High-throughput sequencing of plasmid and BAC DNA prepared using the R.E.A.L.™ Prep 96 Plasmid Kit

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Since the launch of capillary electrophoresis (CE) sequencers such as the ABI PRISM® 3700 (PE Biosystems) and the MegaBACE™ 1000 (Amersham Pharmacia Biotech), sequencing capacities and capabilities have increased considerably. Both of these systems have an array of 96 capillaries, and for most sequencing applications on these instruments, high sample quality is essential. After intensive use of such sequencers in QIAGEN laboratories, a large dataset, giving statistical data on the quality and performance of DNA purified using the R.E.A.L.™ Prep 96 Plasmid Kit, is available.

The R.E.A.L. Prep 96 system provides a fast, simple, and cost-effective method for small-scale purification of plasmid DNA for use in routine molecular biology applications. The procedure is based on a modified alkaline lysis of bacterial cells, followed by clearing of the lysates using the unique QIAfilter™ module and a vacuum manifold. Further purification and concentration of the DNA is achieved by an isopropanol precipitation. The DNA obtained is resuspended in a small volume of Tris buffer and is ready for use (Figure 1). All DNA purification steps are carried out without the use of phenol, chloroform, CsCl, or ethidium bromide.

The R.E.A.L. Prep 96 procedure has been further optimized for the purification of low copy number plasmids, cosmids, BACs, PACs, and P1s. In addition, the procedure can be automated on QIAGEN® BioRobot® Systems. A special kit format optimized for use with BioRobot Systems is available.

We report here on the quality and the suitability of DNA obtained using the R.E.A.L. Prep 96 procedure for high-throughput capillary sequencing. In addition, an optimized protocol for the purification of BAC DNA suitable for end sequencing on CE sequencing machines is presented.

Materials and methods

Preparation of plasmid DNA from shotgun clones

DNA was purified according to the protocol described in the R.E.A.L. Prep 96 Handbook, supplied with every R.E.A.L. Prep 96 kit.

Reproducible Plasmid Purification

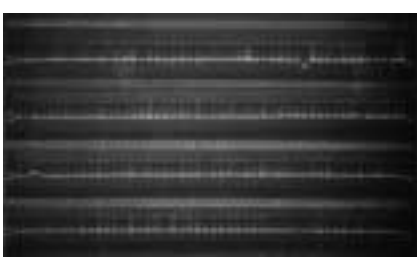

Figure 1 Agarose gel analysis of shotgun clones from an S. pombe shotgun library purified using the R.E.A.L. Prep 96 procedure. Approximately 200 ng DNA was loaded per lane onto a 1% TAE agarose gel. Outer lanes: markers.

Preparation of BAC DNA for capillary end sequencing

Four 48-well growth blocks were filled with medium, and each clone was inoculated into two wells. After cultivation and subsequent sedimentation of the cells, each cell pellet was resuspended in 200 µl Buffer R1. The duplicate resuspended clones were combined in one well of a 48-well block. The usual lysis and purification protocol described in the R.E.A.L. Prep 96 Handbook was used, with the following exceptions: 400 µl Buffer R2 and Buffer R3 was used, and 840 µl ▶

isopropanol was used for the precipitation step. After resuspension in 10 mM Tris·Cl, pH 8.5, an extra cleanup step was added in which the entire BAC DNA preparation was loaded onto a DyeEx™ 96 plate and spun through. Figure 2 shows a flowchart of the steps involved in this procedure.

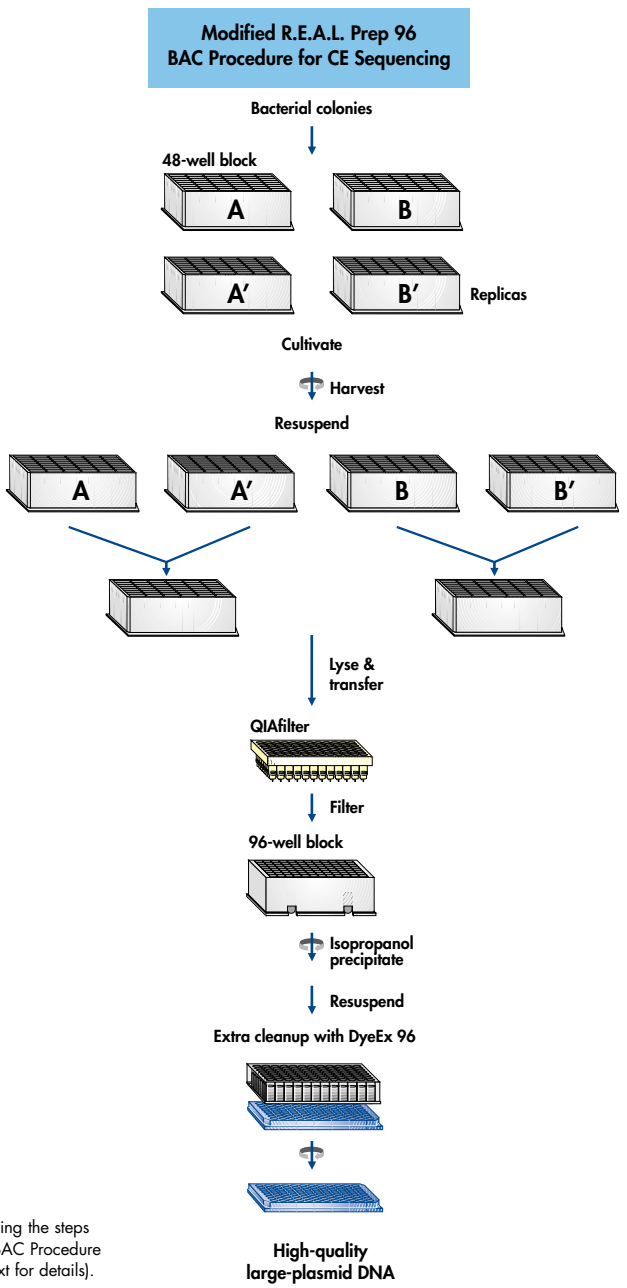

Figure 2 Flowchart showing the steps involved in the modified BAC Procedure for CE sequencing (see text for details).

Results and discussion

Sequence read lengths

The QIAGEN DNA Sequencing & Genomics group (1) uses the R.E.A.L. Prep 96 System for the purification of plasmid shotgun and BAC clones in a high-throughput format. Each sequence is quality checked using the Phred program (2) with a standard quality setting of q>20, equivalent to 99% sequence accuracy.

Figure 3 shows the read length scored by the Phred program plotted versus the percentage of samples sequenced using different gel matrices on an ABI PRISM 3700 CE sequencer.

As shown in Figure 3, obtained read lengths generally range around 600 bases. Since the results were obtained from two sets of shotgun clones derived from two unrelated organisms with differing genomic DNA GC-contents (a Gram-positive bacterium and a small fungus), the results for both gel matrices cannot be directly compared. However, the consistency between the two data sets suggests that such read lengths are regularly obtained, regardless of sample GC-content or gel matrix.

These findings indicate that DNA prepared using the R.E.A.L. Prep 96 System is well suited for high-throughput CE DNA sequencing. Similar results in terms of useabilty, reliability, handling, and performance were obtained using the MegaBACE 1000 system in combination with "half term" DYEnamic™ ET Terminator reactions (3; data not shown).

In all analyses carried out so far at QIAGEN the performance of the capillaries when using DNA obtained with the R.E.A.L. Prep 96 Plasmid Kit exceeded our expectations. Capillary arrays are replaced on average after 450 sequencing runs as standard, but feedback from users of the R.E.A.L. Prep 96 system indicates that capillary sets can be used well beyond 500 runs.

Large construct minipreps

Modified protocols for the high-throughput DNA isolation of large plasmid constructs such as BACs, PACs, and P1s are also provided in the R.E.A.L. Prep 96 Handbook. The procedure allows up to 96 samples to be

processed in parallel in just 60–75 minutes. Up to 800 ng large-construct DNA can be reliably obtained from a 2.5 ml bacterial culture using the R.E.A.L. Prep 96 BAC purification procedure. These purified BAC DNAs are also suitable for use in high-throughput applications such as restriction digestion (Figure 4) and for fluorescent BAC DNA end sequencing on ABI PRISM 377 vertical slab-gel sequencers.

Due to the different loading procedures of slab-gel and CE sequencers, increased amounts of BAC DNA must be used for BAC end sequencing on CE sequencers. The reason for this is that only a small amount of the reaction is injected into the capillary during electrokinetic injection, whereas on a slab gel sequencer the entire reaction can be loaded onto the gel.

In order to enable BAC end sequencing on the ABI PRISM 3700 DNA Analyzer and to overcome yield problems for difficult BAC libraries, we have developed an optimized BAC preparation procedure using the R.E.A.L. Prep 96 Kit (Figure 2). The major differences to the previously described ▶

Restriction Analysis of BAC Clones

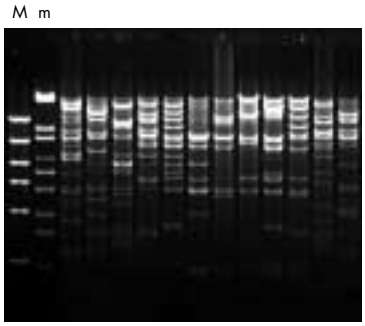

Figure 4 Agarose gel analysis of restricted BAC clones purified using the modified R.E.A.L. Prep 96 BAC procedure. **M**: High molecular weight mass ladder. **m**: marker lambda-StyI.

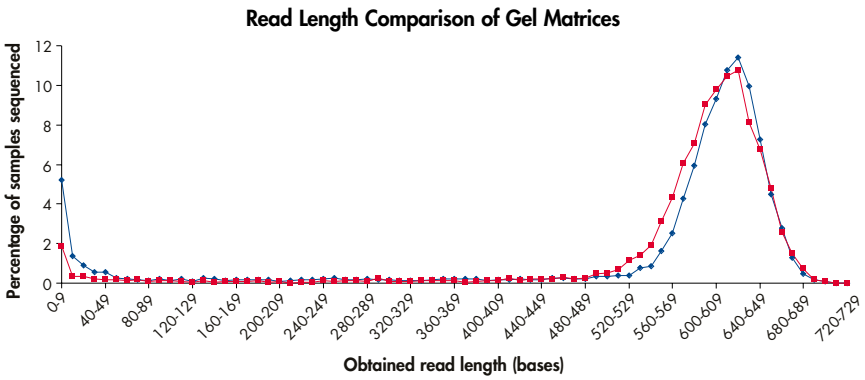

Figure 3 Phred Analysis (q>20). **In blue:** 11808 plasmid shotgun sequences derived from a bacterial genome prepared with the R.E.A.L. Prep 96 System, were sequenced with BigDye™ Terminator sequencing chemistries (quarter reactions) and analyzed on an ABI PRISM 3700 DNA Analyzer with the POP-5™ Gel matrix. **In red:** 6025 Schizosaccharomyces pombe shotgun plasmid clones prepared with the R.E.A.L. Prep 96 System, were sequenced with BigDye Terminator sequencing chemistries (quarter reactions) and analysed on an ABI PRISM 3700 DNA Analyzer with the POP-6™ Gel matrix.

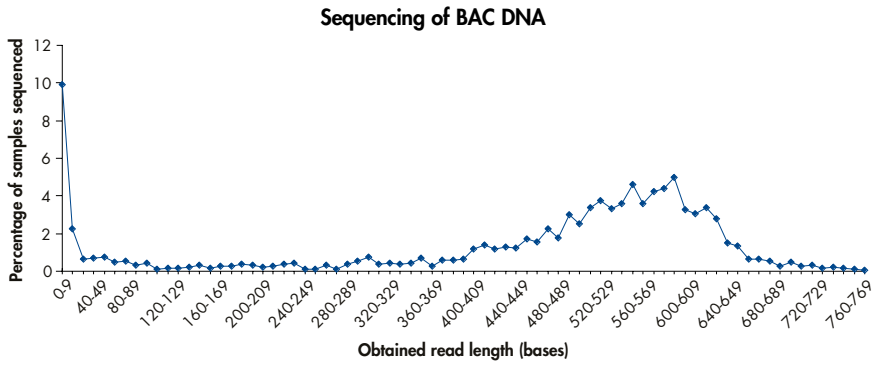

Figure 5 BAC end sequencing Phred Analysis (q>20) of 1902 BAC clones prepared with R.E.A.L. Prep 96, sequenced with BigDye Terminator II sequencing reactions, and analyzed on the ABI PRISM 3700 using the POP-5 Gel matrix.

References

1. www.qiagen.com/sequencing/
2. www.codoncode.com
3. www.megabace.com/products/MegaBACE/
4. Zhao, S., Malek, J., Mahairas, G., Fu, L., Nierman, W., Venter, J., and Adams, M. (2000) Human BAC Ends Quality Assessment and Sequence Analyses. Genomics **63**, 321.

Table 1. Statistical overview of the BAC end sequencing results on the ABI PRISM 3700

Number of reactions	1902
Number of sequences with acceptable quality (read length ≥120 bases)	1591 (83.6%)
Number of sequences below acceptable quality (read length <120 bases)	311 (16.4%)
Average read length (bases)	463

DNA was obtained from BAC clones grown using the modified BAC protocol for CE sequencing described above.

protocol are a doubling of the culture volume (e.g. four 48-well growth blocks for 96 clones) and an additional cleanup step using DyeEx 96. The new, optimized BAC DNA purification protocol from QIAGEN makes it possible to determine end sequences of BAC clone inserts on CE sequencers, which up to now has proven very difficult due to the limitations imposed by CE sequencer electroinjection techniques. The success rate and read length (Fig. 5, Table 1) is consistent with published data (4). However, we found that the success rate can vary considerably between BAC libraries derived from different organisms. If difficulties in end sequencing with the established BAC purification protocol emerge, it is often possible to overcome these difficulties by using the new optimized BAC purification protocol and sequencing recommendations.

Conclusions

The R.E.A.L. Prep 96 system provides a very versatile high-throughput method for the purification of a range of DNAs, from small insert plasmids up to large constructs such as BACs, PACs and P1s. The DNA quality is very well suited for fluorescent sequencing using CE sequencers. For BAC end sequencing applications, two QIAGEN approved protocols for BAC DNA purification are now available.

Reader Inquiry No. 00503

Ordering Information

Product	Contents	Cat. No.
R.E.A.L. Prep 96 Plasmid Kit (4)*†	4 QIAfilter 96 Plates, for 4 x 96 rapid extraction alkaline lysis minipreps	26171
R.E.A.L. Prep 96 Plasmid Kit (24)*†	24 QIAfilter 96 Plates, for 4 x 96 rapid extraction alkaline lysis minipreps	26173
Related products		
QIAvac 96	Vacuum manifold for processing QIAGEN 96-well plates	19504
48-well blocks (24)	48-well blocks with 5 ml wells, 24 per case	19577
DyeEx 96 Kit (4)	4 DyeEx 96 Plates; 4 Collection Plates, 48-well	63181

* Requires use of QIAvac 96

† 48-well blocks required for optimal cultivation of BAC clones are not included in R.E.A.L. Prep 96 Kits and must be purchased separately.