

Benchmarks

Rapid DNA Fiber Technique for Size Measurements of Linear and Circular DNA Probes

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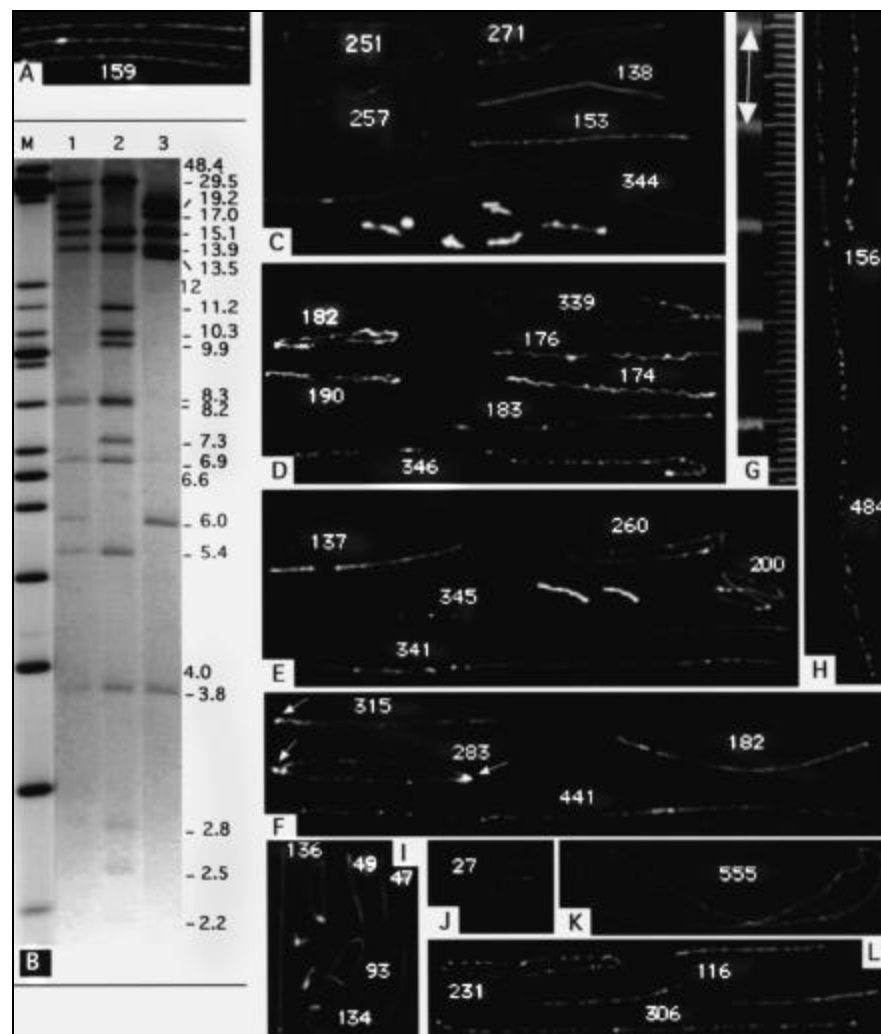
DNA size measurements are an integral part of laboratory procedures, such as physical mapping, subcloning, or sequencing. The large variety of vectors in use today allows the cloning and propagation of DNA inserts of various sizes: 0.1–10 kb in plasmids; 5–25 kb in phages; 30–45 kb in cosmids; 80–300 kb in P1 clones, P1 artificial chromosomes (PACs), or bacterial artificial chromosomes (BACs); and up to 2000 kb in yeast artificial chromosomes (YACs). Some of these recombinant DNA molecules are circular (e.g., plasmids, cosmids, PACs, and BACs), whereas others are linear (e.g., phages and YACs). With the exception of YACs, which are propagated in yeast, all vectors are propagated by transforming bacterial hosts, which are usually strains of *E. coli*.

Short of direct sequencing, two main approaches are used today to measure the size of DNA probes: (i) restriction enzyme digestion, followed by gel-electrophoresis and sizing of DNA fragments by comparison with known molecular weight markers (Figure 1B), and (ii) DNA fiber procedure(s). In fluorescence in situ hybridization (FISH) on DNA fibers, a DNA template is stretched on a slide (1,8,13); a labeled DNA probe is then hybridized onto it, and the physical length of the hybridization signals is measured (3,9, 12). The hybridization signals appear as an array of closely spaced dots resembling beads on a string (Figure 1H). The sizing is done by measuring the length (pixels and microns) of the signal array and comparing it with a known standard (micrometer). However, if the stretching technique involves subjecting whole cells to chemical reagents to release the DNA directly onto the slide, individual fibers are stretched to different degrees, depending on how much residual protein is still attached to particular DNA strands. Fiber-FISH is a valuable tool for map-

ping DNA probes relative to one another and for measuring the size of small microdeletions. DNA of any size can be resuspended in a solution at appropriate concentration and pH and stretched on chemically pretreated, positively charged slides using one of the procedures collectively known as molecular combing (2,8). The DNA is stained with a green fluorescent dye (YOYO[®]-1; Molecular Probes, Eugene, OR, USA) and visualized using a fluorescence microscope (Figure 1). Various DNA fiber techniques have been previously published (6,7,10), but the stretched DNA was usually subjected to hybridizations or was enzymatically cut to yield restriction maps (e.g., optical mapping) (4,5,11).

Here, we describe a molecular combing protocol that allows DNA molecules to be stretched on silanized

glass slides in seconds and can be used with DNA ranging from less than 10 to 300 kb or longer in size. To our knowledge, this protocol appears to be the fastest DNA-stretching procedure described to date. We show that the DNA can be stretched in various degrees on a chemically treated glass surface, especially when the DNA molecule is circular. The procedure requires only minute amounts of DNA (10–20 ng) and can be performed in about 15 min in any laboratory with access to a fluorescence microscope. Visual inspection with a microscope is very simple because the DNA fibers are bright and very visible, especially with a 63× or a 100× objective. DNA fiber measurements (Figure 1, panels D–F) were compared with results from restriction-digest gel electrophoresis (Figure 1B) and direct-sequencing (Figure 1K) information. For



example, *Bam*HI-digested DNA of three different but partially overlapping PACs was separated on an agarose gel and was stained with ethidium bromide (Figure 1B). With that enzyme, PAC no. 1 was 137 kb, PAC no. 2 was 139 kb, and PAC no. 3 was 91 kb. Several other single or double restriction digests (*Xho*I, *Sfi*I, *Eco*RI, *Hind*III, and *Not*I) of the same PACs yielded length values that could vary by as much as 10–15 kb (about 10%) for the same probe. The same DNA was then stretched and measured on glass slides. Based on these and other similar comparisons, we derived simple computations, using correction factors that allow the conversion of size measurements in DNA length from microns to kilobases.

DNA probes are prepared according to the standard laboratory protocols (usually alkaline lysis). Vortex mixing should be avoided because it will shear larger DNA molecules. A small aliquot of isolated DNA is diluted to 2–5 ng/ μ L in 10 mM aminomethyl propanediol buffer (Sigma, St. Louis, MO, USA), pH 8.2–8.5. After gentle mixing, 7–8 μ L DNA solution are pipetted close to the free (unfrosted) end of a silanized, positively charged slide (Sigma). One end of a 24 \times 40 mm coverslip is positioned so that it touches the edge (free end) of the slide. The angle between the coverslip and slide will be gradually decreased, by tilting the coverslip progressively until it comes in contact with the drop of DNA solution. At that mo-

ment, the drop will spread laterally on the slide along the edge of the coverslip. Then, holding the touching edges of the slide and coverslip between the thumb and index finger, the coverslip is gradually pressed down toward the slide until it covers the slide. This move will spread the liquid between the slide and coverslip from the free end toward the frosted end of the slide. The liquid flow between the slide and coverslip is the force that stretches the DNA. Continuous pressure between two fingers on the slide/coverslip assembly should last several seconds, until the liquid spreads completely. Pressure should be applied for another 10–15 s to make sure that all excess liquid is squeezed out from between the slide and coverslip. The coverslip is lifted with sharp forceps and removed, and the slide is air-dried. The DNA can be stained in several ways: (i) 10^{-7} YOYO-1 dye can be added to the DNA solution before stretching. After DNA stretching, the slide is air-dried, mounted with anti-fade solution, and examined. (ii) After stretching, 50–100 μ L 10^{-7} M YOYO/20 mM β -mercaptoethanol solution can be placed on the slide, covered with a coverslip, incubated for 5 min at room temperature to stain the DNA, and then visualized with a microscope. Alternatively, the coverslip can be removed, and the slide can be rinsed in water for a few seconds, air-dried, and mounted with anti-fade. (iii) Anti-fade solution containing 10^{-7} M YOYO-1 can be used to mount the

slide before microscopy. After 3–5 min, slides are examined with a fluorescence microscope using a common FITC filter (for YOYO). Although we primarily use the first procedure, all three staining protocols yield the same results, and it is up to the researcher to choose which one to use.

DNA fiber size is measured using any software that calculates the number of pixels between two points of a digital image (Segmented Ruler[®]; Adobe[®] Photoshop[®]). The pixel-to-kilobase conversion is obtained by imaging a microscopic ruler using the same objective/magnification (Figure 1G). In our case, 10 μ m = 73.5 pixels when using the 100 \times objective. At this magnification, the theoretical size of a 100-kb (34- μ m)-long DNA molecule should be 250 pixels, if the DNA molecule is stretched to its full length. In our experiments, we measured DNA molecules between 8 (Figure 1J) and 220 kb (Figure 1K). It is conceivable that the procedure would be capable of handling larger DNA molecules of approximately 300 kb or even longer. To find the optimal relationship between the degree of DNA stretching and the actual size in kilobases, we performed numerous measurements using DNA probes of known sizes either by sequencing or restriction digest.

After stretching, all tested circular DNA probes yielded DNA fibers with a variety of geometrical shapes (Figure 1C). Depending on the number of kinks in a molecule, the absolute size can vary

Figure 1. Microscopic measurements of DNA fibers. All microscopic images (including the ruler in panel G) were captured with the 100 \times objective and are shown at the same scale. Panel A shows several λ DNA fibers, whereas panel C shows a summary of the most frequent geometric forms taken by the DNA fibers on a slide. For comparison purposes, the gel image shown in panel B (restriction digest) includes the same three BACs depicted as DNA fibers in Figures D, E, and F. Figures I, J, K, and L show DNA fibers of various sizes (cosmid, plasmid, BAC, and PAC). With the exception of panel B, all numbers indicate the length of DNA fibers in pixels. The multiple breaks seen in some fibers were induced by light energy during image visualization and capturing. In assessing DNA length, note that all DNA probes measured included the respective vector DNA. (A) λ -phage DNA (48.5 kb) used as a control, with a measured size of 158–159 pixels. (B) *Bam*HI digestion of three different but partially overlapping PACs, on agarose gel stained with ethidium bromide. Based on gel analysis, PAC no. 1 was 137 kb, PAC no. 2 was 139 kb, and PAC no. 3 was 91 kb. M, marker [1-kb ladder (Life Technologies, Rockville, MD, USA) + λ -*Hind*III + uncut λ DNA]. Numbers close to small lines indicate fragment sizes (kb), whereas numbers without lines indicate marker sizes. Compare with panels D–F. (C) Examples of fiber shapes seen when stretching any circular DNA molecule (P1 DNA shown). Closed circles (251, 257, and 271) have comparable lengths, whereas collapsed circles (138 and 153) have shorter, thicker fibers. Bends in the fiber indicate less pulling force on the DNA and result in less stretching of the molecule. Five fully knotted DNA molecules are also shown. (D) PAC no. 1 fibers (see Figure 1B). The sizes of the circular and collapsed circle DNAs of PAC no. 1 correspond to a 135-kb molecule, similar to the restriction digest value(s). The longest linear molecule found on this slide (346 pixels) was shorter than expected, indicating that the DNA was mostly sheared during isolation. The 182- and 190-pixel DNAs are actually collapsed circles folded back onto themselves (not usable for sizing). (E) PAC no. 3 fibers (see Figure 1B). The closed circle (260) is longer than the twisted circle (200), indicating a higher degree of DNA stretching. A nick in the DNA molecule of the collapsed circle (137) shows clearly that in this geometrical figure, the DNA molecule folds back onto itself. Size measurements correspond to a 105-kb molecule. (F) PAC no. 2 fibers (see Figure 1B). The sizes of the collapsed circle (182) and the linear fiber (441) correspond to a DNA of approximately 138 kb. The small knotted areas (arrows) make the closed circles appear somewhat shorter than expected. (G) Microscopic ruler photographed through the 100 \times objective. The double-headed arrow corresponds to 10 μ m. (H) Fiber-FISH of a BAC probe labeled with Cy3 and hybridized onto genomic DNA fibers obtained by SDS-incubation of cells directly on slides. Because the SDS protocol stretches genomic DNA fibers to different degrees, the same BAC probe yields arrays of hybridization signals varying tremendously in length. (I) Cosmid DNA fibers, corresponding to 38–40 kb. (J) Linear fibers of an 8.6-kb plasmid (vector + insert). (K) The twisted, circular molecule of a sequenced BAC clone (211.3 kb). The 555 pixels corresponds to a DNA molecule of approximately 222 kb (insert + vector), which is close to the real value. (L) DNA fibers of a 95-kb PAC clone, showing numerous breakages induced by light, during image capturing at the microscope.

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from one measured molecule to another. These kinks are produced by the direction of the liquid flow during stretching and the number of attachment points of the DNA molecule to the glass. For the purposes of this work, four classes of shapes are more important—linear fibers, closed circles, collapsed circles, and knotted molecules. Molecular distribution among these classes depends primarily on the age of the DNA preparation and the care with which DNA molecules are handled. In a fresh DNA preparation, the majority of molecules (60%–70%) stretch on the slide similar to circles or knots. In an older preparation, one that was subjected to repeated centrifugations or cycles of freezing and thawing, large molecules such as PACs and BACs break. Such DNA will stretch similar to linear fibers of multiple sizes with very few circles.

Linear DNA fibers vary greatly in size for the same DNA probe because the stretching force of the liquid flow often breaks the DNA molecules into fragments of various sizes. To assess the degree of stretching of a linear molecule, we used as a control a DNA of known size (λ phage DNA). Theoretically, this 48.5-kb molecule should be 16.5- μm -long and should span about 122 pixels. However, when λ DNA was stretched onto silanized slides, fibers of various sizes were visible after YOYO-1 staining. This suggests that the DNA breaks in solution, during stretching, or both. To calculate the average size of a full-length λ molecule (48.5 kb), we measured all reasonably long fibers (132–161 pixels) in five microscopic fields on different areas of the slide (Figure 2). Because of random breakages in the DNA molecules, only the longest of these fibers were considered when calculating the average size of a molecule (i.e., only fibers with 156–161 pixels). In Figure 2, the average size of the 15 seemingly intact DNA molecules was 159 pixels (Figure 1A). The slight differences may be due to the way the DNA attaches to the slide. Sometimes one or both ends of a molecule show an increase in thickness, similar to when the molecule breaks. This suggests that the “thicker” areas close to a free end in the DNA molecule may not be fully stretched. These data indicate that, at least in our

conditions, linear DNA molecules are over-stretched to 130% of the theoretical DNA size. A similar value was obtained with molecules as long as 190–220 kb. Therefore, to correct for over-stretching, the measured size (pixels or microns) of a linear fiber needs to be multiplied by 0.77 to convert it to the actual length. Because of this factor, the real correspondence between the physical measurement and the size of a linear fiber is 100 kb = 44.2 μm = 325 pixels. Therefore, the measured length (pixels) of a linear fiber should be divided by 3.25 ($130/100 \times 2.5$) to convert the DNA size into kilobases. Especially for large probes (more than 180–200 kb), full-length linear fibers may be more difficult to use for calculations because the molecules break easily. Restriction digest of a circular DNA probe before stretching would linearize all of the molecules but would eliminate the various geometrical forms described below, which can provide valuable size information. For linear molecules, the longest fibers found on the slide should be measured.

Closed circles and ovals. A circular DNA is rarely stretched smoothly; instead, the fiber makes numerous and irregular little turns. The measurements of DNA probes of known size show that circular DNA is stretched closest to or at its theoretical size (100 kb = 34 nm = 250 pixels). Therefore, to convert

the size of a circle (pixels) into the actual DNA size (kb), the pixel number of a circular fiber is divided by 2.5 (250/100).

Collapsed circles. In this case, the DNA fiber is short and looks thicker compared to other circular or linear fibers on the slide. This increased thickness is due, to some degree, to the twisting of the “sides” of the circular molecule around each other. The collapsed circles are very convenient to use for sizing DNA probes because they are easier to measure, especially in the case of large DNA molecules (200–300 kb). Our measurements indicated that DNA in collapsed circles is somewhat over-stretched to approximately 104% of the theoretical values. To convert the measured size (in pixels) of a collapsed circle into theoretical size (in pixels), the measured value is multiplied by 1.925 (2.5×0.77). The 2.5 value is the factor required to convert the size of the collapsed circle to the measured size of the linear DNA fiber, if the same molecule were stretched completely on the slide. To convert the size of a collapsed circle from pixels directly into kilobases, the measured value is multiplied by 0.77.

Knotted DNA molecules. Whether originating from circular or linear molecules, they appear like small knots of DNA (Figure 1, panels C, E, and I) and are unusable for sizing.

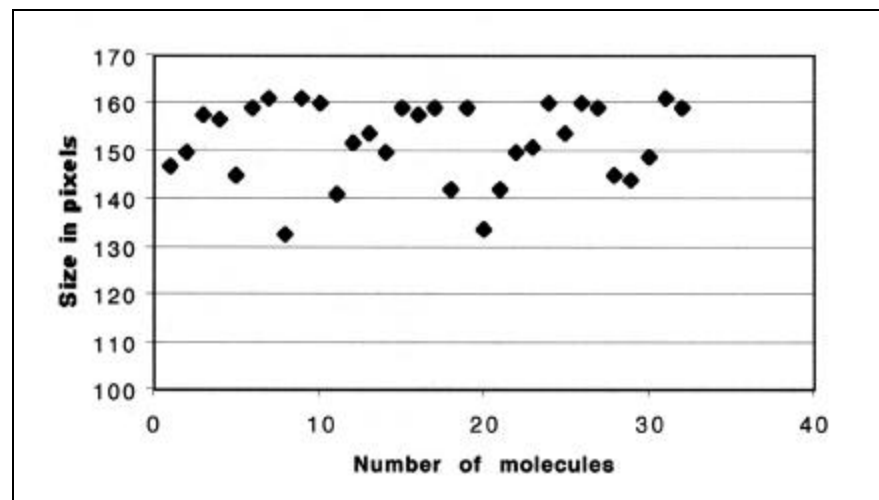


Figure 2. Size distribution of 32 λ -phage DNA fibers measured from five different microscopic fields (using a 100 \times objective). Because of the random breakages in the DNA molecules, only the longest fibers were considered when measuring the average size of an intact λ molecule (i.e., only the 15 fibers between 156 and 161 pixels). The average size of an intact DNA molecule was 159 pixels or 130% over-stretching. This number was subsequently verified by checking other molecules with known lengths.

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Fiber DNA measurements were performed using two different microscope settings (Leica and Olympus) in two different laboratories with identical results. Our data indicate that this fast and simple procedure allows DNA size measurements that are as accurate or more accurate (5%–10%) than those taken by gel electrophoresis.

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Method to Introduce Stable, Expanded, Polyglutamine-Encoding CAG/CAA Trinucleotide Repeats into CAG Repeat-Containing Genes

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Expansion of CAG trinucleotide repeats coding for polyglutamine causes eight progressive neurodegenerative diseases, including Huntington's disease (HD) and a number of inherited spinocerebellar ataxias (SCA) (1). While these disorders are only slowly progressive, investigation of the effects of elongated polyglutamine in cell lines and

transgenic animals can be accelerated by using constructs containing long CAG repeats expanded even beyond the pathological size. Often, the expanded repeats are obtained from patient's material; however, native CAG tracts are unstable in bacteria (4), as well as in stably transfected cell lines (3) and transgenic mice (8). PCR-based methods were developed to enlarge CAG repeats within cDNAs (9), some also introducing CAA interruptions (6), which increase the stability of CAG repeats, while still coding for glutamine. However, misincorporation errors can occur during PCR, and the final repeat size is not always predictable. Recently, Kazantsev et al. (5) described the production of alternating CAG/CAA repeats, (5'-CAACAGCAGCAACAGCAA-3')_n, ranging in size from 25 to 300 triplets, which remained stable in bacteria and in several different eukaryotic systems (unpublished data). Here, we describe a straightforward method to introduce these repeats (20, 41, 60, 67, 98, 185, or 264 units) into the native CAG repeat of any gene. Our strategy is advantageous because the resulting repeats are stable, have a predictable size, and the amino acid sequence surrounding the polyglutamine tract is preserved. While our method can be used for any gene, we elongated the SCA7 CAG repeat to encode 107 and 273 glutamines.

The strategy is outlined in Figure 1. Four bases—GTAC—were introduced into the SCA7 CAG repeat by in vitro mutagenesis to create a *KpnI* site (GGTACC) (Figure 1, left). Since the SCA7 cDNA already contains a *KpnI* site, only a part of the gene [bases 1–654 of the SCA7 open reading frame (ORF) with 9 CAG] was *EcoRI* cloned into pBluescript® SK II(+) vector (Stratagene, La Jolla, CA, USA), in which we previously removed the *KpnI* site from the polylinker (pBSSKII/*KpnI*-). The in vitro mutagenesis was performed using primers SCA7CAG *KpnI* [5'-CGG(CAG)₄GTAC(CAG)₅CCG-3'] and SCA7CTG*KpnI* [5'-CGG(CTG)₅GTAC(CTG)₄CCG-3'], as follows. The SCA7 segments upstream and downstream of the CAG repeat were amplified separately, using SCA7CTG*KpnI* and SCA7CAG*KpnI* primers in combination with vector primers, M13 Reverse and Forward, re-