A rapid procedure for the isolation of C_0t-1 DNA from plants

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Abstract: In situ hybridization (ISH) for the detection of single- or low-copy sequences, particularly large DNA fragments cloned into YAC or BAC vectors, generally requires the suppression or "blocking" of highly-repetitive DNAs. C_0t-1 DNA is enriched for repetitive DNA elements, high or moderate in copy number, and can therefore be used more effectively than total genomic DNA to prehybridize and competitively hybridize repetitive elements that would otherwise cause nonspecific hybridization. C_0t-1 DNAs from several mammalian species are commercially available, however, none is currently available for plants to the best of our knowledge. We have developed a simple 1-day procedure to generate C_0t-1 DNA without the use of specialized equipment.

Key words: C_0t-1 DNA, in situ hybridization, BACs, plants.

Résumé : Lorsque pratiquée en vue de détecter des séquences présentes à raison d'une ou quelques copies, l'hydridation in situ (ISH) exige la suppression ou le masquage des ADN hautement répétés et ce particulièrement lorsque de grands fragments d'ADN clonés dans des YACs ou des BACs sont employés. L'ADN C_0t-1 est enrichi en éléments d'ADN hatuement ou modérément répétés et ainsi, lors de la préhybridation, il peut masquer de façon plus efficace que l'ADN génomique total les séquences répétées qui, autrement, causent une hybridation non-spécifique. L'ADN C_0t-1 de plusieurs espèces de mammifères est disponible commercialement tandis qu'aucun n'est disponible chez les plantes. Les auteurs ont mis au point une technique simple qui permet d'obtenir de l'ADN C_0t-1 en une journée et ce sans devoir recourir à des équipements spécialisés.

Mots clés : ADN C_0t-1, hybridation in situ, BACs, plantes.

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Introduction

The genomes of eukaryotic species contain numerous types of highly to moderately repetitive DNA elements, such as the *Alu* sequence in humans (Daniels et al. 1983), *B1* in mouse (Krayev et al. 1980), *L1* in mammals (Burton et al. 1986), *del2* in *Lilium* spp. (Leeton et al. 1993), *Tourist* in cereals (Bureau and Wessler 1994), and *KpnI* in *Pennisetum* spp. (Ingham et al. 1993). In many plant species of economic importance, repeated sequences comprise a large proportion of the genome. For example, the rice genome is composed of 50% repeated sequences (McCouch and Tanksley 1991), maize of 78%, wheat of 83%, rye of 92%, and onion of 95% (Flavell et al. 1974). Closely related species can differ considerably in content with respect to specific repeated elements (Ganal et al. 1986; Zhao et al. 1989), whereas more distantly related species can have similar distributions of other specific repetitive elements, for example, telomeric repeats and rDNAs. Repeated sequences can hinder analysis involving in situ hybridization (ISH) by binding labeled probe and increasing chromatin-associated signal background. The functional importance of such binding tends to be greatest for probes made from larger DNA clones, owing to their propensity to include repetitive elements.

C_0t-1 DNA (DNA enriched for highly and moderately repetitive DNA sequences) can be used to compete with labeled probe for repetitive sequences in the specimen, i.e., to eliminate or "block" nontarget-specific binding. For a review of C_0t-1 DNA and its kinetics, see Britten and Kohne (1968). Barrett (1992) demonstrated the use of hamster C_0t-1 DNA to suppress hybridization of repetitive DNA sequences in a labeled probe from a 25-kb clone. Chromosomes subjected to ISH with probe but no C_0t-1 DNA showed a high degree of nonspecific hybridization, whereas chromosomes hybridized with probe and hamster C_0t-1 DNA showed specific signals with little background. Nisson et al. (1991) also demonstrated that C_0t-1 DNA is more effective in blocking *Alu* repeats than total genomic human DNA in ISH. Since C_0t-1 is highly enriched for repetitive DNAs and devoid of unique sequences (opposed to total genomic DNA), it competes for repetitive sequence hybridization sites of the probe or target, but does not inhibit or block hybridization to low copy or unique sequence sites. Green and Veltzen (1992) demonstrated the ex situ use of hamster C_0t-1 DNA as probe and found it superior to total genomic DNA when screening human chromosome specific yeast artificial chromosome (YAC) libraries generated from hamster–human cell lines. Southern hybridization to yeast clones of unblocked hamster total genomic DNA

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probe produced high background and nonspecific hybridization, making it difficult to discriminate between clones containing human or hamster DNAs. The use of hamster C_0t-1 DNA to block probe hybridization to clones containing human DNA, eliminated most of these problems, therefore making the task of differentiating the clones more reliable. It may be surmised that C_0t-1 DNA is critical to ISH and can also aid in ex situ hybridization.

Commercial sources of C_0t-1 DNA currently exist for at least three mammalian species, i.e., hamster, human, and mouse, but are unavailable for most species, including all plants. To overcome this problem, we have developed a technique that generates high-quality C_0t-1 DNA in 1 day and eliminates the need for special DNA-shearing equipment, such as a French press or sonicator. C_0t-1 DNA fractions have been obtained from maize, cotton, sorghum, wheat, tomato, and rice, i.e., from both dicotyledenous and monocotyledenous angiosperms. In all these species, the C_0t-1 DNA was used successfully in ISH procedures to block binding of repetitive sequences.

Materials and methods

Since S1 nuclease degrades linear single-stranded DNA, final yields typically range between one-tenth and one-twentieth of the original starting concentration of genomic DNA, depending on the number of repetitive sequences present in the genome. We recommend that this procedure be carried out with a minimum of 2 mg of RNase-treated DNA to minimize any potential problems associated with RNA contamination. We prepared cotton DNA by the methods of Paterson et al. (1993) and all other DNAs as described by N.M. Ayres, A.M. McClung, P.D. Larkin, H.J.F. Bligh, C.A. Jones, and W.D. Park (submitted for publication).[2] In brief, plant tissues were ground in liquid nitrogen, and approximately 20 mL of powered tissue was added to a 50 mL falcon tube. Twenty millilitres of extraction buffer (6.25 mM potassium ethyl xanthogenate (Fluka), 0.7 M Tris–HCl (pH 7.5), 0.7 M NaCl, plus 10 mM EDTA, pH 8.0) was added to each tube and mixed. Tubes were incubated in a water bath for 45 min at 65°C. The samples were extracted once with an equal volume of chloroform – isoamyl alcohol (24:1) and the supernatant was precipitated with a 0.1 volume of 3 M sodium acetate, pH 5.2 and 2 volumes of ice-cold ethanol. Samples were left at room temperature for 30 min and the DNA was spooled using a glass rod. The DNA was washed in 70% ethanol, dehydrated, resuspended in an appropriate volume of TE (10 mM Tris–HCl plus 1 mM EDTA, pH 8.0), and RNase treated (approximately 50 ng RNase per millilitre of sample). Samples were then extracted with phenol–chloroform, precipitated with ethanol, dehydrated, resuspended in TE, and quantitated on a fluorometer.

Equipment needed

Fluorometer or UV spectrophotometer
Autoclave, adjustable for liquid cycles of 5 minutes
Agarose electrophoresis apparatus
Water baths at 37, 65, and 95°C
Bench-top swinging-bucket centrifuge, preferably refrigerated, capable of 2700 × *g* or greater, and able to hold 15-mL tubes
Liquid nitrogen container and ice buckets

Solutions

S1 nuclease (Boehringer Mannheim, Indianapolis, Ind.) 10 000 U at a concentration of 400 U/μL)
5 M NaCl
10× S1 nuclease buffer (0.5 M NaOAc (pH 4.5), 45 mM $ZnSO_4$ (Sigma, St. Louis, Mo.)) filter sterilized.

Procedures

Preparation of genomic DNA

1. Quantitate clean RNA-free genomic DNA. Concentration is the key to determining C_0t-1 reannealing time.
2. Dilute the genomic DNA to a concentration between 100–500 ng/μL using 5 M NaCl and double-distilled H_2O to a final concentration of 0.3 M NaCl. The salt concentration influences the rate of reassociation of single-stranded DNA, and most C_0t values are standardized at 0.3 M NaCl. DNA concentrations within the suggested range give the most practical reannealing times, from 678 to 3389 s.

DNA shearing

3. Note the volume of DNA and aliquot 1-mL portions into 1.5 mL screw cap tubes and cap lightly. Place all the tubes into a microfuge rack and add water to the remaining unoccupied holes of the rack. Autoclave the samples 3 times on a liquid cycle (5 min each). After 3 cycles, remove 500 ng of DNA and determine its size by electrophoresis in a 1% agarose gel. Ideally, the DNA fragments should range between 100 and 1000 bp in size. If not, autoclave the DNA, as above, until the proper size range is obtained.
4. Once sheared, combine the DNA samples in a 15 mL polypropylene tube. Put on ice.

Reannealing

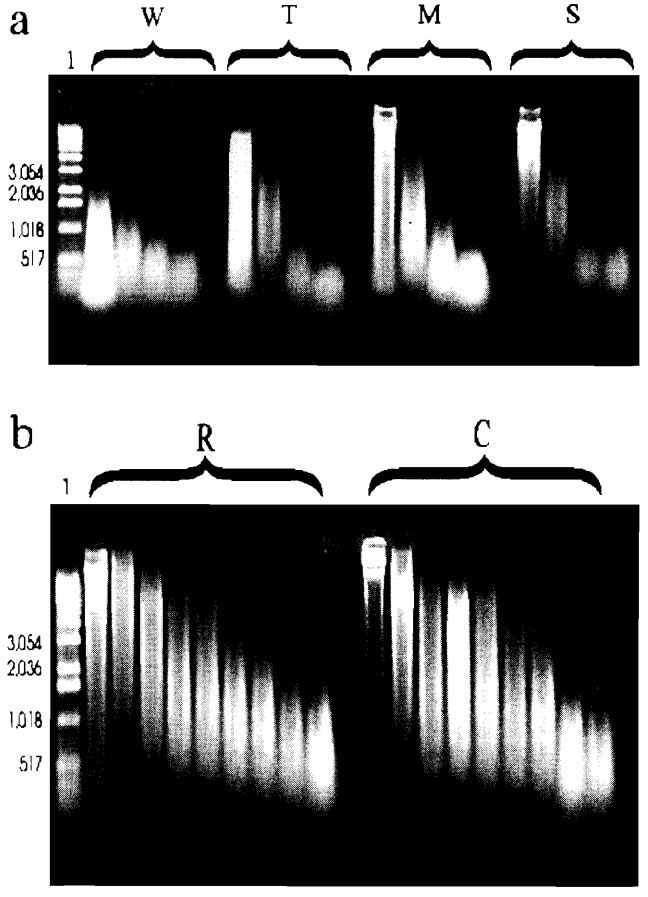
5. Calculate the time needed for the reannealing reaction using the formula $C_0t = 1 = \text{mol/L} \times T_s$, where the initial concentration (C_0) is calculated in moles of nucleotides per litre and time is in seconds. Assume an average molecular weight for a deoxynucleotide monophosphate to be 339 g/mol (gram formula weight from Sigma). For example, if the sheared DNA is at a concentration of 300 ng/μL, this is equivalent to 0.300 g/L. Using 1 mole of dNTP equal to an average of 339 g/mol, then moles of sheared DNA is equal to (0.300 g/L)/(339 g/L) = 8.85×10^{-4} mol/L. By using the formula we can determine that the reaction must therefore proceed for 1130 s in order for C_0t to equal 1. (Note: the molecular weight may be recalculated if the G/C–A/T ratio of the DNA being used is drastically different from 1:1.)
6. Based on the volume of DNA, calculate the amount of 10× S1 nuclease buffer needed to equal a 1× final working volume (including enzyme). Calculate this using an increased volume from that of the DNA volume. The S1 nuclease concentration is 1 U S1 per microgram of DNA. *Do not add the buffer or enzyme at this step.*
7. Once all the calculations have been made, the DNA is denatured by placing the 15-mL tube in a 95°C water bath for 10 min.
8. Remove the tube, cool it by swirling in ice water for 10 s, and place it in the 65°C water bath. Start timing the reannealing period for the calculated C_0t-1 time.

S1 nuclease digestion

9. Following the time allotted for reannealing, remove the tube, add the calculated amount of 10× S1 buffer, and mix thoroughly. Add the S1 enzyme and again mix the solution thoroughly, but gently. Immediately place the tube in the 37°C water bath for 8 min.
10. Stop the reaction by immediate phenol extraction using equal volumes of TE-equilibrated phenol. If this cannot be done, quick freeze the tube(s) in liquid nitrogen and keep frozen until the sample can be extracted with TE–phenol.

[2] N.M. Ayres, A.M. McClung, P.D. Larkin, H.F.J. Bligh, C.A. Jones, and W.D. Park. Microsatellites and a single nucleotide polymorphism differentiate apparent amylose classes in an extended pedigree of U.S. rice germplasm. Submitted for publication.

Fig. 1. (*a*) Autoclave shearing of total genomic DNAs of wheat (W), tomato (T), maize (M), and sorghum (S). (*b*) Autoclave shearing of total genomic DNAs of rice (R) and cotton (C). A 1 kb ladder DNA marker is shown in lane 1 of both *a* and *b*. For each species, the left-most lane is unsheared total genomic DNA, whereas those to its right were loaded with DNA that has been progressively sheared by autoclaving in 5-min increments. The DNA was repeatedly sheared until it was less than 1 kb in size.

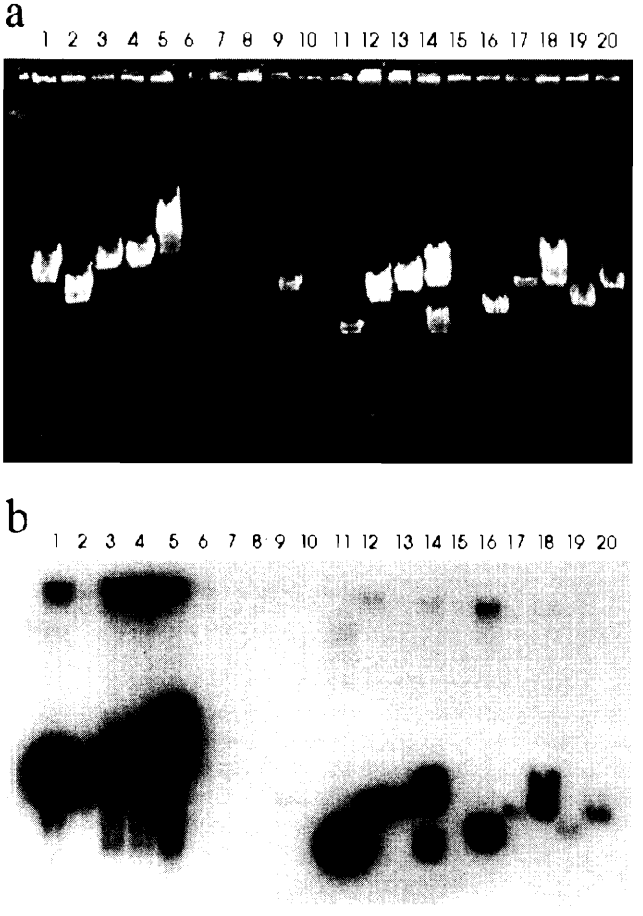


11. Further extract the supernatant with an equal volume of phenol–chloroform (chloroform – isoamyl alcohol (IAA) 24:1) followed by an equal volume of chloroform (chloroform–IAA 24:1).
12. Precipitate the DNA overnight using 2.5 volumes of 100% ethanol.
13. Spin down the pellet, dry, and resuspend in approximately 100–200 μL of TE.
14. Quantify the C_0t-1 DNA.
15. Store DNA at −20°C until needed.

Results and discussion

Several contempary methods of ISH require the use of species-specific C_0t-1 DNAs. Early methods of C_0t-1 DNA isolation required the use of hydroxyapatite columns, but these are expensive and yields vary owing to their sensitivity to temperature fluctuations and varying salt concentrations (Britten and Kohne 1968). We have developed a method based on S1 nuclease to isolate C_0t-1 DNA from plants. The method is inexpensive, fast, simple, and reliable.

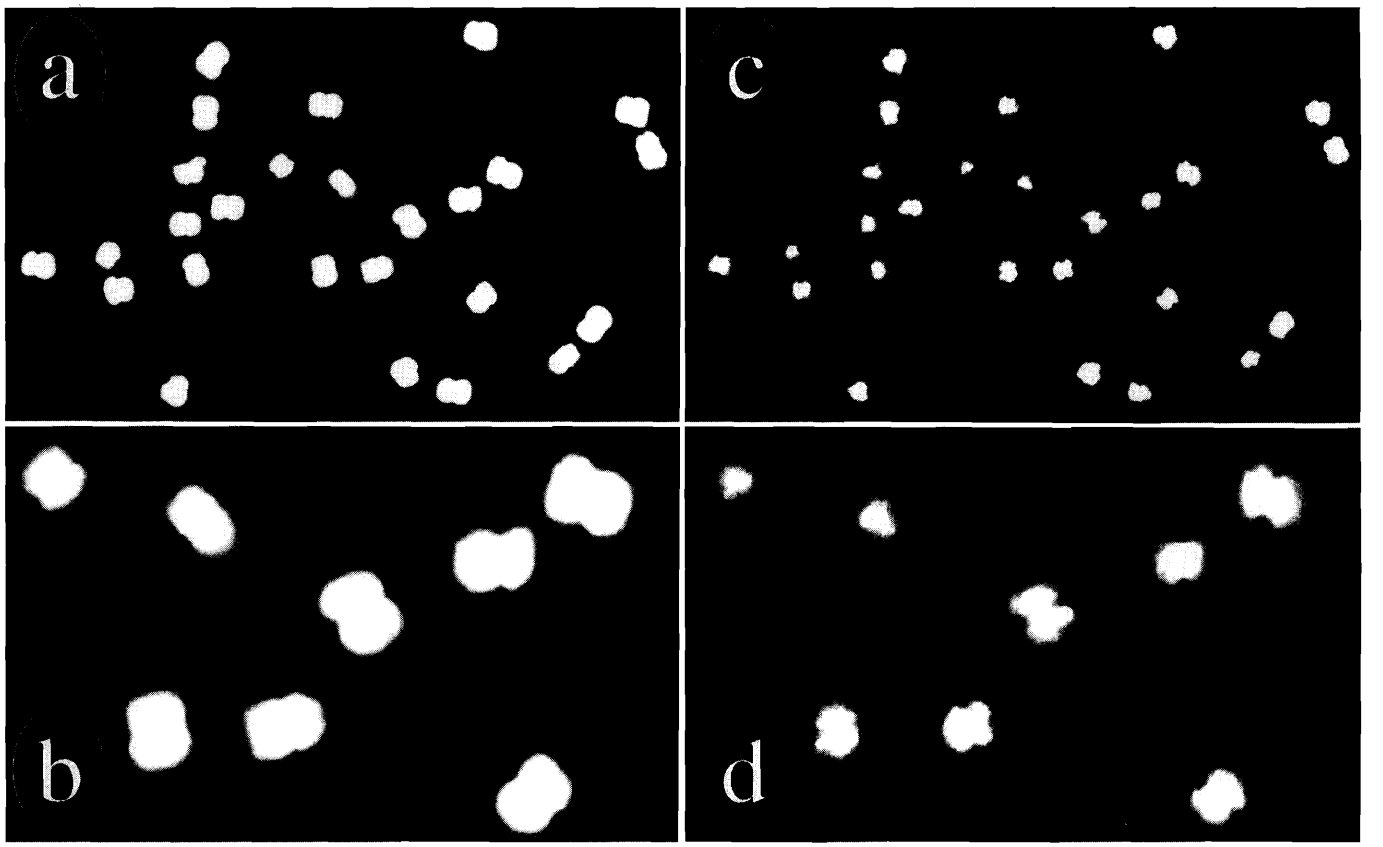
Fig. 2. (*a*) Ethidium bromide stained CHEF (contour-clamped homogeneous electric field) gel of *Gossypium hirsutum* BAC clones 1339–1358 (labeled 1–20, respectively). BAC DNA was isolated by alkaline lysis plasmid minipreparations. Inserts were excised with *Not*I restriction endonuclease digestion prior to electrophoresis. (*b*) Southern blot of *a* hybridized with radio-labeled cotton C_0t-1 DNA ([^{32}P]dCTP) (labeled 1–20). BACs containing highly repetitive sequences produce intense signals (e.g., lanes 1 and 3–5) and are less suitable for ISH owing to a higher dispersion of signal on the chromosomes. BACs that show little hybridization (e.g., lanes 2, 17, 19, and 20) harbor a low proportion of repetitive sequences and require a minimum of 20× blocking C_0t-1 DNA to inhibit the hybridization of the repetitive DNA sequences. BACs to which C_0t-1 DNA did not hybridize (e.g., lanes 9 and 10), contain unique DNA sequences and can be used for ISH with little or no blocking.



We have found that the yields of C_0t-1 DNA are relatively high for large genomes, which typically contain fairly large proportions of repetitive DNA: for example, approximately one-tenth the concentration of C_0t-1 DNA was obtained from the genomic DNAs of bread wheat, with a genome size of 16 695 – 17 423 Mbp[3] (Bennett and Smith 1976; Michaelson et al. 1991); of tetraploid cotton, with a genome size of 2702–3136 Mbp (Michaelson et al. 1991; Gomez et al. 1991; Bennett and Smith 1976); and of maize, with a genome size of

[3] Genome size in megabase pairs was calculated from picogram values based on 1pg = 965 Mbp per 1C value (Strauss 1971).

Fig. 3. FISH painting of propidium iodide (PI) stained (red) *Gossypium arboreum* (A genome) chromosomes using fluorescein (green) labeled *G. arboreum* C_0t-1 DNA. A color image (24-bit RGB (red, green, blue)) was digitally separated into red, blue, and green layers and the red and green layers were converted to 8-bit greyscale using Corel Photo-Paint. (*a*) The converted red layer shows chromosome morphology analogous to PI stained chromosomes. (*b*) An enlargement of a section of the image in *a*. (*c*) Bright regions of the converted green layer show the localization of signal on the chromosomes. Hybridization signal was far stronger in pericentric regions and less intense in distal regions of the chromosomes. (*d*) An enlargement from *c* of the same area as *b* showing hybridization signal of the C_0t-1 DNA.



2369–2939 Mbp (Rayburn et al. 1985; Laurie and Bennett 1985). Species having smaller genome sizes and lower proportions of repetitive DNAs, for example, rice, with a genome size of 386–579 Mbp (Bennett and Smith 1976, 1991); sorghum, with a genome size of 753–840 Mbp (Laurie and Bennett 1985, Michaelson et al. 1991); and tomato, with a genome size of 917–1062 Mbp (Bennett and Smith 1991; Michaelson et al. 1991) typically yielded about one-twentieth the concentration of genomic DNA. Therefore large quantities of genomic DNA are necessary.

The use of an autoclave for shearing genomic DNA is non-specialized, rapid, and cost-effective. The number of cycles needed is dependent on the nature of the DNA (i.e., species, A/T–G/C content), concentration, and the initial molecular weight. Sorghum, maize, tomato, and wheat were sheared after only 3 cycles, whereas rice and cotton required a minimum of 8 cycles (Fig. 1). We have found that autoclaving DNA in small volumes (1 mL) gives more uniform shearing than preparation in larger volumes. Experiments have shown variation in the number of cycles required for the shearing of DNA among different preparations of the same species. Thus, cyclic autoclave treatments should be intermittently analyzed for size.

C_0t-1 DNAs generated using this protocol have performed well for in situ suppressive hybridization of large genomic clones (bacterial artificial chromosomes (BACs)) in the several crops tested. In the case of ISH using BACs, where the probes range in size from 100–300+ kb, we have found that some largely unique probes require no blocking and that the amount of C_0t-1 DNA needed (from 20× to 100× C_0t-1 DNA) increases proportionately with the amount of repetitive DNA in the BAC. For probes containing largely unique sequences and few repetitive sequences, such as certain BACs, a lower relative concentration (e.g., 20×) of C_0t-1 DNA to probe DNA is optimal for blocking (Hanson et al. 1995). Other probes may require 100× C_0t-1 DNA blocking to obtain discrete ISH signals only at low-copy DNA target sites.

C_0t-1 DNA isolated by the described procedure is amenable to both random labeling, as shown by Southern hybridization to cotton BACs (Fig. 2), and nick translation, for use in genomic or chromosome painting. To demonstrate genomic painting, C_0t-1 DNA from the diploid A-genome cotton *Gossypium arboreum* L. was biotinylated by nick translation and hybridized to *G. arboreum* metaphase chromosomes (Fig. 3). Chromosomes show FISH signals near the centromere with little to no signal toward the telomeres. This result was concordant with our initial results that five BAC probes, each containing a mostly low-copy segment, hybridized to the

distal regions of chromosomes in cotton. C_0t-1 DNA can also be used in genomic in situ hybridization (GISH). For example, in cotton, C_0t-1 DNA derived from *G. arboreum*, an A-genome diploid species ($2n = 2x = 26$), can be used to paint the A subgenome of *Gossypium hirsutum* L., an AD disomic tetraploid species ($2n = 4x = 52$), thus allowing differentiation of the chromosomes of the A and D subgenomes. GISH has been conducted in *G. hirsutum* using biotin-labeled total genomic *G. arboreum* DNA (A genome) as probe, blocked with a 20× excess of unlabeled total genomic *Gossypium raimondii* Ulbr. (D genome) DNA. Painting of the A subgenome was intense, with only the highly conserved 18S–28S rDNA sites in the D subgenome producing signal (Hanson et al. 1996).

The described procedure produces high quality C_0t-1 DNA that can be used as a probe, but more often as a blocking agent, in suppressive, Southern, and in situ hybridizations. It is rapid, cost effective, requires no special equipment, and allows for the isolation of species-specific C_0t-1 DNA that is otherwise commercially unavailable.

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