Benchmarks

Thermal Asymmetric Interlaced PCR Amplification of YAC Insert End Fragments for Chromosome Walking in Plasmodium falciparum and Other A/T-Rich Genomes


The DNA of Plasmodium falciparum, the major causative agent of human malaria, has an unusually high average A+T content of 82%, and this value frequently exceeds 90% in non-coding regions (14,16). This property renders the DNA unstable in Escherichia coli-based cloning systems and has severely limited molecular genetic analysis of the parasite. A genome project involving the assembly of overlapping cloned DNA fragments to reconstitute entire chromosomes (5) only became possible after the observation that the DNA of P. falciparum was stable when cloned as yeast artificial chromosomes (YACs) (18). However, a chromosome walk in which overlapping clones are identified involves deriving sequence from the end of one YAC clone for use as a probe to rescreen the entire library. Direct sequencing of YAC insert ends with total yeast DNA has been reported using a cycle sequencing approach (3); however, this strategy does not generally give high-quality reads of sufficient length for production of new probes, and therefore has not been widely used. Methods such as inverse polymerase chain reaction (PCR) (12,19), one-sided or hemispecific PCR (6,13,15) and plasmid rescue (1) have been developed for the rescue of ends of cloned DNA fragments; however, they are generally time-consuming and inefficient. These approaches all require Southern analysis, endonuclease digestion, ligation or tailing reactions before end-rescue and/or screening methods to identify the desired product among a background of nonspecific products afterwards. Furthermore, the high A+T content of P. falciparum DNA makes plasmid rescue impracticable.

As part of the P. falciparum genome project, we have adapted a relatively new method, thermal asymmetric interlaced PCR (TAIL-PCR) (10), to derive sequence tagged site (STS) markers from YAC end fragments to facilitate the assembly of YAC contigs and STS maps of the chromosomes. TAIL-PCR is a hemispecific PCR method that uses vector-specific primers with high annealing temperature (T_m) values in combination with a degenerate primer of low T_m for the isolation of insert end fragments from large clones (e.g., P1 and YACs). By interspersing symmetric low-temperature PCR cycles (in which both primers function) with asymmetric cycles (in which only the nested vector arm-specific primers with high T_m values can function) the true target molecule is preferentially amplified relative to the various nonspecific products. Unlike other methods for end-rescue, TAIL-PCR requires no manipulation of the DNA before amplification and no subsequent screening to identify genuine product. The method is also inexpensive, easily automated for high-throughput and quick, allowing, for example, isolation and sequencing of DNA that flanks regions of known sequence (e.g., YAC vector arm) within 3–4 days of initiating the yeast culture. Products generated by TAIL-PCR can be sequenced directly without purification to remove unincorporated primers and dNTPs and without the need for end-labeled sequencing primer. The sequence-specific primer from the tertiary PCR is used directly in a cycle-sequencing approach with tertiary PCR product serving as template (9–11). In certain cases, even the secondary PCR product is sufficiently free from nonspecific product to serve as template for DNA sequencing.

The specificity and ease of TAIL-PCR, coupled with convenient direct sequencing methods, lead us to adapt the method for the genome mapping studies that are in progress for P. falciparum. For this, primers with high T_m values, based on the most commonly used YAC vector, pYAC4, were designed together with degenerate primers of low T_m values and biased towards the A + T-rich DNA of P. falciparum such that PCR products were typically of a few hundred base pairs. The asymmetric PCR tailing was amended to accommodate these degenerate primers and to prevent melting of the P. falciparum DNA during PCR extensions.

For end-rescue of P. falciparum YAC inserts, the following pYAC4-specific primers were designed:

**Left arm:**
- YL1: ATGCGGTAGTTTATACAGTTAA (T_m = 55.3°C)
- YL2: AAGTACTCTCGGTAGCCAAG (T_m = 57.3°C)
- YL3: GCCAAGTTGGTTTGAAGCGG (T_m = 59.4°C)

**Right arm:**
- YR1: ATCATCGTCGCTCCGATCCAGCCA (T_m = 63.7°C)
- YR2: AGTCGAACGCCCAGTCCTCAA (T_m = 59.4°C)
- YR3: GCCCGATCTCAAAGTACCCAG (T_m = 56.7°C)

where the number after YL or YR indicates use in the primary, secondary or tertiary PCR, and T_m is calculated according to the formula described previously (11).

These specific primers were paired with the following 256-fold degenerate primers in separate reactions:

**TAIL1:** TTAATA(T/A)ANANATN(T/A)GGATCTCAAAGTACCCAG

**TAIL2:** TTAATA(T/A)ANANATN(T/A)CCA

The T_m of these primers is calculated as 41.2°C, and therefore at least 14°C below that of any of the YAC-specific primers. If the N positions are actually A or T, as is likely with P. falciparum DNA, then the real T_m would be 37.7°C. Culture of yeast cells, subsequent spheroplasting and preparation of PCR mixtures were as described earlier (10), while the cycling conditions on an OmniGene® Thermal Cycler (Hybaid, Ashford, Middx, UK) were adapted to account for the different T_m of the primers used in this study and the low melting temperature of P. falciparum DNA (see Table 1). Products resulting from the primary and secondary PCRs were diluted 100-fold with sterile water before establishing the secondary and
tertiary reactions.

For rescue of sequence flanking the right arm of the pYAC4 vector, the high-temperature annealing steps were increased to 62°C to account for the higher \( T_m \) values of the YAC right-arm-specific primers. The extension steps were performed at 62°C (left arm) and 64°C (right arm), because it is believed that melting of A+T-rich tracts of \( P. falciparum \) DNA occurs at 72°C, preventing amplification of such sequences (17). We have successfully amplified the ends of fragments of \( P. falciparum \) DNA cloned in YACs using the above methods (see Figure 1a). PCR products ranged from 100 bp to approximately 2 kb, with an average size of 600 kb, making them suitable for use as STS markers. In our hands, the combination of the YAC left-arm primers and the degenerate primer, TAIL1, was most efficient, giving product in 70% of reactions at the first attempt. The efficiency of the other combinations (between 5% and 25% at the first attempt) might be improved by modifications to the primers or by changes to the cycling parameters. However, because the YAC library has approximately 10-fold genome coverage, the left-arm primers were used to walk in both directions along the chromosome by rescuing ends of fragments cloned in both orientations. Because of the lack of DNA manipulation before PCR and lack of screening for product after amplification, we routinely process YACs in batches of 48. The procedure could be scaled up further by use of microplates and multichannel pipets. The PCR products are sequenced directly (9,11) to provide new STSs that allow assembly of contigs by identification of overlapping YAC clones, while simultaneously generating an STS map of the chromosome. In genome projects in which a STS map is not one of the primary goals, the tertiary PCR product could be used as a hybridization probe on gridded arrays of the library clones, thereby eliminating the need to sequence the product to produce a new STS for PCR screening of the library.

In addition to its use in isolation of DNA from a segment bordering a known vector sequence, TAIL-PCR can also be used for cloning insertion tagged genes, recovery of regulatory sequences associated with cloned cDNA and general walking strategies (10). We have also used TAIL-PCR, using these degenerate primers together with specific primers based on known sequence, to extend partial gene sequences already mapped to central regions of YAC clones (Figure 1b) when other methods for extending the sequence or cloning strategies have failed. TAIL-PCR is applicable to the DNA of any organism cloned into any vector. It has also been used to recover single-copy sequences from genomic DNA of plants having complex genomes (8). Our adaptations to the original methods have provided an efficient means of rescuing end fragments.

### Table 1. Cycling Conditions for TAIL-PCR of \( P. falciparum \) DNA Flanking pYAC4 Left Arm

<table>
<thead>
<tr>
<th>Reaction No.</th>
<th>Segment No.</th>
<th>No. Cycles</th>
<th>Thermal Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary</td>
<td>1</td>
<td>1</td>
<td>92°C (2 min), 95°C (1 min)</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>94°C (15 s), 60°C (1 min), 62°C (2 min)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>94°C (15 s), 30°C (3 min) ramping to 62°C over 3 min, 62°C (2 min)</td>
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</tr>
<tr>
<td>4</td>
<td>10</td>
<td>94°C (5 s), 35°C (1 min), 62°C (2 min)</td>
<td></td>
</tr>
<tr>
<td>5</td>
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</tr>
<tr>
<td>6</td>
<td>1</td>
<td>62°C (5 min)</td>
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<tr>
<td>Secondary</td>
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<td>1</td>
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<tr>
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<tr>
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<tr>
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<td>1</td>
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<tr>
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</tr>
<tr>
<td>3</td>
<td>1</td>
<td>62°C (5 min)</td>
<td></td>
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**Figure 1.** Agarose gel electrophoresis of TAIL-PCR products. Products amplified from the pYAC4 vector-insert boundary of 6 different \( P. falciparum \) YACs (a) and from a known sequence located centrally on a \( P. falciparum \) YAC (b) are shown. Products from secondary [2] and tertiary [3] PCR amplifications are shown alongside a 1-kb ladder molecular-weight marker, m.
of \textit{P. falciparum} DNA cloned in YACs, which would not be readily subcloned into \textit{E. coli}-based systems for further analysis. Fragments showing YAC vector sequence with the common EcoRI cloning site, followed by A+T-rich DNA typical of \textit{P. falciparum} DNA, have been recovered. These YAC insert end sequences, some of which match known \textit{P. falciparum} genes, have permitted reconstruction of chromosomes through a targeted walking approach whereby the ends of YAC inserts serve as STS probes with which to rescreen the YAC library for overlapping clones and so on. The provision of ordered arrays of YAC clones, together with STS and restriction maps for entire chromosomes, prompted a full genome sequencing project \cite{4,7}. This is being achieved by a modified shotgun sequencing approach for individual chromosomes with approximately 10-fold sequencing redundancy to obtain consensus sequence for the \textit{P. falciparum} DNA, which is inherently unstable in the M13 and plasmid vectors used for shotgun library construction. In some cases, YAC clones themselves are shotgun sequenced in a similar manner. The YAC maps and ordered STS markers for each chromosome are used to identify sequence contigs from the same part of the chromosome, aid in gap closure and confirm colinearity of the assembled sequence \cite{4,7}. Genome projects are underway for several other organisms, including a number of parasite species \cite{2}, and it is anticipated that similar modifications to the original TAIL-PCR method could be successfully applied to these mapping programs. This is already proving to be the case for the filarial nematode, \textit{Brugia malayi} (75\% A/T; J. Daub, D. Guiliano and J. Foster, unpublished) and might be particularly true for additional organisms having DNA of high A+T content \cite{e.g., the parasitic blood fluke, \textit{Schistosoma mansoni}} in which much of the genome may be refractory to standard methods.

\section*{References}


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Jeremy M. Foster, Zoe Christodoulou, GILL M. Cowan and Christopher I. Newbold

John Radcliffe Hospital

University of Oxford

Oxford, England, UK