Improved Method of BAC DNA preparation for sequencing

Using TempliPhi DNA Amplification Kits

Key words: BAC, alkaline lysis, heat lysis, TempliPhi, PicoGreen, agarose gel, cycle sequencing, DYEnamic ET Terminators, BigDye v3.0 terminators, capillary electrophoresis

Bacterial artificial chromosomes (BACs) are cloning vectors based on the F factor plasmid of Escherichia coli. They are capable of maintaining cloned DNA fragments up to 300 kb (1). Sequencing of BAC inserts, and in particular, the ends of BAC inserts, represents a key step in large-scale chromosomal sequencing projects. The preparation and sequencing of BAC DNA can be challenging. Clones based on cosmids and fosmid vectors pose similar difficulties. Success rates heavily depend on the purity and quantity of DNA in the reactions, and considerable effort has been devoted to the development of reliable methods for BAC isolation and purification. Unlike high-copy number plasmids and M13, isolation of BAC or fosmid-based constructs directly from colonies and cultures is relatively inefficient. This is because protocols that enable the release of BAC also release chromosomal DNA. Additionally, BAC and fosmid-based constructs are only present as a single copy per cell.

TempliPhi™ DNA Amplification Kit uses rolling circle amplification (RCA) catalyzed by bacteriophage Phi29 DNA polymerase to efficiently prepare DNA sequencing templates. Circular DNA is amplified exponentially in an isothermal process lasting from 4 to 18 h, depending on the nature of the template and the degree of amplification desired. The use of random sequence hexamers in the reaction eliminates the requirement for custom primers. Following incubation at 30 ºC for the desired time, the enzyme is inactivated at 65 ºC for 10 min, and the amplified DNA is ready for sequencing with no additional purification steps required.

A modified TempliPhi protocol for the preparation of large circular constructs for sequencing is described below. The method is efficient, streamlined, and generates approximately 10-15 micrograms of DNA—equivalent to amounts obtained from the best current methods for BAC DNA preparation. The amplification reaction requires a minimum quantity of 10 nanograms of purified or partially purified BAC DNA. Partially purified BAC DNA can be readily obtained in sufficient quantity from an overnight 1.5 ml culture, followed by a standard mini-alkaline lysis protocol, such as the one recommended below. Additionally, two separate methods for liberating sufficient BAC DNA for amplification from single colonies are described.

Products used

- TempliPhi 100 Amplification Kit
- TempliPhi 500 Amplification Kit
- LB Agar
- Chloramphenicol
- TE Buffer, 50x
- DYE™ ET Terminator Cycle Sequencing Kit
- MegaBACE™ Loading Solution
- Ribonuclease A
- MegaBACE 1000 DNA Analysis System

Other materials

- ABI PRISM™ 3700 DNA Analyzer (Applied Biosystems)
- ABI PRISM 3100 Genetic Analyzer (Applied Biosystems)
- Peltier Thermal Cycler System (PTC-200/PTC-225) (MJ Research)
- Centrifuge 4K-15C (Qiagen™ Corp)
- Eppendorf microcentrifuge Model 4515C
- Microamp™ Optical 96-well Reaction Plates (Applied Biosystems)
- PicoGreen™ dsDNA Quantitation Kit (Molecular Probes Inc)
- DNA Sequencing Primers (Qiagen-Operon)
- Ethanol
- Isopropanol
- Potassium acetate
- MilliQ purified water
Protocol

Three BAC clones were used in this study:

<table>
<thead>
<tr>
<th>Clone</th>
<th>Insert size (kb)</th>
<th>Vector</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>89N6</td>
<td>92</td>
<td>pBeloBAC11</td>
<td>Research Genetics</td>
</tr>
<tr>
<td>135A24</td>
<td>163</td>
<td>pBACe3.6</td>
<td>Elaine Mardis Washington University</td>
</tr>
<tr>
<td>135M8</td>
<td>46</td>
<td>pBACe3.6</td>
<td>Elaine Mardis Washington University</td>
</tr>
</tbody>
</table>

Glycerol stocks of the BAC clones were each streaked onto LB plates containing 20 µg/ml chloramphenicol and BAC DNA prepared from cultures of individual colonies using the methods below.

Highly purified preparations of the BAC DNAs listed above were obtained from Nature Technology Corporation, Lincoln, NE.

1. **BAC Preparation methods**

   **A. Mini alkaline lysis method for BAC purification from 1.5 ml culture**

   NOTE: Reagents supplied by commercial vendors such as Qiagen Corp. are appropriate for this mini-alkaline lysis method. Alternatively, the user may choose to prepare their own reagents. See Appendix 1 for suggested recipes.

   NOTE: Once bacterial cells are lysed, it is important that BAC DNA be treated gently in order to prevent shearing.

   1.1 Inoculate LB media containing the appropriate antibiotic with a single BAC colony. Incubate at 37 °C overnight (≥ 16 h).

   1.2 Transfer 1 ml of an overnight BAC culture to a 1.5 ml microcentrifuge tube. Centrifuge for 1 min at 16,000 x g in a microcentrifuge to pellet the bacterial cells. Discard the supernatant.

   1.3 Resuspend the bacterial pellet in 200 µl of Resuspension Buffer containing 100 µg/ml RNase A. Vortex to mix well.

   1.4 Add 200 µl Lysis Buffer, and mix by gently inverting tubes 5–6 times. Do not vortex. Incubate at room temperature for no more than 5 min.

   1.5 Add 200 µl of ice cold Neutralization Buffer, and mix by gently inverting tubes 5–6 times. Do not vortex. Incubate on ice for 10 min.

   1.6 Centrifuge at 16,000 x g for 10 min. Transfer BAC-containing supernatant to a fresh tube. If necessary, perform an additional centrifugation step to remove remaining particulate matter in the supernatant, and transfer supernatant to a fresh tube.

   1.7 Add 1 ml of 95% ethanol to cell extract. Invert to mix. Do not vortex. Centrifuge at 16,000 x g for 10 min to recover BAC DNA pellet. Remove supernatant.

   1.8 Wash pellet with 1 ml of 70% ethanol. Centrifuge at 16,000 x g for 5 min. Discard supernatant.

   1.9 Air-dry at room temperature for 5–10 min. Gently dissolve the pellet in 50 µl of TE. Incubate at least 10 min at room temperature, and mix the contents by gentle tapping with a finger. Do not vortex as this may shear the BAC DNA and render the TempliPhi reaction inefficient. Estimate DNA concentration and prepare a working stock of 10 ng/µl for use in TempliPhi reactions.

   Continue to Section 2: TempliPhi amplification protocol for BAC and other large constructs.

   **B. Heat-lysis method for obtaining DNA from a BAC colony**

   1.1 Pick an entire BAC-containing colony into a reaction tube containing 20 µl of deionized water. The whole colony must be picked to liberate enough starting material. Vortex thoroughly to resuspend cells.

   1.2 Heat at 95 °C for 3 min.

   1.3 Incubate cell suspension at -20 °C or below for 2–3 hours, until frozen. This step allows for any released chromosomal DNA to precipitate preferentially while retaining BAC DNA in solution.

   1.4 Thaw cells suspension slowly. Centrifuge at 4 °C for 15 min. This step pellets cell debris and chromosomal DNA.

   1.5 Transfer BAC-containing supernatant to a new tube, and vacuum-dry the entire sample. Do not over-dry.

   1.6 Add 50 µl of Sample Buffer to the dried DNA. Resuspend by gentle tapping and proceed with Step 2.2.

   **C. Mini-alkaline lysis method for BAC colonies**

   NOTE: Reagents supplied by commercial vendors such as Qiagen Corp. are appropriate for this mini-alkaline lysis method. Alternatively, the user may choose to prepare their own reagents. See Appendix 1 for suggested recipes.

   1.1 Pick an entire BAC-containing colony into 50 µl Resuspension Buffer containing 100 µg/ml RNase A. Vortex to mix well.

   1.2 Add 50 µl Lysis Buffer, and mix by gently inverting tubes 5–6 times. Do not vortex. Incubate at room temperature for no more than 5 min.

   1.3 Add 50 µl of ice-cold Neutralization Buffer, and mix by gently inverting tubes 5–6 times. Do not vortex. Incubate on ice for 10 min.

   1.4 Centrifuge at 16,000 x g for 10 min in a microcentrifuge. Transfer BAC-containing supernatant to a fresh tube. If necessary, perform an additional centrifugation step to remove remaining particulate matter in the supernatant, and transfer supernatant to a fresh tube.
1.5 Add 0.6 volumes of isopropanol to tube (90 µl), and mix by gentle
inversion. Do not vortex.

1.6 Centrifuge at 16 000 × g for 10 min to recover BAC DNA pellet. Remove supernatant.

1.7 Wash pellet with 200 µl of 70% ethanol. Centrifuge at 16 000 × g
for 5 min. Remove excess ethanol and air-dry pellet.

1.8 Dissolve DNA in 50 µl of TempliPhi Sample Buffer. Resuspend DNA by tapping gently. Proceed with Step 2.2.

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TempliPhi amplification protocol for BAC and other large constructs

The partially purified BAC DNA generated by any of the
methods described above, is ready to be processed according
to the modified procedure recommended below. The final yield
of amplified DNA from this modified protocol is typically
10–15 µg per reaction tube.

NOTE: Reactions must be prepared on ice to prevent non-
specific enzymatic activity by Phi29 polymerase.

2.1 Transfer 1 µl (10 ng/µl) of BAC DNA to 50 µl TempliPhi
Sample Buffer.

2.2 Denature at 95 ºC for 3 min, then incubate at 4 ºC until ready to use.

2.3 Prepare TempliPhi premix.

NOTE: TempliPhi amplification of BAC DNA requires 10 ×
the reaction volume of standard controls in order to generate
optimal yield. Scaled-down versions of this reaction are hypo-
thetically feasible but must be optimized by the researcher.

2.4 For each BAC sample, combine 50 µl Reaction Buffer and 2 µl
Enzyme Mix in an appropriate tube. If desired, a master mix may be
prepared by combining sufficient reagents for the required number
of amplification reactions. The prepared TempliPhi premix should be kept
in ice until ready for use.

NOTE: Any unused premix must be discarded and not stored
for reuse.

2.5 Transfer 50 µl of prepared TempliPhi premix (Reaction Buffer +
Enzyme Mix) to the denatured sample from Step 2.2.

2.6 Incubate the reaction tubes at 30 ºC for ≥ 18 h.

NOTE: A minimum incubation of 18 h is necessary for complete
DNA amplification of BAC and other large constructs.

2.7 Heat-inactivate the Phi29 DNA polymerase by incubating completed
reactions at 65 ºC for 10 min. Store reaction tubes at 4 ºC or -20 ºC
until ready for use.

2.8 Precipitate BAC amplified products.

NOTE: Amplified BAC DNA should be precipitated by standard
isopropanol or ethanol addition following the recommendations
below. This is an essential step to ensure the removal of salt,
excess dNTPs, and random hexamers that are present in the
scaled-up TempliPhi amplification reaction. This step also
concentrates the amplified products. Typical sequencing pro-
tocols for large constructs require higher amounts (2–5 µg)
of input DNA.

2.8.1 Add equal volume isopropanol (1:1 ratio) to completed reactions. Alternatively, add 3 × volume 95% ethanol. Invert several times to mix.
DNA pellet should be clearly visible.

2.8.2 Pellet DNA at 5000 × g for 5–10 min.

2.8.3 Remove supernatant. Add 500 µl 70% Ethanol to wash pellet. Vortex well.

2.8.4 Centrifuge at 5000 × g for 2 min. Remove as much supernatant
as possible.

2.8.5 Air-dry pellet for 5–10 min. Do not over-dry. Check pellets
frequently to avoid over-drying.

2.8.6 Resuspend pellet in TE. Vortex vigorously. Resuspend
in 1/4 of the original volume (i.e. if your reaction volume was 100 µl,
resuspend precipitated DNA in 25 µl).

NOTE: Resuspended DNA may appear cloudy. This DNA will
still generate good quality sequence data. Do not centrifuge
this DNA solution before quantitation or sequencing, as this
will affect the results.

NOTE: Although a total of 10–15 µg of DNA is generated from
amplification, some loss of DNA occurs following alcohol
precipitation. Between 2–5 µg of amplified BAC DNA is
required per sequencing reaction. Determine the concentra-
tion of amplified BAC DNA post-precipitation. Typical yields
following resuspension will result in about 100 ng/µl of
amplified DNA. We highly recommend estimating DNA yield
using PicoGreen reagents, using recommended protocols
from the manufacturer, prior to use in sequencing.

2.9 Transfer 2–5 µg of the amplified material to a sequencing reaction
according to sequencing protocols specific for BAC and other large
constructs shown below.
Sequencing of BAC DNA

NOTE: Many factors determine success rates of BAC and other large construct sequencing. Basic guidelines include: increasing template amount, sequencing primer, number of cycles, or extension time as well as doubling sequencing reaction volume and performing a rigorous post-sequencing terminator removal step.

NOTE: The protocol described below is a starting point for obtaining BAC sequencing data. The user will have to optimize the reaction to successfully sequence the BAC of interest.

Sequencing of BAC DNA must be performed using a 2× reaction volume.

A. Recommended protocol for MegaBACE, ABI PRISM 3700, and ABI PRISM 3100 DNA Analysis Systems using DYEnamic ET terminator chemistry

3.1 For each BAC to be sequenced, prepare the following mix:
- TempliPhi amplified BAC DNA 2–5 µg
- Sequencing primer 10–20 pmoles
- dh2O up to 24 µl

3.2 Heat to 95 ºC for 10 min, and then snap cool to 4 ºC.

NOTE: Do not perform this extended denaturing step in the presence of sequencing premix, as this will inactivate the polymerase.

3.3 Add 16 µl DYEnamic ET Terminator Sequencing Kit premix. Vortex and quick spin. The final sequencing reaction volume should be 40 µl.

3.4 Cycle 80× as follows:
- 95 ºC — 30 s
- 50 ºC — 10 s
- 60 ºC — 4 min

Completed reactions should be stored at 4 ºC for 10 min, and then snap cool to 4 ºC.

3.5 Post-sequencing reaction processing

3.5.1 Add the following to the completed sequencing reactions:
- 4 µl (1/10 volume) 1.5 M sodium acetate/250 mM EDTA buffer (pH 5.2)
- 160 µl (4 volumes) 95% ethanol

3.5.2 Centrifuge plates for 30 min at 5000 × g. Perform a quick invert spin to remove supernatant (1 min, 800 rpm).

3.5.3 Add 200 µl of 70% ethanol, Centrifuge 3 min at 5000 × g, and discard supernatant. Perform quick invert spin to remove supernatant (1 min, 1000 × g).

3.5.4 Air-dry pellets for 10–15 min.

3.5.5 Add 20 µl of MegaBACE Loading Solution and vortex vigorously for 10–20 s to ensure complete pellet resuspension.

3.6 Load on capillary electrophoresis DNA analyzer with the right spectral calibration files and mobility files created for use with DYEnamic ET Terminator chemistry.

B. Recommended protocol for ABI PRISM 3700/3100 DNA analysis systems using ABI PRISM BigDye™ Terminator v3.0

3.1 For each BAC to be sequenced, prepare the following mix (Final volume = 40 µl):
- TempliPhi amplified BAC DNA 2–5 µg
- Sequencing primer 10–20 pmoles
dh2O up to 24 µl
- Terminator Ready Reaction Mix 16 µl

3.2 Heat to 95 ºC for 5 min.

3.3 Cycle 80× as follows:
- 95 ºC — 30 s
- 50 ºC — 10 s
- 60 ºC — 4 min

Completed reactions should be stored at 4 ºC until ready to continue.

3.4 Perform post-sequencing reaction processing and loading as indicated by manufacturer.
Sequencing results

Appearance of sequencing traces

Figures 1 and 2 show sequencing traces from MegaBACE 1000 of purified, non-amplified BAC 89N6 and purified, TempliPhi amplified BAC 89N6. BAC 89N6 DNA was purified by Nature Technology Corporation using a proprietary chromatographic method. Note the similarity in quality between the traces.

Figure 3 shows a sequencing trace from ABI PRISM 3100 of BAC 89N6 which had been TempliPhi amplified from a culture miniprep (Method A above). This represents a typical sequencing result on all instrument platforms for TempliPhi amplified BACs using this miniprep method.

Quality of sequencing results

Figure 4 contains a summary of the sequencing results (% pass rate) obtained for all BACs on all instrument platforms using five different preparation methods. For purified BAC DNA, purified TempliPhi amplified BAC DNA, and TempliPhi amplified miniprep BAC DNA, greater than 100 sequencing reactions were performed for each, and for each of the colony prep methods, approximately 50 sequencing reactions were performed. Note that pass rates by our criterion were nearly identical—60% for purified BAC DNA (amplified and non-amplified) and TempliPhi amplified miniprep BAC DNA. Pass rates for both colony prep methods (TempliPhi amplified) were approximately half that of the miniprep method (~30%).

Fig 1. Sequencing trace from the MegaBACE 1000 showing sequence of purified BAC 89N6 (insert size 92 kb). One microgram of purified, non-TempliPhi amplified BAC DNA was used in the sequencing reaction.
Fig 2. Sequencing trace from MegaBACE 1000 showing sequence of purified, TempliPhi amplified BAC 89N6. Ten nanograms of purified BAC 89N6 was used as input to the TempliPhi reaction, and 2 µg of amplified DNA was used in the sequencing reaction.
Fig 3. Sequencing trace from the ABI PRISM 3100 showing sequence of TempliPhi amplified BAC 89N6 obtained from a culture miniprep. Two micrograms of amplified BAC DNA was used in the sequencing reaction.

Fig 4. Sequencing pass rates for purified BAC DNA as well as TempliPhi amplified BAC DNA prepared by each of the methods presented in the text. Data from all BACs tested (89N6, 135A24, and 135M8) and all instruments (MegaBACE 1000, ABI PRISM 3700, and ABI PRISM 3100) is included in the figure. For pure BAC DNA, TempliPhi amplified pure BAC DNA, and BAC DNA culture mini-prep, more than 100 sequencing reactions of each were performed. For each of the colony preparation methods, approximately 50 sequencing reactions were performed. The error bars represent the standard deviation for all BACs on all instruments except for the colony mini-prep BAC DNA where only data from the ABI PRISM 3100 is included. A run is considered to have passed if over 100 bp of readable sequence is obtained.
Conclusions

- TempliPhi DNA Amplification Kits can be successfully used to amplify BAC DNA prepared by several methods—from cultures or colonies.
- Approximately 10–15 micrograms of TempliPhi amplified BAC DNA are produced from as little as 10 nanograms of input DNA.
- TempliPhi amplified BAC DNA obtained from minipreps give sequencing success rates equivalent to that of the best current methods for BAC DNA preparation.

Acknowledgment

We thank Dr. Elaine Mardis of Washington University School of Medicine for generously providing two of the BAC clones used in this work.

Reference


Appendix 1: Alkaline lysis solutions

Resuspension buffer
25 mM Tris-Cl, pH 8
10 mM EDTA
50 mM glucose

Lysis buffer
0.2 N NaOH
1% SDS

Neutralization buffer
3 M KOAc, pH 5.5