Real-Time PCR-Based Method for Assaying the Purity of Bacterial Artificial Chromosome Preparations

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Large-scale genomic sequencing projects (such as that being undertaken for the human genome project) often prepare large insert (100–200 kb) libraries in bacterial artificial chromosome (BAC) vectors. Short insert libraries are then constructed from individual isolated BACs, and clones from the short insert libraries are randomly selected for sequencing to the requisite coverage. BAC DNA is first purified from E. coli host bacteria, digested and ligated into an appropriate sequencing vector such as M13mp18. To avoid creating large numbers of clones within the library containing E. coli genomic DNA, the BAC DNA must be extensively purified from the host DNA. Because BACs are present at 1–2 copies/cell, BAC DNA must be enriched greater than 400-fold to generate a library with less than 10% E. coli clones (assuming a BAC insert size of 100 kb).

Several methods have been used to purify BAC DNA (1,2,7,9,13,14) (http://www-seq.wi.mit.edu/protocols/BAC.html). However, because of differences in BAC insert size and individual technique, fairly large variations have been observed in the purity of BAC DNA obtained by these methods. Because library construction and evaluation are expensive and time consuming, it is desirable to assess the purity of a BAC DNA preparation before library preparation. The large size of BACs and the fact that they are often sheared along with the contaminating genomic DNA make their purity especially difficult to assess by agarose gel electrophoresis. We have therefore devised a quantitative PCR method for determining the purity of BAC preparations.

We monitored PCR amplification in real time because the potentially large differences in the copy numbers of the BAC DNA and the E. coli DNA required a broader dynamic range of detection than is conveniently afforded by end-point analysis of PCR products. The method we have developed makes use of the TaqMan® technology (PE Biosystems, Foster City, CA, USA) in which PCR-mediated amplification of selected sequences from the BAC vector and the host E. coli DNA are simultaneously monitored using sequence-specific fluorogenic probes (4–6,8,10,11). The fluorogenic probe for each sequence consists of an oligonucleotide with both a reporter and a quencher dye attached. Each probe anneals specifically between the forward and reverse amplification primers. When the probe is cleaved by the DNA polymerase, the reporter dye is separated from the quencher dye, and a sequence-specific signal is generated. With each cycle, additional reporter dye molecules are cleaved from their respective probes, and the fluorescence intensity is monitored during the PCR. The ratio of BAC-specific and E. coli-specific template signals reflects the purity of the BAC preparation.

Probes and primers were designed using Primer Express® software (PE Biosystems). The bacterial chromosomal primers and probe derive from the E. coli TRPE gene. TRPforward: 5'-CACG TACAACATTTTTTGA-3', TRPreverse: 5'-GGCTTCTAGCTCCCGTG GAT-3', TRPprobe: 5'-VIC-AACA-ACGCAC TACGCGCACC-3'. The BAC vector primers and probes amplify nucleotides 6818–6893 of pBACe3.6 (3) (GenBank accession no. U80929). BACforward: 5'-CACG TACAACATTTTTTGA-3', BACreverse: 5'-GGCTTCTAGCTCCCGTG GAT-3', BACprobe: 5'-VIC-AACA-ACGCAC TACGCGCACC-3'.

To allow each sample to be internally controlled, multiplex PCR conditions were established to amplify both bacterial and BAC vector sequences within the same reaction. Forward and reverse primers and probes corresponding to each target were titrated to establish the concentrations at which the R_N (effectively the amount of PCR product generated) is reduced and the C_T value (the threshold cycle at which exponential growth of the PCR product is detected) is unaffected. Optimizing the concentration of the primers minimizes competition between the two amplification reactions (ABI PRISM® model 7700 user bulletin no. 5; PE Biosystems). The optimal concentrations were found to be 270 nM TRPforward, 270 nM TRPreverse, 80 nM BACforward, 80 nM BACreverse, 100 nM TRPprobe and 100 nM BACprobe. For high-throughput testing of BAC preparations, we found it convenient to make up a primer and probe master mixture (PPMM) containing 586 nM TRPforward, 586 nM TRPreverse, 174 nM BACforward, 174 nM BACreverse, 217 nM TRPprobe and 217 nM BACprobe. Large volumes of PPMM were prepared and frozen in aliquots. Reactions were set up by combining 23 µL PPMM, 25 µL 2x TaqMan Universal PCR Master Mix (PE Biosystems) and 2 µL (0.02–15 ng) template DNA. Samples were heated at 50°C for 2 min followed by 95°C for 10 min and then subjected to 40 cycles of 95°C for 15 s and 60°C for 1 min. Fluorescent signal was collected every cycle on an ABI PRISM model 7700 sequence detection system (PE Biosystems).

A standard curve was generated using purified E. coli DNA (Figure 1). A linear relationship was observed (R² = 0.98) between the log of the copy number of E. coli DNA added to each reaction and the C_T value observed for the VIC-labeled probe. Various amounts of purified pBACe3.6 (without insert) were also analyzed, and the amount of contaminating E. coli DNA was deter-
mined using the *E. coli* standard curve to be 6.1 ± 1% (by mass). A standard curve for pBACe3.6 DNA was then generated after subtracting 6.1% from the total mass of DNA applied to each well. A linear relationship was also observed between the log of the derived copy number of BAC DNA added and the C\textsubscript{T} value observed for the FAM-labeled probe (R\textsuperscript{2} = 0.99) (Figure 1).

This preparation of pBACe3.6 was then used as a standard for comparison for other BAC preparations. To arrange this procedure for high throughput, we have frozen aliquots of serial dilutions of pBACe3.6 and *E. coli* DNA. These stocks are used to create standard curves for each 96-well plate analyzed.

This method was used to compare the purity of an approximately 108-kb BAC 89N6 (GenBank accession no. AC003118) prepared by several different methods. Total DNA was analyzed from *E. coli* (strain DH10B) harboring BAC 89N6, BAC 89N6 partially purified by alkaline lysis or BAC 89N6 further enriched either by passing it over a Qiagen\textsuperscript{\textregistered} column (tip 500) (Qiagen, Valencia, CA, USA) or by double acetate precipitation (http://www-seq.mit.edu/protocols/BAC.html). The Qiagen protocol was modified by using 2.5-fold less cell culture volume than is normally recommended for plasmids (200 mL rather than 500 mL) and by heating buffer QF to 65°C and eluting in five 3-mL aliquots. C\textsubscript{T} values were determined in triplicate (except in the alkaline lysis sample) and were used to calculate the copy number of BAC and *E. coli* DNA present in each sample using standard curves analogous to those in Figure 1.

As shown in Table 1, this analysis confirms BAC 89N6 occurs at 1–2 copies/*E. coli* chromosome in accordance with expectations (12). Alkaline lysis enriches the preparation for BAC 89N6 approximately 75-fold. A further two- to five-fold enrichment is obtained by passing the lysate over a Qiagen column or by additional acetate precipitations and phenol extraction. This level of enrichment is predicted to yield 6%–10% *E. coli* DNA-containing clones in libraries prepared from these preparations. These values are in agreement with values obtained by directly sequencing libraries derived from BACs.

The described method provides a convenient means of assessing the amount of *E. coli* DNA contaminating a

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**Table 1. Use of a Real-Time Quantitative PCR Method to Evaluate BAC Purity**

<table>
<thead>
<tr>
<th>BAC Name and Purification Method</th>
<th>Copy Number (BAC/chromosomal)</th>
<th>Predicted <em>E. coli</em> DNA clones</th>
<th>Observed <em>E. coli</em> DNA clones</th>
</tr>
</thead>
<tbody>
<tr>
<td>89N6 unpurified (total cellular DNA)</td>
<td>1.47 ± 0.12</td>
<td>96 ± 7.9</td>
<td>ND</td>
</tr>
<tr>
<td>89N6 alkaline lysis</td>
<td>111</td>
<td>26</td>
<td>ND</td>
</tr>
<tr>
<td>89N6 alkaline lysis/Qiagen column</td>
<td>594 ± 50.6</td>
<td>6 ± 0.5</td>
<td>ND</td>
</tr>
<tr>
<td>89N6 double acetate precipitation</td>
<td>346 ± 133</td>
<td>10 ± 3.9</td>
<td>ND</td>
</tr>
<tr>
<td>RP11-460M01; alkaline lysis/Qiagen column</td>
<td>769 ± 51</td>
<td>3 ± 0.02</td>
<td>3</td>
</tr>
<tr>
<td>RP11-0055M17; alkaline lysis/Qiagen column</td>
<td>315 ± 30</td>
<td>7 ± 0.5</td>
<td>8</td>
</tr>
</tbody>
</table>

The ratio of BAC copy number to *E. coli* chromosomal DNA copy number is reported ± SD. The projected percent of *E. coli* DNA-containing clones in a library prepared from each of these preparations is reported in the third column. The actual percent of library clones found by sequencing to contain *E. coli* DNA is reported in the righthand column. ND: not done.

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**Figure 1.** C\textsubscript{T} values as a function of the copy number of input DNA. Purified *E. coli* genomic DNA and purified pBACe3.6 were serially diluted in triplicate. *E. coli* DNA and BAC DNA were mixed and analyzed by the TaqMan assay as described in the text. The C\textsubscript{T} values were determined on an ABI PRISM model 7700 sequence detection system using PE Biosystems’ sequence detection software. Squares: C\textsubscript{T} values obtained using the VIC fluorescent tag corresponding to amplification of *E. coli* DNA substrate. Diamonds: C\textsubscript{T} values obtained using the FAM fluorescent tag corresponding to amplification of pBACe3.6 substrate.
Benchmarks

BAC preparation before preparing a library. It is performed in a 96-well format, takes 2–3 h and costs $2–$3/sample, far less than the cost of preparing a library, picking and purifying template, running sequencing reactions and sequencing a test plate for a given BAC. Highly contaminated preparations are readily identified and may be removed from production. This method may therefore be a useful procedure to incorporate into the “front end” of sequencing projects that require BAC purification.

REFERENCES


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Simple Devices to Facilitate the Analysis of Collagen Contraction by Cells

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Collagen contraction is a cellular function of fundamental importance to the formation and remodeling of tissues during embryonic development and tissue repair. The contraction of collagen gels by fibroblasts in wound healing provides an important motive force for scar formation and remodeling (1). Moreover, collagen contraction may provide important mechanical cues to neighboring cell types, such as endothelial cells, during angiogenesis (7).

Many assays have been used by investigators to measure collagen or fibrin gel contraction by cells in vitro. Perhaps the most common of these are analyses of floating matrices in which collagen gels that contain cells are rimmed at the perimeter of a tissue culture well or dish at the onset of an experiment (3,5). Decreases in the diameter of the gel are then measured over intervals of several days.

In this system, mechanical tension is distributed isotropically in the floating matrices, and it is thought that these floating matrices emulate a mechanically relaxed tissue, such as normal dermis (1). Alternatively, if gels are not released by rimming, but instead are permitted to develop stress during the incubation, strain is distributed anisotropically. If these gels are released at the end of the experiment, rapid decreases in the gel diameter are observed, owing to stress developed while the matrix was anchored and dissipated when the matrix was released (6). These stress-relaxed matrices are thought to represent the transition that occurs in wound healing as granulation tissue produces a scar, which is gradually remodeled to a point at which mechanical strain is reduced (1).

Finally, if small hemispheric gels are prepared at the bottom of a culture vessel and anchored only at the bottom, decreases in gel height occur as a result of anisotropic tension. In contrast to the stress-relaxed model, changes in anisotropy can be measured throughout the experiment and can be done with cells either inside or on top of the gel (1). The advantages of carrying out continuous measurements of collagen contraction in a microwell format prompted us to develop two tools. The first device is a punch to reproducibly score the bottom of tissue culture wells, either in a 24- or 96-well format. The second device permits a low-cost, noninvasive adaptation of an existing inverted microscope.

The punch device was designed to carry out circumferential scoring on the bottom surface of the plate well (Figure 1). The punches are constructed of a 400 series heat-treatable stainless-steel bar stock in which the outside diameter at one end is reduced by turning on a