Nucleic Acid Chemistry Laboratory
Automated DNA Sequencing Manual

Revised 5-1-04
To DNA Sequencers:

This packet contains lots of information that will help you optimize your automated DNA sequencing samples. We have included the kit protocol for BigDye™ Chemistry as well as some suggestions for designing primers, purifying templates and purifying reaction products. Please take a few minutes to look at our guidelines and the services we provide. Also, please review all the handouts enclosed, even if you are already familiar with automated DNA sequencing. As cycle-sequencing technology has evolved, protocols have changed too. If you have any questions, please feel free to stop by the lab (Room 406 Biotechnology Building) or give us a call anytime Monday - Friday between the hours of 8 a.m. and 5 p.m.

Thank you for your interest in the Nucleic Acid Chemistry Laboratory's DNA Sequencing Facility.

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**TEMPLATE BASICS**

**Template Quality**

One of the most important factors in fluorescent DNA sequencing is the quality of template. It is a common misconception that if a template works for manual sequencing or for PCR amplification, it should work for automated sequencing. Automated sequencing is much more sensitive to many contaminants, including RNA, protein, carbohydrate, lipid and common buffer salts. Often, templates used successfully for other molecular biology protocols are not clean enough for fluorescent sequencing methods and will result in little or no useable data.

Choice of a host strain for cloned DNA can affect DNA quality and sequence results. Experience recommends some strains and warns against others:

<table>
<thead>
<tr>
<th>Recommended Strains</th>
<th>Not Recommended</th>
</tr>
</thead>
<tbody>
<tr>
<td>DH5 alpha</td>
<td>JM101</td>
</tr>
<tr>
<td>HB 101</td>
<td></td>
</tr>
<tr>
<td>XL – Blue</td>
<td></td>
</tr>
<tr>
<td>JM 109</td>
<td></td>
</tr>
<tr>
<td>MV 1190</td>
<td></td>
</tr>
</tbody>
</table>

When sequencing plasmid DNA or BAC DNA, use a fresh culture prepared from a fresh colony for best results. Follow the purification protocol carefully so that you have a template that is free of contaminating RNA, chromosomal DNA and cellular proteins or other debris, and free of residual salts, organic chemicals and detergents.

When sequencing PCR fragments, for best results you should cut the desired fragment from an agarose or polyacrylamide gel and then elute the DNA from the gel. *Make sure that you cut only one band!*

Although it is possible to sequence DNA fragments as short as 100 bases, better sequence data will be obtained using fragments larger than 200 bases.

A "dirty" template can sometimes be cleaned up using one or more of the following methods:

- Purify the DNA by ultrafiltration.
- Purify by chloroform extraction / PEG precipitation.
Template Quantity

Another important factor is the amount of template used in sequencing reactions. This will vary with the size of the template. It is very important to know how much DNA you are using in order to ensure reliable, reproducible data with a minimum number of sequencing reactions. It also helps to know DNA quantity when troubleshooting poor sequence data. Too much template results in top-heavy data with off-scale peaks early and sequence that fades rapidly. Too little template reduces the signal strength and peak height and increases the affect of baseline noise. We prefer that you quantify your DNA using a spectrophotometer or fluorometer since these are more reliable methods for determining DNA quantity than estimating concentration based on the size of bands in an agarose gel.

Some Common Methods Used to Quantify DNA:

1. Fluorometer readings are usually accurate.
2. Using a spectrophotometer to determine $\text{OD}_{260} / \text{OD}_{280}$ ratio gives you both DNA quantity and quality. A 260/280 ratio of 1.8 to 2.0 indicates highly pure DNA. This ratio should be at least 1.7 to ensure high quality sequence data.
3. Gels are important for checking DNA purity but determining quantity is very subjective. Short wavelength UV lights frequently used to visualize the bands will nick DNA. If the DNA from that band will be used for sequencing, be sure to use a long wave UV light.
4. Dipsticks just aren’t accurate enough

To calculate DNA concentration, use the following formulas derived from Beer's Law (Ausubel et al., 1998):

\[
\text{One OD}_{260} \text{ unit of single stranded DNA} = 33 \text{ ng/\mu l}
\]

\[
\text{One OD}_{260} \text{ unit of double stranded DNA} = 50 \text{ ng/\mu l}
\]

Note: Absorbance measurements of very concentrated DNA (OD > 1.0) or very dilute DNA (OD < 0.05) are frequently inaccurate.
Methods for Template Preparation

Plasmid DNA:
It is very important to use high quality plasmid prep columns and elute the DNA with either sterile water or sterile Tris, pH 8.0 - 8.5. **No EDTA, please!** In general Qiagen mini and maxi preps result in good, clean plasmid DNA. If you use Promega Wizard preps or Qiagen midi preps, please ethanol precipitate the DNA after elution and resuspend the pellet in water or Tris buffer before sequencing. Alternatively you can use an old-fashioned alkaline lysis/PEG precipitation, which usually results in very clean plasmid DNA.

Recommended Commercial Plasmid Prep Kits
- ABI Prism Plasmid Miniprep Kit
- Qiagen Plasmid Prep Kits
- Edge Biosystems AGCT columns
- Promega Magic or Wizard Kits (followed by an ethanol precipitation to ensure clean DNA)

Recommended "HomeMade" Methods
- Cesium Chloride Banding
- Modified Alkaline Lysis / PEG Precipitation

BAC DNA:
With very large DNA templates, such as bacterial artificial chromosomes, quality of the DNA is extremely important to successful sequencing. Two "HomeMade" methods usually give good sequencing results. Store BAC DNA in sterile Tris, pH 8.0 at 4°C. Do not freeze BAC DNA since it may precipitate to form an insoluble pellet.

Recommended "HomeMade" Methods
- Alkaline Lysis, with extra phenol extraction followed by isopropanol precipitation
- Cesium Chloride Banding

Recommended Commercial Kits for BAC DNA Preparation
- LigoChem ProPrep BAC Kits
- Qiagen-tip 100 and Qiagen-tip 500 Kits
Bacteriophage \(\lambda\)-DNA and M13 phage DNA:

It is possible to get good sequence data for \(\lambda\)-DNA and phage DNA. However, signal strengths for these templates are generally weak. Template purity and quantitation is crucial. Do not freeze \(\lambda\)-DNA or phage DNA before sequencing, because it may form insoluble pellets.

Recommended Methods for \(\lambda\)-DNA and M13 Phage DNA Purification

- Alkaline Lysis/PEG precipitation/chloroform extraction.
- Qiagen Lambda System
- Qiagen QIAprep M13 System

Sequencing Conditions for Various Template Types

<table>
<thead>
<tr>
<th>Template</th>
<th>Plasmid</th>
<th>BAC</th>
<th>Microbial Genomic DNA</th>
<th>(\lambda)-DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size</td>
<td>&lt;10Kb</td>
<td>50-300Kb</td>
<td>750Kb</td>
<td>50-300kb</td>
</tr>
<tr>
<td>Reaction Mix</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Big Dye Terminator</td>
<td>8 (\mu)l</td>
<td>8 (\mu)l</td>
<td>16 (\mu)l</td>
<td>16 (\mu)l</td>
</tr>
<tr>
<td>Primer</td>
<td>3.2 pmoles</td>
<td>10 pmoles</td>
<td>12-13 pmoles</td>
<td>6.4 pmoles</td>
</tr>
<tr>
<td>Template DNA</td>
<td>50-100ng/kb</td>
<td>800ng-1(\mu)g</td>
<td>2-3 (\mu)g</td>
<td>0.5-1.0 (\mu)g</td>
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<tr>
<td>DMSO</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>5% v/v Glycerol</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5% v/v DMSO</td>
</tr>
<tr>
<td>Total Volume</td>
<td>20 (\mu)l</td>
<td>20 (\mu)l</td>
<td>40 (\mu)l</td>
<td>40 (\mu)l</td>
</tr>
<tr>
<td>Cycling Conditions</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial Denaturation</td>
<td></td>
<td>95(^\circ)C, 5 min</td>
<td>95(^\circ)C, 5 min</td>
<td>95(^\circ)C, 5 min</td>
</tr>
<tr>
<td>Denaturation</td>
<td>96(^\circ)C, 10 sec</td>
<td>96(^\circ)C, 30 sec</td>
<td>96(^\circ)C, 30 sec</td>
<td>96(^\circ)C, 30 sec</td>
</tr>
<tr>
<td>Annealing</td>
<td>55(^\circ)C, 10 sec</td>
<td>55(^\circ)C, 20 sec</td>
<td>55(^\circ)C, 20 sec</td>
<td>50-55(^\circ)C, 10 sec</td>
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<tr>
<td>Extension</td>
<td>60(^\circ)C, 4 min</td>
<td>60(^\circ)C, 4 min</td>
<td>60(^\circ)C, 4 min</td>
<td>60(^\circ)C, 4 min</td>
</tr>
<tr>
<td>Number of Cycles</td>
<td>25-50</td>
<td>50-99</td>
<td>45-99</td>
<td>30 or more</td>
</tr>
<tr>
<td>Hold (Soak)</td>
<td>4(^\circ)C</td>
<td>4(^\circ)C</td>
<td>4(^\circ)C</td>
<td>4(^\circ)C</td>
</tr>
<tr>
<td>Rxn Clean Up Method</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
</tr>
</tbody>
</table>

* Most reliable method and preferred by DNA Sequencing Laboratory
PCR Fragments:
It is very important to use a purified PCR fragment for sequencing. The recommended method is to gel purify the PCR fragment before sequencing. However, if you are sure you have a single extension product based on gel analysis, there are two options for direct sequencing: (1) You can do an Exonuclease I – Shrimp Alkaline Phosphatase digestion to remove residual PCR primers and dNTPs. (2) You can optimize PCR conditions to limit the amounts of primers and dNTPs in the reaction so that most of the primers and dNTPs are exhausted during amplification of the fragment. Alternatively, you can use column purification to desalt the PCR fragment and remove residual primers and dNTPs. As with plasmid DNA, PCR fragments should be in a buffer which does not contain EDTA.

Recommended Products for Purifying PCR Fragments
- Qiagen QIAquick PCR Purification Kit
- Qiagen QIAquick Gel Extraction Kit
- Millipore Centricon 100 or Microcon 50 ultrafiltration units

Pros and Cons of Different Clean Up Methods for PCR Products

<table>
<thead>
<tr>
<th>Method</th>
<th>Pro</th>
<th>Con</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exonuclease I / Shrimp Alkaline Phosphatase (a.k.a. Exo/Sap) Digestion</td>
<td>Easy, cheap, cleans many samples at a time</td>
<td>Exo/Sap will not eliminate multiple PCR products</td>
</tr>
<tr>
<td>Column Purification: Qiagen's QIAquick Kits Centricon 100 Columns</td>
<td>Quick, easy, and a high reproducibility</td>
<td>Will not remove contaminating PCR products with high molecular weight but will remove primer-dimers.</td>
</tr>
<tr>
<td>Gel Purification</td>
<td>Isolates the fragment of interest away from contaminants</td>
<td>Time consuming. Quantity of DNA obtained can be variable. May not separate contaminants of similar size to PCR fragment.</td>
</tr>
<tr>
<td>Direct sequencing of PCR product</td>
<td>Saves time and money for multiple samples with same target</td>
<td>Optimization of PCR amplification conditions and characterization take time</td>
</tr>
</tbody>
</table>
MgCl$_2$ is a cofactor for the DNA polymerase enzyme. Since EDTA chelates Mg, the presence of EDTA in the cycle sequencing reaction can dramatically affect reaction efficiency. Many common buffers used to elute DNA from plasmid prep kits (e.g., TE) contain EDTA. Therefore, always use sterile distilled water or sterile Tris, pH 8.0 for the elution step when you plan to sequence that DNA. Also, always prepare your primer stocks in sterile distilled water or sterile Tris, pH 8.0.
PRIMER BASICS

Desirable primer-template interaction is based on many parameters. The length, base composition and sequence of the primer determine the conditions used to denature and anneal in a cycle sequencing PCR reaction. In general, primers should be selected to have one or more Gs or Cs at the 3'-end, a base composition of approximately 45 - 55% GC and no inverted repeats or homopolymeric regions. Primers should be 18 - 25 nucleotides in length with a calculated Tm greater than 45ºC.

Primer sites should be selected only from unambiguous sequence regions. As few as three mismatches between the primer and the primer binding site can reduce the stability of the complex formed, causing the sequence reaction to fail.

When a DNA sequencing strategy such as primer walking is involved, primer site selection is important in order to achieve a balance of minimized redundancy and assured quality of the sequencing data.

With proper preparation and the correct template/primer ratio, the BigDye terminator cycle sequencing method regularly yields 600 - 1000 bases of sequence information with greater than 98% accuracy for single-stranded DNA and 500 - 800 bases with greater than 98% accuracy for double-stranded DNA.

It is important to remember that the first 50 – 60 bases from the priming site may not yield reliable sequence data. Therefore, priming sites should be chosen to take that into account so that critical sequence information from an important region of the template will not be unreadable.

Common Primer-Related Sequencing Problems

1. Primer purity
2. Inhibitors, especially EDTA
3. Mismatch due to error in the sequence of the primer or template
4. Presence of a secondary hybridization site
5. No hybridization site
6. Primers with unsuitable Tm

Recommendations

1. Use fresh working stock of properly stored primers. Avoid multiple freeze/thaw cycles.
2. Design primers based on reliable, known sequence of template and check for possible secondary hybridization site.
3. Quantity is important. Use 3 - 4 pmol of primer unless your template is very large. Do not use too much primer.

4. Avoid primers with long runs of a single base (i.e., more than 3 or 4, especially G or C).

5. Primers should generally be at least 18 bases long with a 3’ GC clamp to ensure good hybridization. Primers with 20 -24 bases work best.

6. For cycle sequencing, primers with melting temperatures above 45ºC generally produce better results than primers with lower melting temperatures. If necessary, adjust annealing temperature based on Tm of primer. Determine the calculated Tm for best results. Avoid estimating Tm.

7. For primers with a GC content of less than 50%, it may be necessary to extend the primer sequence beyond 18 bases to keep the melting temperature above the recommended lower limit of 45ºC.

8. Use of primers longer than 18 bases also minimizes the chances of encountering problems with a secondary hybridization site on the template.

9. Avoid primers that have secondary structure, or that can hybridize to form dimers.

10. All primers should ideally be prepared in dH₂O since salts, particularly EDTA, inhibit the sequencing reaction. Tris, pH 8.0 – 9.0 can be used instead of water. Please see other enclosures for additional information.

Several computer programs for primer design are available for use through the internet (try idtdna.com or qiagen.com). However, these programs address a limited set of problems. They are chiefly useful for calculating an actual Tm (rather than an estimated Tm), identifying potential secondary structure problems and determining if there is a secondary hybridization site on the vector or in known portions of the insert sequence. Take a look at the following formula and you will see how convenient it can be to use a computer program to calculate the actual primer Tm.

**Estimating Primer Melting Temperature**

\[ Tm = 4 \times (G+C) + 2 \times (A+T) \quad \text{or} \quad Tm = 81.5 + 0.41(\%GC) - 500/L + 16.6 \times \log[M] \]

L = length of primer  
[M] = concentration of monovalent cation

**Calculating Actual Primer Melting Temperature**

\[ Tm = \left\{ \frac{\Delta H}{\Delta S + R \ln(C)} \right\} - 273.15 + 12.0 \times \log[Na+] \]

\( \Delta H \) = change in enthalpy  
\( \Delta S \) = change in entropy  
R = molar gas constant (1.987 cal.K⁻¹ mole⁻¹)  
C = molar concentration of primer
Calculating Primer Concentration

MW ÷ grams/L of DNA = Molar Concentration

Assumptions: One OD unit = 33 g/ml (or 0.033 g/L)
Average MW = 330 g/mol/base

Determine OD$_{260}$ for the primer

(330 g/mol x number of bases) ÷ (OD$_{260}$ x 0.033 g/L) = concentration of primer

For Example: An 18 base primer at OD$_{260}$ of 0.50
MW = 18 x 330 g/mol = 5940 g/mol
g/L of DNA = 0.5 ODU x 0.033 g/L = 0.0165 g/L
Concentration = 0.0165 g/L ÷ 5940 g/mol
Concentration = 2.77 x 10$^{-6}$ M or 2.77 µM
2.77 µM = 2.77 pmol/µl

Universal Sequencing Primers

<table>
<thead>
<tr>
<th>Primer</th>
<th>Length</th>
<th>Tm</th>
<th>Sequence (5' - 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>-21 M13 Forward</td>
<td>18</td>
<td>54</td>
<td>TGT AAA ACG ACG GCC AGT</td>
</tr>
<tr>
<td>-40 M13 Forward</td>
<td>17</td>
<td>52</td>
<td>GTT TTC CCA GTC ACG AC</td>
</tr>
<tr>
<td>M13 Reverse</td>
<td>17</td>
<td>50</td>
<td>CAG GAA ACA GCT ATG AC</td>
</tr>
<tr>
<td>SP6 Promoter</td>
<td>18</td>
<td>48</td>
<td>ATT TAG GTG ACA CTA TAG</td>
</tr>
<tr>
<td>T7 Promoter</td>
<td>18</td>
<td>48</td>
<td>TAA TAC GAC TCA CTA TAG</td>
</tr>
<tr>
<td>T3 Promoter</td>
<td>20</td>
<td>56</td>
<td>ATT AAC CCT CAC TAA AGG GA</td>
</tr>
<tr>
<td>T7 Terminator</td>
<td>19</td>
<td>54</td>
<td>GCT AGT TAT TGC TCA GCG G</td>
</tr>
</tbody>
</table>
Why Is It So Important to Clean Up Those Sequence Reactions?

During analysis of sequence data, the Y-axis of the electropherogram is automatically scaled to the largest peak found by the analysis software. The software does not allow this parameter to be set manually. When the largest peak happens to be a gargantuan (relatively speaking) unincorporated dye terminator peak, the Y-axis scale is so large that the smaller sequence peaks may not be resolved from the baseline noise. In some cases, the true sequence peaks can be seen underneath the contaminating dye terminator peaks. Where these dye terminator peaks appear in the sequence, the sequence analysis basecaller may call incorrect bases or it may call Ns. Samples that have relatively low signal strengths are affected more than samples that have higher signal strengths.

The best method for removing unincorporated dye terminators from your cycle sequencing reaction is to use a spin column. Unlike precipitation protocols, spin columns remove all unincorporated terminators from the extension products. Your sequencing results will be much more accurate and you will get longer reads.

The electropherograms below are from three samples recently analyzed in the DNA Sequencing Lab. These three samples all have relatively high signal strengths (G = 300 - 500). Figure 1 contains data from a sample that was cleaned up using a dye terminator removal spin column. Figure 2 and Figure 3 contain data from two different samples both cleaned up using an ethanol precipitation protocol that was followed carefully. The very large red, blue and black background peaks between 0 and 110 bases in Figure 2 and Figure 3 were caused by aggregated unincorporated dye terminators that were precipitated with the DNA during the ethanol precipitation clean up protocol. Dye terminator peaks such as these are rarely seen in samples cleaned up with spin columns. Take a look at the units for the Y-axis for each of these electropherograms. For Figure 1, the Y-axis = 1070. For both Figure 2 and Figure 3, the Y-axis = 1632.

**Recommended Dye Terminator Removal Columns & 96-Well Plates**

- **Edge Biosystems Gel Filtration Columns**
  - Catalog # 42453
  - [http://edgebio.com](http://edgebio.com)

- **Qiagen DyeEx Spin Kit**
  - Catalog # 63204
  - [http://www.qiagen.com](http://www.qiagen.com)

- **Sigma SigmaSpin Post-Reaction Clean Up**
  - Catalog # S 5059
  - [http://www.sigma-aldrich.com](http://www.sigma-aldrich.com)
Figure 1. Spin column clean up.

Figure 2. Ethanol precipitation clean up.

Figure 3. Ethanol precipitation clean up.
**GENERAL GUIDELINES FOR AUTOMATED SEQUENCING WITH BIGDYE TERMINATORS**

**Plasmid DNA**

**Template Quantity:**
If you are using the standard 1X sequencing reaction, use approximately 50-100 ng of template DNA per kb of total construct size. For example, if your vector + insert is around 5.5 kb, try to use about 250-550 ng of plasmid DNA. In some cases you may be able to use less DNA.

If you are using a dilution of the standard reaction, you may need to use less DNA so that you do not use up all of the terminators making small extension products. This results in a short read length, usually around 120 to 150 bases.

**Primer Quantity:**
Always use between 3 and 4 pmol of primer for the sequencing reaction. Do not cut primer amount in half when you use the 0.5X reaction!

The only exception to this is when you are sequencing a very difficult template or sequencing a very large plasmid (greater than 10 kb). In these cases, you may need to increase primer to 5 to 6 pmol.

**PCR Products (direct sequencing)**

**Template Quantity:**
Use about 1 ng of PCR product per 100 bp for either the standard 1X sequencing reaction or the 0.5X reaction. For example, if you have a PCR product of around 350 bp, use 3 to 4 ng of that PCR product in the reaction.

**Primer Quantity:**
Always use 2 to 3 pmol of primer.

**Very Large Templates or Very Difficult Templates**
If you are sequencing very large plasmids (larger than 15 kb) or sequencing bacterial genomic DNA, BAC DNA or λ-DNA, you should always use at least the standard 1X sequencing reaction and may sometimes need to use the 2x reaction. Please give me a call if you need specific protocols for these templates.

<table>
<thead>
<tr>
<th><strong>Standard 1X Sequencing Reaction</strong></th>
<th><strong>0.5X Sequencing Reaction</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Template</td>
<td>X ng</td>
</tr>
<tr>
<td>Primer</td>
<td>3 - 6 pmol</td>
</tr>
<tr>
<td>BigDye Terminator</td>
<td>8 µl</td>
</tr>
<tr>
<td>Final volume</td>
<td>20 µl</td>
</tr>
</tbody>
</table>
TEMPLATE AND PRIMER REQUIREMENTS FOR CYCLE SEQUENCING REACTIONS TO BE RUN BY THE DNA SEQUENCING LAB

In order to provide the best possible sequencing data, we request the following materials. Please read this carefully before submitting template DNA and primers for sequencing by the DNA Sequencing Laboratory.

Template Quality

Plasmid DNA:
Please provide high quality plasmid prep DNA that is resuspended in either sterile water or sterile Tris, pH 8.0 - 8.5. **No EDTA, please!** In general Qiagen mini and maxi preps result in good, clean plasmid DNA. If you use Promega Wizard preps, please ethanol precipitate the DNA after elution and resuspend the pellet in water or Tris buffer before submitting it for sequencing. Please check the $\text{OD}_{260} / \text{OD}_{280}$ ratio for your plasmid preps. A ratio under 1.7 indicates a significant amount of contaminant and rarely results in good sequencing data.

PCR Products:
Please provide a purified PCR product. If you have a single band on your gel, you can simply perform an Exo-SAP digest to remove residual PCR primers and dNTPs. Alternatively, you can use a PCR clean up column such as Qiagen MinElute Gel Extraction or PCR purification columns. As with plasmid DNA, your PCR product buffer should not contain any EDTA.

Larger Templates:
If you are sequencing very large templates such as BAC DNA and Lambda DNA, please call the lab for specific protocols.

Template Quantity

The amount of template used in sequencing reactions varies with the size of the template. We prefer that you determine the concentration of your DNA using a spectrophotometer since that usually is a reliable way to estimate quantity. If you cannot give us a reliable estimate of DNA concentration, we cannot guarantee good sequence data.

If you are sequencing a difficult template (GC rich, repeats, homopolymers), please let us know so we can adjust the reaction conditions as necessary.
**Template Quantity (cont’d)**

**Plasmid DNA:**
In general for plasmid DNA we like to use 100 ng per kb total construct size. For example, if your vector + insert is about 5.5 kb total, we would use 550 ng of DNA in each sequencing reaction. Please adjust DNA concentration to between 200ng/µl and 500 ng/µl. For templates that are 10 kb or larger, you may adjust the concentration to 1.0 µg/µl or higher.

**PCR Products:**
For PCR products, a good general rule of thumb is 1.0 ng of PCR product per 100 bases, i.e. 10 ng of a 1000 bp PCR product for each sequencing reaction. Please adjust DNA concentration to between 5 ng/µl and 10 ng/µl for shorter PCR products. For longer PCR products, you may set the concentration proportionally higher.

**Primers**
We routinely use 3 pmol to 5 pmol of primer per sequencing reaction, depending on DNA template size. Please adjust the concentration of your primer(s) to between 3 pmol/µl and 10 pmol/µl. We need a molar concentration. Please do not give us a µg/ml concentration. If your primer(s) are not 15-20 bases long or have an unusual base composition, please let us know, as this will affect the cycle sequencing conditions. It is also helpful for us to have the primer sequence in these cases so we can determine a Tm.

**Template + Primers Provided Combined in a PCR Tube**
Please combine your DNA Template and Primer so that the final volume equals 12 µl. We can then add Sequence Premix and buffer to meet our volume requirements without having to either guess your volume or measure it (which will waste some of your sample).

We use 0.2 ml Thin-Wall PCR tubes in our PCR machines. If your tubes are larger we will need to transfer your sample into a smaller tubes so please pipet generously.
**Protocol for diluting BigDye™ Terminator Premix**

2.5X Sequencing Buffer          200 mM Tris, pH 9.0
                               5 mM MgC\(_2\)

For the standard BigDye™ sequencing reaction, 8 µl of Ready Reaction premix is used in a 20 µl reaction to make a final concentration of 80 mM Tris, pH 9.0 + 2 mM MgCl\(_2\). Since BigDye™ chemistry results in such a bright signal, it is possible to decrease your sequencing costs by diluting the amount of premix used in the reaction. To keep buffer and cofactor concentration optimal use this 2.5X concentration sequencing buffer and add a sufficient amount of buffer to bring the Ready Reaction premix + buffer volume to a total of 8 µl.

**Note:** If you would prefer to use a 10 µl reaction volume, the premix volume + 2.5X buffer volume should equal 4 µl.

**Important points to remember:**

When sequencing new templates, always start by using the standard reaction protocol. If your signal levels are good, you can decrease the amount of BigDye™ premix you use in subsequent reactions. Short PCR products and smaller plasmid DNA constructs (≤ 5 kb) are more likely to sequence well with less premix. Large templates, such as plasmid constructs > 10 kb or BACs, rarely sequence well with diluted premix.

BigDye™ Terminator Premix is most stable undiluted. Do not make a diluted stock of premix + 2.5X Sequencing Buffer for long-term storage. This will result in decreased shelf-life of the premix!

### BigDye™ Dilution for 20 µl Reaction

<table>
<thead>
<tr>
<th>Premix (µl)</th>
<th>Buffer (µl)</th>
<th>Reaction (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>4</td>
<td>20</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>20</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>20</td>
</tr>
<tr>
<td>1</td>
<td>7</td>
<td>20</td>
</tr>
</tbody>
</table>
Getting Better Results with BigDye™

These are the guidelines we use here in the DNA Sequencing lab for sequencing different types of templates. Remember that read lengths are likely to decrease as you decrease your premix volumes, particularly for longer templates. These guidelines were set up to achieve a minimum of 500 bp for most template preps.

<table>
<thead>
<tr>
<th>Template</th>
<th>BigDye™ Premix</th>
<th>2.5X Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR Products (&lt; 2000 bp)</td>
<td>2 µl</td>
<td>6 µl</td>
</tr>
<tr>
<td>Plasmids (&lt; 5 kb)</td>
<td>4 µl</td>
<td>4 µl</td>
</tr>
<tr>
<td>Plasmids (6-8 kb)</td>
<td>6 µl</td>
<td>2 µl</td>
</tr>
<tr>
<td>Plasmids (&gt; 8 kb)</td>
<td>8 µl</td>
<td>none</td>
</tr>
<tr>
<td>BAC DNA</td>
<td>8 µl</td>
<td>none</td>
</tr>
<tr>
<td>Genomic DNA</td>
<td>8 µl</td>
<td>none</td>
</tr>
</tbody>
</table>

Properly quantitate the DNA. Remember the amount of template and primer may vary based on your template type and size.

<table>
<thead>
<tr>
<th>DNA Type</th>
<th>Amount to Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single-Stranded</td>
<td>25 - 50 ng/kb</td>
</tr>
<tr>
<td>Double-Stranded (Plasmid)</td>
<td>50 - 100 ng/ kb total length</td>
</tr>
<tr>
<td>PCR Product</td>
<td>1 ng/100 base pairs</td>
</tr>
<tr>
<td>Cosmid, BAC</td>
<td>800 - 1000 ng</td>
</tr>
<tr>
<td>Bacterial Genomic DNA</td>
<td>1000 - 2000 ng</td>
</tr>
</tbody>
</table>

There are several commercial sequence reaction dilution buffers available - ABI, Edge Biosystems, Sigma, The Gel Company, and others all have their own versions. Most of these are at 5X strength and some (but not all) contain simply Tris and MgCl₂. Most of the time the homemade 2.5X Buffer (which is an ABI recipe) will work just fine as a dilution buffer. If you have problems getting good sequence results with your homemade buffer, you may need to consider purchasing one of the commercial buffers, such as "Better Buffer" from The Gel Company.
Ethanol Precipitation for BigDye™ Sequencing Reactions

1. Pipet entire contents of extension reactions into clean 0.5 ml or 1.5 ml tubes:
   For 20 µl reactions add: 16µl deionized water
   64µl non-denatured 95% ethanol at RT.
   For 10µl reactions add: 100µl of non-denatured 70% ethanol at RT.
   The final ethanol concentration should be 60 ± 3%. Do not exceed 63% ethanol.

2. Vortex briefly. Leave tubes at room temperature for 15-20 minutes to precipitate extension products.
   It is not necessary to use salt or a carrier for this precipitation. Your extension products will precipitate without either of those additives.
   Commercial dyes used to visualize DNA pellets may cause background problems on sequencing gels and affect your data. Samples containing these dyes cannot be run on our Capillary Electrophoresis Sequencers. While we will not refuse samples prepared with these dyes, your data may be delayed. The most commonly used product is Pellet Paint NF™ from Novagen.
   Precipitation times <15 minutes will result in the loss of very short extension products. Precipitation times >24 hours will increase precipitation of unincorporated dye terminators.

3. Spin tubes in microfuge at maximum speed and at room temperature for 20 minutes. Proceed to the next step immediately or spin again.

4. Carefully aspirate supernatants with a pipet tip.
   Remove as much of the liquid as you can. The more residual supernatant left in the tubes, the more unincorporated terminators will remain in the samples.
   Do not dump the supernatants out of the tubes. The unincorporated terminators will be spread along the sides of the tubes and will then precipitate with the extension products in the wash step.

5. Add 250 µl of 70% ethanol at room temperature to the tubes and vortex briefly.

6. Spin in microfuge at maximum speed and at room temperature for 10 minutes.

7. Aspirate the supernatants carefully.

8. Dry pellets in a vacuum-centrifuge for 10-15 minutes on low heat or to dryness. Alternatively, air dry for an hour or so.

Note: Other versions of EtOH precipitation protocols are not suitable to use with BigDye™ sequencing reactions due to the nature of the dye compounds.
PREPARING EXTENSION PRODUCTS FOR CAPILLARY ELECTROPHORESIS BY ISOPROpanol PRECIPITATION (96-Well Plate Format and Microcentrifuge Tube Format)

96-Well Column Purification Recommendations
The following 96-well purification plates are recommended by ABI for good results on capillary sequencers. Refer to manufacturer's instructions for procedures.

96-well Spin Columns, Gel Filtration Kit (Edge Biosystems, P/N 94880)
Arrylt (Telechem, P/N DTC-96-100)
Centri-Sep 96 plate (Princeton Separations, P/N CS-961)
Multiscreen 96-well Filter Plates (Millipore, P/N MadyEkit1)

Isopropanol Precipitation Protocol for 96-Well Plates
You will need the following equipment and reagents:

- Variable speed table-top centrifuge with microtiter plate rotor, capable of reaching at least 1400 X g.
- Strip caps for 96-well PCR plates or adhesive-backed aluminum foil tape (3M Scotch Tape 431 or 439)
- 75% isopropanol or 100% (anhydrous) isopropanol at room temperature

Note: This protocol does not use salt.

1. Remove the 96-well reaction plate from the PCR machine. Remove the caps.
2. Add one of the following:
   - 80 µl of 75% isopropanol
   - 20 µl of DI water and 60 µl of 100% isopropanol

   Final isopropanol concentration should be 60 ± 5%.
3. Seal the plate with strip caps or adhesive-backed aluminum foil tape.
4. Invert the plate a few times to mix, or vortex.
5. Leave the plate at room temperature for 15 minutes to precipitate the extension products.
6. Place the plate in centrifuge and spin at the maximum speed, which must be at least 1400 x g but less than 3000 x g:
   - 1400-2000 x g for 45 minutes
   - 2000-3000 x g for 30 minutes
7. Without disturbing the precipitates, remove the adhesive tape or caps and discard the supernatant by inverting the plate onto a paper towel. Remove as much of the supernatant as possible.
8. Rinse the pellet by adding 150 µl of 70% isopropanol to each well.
9. Seal the plate with adhesive tape and invert a few times to mix.
10. Place the plate in the centrifuge and spin at 2000 x g for 10 minutes.
11. Remove the adhesive tape. Discard the wash onto a paper towel that is folded to the size of the plate.
12. Place the inverted plate with the paper towel into the centrifuge and spin at 700 x g for 1 minute.
13. Remove the plate and discard the paper towel.
14. Pellets may or may not be visible. Vacuum drying the samples is not necessary.
**Isopropanol Precipitation for Microcentrifuge Tubes**

**Note:** This protocol does not use salt.

1. Pipet the entire contents of each extension reaction into a 1.5 ml microcentrifuge tube.

2. Add **one** of the following:
   - 80 µl of 75% isopropanol - or-
   - 20 µl of DI water and 60 µl of 100 % isopropanol

   *The final isopropanol concentration should be 60 ± 5%.*

3. Close the tubes and vortex briefly.

4. Leave tubes at room temperature for 15 minutes to precipitate the extension products.

   Precipitation times shorter than 15 minutes will result in the loss of very short extension products. Precipitation times longer than 24 hours will increase the precipitation of unincorporated terminators.

5. Place the tubes in a microcentrifuge and mark their orientations. Spin the tubes for 20 minutes at maximum speed at room temperature.

6. Carefully aspirate the supernatants with a separate pipette tip for each sample and discard. Remove as much of the supernatant as possible. Pellets may or may not be visible.

   The supernatants must be removed completely, as unincorporated dye terminators are dissolved in them. The more residual supernatant left in the tubes, the more unincorporated terminators will remain in the samples. Capillary electrophoresis is extremely sensitive to "dirty" samples, which clog the capillaries and significantly decrease the life of the capillaries.

   **VERY IMPORTANT:** Do not use any co-precipitants such as salt, glycogen, tRNA or "Pellet Paint." These drastically clog the capillaries and will result in immediate capillary failure!

7. Add 250 µl of 75% isopropanol to the tubes, and vortex them briefly.

8. Place the tubes in the microcentrifuge in the same orientation as marked and spin for 5 minutes at maximum speed.

9. Aspirate the supernatants carefully.

10. Dry the samples in a vacuum centrifuge for 10 to 15 minutes or to dryness.
Plasmid DNA Purification by Alkaline Lysis/PEG Precipitation

This protocol usually yields a very clean prep of plasmid DNA free of contaminating protein, genomic DNA and RNA. Do not use a vortexer to minimize shearing the chromosomal DNA.

Reagents Needed:
GET Buffer: 50 mM Glucose
          10 mM EDTA
          25 mM Tris, pH 8.0
3.0 M Potassium Acetate, pH 4.8
2 N NaOH
10% SDS
4M NaCl
PEG 8000 13% w/v, autoclaved and cooled to RT
100% Isopropanol
RNase A (DNase-free)
Chloroform
70% Ethanol
Sterile ultrapure water

1. Pellet 1.5 ml aliquots of overnight culture in microfuge for 1 minute at maximum speed. 
   Note: A total culture volume of 4.5 ml can be pelleted per tube without changing volumes 
   in this protocol. This allows a larger yield without the need for extra tubes.
2. Remove the supernatant by aspiration.
3. Resuspend the bacterial pellet in 200 µl of GET buffer by pipetting up and down.
4. Add 300 µl of freshly prepared 0.2 N NaOH / 1% SDS. Mix the contents of the tube by 
   inversion. Incubate on ice for 5 minutes.
5. Neutralize the solution by adding 300 µl of 3.0 M potassium acetate, pH 4.8. Mix by inverting 
   the tube. Incubate on ice for 5 minutes.
6. Remove cellular debris by spinning in a microfuge at maximum speed for 10 minutes at room 
   temperature. Transfer the supernatant to a clean tube.
7. Add RNase A (DNase free) to a final concentration of 20 µg/ml. Incubate the tube at 37° C for 
   20 minutes.
8. Extract the supernatant twice with chloroform: 
   a. Add 400 µl of chloroform.
   b. Mix the layers by inversion for 30 seconds.
   c. Centrifuge the tube for 1 minute to separate the phases.
   d. Transfer the upper aqueous phase to a clean tube.
9. Add an equal volume of 100% isopropanol. Mix the contents of the tube by inversion.
10. Spin the tube in a microfuge at maximum speed for 10 minutes at room temperature.
11. Remove the isopropanol completely by aspiration.
12. Wash the DNA pellet with 500 µl of 70% ethanol. Dry under vacuum for 3 minutes.
13. Dissolve the pellet in 32 µl of sterile water.
14. Add 8.0 µl of 4M NaCl, then 40 µl of autoclaved 13% PEG 8000.
15. Mix thoroughly, then leave the sample on ice for 20 minutes.
16. Pellet the plasmid DNA by spinning in a microfuge at maximum speed for 15 minutes at 4° C.
17. Carefully remove the supernatant. Rinse the pellet with 500 µl of 70% ethanol.
18. Resuspend the pellet in 20 µl of sterile water or 10 mM Tris, pH 8.0.
20. Store DNA at - 20° C.
DyeDeoxy Cycle Sequencing of Agarose Gel Purified PCR Products after β-Agarase Digestion

Gel Electrophoresis and DNA Isolation:

Load 30 to 50 µl of the PCR product into a single well of an appropriate concentration of NuSieve GTG™ or SeaPlaque GTG™ agarose gel in 1X TAE buffer. Separate by electrophoresis, stain the gel with ethidium bromide, then photograph the gel. Estimate PCR yield by comparison with standards on the gel. Carefully excise the band of interest and trim away the excess agarose.

β-Agarase Digestion:

Place the trimmed gel slice in a microcentrifuge tube and incubate at 65°C for 5 minutes, briefly centrifuge the tube, incubate an additional 5 minutes at 65°C, and estimate the gel volume. Cool the agarose to 40°C for 5 minutes. Add an appropriate amount of β-agarase and incubate at 40°C for 30 minutes to overnight.

Cycle Sequencing:

Use an appropriate volume (1.0 to 9.5 µl, for 5 to 50 ng of DNA) of the resulting liquefied gel slice in the ABI cycle sequencing protocol. After cycling is complete, use a spin column to purify the extension products away from the DyeDeoxy terminators, β-agarase, and agarose-derived oligosaccharides.

Reference:

PCR Cleanup with Exonuclease I and Shrimp Alkaline Phosphatase

This enzymatic cleanup of a PCR fragment is performed just prior to sequencing the PCR fragment, and acts to remove excess oligonucleotide primers and dNTPs. It can be used as an alternative to Qiagen QIAquick™ PCR Purification columns (or other PCR product cleanup columns).

1. Amplify the desired PCR fragment using the appropriate primer set in a 50 µl reaction volume. It is always good practice to check the fragment following PCR by running 1-5 µl on an agarose gel with a size standard.
   
   Hint: This method will not help "clean up" sequencing reactions for PCR reactions which yield more than one product. If you have more than one PCR fragment band on a gel, you will need to do a gel extraction, such as the Qiagen QIAquick™ Gel Extraction Kit, to remove the contaminating bands.

2. If a single band has been obtained, remove a 5 µl aliquot from each PCR reaction into a clean 0.2 ml thin walled (or other PCR) tube.

3. Add 1 µl of exonuclease I (10 U/µl - Amersham product # E70073) and 1 µl Shrimp Alkaline Phosphatase (SAP - Amersham product # E70092) (2 U/µl) to each of the PCR fragment aliquots.

4. Program the thermal cycler as follows:
   
   37° C for 15-30 minutes
   80° C for 15 minutes
   Cool to 4° C

5. Place the PCR fragment + enzyme-containing tube from step 3 into the thermal cycler and run the program from step 4 (above).

6. Once the thermal cycler has completed the program outlined in step 4, the fragment is ready for sequencing.
   
   Note: We typically add 2-3 µl of undiluted reaction mixture to sequence with BigDye™ terminators successfully. Of course, the amount used will depend on the initial PCR fragment yield.
**Sequencing BACs**

Some BACs prove fairly easy to sequence. Unfortunately, for some BACs it has proven almost impossible to obtain good sequencing data. The only way to know if you will get usable data from a BAC is to give it a try. Here are some guidelines for sequencing from a BAC.

It is very important to have a very clean template preparation. Qiagen Tip 100s or Qiagen Tip 500s are two of the best kits for BAC purification. If you prefer, you can do a standard alkaline lysis followed by an extra phenol extraction and then two extra isopropanol extractions to obtain clean BAC DNA.

First try a 1X reaction as follows:

8 µl BigDye™ Terminator Cycle Sequencing Premix  
1 µg BAC DNA  
10 pmol primer  
ultra pure water to make 20 µl final volume

**Cycling Conditions:**

Hot start 95°C for 5 minutes  
Then 50 cycles of:  
96°C for 30 seconds  
50°C for 20 seconds  
60°C for 4 minutes  
and finally:  
Hold 4°C.

If you get a low signal with 50 cycles, increase cycle number to 75 or 100. This may increase your signal level.

Occasionally it is necessary to use a 2X reaction:

16 µl BigDye™ Terminator Cycle Sequencing Premix  
2 µg BAC DNA  
6-13 pmol primer  
ultra pure water to make 40 µl final volume

**Reagents for BAC Sequencing:**

If we will be running your cycle sequencing reactions, please provide the following:  
BAC DNA purified by one of the methods listed above.  
- Please provide enough BAC DNA to run all reactions in duplicate, if possible.  
- Concentration of BAC DNA should be 0.5 to 1.0 µg/ml.  
Custom Primer(s) - concentration of primer should be 5 to 10 pmol/µl.

We will provide standard primers (eg, T7, T3, Sp6, M13 forward and reverse).
**Tips for Sequencing Cosmid DNA, BAC DNA, etc**

1. Use Applied Biosystem's Modified alkaline lysis/PEG precipitation protocol to isolate the cosmid or BAC DNA. Follow with an extra phenol extraction and ethanol precipitation for very clean DNA. Sequence 1-5 µg DNA depending on template size.

2. **DO NOT FREEZE** the DNA before sequencing. Store at 4°C.

3. Always use purified primers (OPC or HPLC purified).

**If necessary:**

4. Reduce annealing temperature for cycle sequencing as low as 45°C.

5. Add a 2 min soak at 95°C before cycling.

6. Add 5% DMSO (by volume) to sequencing reaction.

**References:** Biotechniques, Vol 13, No 1 (1992), pg. 46-47.  

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**2X Cycle-Sequencing Protocol for Large Templates**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Big Dye™ Terminator Premix</td>
<td>16 µl</td>
</tr>
<tr>
<td>Primer</td>
<td>6-8 pmol</td>
</tr>
<tr>
<td>Template DNA</td>
<td>1-2 µg (Cosmid)</td>
</tr>
<tr>
<td></td>
<td>2-5 µg (Microbial Genomic, BACs)</td>
</tr>
<tr>
<td>Total Volume</td>
<td>40 µl</td>
</tr>
</tbody>
</table>

**Cycling conditions**  
95°C for 5 min preincubation  
30 - 75 cycles of  
95°C for 30 sec  
55°C for 20 sec  
60°C for 4 min  
4°C soak
Direct Sequencing of Bacteriophage λ-DNA

Reaction Mixture:

Template 0.5 to 1.0 µg
Primer ratio 6.4 pmol/µg DNA
DMSO and Glycerol 5%/5%
BigDye™ Premix 16 µl
DI water q.s. 40 µl

Cycle Sequencing Conditions:

95°C for 5 minutes (Hot Start)
followed by 30 cycles* of:

95°C for 30 sec.
50-55°C for 10 sec.
60°C for 4 min.

4°C hold

*May need to increase number of cycles to get better results.

General Guidelines for λ-DNA:

Template purity is crucial.
Template quantitation is crucial.
Do not freeze DNA before sequencing.
Signal is generally weaker than with plasmids.