20°C for 20 min before spinning at 14,000 rpm for 20 min. After the supernatant was discarded the pellet was resuspended in 60.0 µl of T0.1E (pH 7.4) and 60.0 µl of blue dextran formamide dye (9.8 ml deionized formamide, 200 µl of 0.5 M EDTA, 0.01 grams of blue dextran), and mixed before use.

Fluorescent Fingerprinting

Reactions were carried out individually in wells of a U-bottom microtiter plate (Greiner) as follows. The fingerprinting mix for each fluorescent labeling reaction constituted 2.8 units of *Hin*dIII (20 U/µl), 3.7 units of Thermo Sequenase (32 U/µl) [or *Taq* FS (8 U/µl)], 0.14 µl of fluorescent ddA (10 µM) (HEX 403036c, NED 402997c, TET 402998c), and 3 units of *Sau*3AI (30 U/µl). Twenty microliters from the reaction mix was added to the clone DNA sample using a Hamilton repeat dispenser and incubated at 37°C for 1 hr. Seven microliters of 0.3 M Na acetate and 40.0 µl of 96% ethanol were added to each well, and appropriate samples (three differently labeled fragments) were pooled and the microtiter plate spun at 3200 rpm at 20°C for 20 min. The pellet was washed, by the addition of 100 µl of 70% ethanol, spun for 20 min at 20°C, and dried and resuspended in 5 µl of T0.1E. Two microliters of the marker solution was added to each well. The samples were denatured on a heated block for 10 min at 80°C prior to loading on a 377 Automated DNA Sequencer using a 0.2-mm, 12-cm, well-to-read 4.5% denaturing polyacrylamide gel and 64 lane combination. Reaction products were stored overnight at −20°C, when necessary, prior to the addition of the marker.

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