



Protocols

High Throughput BAC DNA Isolation for Physical Map Construction of Sorghum (*Sorghum bicolor*)

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Abstract. With the aim of constructing a physical map of sorghum, we developed a rapid, high throughput approach for isolating BAC DNA suitable for restriction endonuclease digestion fingerprinting, PCR-based STS-content mapping, and BAC-end sequencing. The system utilizes a programmable 96 channel liquid handling system and associated accessories that permit bacterial cultivation and DNA isolation in 96-well plate format. This protocol details culture conditions that optimize bacterial growth in deep-well plates and criteria for BAC DNA isolation to obtain high yields of quality BAC DNA. The system is robust, accurate, and relatively cost-effective. The BAC DNA isolation system has been tested during efforts to construct a physical map of sorghum.

Key words: BAC, BAC-end sequencing, DNA isolation, sorghum

Abbreviations: BAC, bacterial artificial chromosome; SSR, simple sequence repeat; STS, sequence-tagged site; TB, terrific broth.

Introduction

Developing contiguous sets of overlapping genomic DNA clones (contigs) is one of several necessary steps for construction of a physical map of any genome. Large-insert DNA libraries are necessary for construction of overlapping contigs of complex genomes as they provide long-range continuity for the genome and further enable the physical and genetic maps to be aligned. Bacterial artificial chromosomes (BACs) have become the preferred large-insert cloning system for genomic analysis because such libraries are characteristically stable, show high-fidelity, and are compatible with common automated DNA purification procedures (Schmitt et al., 1996; Boysen et

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al., 1997). BACs are proven substrates for sequencing when subcloned into plasmids or when used directly as a template for BAC-end sequencing.

To validate a physical map, several independent approaches must be utilized to assemble contigs (Balding, 1994). Several commonly used approaches to assemble contigs include: DNA restriction endonuclease fingerprint analysis (Sulston et al., 1988, 1989), PCR-based sequence-tagged site (STS) content mapping (Green and Olson, 1988; Olson et al., 1989), BAC-end sequencing to generate new STS probes specific for BAC ends (Boysen et al., 1997), and colony hybridization of BAC libraries with labeled-DNA probes (Evan and Lewis, 1989). Of these techniques, the first three require the isolation of high-quality BAC DNA from potentially thousands of BAC clones in order to generate a set of overlapping contigs. Hence, the development of a robust, high throughput system for isolating high quality BAC DNA is a necessary initial requirement for physical map construction.

To the end of assembling an integrated sorghum physical map, we developed a rapid, high throughput technique for isolating BAC DNA in a 96-well format in sufficient quantity and quality for physical map construction. A key to the efficiency of this method is a semi-automated 96 channel liquid handling system used in conjunction with associated equipment (e.g. vortexer, centrifuge with appropriate rotor assembly) designed for deep-well plates. In addition, we have optimized several parameters of bacterial growth in deep-well plates to insure consistent high yields of quality BAC DNA. Utilizing this system, 1152 BAC DNA minipreps (12, 96-well plates) can be processed in eight hours with DNA yields of 1.0–2.0 μg (from a 1.5 mL culture). In addition to being robust, this semi-automated DNA isolation system eliminated common pipetting errors that can plague non-automated DNA isolation procedures.

Materials and Methods

Equipment and supplies

- Quadra 96 Model 220 liquid handling system (TomTec)¹
- Beckman GS-6KR centrifuge with PTS-2000 canisters for deep-well plates
- Multi-Tube vortexer (VWR)
- Mini-Orbital shaker (Bellco)
- Replicator pin-tool for 96-well plates (Boekel)
- AirPore Tape Sheets (Qiagen)
- 2 mL capacity, 96-well Uniplates (Whatman)
- Seal and Sample Aluminum Foil sealants (Beckman)
- ProCipitate protein-binding resin (LigoChem)

- RNase Cocktail (Ambion)
- Plexiglas clamp with silicone rubber liner

Note

¹Mention of trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products or vendors that may be suitable.

Bacterial cell growth in deep-well plates

1. Inoculate microtiter plate containing 150 μ L media (LB plus 12.5 μ g/mL chloramphenicol) with BAC library frozen stocks using a replicator pin-tool. Grow cultures stationary for 14–16 h at 37 °C.
2. Add 1.4 mL of TB media plus chloramphenicol (12.5 μ g/mL) to each well of a 96-well Uniplate (2 mL capacity deep-well plate).
3. Inoculate deep-well plate with freshly replicated cultures using a replicator pin-tool.
4. Cover plates with AirPore gas permeable plate sealant.
5. Shake plates on Mini-Orbital shaker equipped with a platform designed for micro titer plates (setting of 5.5). Grow for 18–22 h at 37 °C with constant shaking.

BAC DNA isolation for restriction endonuclease fingerprinting and STS-content mapping

All liquid pipetting for BAC DNA isolation was done with a Quadra 96 liquid handling system.

1. After bacterial growth, place deep-well plates on ice for 15 min.
2. Centrifuge deep-well plates in Beckman bench top centrifuge for 15 min at maximum g -force of $2,500 \times g$ (4 °C).
3. Drain bacterial pellets by inverting plates and repeatedly tapping on paper towels.
4. Centrifuge deep-well plates again for five min to re-pellet cells and collect residual fluid.
5. Drain bacterial pellets completely by tapping repeatedly on paper towels.
6. Add 150 μ L of solution I (50 mM Tris-HCl, pH 7.5; 50 mM EDTA, pH 7.5) to each well using the Quadra 96. Cover plates with Aluminum foil plate sealants.
7. Resuspend bacterial pellets utilizing a multi-tube vortexer (setting 5) for 5–10 min at room temperature.
8. Add 300 μ L of solution II (0.2 M NaOH, 1% SDS, freshly prepared).

9. Cover plates with aluminum foil sealants, clamp tightly in rubber-lined Plexiglas clamp and invert four times. Use of the rubber-lined Plexiglas clamp insures a tight seal at the opening of each well during inversion and thus prevents well leakage and cross-contamination. Let stand at room temperature for five min. Unclasp plates and centrifuge briefly to remove fluid from plate cover.
10. Add 225 μL of ice-cold solution III (3 M potassium acetate, pH 5.2). Seal plate with aluminum foil sealant, clamp firmly in rubber-lined Plexiglas clamp, and invert four times. Place plates on ice for 15 min.
11. Centrifuge deep-well plates at 30 °C at 2,500 $\times g$ for 30 min.
12. Transfer clarified supernatant to new deep-well plate in two sequential steps (425 μL first transfer, 175 μL second transfer) utilizing the Quadra 96.
13. To insure complete clarification of the supernatant, spin samples again at 2,500 $\times g$ for five min (30 °C).
14. Transfer 445 μL of clarified supernatant to a new deep-well plate containing 1.0 volume of isopropanol. Precipitate BAC DNA overnight at -20 °C.
15. The next day, centrifuge samples at 2,500 $\times g$ for 30–60 min. Drain pellets by inversion, rinse with 800 μL of 70% ethanol, followed by a second rinse with 800 μL of 80% ethanol.
16. Air-dry nucleic acid pellets at 37 °C and resuspend in TE (pH 8.0) containing 10 $\mu\text{g}/\text{mL}$ RNase A. Dissolve nucleic acid at room temperature and then heat to 65 °C for 15 min prior to DNA quantitation by flourometry.

BAC-end sequencing

To directly sequence the ends of BAC clones, additional steps were added to the BAC DNA isolation protocol detailed above to aid in the removal of RNA and protein contaminants. All other operations (pipetting, sample mixing, centrifugation) were as detailed above.

1. Start BAC cultures from a single isolated colony (LB plate containing 20 $\mu\text{g}/\text{mL}$ chloramphenicol). Grow 3 mL cultures (TB media) of each BAC clone in culture tubes (14 h with shaking at 37 °C) and subsequently transfer 1.2 mL of each culture to deep-well plates. Alternatively, grow 1.4 mL cultures (TB media) directly in deep-well plates for approximately 16 h with shaking at 37 °C.
2. Centrifuge deep-well plates and drain bacterial pellets completely as detailed above.

3. Resuspend each pellet in 300 μL of solution A (50 mM Tris-HCl, 10 mM EDTA, pH 8.0 plus 100 $\mu\text{g}/\text{mL}$ RNase A). Incubate five min at room temperature.
4. Lyse cells by adding 300 μL of solution II (see above). Mix by inverting plates five times. Incubate five min at room temperature.
5. Precipitate bacterial debris by adding 300 μL of solution III (see above). Incubate on ice for five min.
6. Add 30 μL of RNase cocktail (Ambion), and incubate at room temperature for 30 min.
7. Add 150 μL of ProCipitate protein-binding resin (LigoChem). Incubate at room temperature for 5 min with several mixings by inversion.
8. Centrifuge at 2,500 g for 30 min (25–30 °C). Transfer 900 μL of clarified supernatant to a new plate containing 0.7 volumes of isopropanol. Incubate at –20 °C for 30 min.
9. Pellet BAC DNA by centrifugation at 2,500 g for one h (room temperature). Rinse the pellet with 450 μL of 80% ethanol. Drain pellet carefully by inversion. Air-dry pellet being careful not to over-dry.
10. Resuspend pellet in 20 μL of water. Yields of sequence-grade BAC DNA were between 25 ng/ μL and 50 ng/ μL .

BAC DNA was sequenced with BigDye terminators basically as described (Rosenblum et al., 1997) except one-half volume reactions were used. The reaction mixture contained five μL BAC DNA (125–250 ng DNA), 1.0 μM of vector-specific primer, 4 μL BigDye Dye Terminator Ready Mix, and brought to volume (10 μL) with water. BAC samples were cycled in a Perkin Elmer 9600 thermocycler according to the following protocol: initial denaturation 95 °C for two min, followed by 100 cycles of 95 °C for 30 s, 50–60 °C (depending on primer annealing temperature) for 20 s and 60 °C for 4 min. Excess terminators were removed using a Sephadex G-50 spin column. Samples were vacuum-dried and resuspended in 1.5 μL EDTA/formamide loading dye (5:1 ratio formamide:25 mM EDTA [pH 8.0] plus 50 mg/mL blue dextran) and the entire sequence reaction was loaded on an ABI Prism 377XL sequencer.

BAC DNA restriction endonuclease fingerprinting

Restriction endonuclease fragmentation analysis of sorghum BAC DNA was conducted as described previously (Quanzhou et al., 1995) except ^{33}P -dATP was substituted for ^{32}P -dATP. Polyacrylamide gel electrophoresis of restriction endonuclease DNA fragments and labeling of lambda DNA markers were essentially as described (Quanzhou et al., 1995). Polyacrylamide gels were run (85 watts, 2.5 h), dried and exposed to X-ray film for 2–4 d.

PCR screening of BAC clones with genetic markers

To screen BAC clones for PCR-based genetic markers, a 'hot start' amplification strategy utilizing *AmpliTaq* Gold DNA polymerase was employed. The reaction conditions were: 1X Perkin Elmer buffer II, 2.5 mM MgCl₂, 200 μM dNTP (minus dATP), 1 μM dATP, 1 μCi ³³P dATP, 12.5 ng of each primer, and 4 ng BAC DNA in a total volume of 12.5 μL. Cycle parameters for all PCR reactions were: 95 °C, 10 min followed by 95 °C one min, 55 °C one min, 72 °C one min for 33 cycles followed by a seven min final extension at 72 °C. Reactions were run in either a Robocycler (Stratagene Inc) or in a Perkin Elmer 9700 thermal cycler. Samples were electrophoresed on 6% polyacrylamide gels containing 7.6 M urea (85 W, 2.5–4 h). Gels were dried and exposed to X-ray film for 1–3 d.

Results and Discussion

BAC DNA extraction

The major aim of this study was to develop an affordable, semi-automated, high throughput BAC DNA isolation system that provides sufficient yield of high quality BAC DNA for physical map construction of cereals utilizing sorghum as a target plant species. Our initial efforts to develop a high throughput BAC DNA isolation system involved adapting our standard BAC DNA protocol to 96-well format. Our BAC DNA isolation protocol is basically a standard alkaline lysis miniprep (Sambrook et al., 1989) in which care is taken to prevent chromosomal contamination and plasmid nicking (see Materials and Methods). Furthermore, we routinely start BAC cultures from freshly replicated micro titer plates (or a single isolated colony) rather than directly from frozen stocks as growth of BAC cultures from aged frozen stocks may result in significant variation in culture growth rates and BAC DNA yields.

Initial efforts identified two major technical difficulties associated with bacterial cultivation and DNA isolation in a deep-well format: (1), suboptimal culture aeration; and (2) fluid transfer errors during manual pipetting. An additional complication was that the time required for manual pipetting restricted the number of clones that could be processed in a given period of time. To address the liquid handling requirements of a high throughput system, a semi-automated 96 channel liquid handling system, the Quadra 96, was utilized. The Quadra 96 is, in essence, a 96 channel P-500 pipettor that can be programmed for the following features: (1) volume of fluid aspirated and dispensed (20–450 μL), (2) the rate at which fluid is aspirated and dis-

pensed, and (3) the height within the well at which fluid is aspirated and dispensed. These features make this liquid handling system ideally suited for conducting DNA minipreps in a 96-well plate format. Utilizing this liquid handling system, common pipetting errors were minimized and the time required for each liquid handling step was reduced drastically. To complete this high throughput system, additional accessory equipment designed for micro titer plates was utilized. A Mini-Orbital shaker equipped with a platform with a capacity of 8 deep-well plates was obtained (Belco). The shaker mixes by a vortex-like action thereby enhancing culture mixing and gas exchange during bacterial cultivation. In addition, a multi-tube vortexer with the capacity of four deep-well plates greatly reduced the time required to resuspend bacterial pellets. Finally, a Beckman PTS-2000 cannister assembly was obtained to permit centrifugation of deep-well plates (capacity of 4 plates).

Several simple modifications of our BAC DNA protocol were necessary to obtain high quality BAC DNA in a deep-well format. Several of the modifications were the result of the relatively low *g*-forces required for centrifugation of deep-well plates. The most critical modification of the DNA protocol involved increasing the time and temperature of centrifugation necessary to completely clarify the supernatant following potassium acetate-induced precipitation of bacterial debris. To fully clarify the supernatant, bacterial debris was pelleted at maximum *g*-force (2,500 *g*) for 30 min at 25–30 °C. Centrifugation at low temperatures (4–20 °C) or for shorter time periods significantly hindered clarification of the supernatant resulting in impure BAC DNA preparations that were difficult to resuspend. Following a 30 min centrifugation, a very tight bacterial debris pellet was formed and the height of the debris pad was very consistent from well to well. The compact nature of the debris pellet further allowed us to program the Quadra 96 to aspirate the supernatant from a preset well depth without disturbing the debris pad. The second adaptation to the standard alkaline lysis DNA protocol required the construction of a rubber-lined Plexiglas clamp to permit inversion of deep well plates for steps requiring mixing. While other methods of mixing can be employed (e.g. mixing with wide-bore pipette tips), mixing deep-well plates by inversion is complete and gentle resulting in less chromosomal shearing. The rubber-lined Plexiglas clamp permitted inversion of deep-well plates without leakage and cross-contamination of individual wells. Utilizing this system, between 8 and 12 deep-well plates (768–1152 BAC minipreps) can be processed in an 8 h period and with no sample cross-contamination or sample handling errors.

Optimization of bacterial growth in deep-well plates

To obtain consistent high yields of BAC DNA, we attempted to optimize bacterial cultivation in deep-well plates. Two parameters were examined that

affected BAC DNA yields in deep-well plates: (1) culture growth media, and (2) gas exchange during bacterial growth. To address the effect of bacterial growth media on BAC DNA yields, cultures were grown in both LB and TB media. Initially, SOC media was also examined but cells were found to lyse in this media after approximately 20 h of growth. To examine the effect of gas exchange on BAC DNA yields, two types of plate sealants were examined: Qiagen AirPore microporous tape sheets and Beckman Seal and Sample Aluminum foil plate sealants. AirPore sealants are gas permeable adhesive sheets designed to promote gas exchange while preventing well spillover. The results of these comparisons are shown in Figure 1.

The most consistent well-to-well bacterial growth and highest BAC DNA yields were obtained using TB growth media in conjunction with AirPore microporous plate sealants. When gas exchange was restricted by covering cultures with aluminum foil adhesive plate sealants, bacterial growth and BAC DNA yields were reduced in comparison to wells covered with AirPore sealants. Additionally, greater well-to-well variation in bacterial growth and BAC DNA yields were observed when gas exchange was restricted by nonporous aluminum sealants. This variability was observed with both LB and TB cultures (as depicted by SD bars). In general, TB-grown cultures reached saturation at a higher cell density and displayed higher BAC DNA yields when compared to LB-grown cultures. It should be noted that a cellular cultivation period greater than 24 h did not increase cell density nor BAC DNA yields under any growth conditions examined (data not shown). Shorter growth periods (e.g. 12–14 h) did reduce the yield of BAC DNA in TB media as cell density had not reached saturation at this time point.

DNA restriction endonuclease fingerprinting

Having optimized several growth parameters affecting BAC DNA yields, it was necessary to determine whether BAC DNA was of sufficient quality for analyses involved in physical map construction. Figure 2A shows the results of restriction endonuclease fingerprinting of BAC DNA. The most noticeable difference in the restriction digest patterns was in the intensity of the signal obtained between cultures grown with AirPore and aluminum foil sealants. When nonporous Aluminum foil sealants were used, the fingerprint signal was variable from sample-to-sample which likely reflects the inconsistent nature of bacterial growth and BAC DNA yields. In contrast, consistent high-quality restriction endonuclease fingerprint signals were obtained with both TB and LB cultures sealed with AirPore adhesive strips. Figure 2B illustrates a random sampling of restriction endonuclease fingerprints of BAC clones cultured in TB growth media and with AirPore plate sealants. High-quality restriction endonuclease fingerprints were obtained with all clones sampled

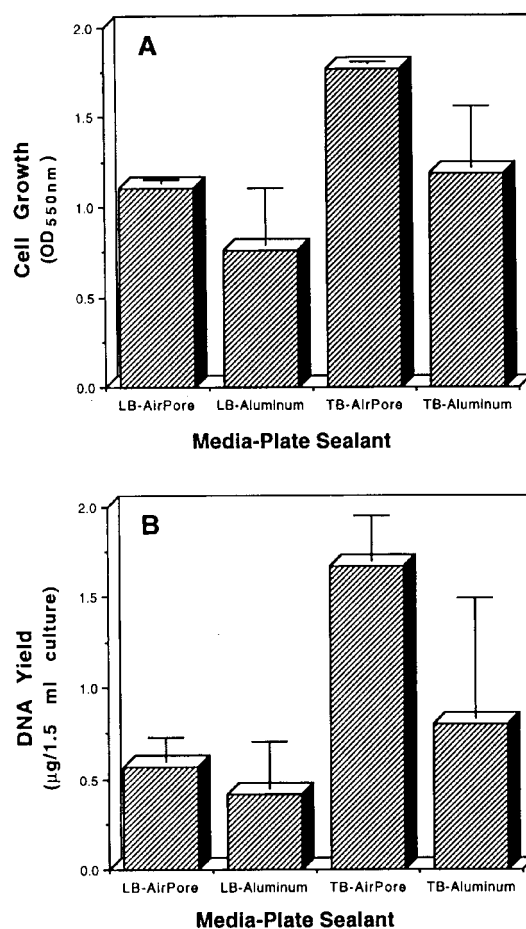


Figure 1. Effect of growth media and plate sealant on bacterial growth and BAC DNA yields in deep-well plates. LB growth media (1.4 mL) was added to one-half of the wells of a 96 well Uniplate and TB growth media was added to the remaining wells. One-half of the plate was covered with AirPore tape sheets and the remaining half covered with aluminum foil (Seal and Sample) sealants. Cultures were grown for 24 h at 37 °C, followed by determination of cell density and isolation and quantitation of BAC DNA.

indicating that the DNA is of sufficient quality to permit contig assembly via BAC DNA fingerprinting. To date, we have fingerprinted approximately 25,000 sorghum BAC clones in a 12 month time period utilizing this protocol. Based on the percentage of BAC clones displaying a readable fingerprint, a throughput success rate of 92% was estimated for this protocol.

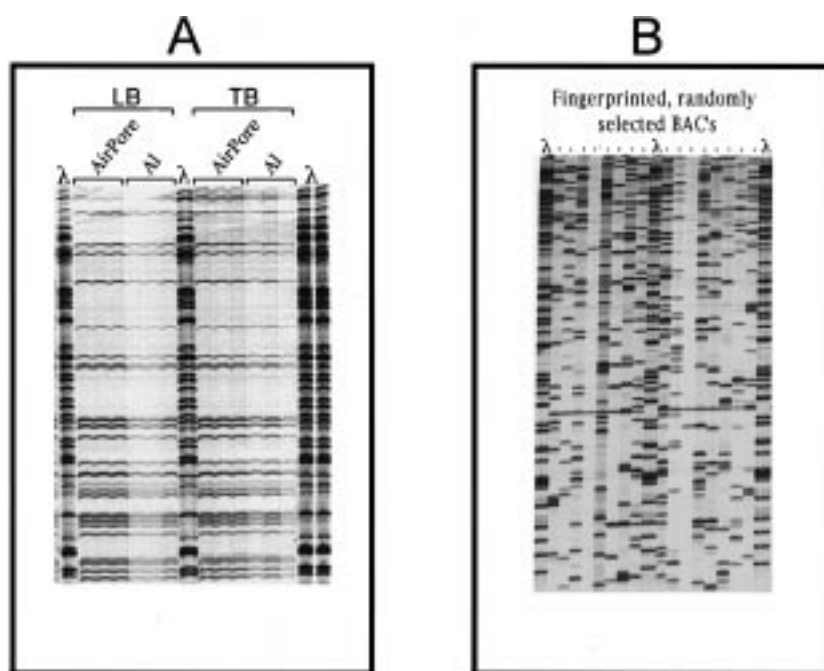


Figure 2. Restriction endonuclease digestion analysis of BAC DNA. (A) A single BAC clone (designation BAC clone 6A3) was used as culture inoculum for all sample wells (1.5 μ L inoculum for 1.5 mL growth media). Cultures were grown and DNA isolated as described for Figure 1. Restriction endonuclease digestion analysis of BAC DNA was conducted and a representative sample of restriction digest fingerprints are shown for each growth condition. (B) BAC clones, randomly sampled from the sorghum BAC library, were grown in TB growth media with AirPore plate sealants. BAC DNA was isolated and restriction endonuclease digestion analysis was conducted on each sample. Each lane represents a fingerprint analysis from a unique sorghum BAC clone with DNA standard lanes appropriately labeled (λ).

Multiplexed amplification of PCR-based genetic markers

We next examined whether BAC DNA was of sufficient quality to permit PCR-based screening of BACs for STS and SSR genetic markers. To increase the efficiency of PCR-based screening, more than one SSR was amplified in each multiplexed PCR reaction. A strong PCR signal was obtained when a single positive BAC clone (4 ng DNA) was used as PCR template (Figure 3A). The BAC DNA was also of sufficient quality to produce a strong, unambiguous PCR signal from a complex pool of BAC DNA that contained a single target BAC clone (Figure 3B). PCR-based genetic marker content mapping of large BAC libraries necessitates a BAC clone pooling strategy to reduce the number of PCR reactions to a manageable number. Hence, BAC DNA isolated from pooled clones must be of sufficient quality to produce a

strong, unambiguous signal in those pools containing a target sequence. Each pool of BAC DNA shown in Figure 3B consisted of 1024 unique clones and a single BAC clone accounted for the PCR signal associated with each positive pool. The unambiguous nature of the signal is due, in part, to the quality of BAC DNA and also to a 'hot start' PCR strategy utilizing Amplitaq Gold. The elimination of nonspecific PCR products especially under multiplexed conditions is critical in STS-content mapping as spurious PCR signals may result in incorrect assignment of BAC clones to contigs and hence reduce the accuracy of the resulting physical map. The present results indicate that the BAC DNA isolation protocol detailed within, when used in conjunction with a 'hot start' multiplexed PCR amplification, is an effective system for STS-content mapping of the sorghum genome.

BAC-end sequencing

An efficient method for contig building and contig confirmation is to obtain and map new STS markers from the end sequences of BAC clones. Generation of new STS markers from the ends of contigs is crucial for map closure of any large-scale physical mapping project. Key to obtaining sequence information from the BAC template is the quality of BAC DNA. Numerous protocols exist for obtaining sequence-grade BAC DNA including organic solvent extraction (protocol guide, Autogen 740 Automated nucleic Acid Isolation System), spooling BAC DNA to physically remove contaminants (Fajas et al., 1997), and use of protein-binding resins to remove protein contaminants (<http://www.tigr.org/softlab/TPFBACmultiprep.052397.html>). Though each of these techniques can yield sequence-grade BAC DNA, it was desirable to develop a protocol that was easily adaptable to our high throughput BAC DNA isolation system and avoided the use of organic solvents. The protocol we decided upon was an adaptation of the protocol detailed by TIGR. The protocol is, in essence, a standard alkaline lysis miniprep that utilizes a protein-binding resin, ProCipitate (Ligochem, Inc.), to remove protein contaminants and an RNase cocktail (RNase A&T₁) to eliminate RNA contamination.

Utilizing this modified alkaline lysis protocol, we were able to consistently obtain sequence-grade BAC DNA from 1.2 mL aliquots of BAC cultures (TB media) grown in culture tubes. The average high-quality read length was approximately 500 base pairs utilizing BigDye terminators (data not shown). Preliminary results further indicate that sequence-grade BAC DNA can be obtained from 1.4 mL TB cultures grown directly in deep-well plates thereby eliminating the need for growing larger cultures (typically 3–20 mL LB) followed by transfer to deep-well plates for DNA isolation. The ability to obtain sufficient BAC DNA from a smaller culture volume should permit the

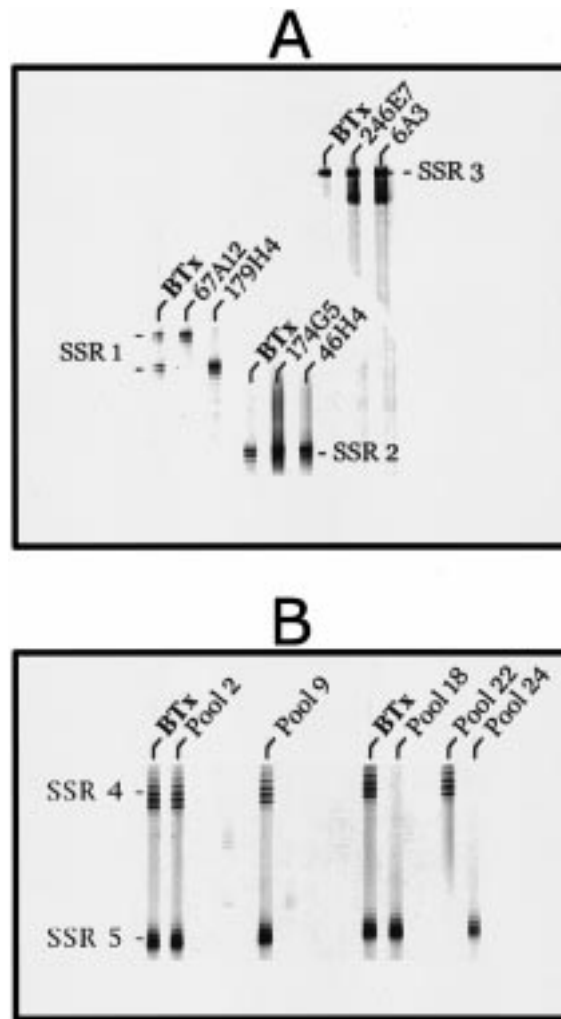


Figure 3. PCR-based screening of BAC DNA for microsatellite genetic markers. (A) Three sorghum microsatellite (SSR) genetic markers (arbitrarily designated SSR1, SSR2, SSR3) previously mapped to the sorghum BAC library were utilized in multiplexed PCR reactions. PCR template was BAC DNA isolated from clones identified as containing microsatellite genetic markers, SSR1-SSR3. Plant genomic DNA was used as PCR template in control reactions (Sorghum cultivar, BTx623). Two alleles of SSR1 are present in BTx623 and are shown in Figure 3A. (B) PCR screening of complex BAC DNA pools for microsatellite genetic markers. BAC DNA isolated from pools of BAC clones (1024 clones per individual pool) was used as template for multiplexed PCR reactions of microsatellites SSR4 and SSR5. Positive BAC pools are identified by legends above the respective lane. Plant genomic DNA (BTx623) was used as PCR template in control reactions.

entire bacterial cultivation and DNA isolation procedure to be performed in the same deep-well plate.

In summary, this manuscript details an affordable, high throughput system for isolating BAC DNA suitable for restriction endonuclease fingerprinting, PCR-based STS-content mapping and for BAC-end sequencing. As the field of plant genomics continues to expand, more plant scientists will be faced with the challenge of isolating DNA from large-insert libraries. The system detailed here may represent a useful alternative to error prone non-automated systems and to expensive automated systems with a much lower throughput capacity.

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