AB Applied Biosystems

DNA Sequencing by Capillary Electrophoresis

Applied Biosystems Chemistry Guide | Second Edition

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DNA Sequencing by Capillary Electrophoresis
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Contents

Chapter 4   Cycle Sequencing

Chapter 5   Purification of Extension Products

Chapter 6   Capillary Electrophoresis

Chapter 7   Data Analysis

Chapter 8 Troubleshooting

Appendix A Product Information

Appendix B Parts List

Bibliography

Glossary

Index

Preface

How to Use This Guide

Purpose of This Guide

This chemistry guide is designed to familiarize you with Applied Biosystems genetic analyzers for automated DNA sequencing by capillary electrophoresis, to provide useful tips for ensuring that you obtain high quality data, and to help troubleshoot common problems.

Chapter 4, "Cycle Sequencing," Chapter 5, "Purification of Extension Products," and Chapter 8, "Troubleshooting" are each bound separately so they can be removed from the binder for easy use in the laboratory. Chapter 4 and Chapter 5 contain many protocols used in DNA sequencing.

Audience

This guide is intended for novice and experienced users who perform automated DNA sequencing.

Assumptions

This guide assumes that your Applied Biosystems genetic analyzer has been installed by an Applied Biosystems technical representative.

This guide also assumes that you have a working knowledge of the Windows operating system.

Text Conventions

This guide uses the following conventions:

- **Bold** text indicates user action. For example:
  Type **0**, then press **Enter** for each of the remaining fields.
- *Italic* text indicates new or important words and is also used for emphasis. For example:
  Before analyzing, *always* prepare fresh matrix.
- A right arrow symbol (▶) separates successive commands you select from a drop-down or shortcut menu. For example:
  Select **File ▶ Open ▶ Spot Set**.
  Right-click the sample row, then select **View Filter ▶ View All Runs**.

User Attention Words

Two user attention words appear in Applied Biosystems user documentation. Each word implies a particular level of observation or action as described below:

Note: – Provides information that may be of interest or help but is not critical to the use of the product.

IMPORTANT! – Provides information that is necessary for proper instrument operation, accurate chemistry kit use, or safe use of a chemical.

Examples of the user attention words appear below:

Note: The Calibrate function is also available in the Control Console.

IMPORTANT! To verify your client connection to the database, you need a valid user ID and password.

Safety Alert Words   Safety alert words also appear in user documentation. For more information, see "Safety Alert Words" on page x.

How to Obtain Support

For the latest services and support information for all locations, go to **www.appliedbiosystems.com**, then click the link for **Support**.

At the Support page, you can:

- Search through frequently asked questions (FAQs)
- Submit a question directly to Technical Support
- Order Applied Biosystems user documents, MSDSs, certificates of analysis, and other related documents
- Download PDF documents
- Obtain information about customer training
- Download software updates and patches

In addition, the Support page provides access to worldwide telephone and fax numbers to contact Applied Biosystems Technical Support and Sales facilities.

Safety and EMC Compliance Information

This section covers:

Safety Conventions Used in This Document

Safety Alert Words

Four safety alert words appear in Applied Biosystems user documentation at points in the document where you need to be aware of relevant hazards. Each alert word—**IMPORTANT, CAUTION, WARNING, DANGER**—implies a particular level of observation or action, as defined below.

Definitions

IMPORTANT! – Indicates information that is necessary for proper instrument operation, accurate chemistry kit use, or safe use of a chemical.

⚠ **CAUTION** – Indicates a potentially hazardous situation that, if not avoided, may result in minor or moderate injury. It may also be used to alert against unsafe practices.

⚠ **WARNING** – Indicates a potentially hazardous situation that, if not avoided, could result in death or serious injury.

⚠ **DANGER** – Indicates an imminently hazardous situation that, if not avoided, will result in death or serious injury. This signal word is to be limited to the most extreme situations.

Except for IMPORTANTs, each safety alert word in an Applied Biosystems document appears with an open triangle figure that contains a hazard symbol. *These hazard symbols are identical to the hazard symbols that are affixed to Applied Biosystems instruments*.

Examples

The following examples show the use of safety alert words:

IMPORTANT! You must create a separate sample entry spreadsheet for each 96-well plate.

⚠ **CAUTION** The lamp is extremely hot. Do not touch the lamp until it has cooled to room temperature.

⚠ **WARNING** **CHEMICAL HAZARD. Formamide**. Exposure causes eye, skin, and respiratory tract irritation. It is a possible developmental and birth defect hazard. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

⚠ **DANGER** **ELECTRICAL HAZARD.** Failure to ground the instrument properly can lead to an electrical shock. Ground the instrument according to the provided instructions.

For Additional Information

Please see the Safety chapters in:

• The protocols for the template preparation, sequencing chemistry and/or extension product purification you use.

• The user guides for the thermal cycler and DNA sequencer you use.

Chemical Safety

Chemical Hazard Warning

⚠️ **WARNING** **CHEMICAL HAZARD.** Before handling any chemicals, refer to the Material Safety Data Sheet (MSDS) provided by the manufacturer, and observe all relevant precautions.

About MSDSs

Chemical manufacturers supply current Material Safety Data Sheets (MSDSs) with shipments of hazardous chemicals to *new* customers. They also provide MSDSs with the first shipment of a hazardous chemical to a customer after an MSDS has been updated. MSDSs provide the safety information you need to store, handle, transport, and dispose of the chemicals safely.

Each time you receive a new MSDS packaged with a hazardous chemical, be sure to replace the appropriate MSDS in your files.

Chemical Waste Safety

Chemical Waste Hazard

⚠️ **CAUTION** **HAZARDOUS WASTE.** Refer to Material Safety Data Sheets and local regulations for handling and disposal.

Biological Hazard Safety

⚠️ **WARNING** **BIOHAZARD.** Biological samples such as tissues, body fluids, infectious agents, and blood of humans and other animals have the potential to transmit infectious diseases. Follow all applicable local, state/provincial, and/or national regulations. Wear appropriate protective equipment, which includes but is not limited to: protective eyewear, face shield, clothing/lab coat, and gloves. All work should be conducted in properly equipped facilities using the appropriate safety equipment (for example, physical containment devices). Individuals should be trained according to applicable regulatory and company/institution requirements before working with potentially infectious materials. Read and follow the applicable guidelines and/or regulatory requirements in the following:

- U.S. Department of Health and Human Services guidelines published in *Biosafety in Microbiological and Biomedical Laboratories* (stock no. 017-040-00547-4; **http://bmbl.od.nih.gov**)
- Occupational Safety and Health Standards, Bloodborne Pathogens (29 CFR§1910.1030; **http://www.access.gpo.gov/ nara/cfr/waisidx_01/ 29cfr1910a_01.html**).
- Your company's/institution's Biosafety Program protocols for working with/handling potentially infectious materials.

Additional information about biohazard guidelines is available at:

http://www.cdc.gov

Introduction to DNA Sequencing

<div style="text-align: right; font-size: 3em;">1</div>

This chapter covers:

DNA Sequencing Basics

This section presents basic synthesis, replication, and sequencing principles that you need to know in order to perform automated DNA sequencing by capillary electrophoresis.

Cell Replication
The process of DNA synthesis and replication in a cell involves DNA helicase, DNA polymerase, DNA template, and deoxynucleotides. DNA replication starts when DNA helicase unravels the double-helix structure to expose single-stranded DNA and form a replication fork. RNA primase introduces a primer that binds to the single-stranded DNA. DNA polymerase then binds to the replication fork and starts DNA synthesis by sequentially adding nucleotides to the 3′-hydroxyl end of the RNA primer bound to the DNA template (Figure 1). The result is the creation of an "extension product."
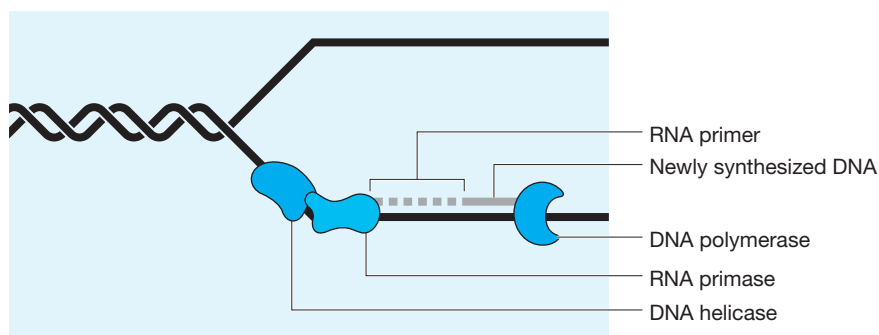
Figure 1   DNA replication fork

The extension product grows in the 5′ to 3′ direction by forming a phosphodiester bridge between the 3′-hydroxyl group at the growing end of the primer and the 5′-phosphate group of the incoming deoxynucleotide (Kornberg and Baker, 2005) (Figure 2).
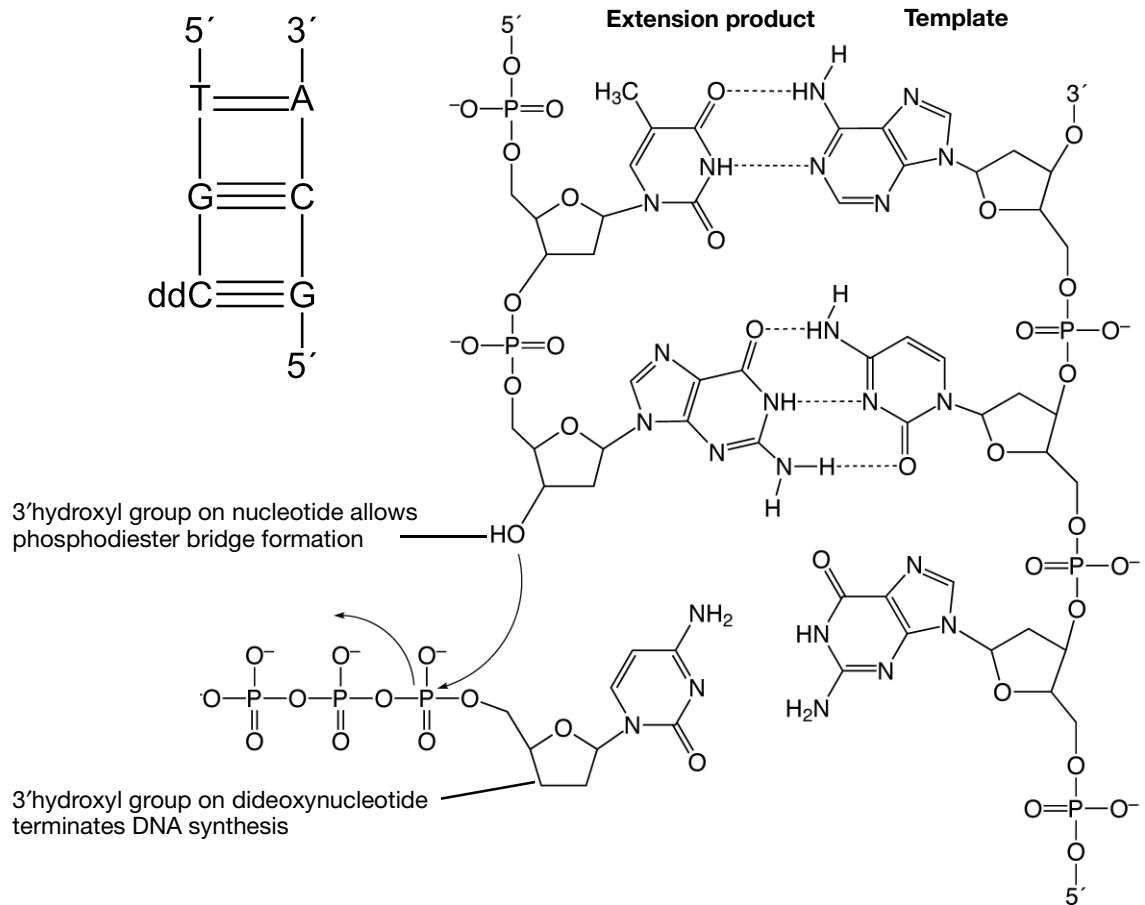
Figure 2   DNA strand synthesis and termination

The DNA sequence is copied with high fidelity because at each base on the DNA template, DNA polymerase incorporates only the nucleotide that is complementary to that base. Thymine (T) is complementary to adenine (A) and guanine (G) is complementary to cytosine (C) because they can form hydrogen bonds with each other (Figure 3).
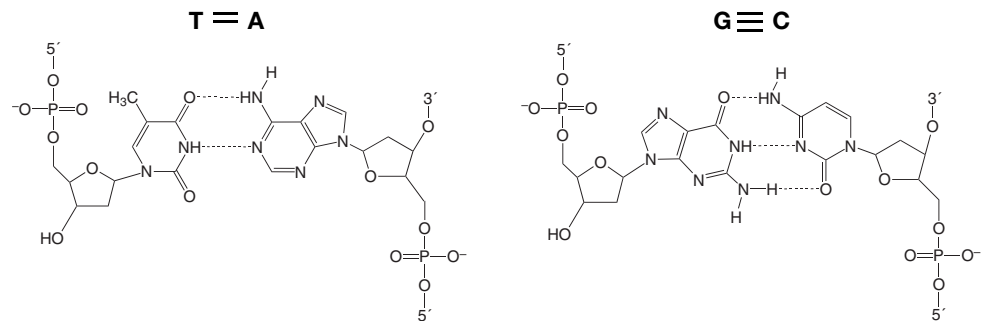
Figure 3   Base complements

History of Sanger Dideoxy Sequencing

The principles of DNA replication were used by Sanger *et al.* (1974) in the development of the process now known as Sanger dideoxy sequencing. This process takes advantage of the ability of DNA polymerase to incorporate 2′, 3′-dideoxynucleotides, nucleotide base analogs that lack the 3′-hydroxyl group essential in phosphodiester bond formation.

Sanger dideoxy sequencing requires a DNA template, a sequencing primer, DNA polymerase, nucleotides (dNTPs), dideoxynucleotides (ddNTPs), and reaction buffer. Four separate reactions are set up, each containing radioactively labeled nucleotides and either ddA, ddC, ddG, or ddT. The annealing, labeling, and termination steps are performed on separate heat blocks. DNA synthesis is performed at 37 °C, the temperature at which the T7 DNA polymerase used has the optimal enzyme activity.

DNA polymerase adds either a deoxynucleotide or the corresponding 2′, 3′-dideoxynucleotide at each step of chain extension. Whether a deoxynucleotide or a dideoxynucleotide is added depends on the relative concentration of both molecules. When a deoxynucleotide (A, C, G, or T) is added to the 3′ end, chain extension can continue. However, when a dideoxynucleotide (ddA, ddC, ddG, or ddT) is added to the 3′ end, chain extension terminates (Figure 2). Sanger dideoxy sequencing results in the formation of extension products of various lengths terminated with dideoxynucleotides at the 3′ end.

Electrophoresis

The extension products are then separated by electrophoresis. During electrophoresis, an electrical field is applied so that the negatively charged DNA fragments move toward the positive electrode. The speed at which a DNA fragment moves through the medium is inversely proportional to its molecular weight. This process of electrophoresis can separate the extension products by size at a resolution of one base.

Applied Biosystems Automated DNA Sequencing

Applied Biosystems fluorescence-based cycle sequencing system is an extension and refinement of Sanger dideoxy sequencing. Applied Biosystems automated DNA sequencing generally follows this flow:

1. Template preparation (Chapter 3)

2. Cycle sequencing (Chapter 4)

3. Purification after cycle sequencing (Chapter 5)

4. Capillary electrophoresis (Chapter 6)

5. Data analysis (Chapter 7)

Cycle Sequencing

Process Overview

Like Sanger sequencing, fluorescence-based cycle sequencing requires a DNA template, a sequencing primer, a thermal stable DNA polymerase, nucleotides (dNTPs), dideoxynucleotides (ddNTPs), and buffer. But unlike Sanger's method, which uses radioactive material, cycle sequencing uses fluorescent dyes to label the extension products and the components are combined in a reaction that is subjected to cycles of annealing, extension, and denaturation in a thermal cycler. Thermal cycling the sequencing reactions creates and amplifies extension products that are terminated by one of the four dideoxynucleotides (Figure 4). The ratio of deoxynucleotides to dideoxynucleotides is optimized to produce a balanced population of long and short extension products.
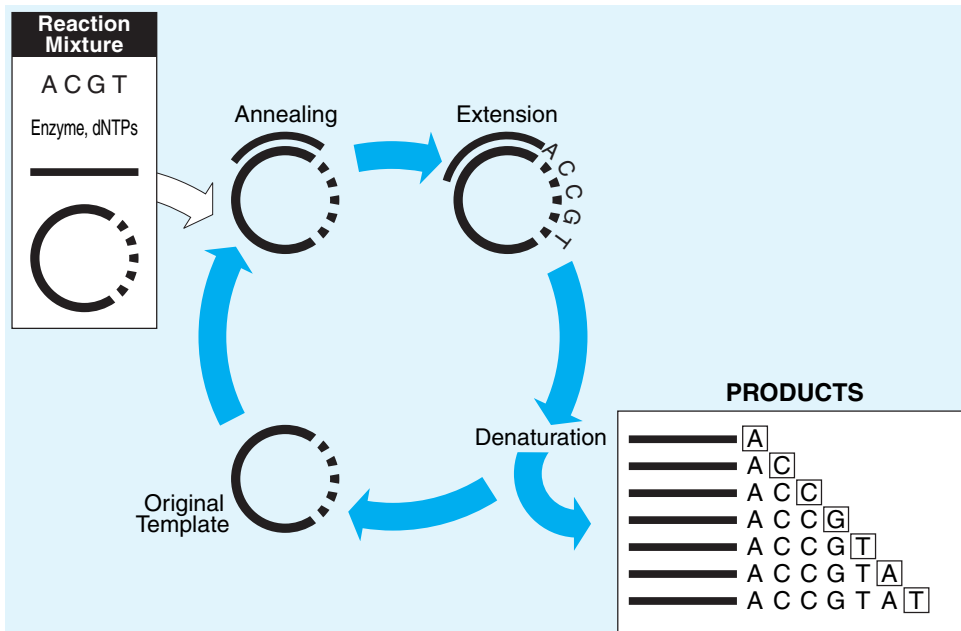
Figure 4   Example cycle sequencing reactions in a thermal cycler

Advantages   There are many advantages to performing cycle sequencing, including:

- Protocols are robust, easy to perform, and effective for sequencing PCR products.
- High temperatures reduce secondary structure, allowing for precise priming, template annealing, and thorough extension.
- The same protocol can be used for double- and single-stranded DNA.
- Difficult templates, such as bacterial artificial chromosomes (BACs), can be sequenced.

How Extension Products Are Labeled   Automated cycle sequencing procedures incorporate fluorescent dye labels using either dye-labeled dideoxynucleotide triphosphates (dye terminators) or dye-labeled primers (dye primers). Both chemistries use four different dyes. Because each dye emits a unique wavelength when excited by light, the fluorescent dye on the extension product identifies the 3′ terminal dideoxynucleotide as A, C, G, or T.

Dye Terminator Chemistry   With dye terminator chemistry, each of the four dideoxynucleotide terminators is tagged with a different fluorescent dye. One reaction is performed, containing the enzyme, nucleotides, and all dye-labeled dideoxynucleotides. The products from this reaction are injected into one capillary (Figure 5).
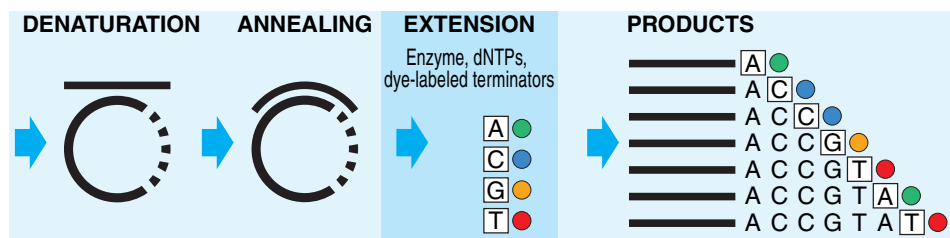
Figure 5   Diagram of dye terminator cycle sequencing

Advantages of dye terminator chemistry compared with dye primer chemistry:

- You can use unlabeled primers, which cost less than labeled primers.
- You can perform reactions in one tube.
- Reactions require fewer pipetting steps than dye primer reactions.
- False stops (fragments not terminated by a dideoxynucleotide) are not detected because no dye is attached.
- BigDye terminators v1.1 and v3.1 and dRhodamine terminators are formulated with dITP in place of dGTP to reduce peak compressions.
- dGTP BigDye terminators are formulated with dGTP for sequencing G-C-rich templates or sequence motifs consisting of Gs and Cs.

Dye Primer Chemistry

With dye primer chemistry, four separate tubes of sequencing primer are each tagged with a different fluorescent dye. Four separate reactions are performed, each containing the enzyme, nucleotides, a specific dye-labeled sequencing primer, and either A, C, G, or T dideoxynucleotides. The products from these four reactions are then combined and injected into one capillary (Figure 6).
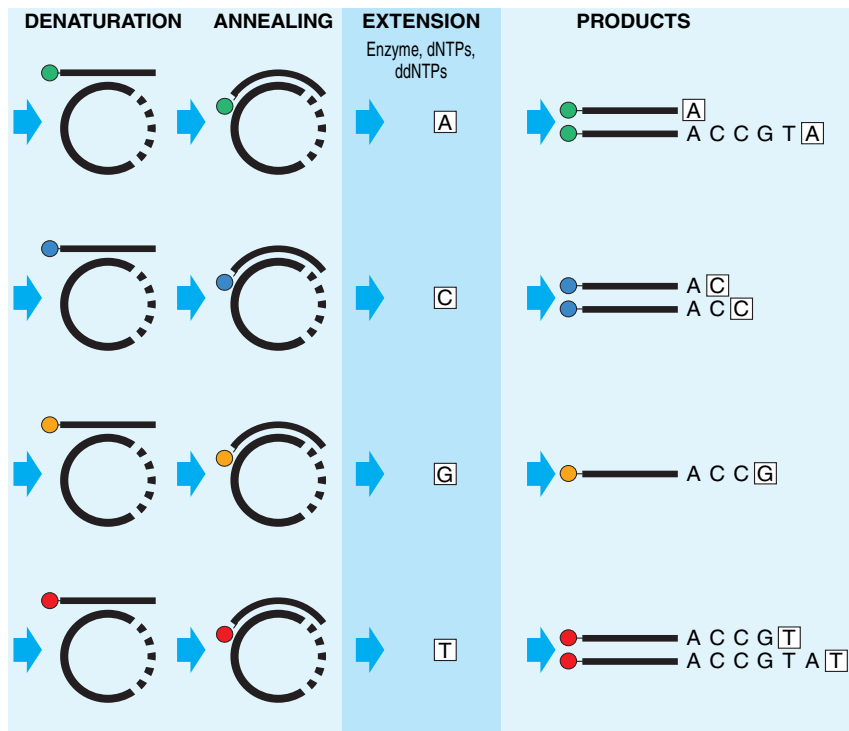
Figure 6   Diagram of dye primer cycle sequencing

Advantages of dye primer chemistry compared with dye terminator chemistry:

- Dye primer chemistries generally produce more even peak heights than dye terminator chemistries.
- Labeled primers are available for common priming sites. Custom primers can also be labeled.

Cycle Sequencing Kits

Applied Biosystems cycle sequencing kits available for both dye primer and dye terminator chemistries:

- BigDye® Terminator v1.1 and v3.1 Cycle Sequencing Kits
- dGTP BigDye® Terminator v1.0 and v3.0 Cycle Sequencing Kits
- dRhodamine Terminator Cycle Sequencing Kits
- BigDye® Primer Cycle Sequencing Kits

See Appendix B, Parts List for product information.

Modified DNA Polymerase

The cycle sequencing reaction is directed by highly modified, thermally stable DNA polymerases. These enzymes have been carefully selected to allow incorporation of dideoxynucleotides, to process through stretches of G-C-rich and other difficult sequences, and to produce uniform peak heights. The modified DNA polymerases are also formulated with a pyrophosphatase to prevent reversal of the polymerization reaction (pyrophosphorolysis).

Emission Spectra of Fluorescent Dyes

The fluorescent dyes used in BigDye® terminators, BigDye® primers, and dRhodamine terminators have narrower emission spectra and less spectral overlap than the rhodamine dyes used in previous sequencing kits. As a result, the dyes produce less noise. Figure 7 shows the normalized emission spectra and spectral overlap of the four dyes in the BigDye Terminator Cycle Sequencing Kit.
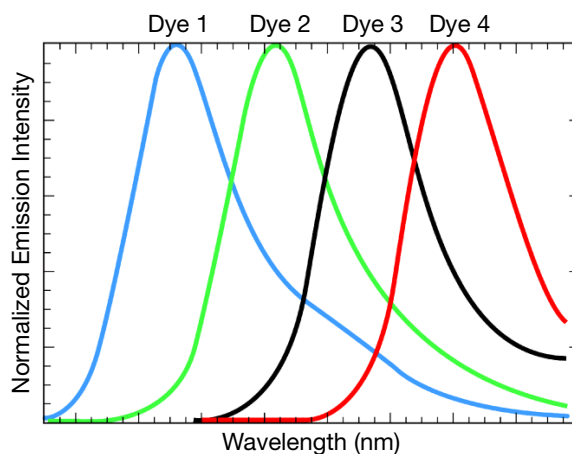
Figure 7   Emission spectra of the four BigDye dyes, where Dye 1 = Big-d110, Dye 2 = R6G, Dye 3 = Big-dTAMRA, and Dye 4 = Big-dROX

Capillary Electrophoresis

Historically, DNA sequencing products were separated using polyacrylamide gels that were manually poured between two glass plates. Capillary electrophoresis using a denaturing flowable polymer has largely replaced the use of gel separation techniques due to significant gains in workflow, throughput, and ease of use.

Fluorescently labeled DNA fragments are separated according to molecular weight. Because you do not need to pour gels with capillary electrophoresis, you can automate DNA sequence analysis more easily and process more samples at once.

Process Overview

During capillary electrophoresis, the extension products of the cycle sequencing reaction enter the capillary as a result of electrokinetic injection. A high voltage charge applied to the buffered sequencing reaction forces the negatively charged fragments into the capillaries. The extension products are separated by size based on their total charge.

The electrophoretic mobility of the sample can be affected by the run conditions: the buffer type, concentration, and pH; the run temperature; the amount of voltage applied; and the type of polymer used.

Shortly before reaching the positive electrode, the fluorescently labeled DNA fragments, separated by size, move across the path of a laser beam. The laser beam causes the dyes on the fragments to fluoresce. An optical detection device on Applied Biosystems genetic analyzers detects the fluorescence. The Data Collection Software converts the fluorescence signal to digital data, then records the data in a *.ab1 file. Because each dye emits light at a different wavelength when excited by the laser, all four colors, and therefore all four bases, can be detected and distinguished in one capillary injection (Figure 8).
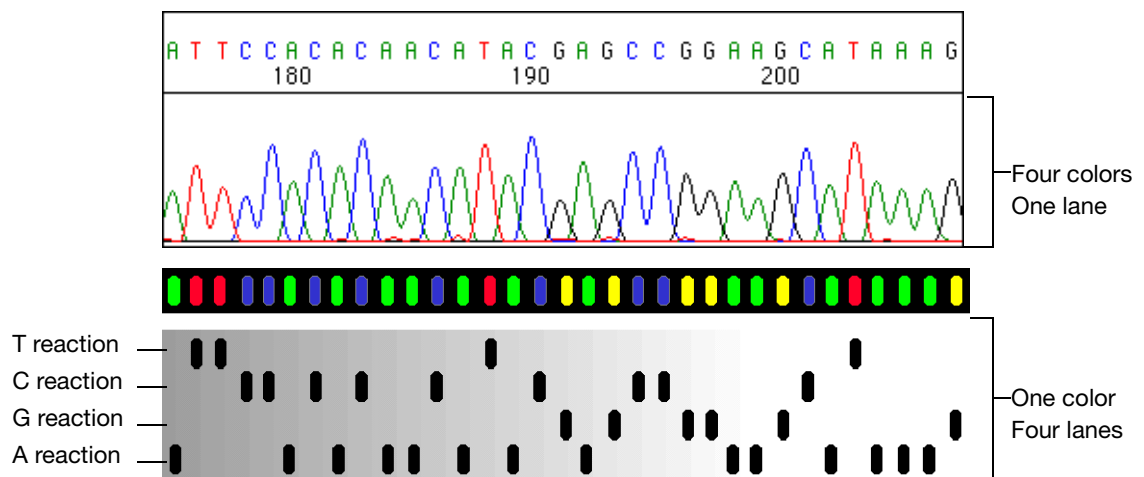
Figure 8   Fluorescent sequencing compared with radioactive sequencing

Available Instruments   Applied Biosystems offers the following automated genetic analyzers (Table 1).

Table 1   Applied Biosystems genetic analyzers

Instrument	Number of Capillaries	Capillary Array Length‡§ (cm)	Polymer Type	Sample Capacity#
Applied Biosystems 3730*xl* DNA Analyzer	96	36, 50	POP-7™	96- and 384-well plates
Applied Biosystems 3730 DNA Analyzer	48	36, 50	POP-7™	96- and 384-well plates
Applied Biosystems 3130*xl* Genetic Analyzer	16	36, 50, 80	POP-7™ POP-4™ POP-6™	96- and 384-well plates
Applied Biosystems 3130 Genetic Analyzer	4	36, 50, 80	POP-7™ POP-4™ POP-6™	96- and 384-well plates
Applied Biosystems 3100 Genetic Analyzer	16	36, 50, 80	POP-4™ POP-6™	96- and 384-well plates
Applied Biosystems 3100-*Avant* Genetic Analyzer	4	36, 50, 80	POP-4™ POP-6™	96- and 384-well plates
Applied Biosystems 310 Genetic Analyzer	1	47, 61	POP-4™ POP-6™	48 or 96 sample tubes

‡ 22-cm capillaries are not listed because they are not used for sequencing applications.

§ For multicapillary instruments (all but the 310 instrument), the capillary array length is the well-to-read length.

Sample capacity is the number of samples or plate types the autosampler can accommodate.

For more information about each instrument, refer to the appropriate instrument user guide.

Data Analysis

Process Overview

Data analysis software processes the raw data in the *.ab1 file using algorithms and applies the following analysis settings to the results:

- **Multicomponent analysis** – Each fluorescent dye emits its maximum fluorescence at a different wavelength, but there is some overlap in the emission spectra (Figure 7 on page 8). Thus a signal generated primarily in one color channel may yield a lower signal in an adjacent color channel. Multicomponent analysis separates the four different fluorescent dye signals into distinct spectral components by mathematically filtering fluorescence signal from dyes with emission spectra overlap

- **Basecalling** – The selected basecaller processes the fluorescence signals, then assigns a base to each peak (A, C, G, T, or N). If the KB™ basecaller is used, it also provides per-base quality value predictions, optional mixed base calling, and automatic identification of failed samples (page 145).

- **Mobility shift correction** – The mobility file (page 145) corrects electrophoretic mobility changes imposed by the presence of different fluorescent dye molecules associated with differently labeled reaction extension products (page 262). The mobility file also corrects for the differences between the dye-to-nucleotide relationships in the raw data and the analyzed data.

- **Quality value determination (QV)** – If the KB basecaller is used for analysis, the software assigns a QV for each base. The QV predicts the probability of a basecall error. For example, a QV of 20 predicts an error rate of 1%. The quality prediction algorithm is calibrated to return QVs that conform to the industry-standard relationship established by the Phred software. If your pipeline involves analysis with Phred software to assign QVs after the data is basecalled, you can simplify your workflow and use the KB basecaller instead. The KB basecaller can perform basecalling and assign QVs. Then, you can generate phd.1 or *.scf files using the KB basecaller to integrate with your downstream pipeline.

Analyzed sample data is displayed as an electropherogram, a sequence of peaks in four colors. Each color represents the base called for that peak (Figure 9).
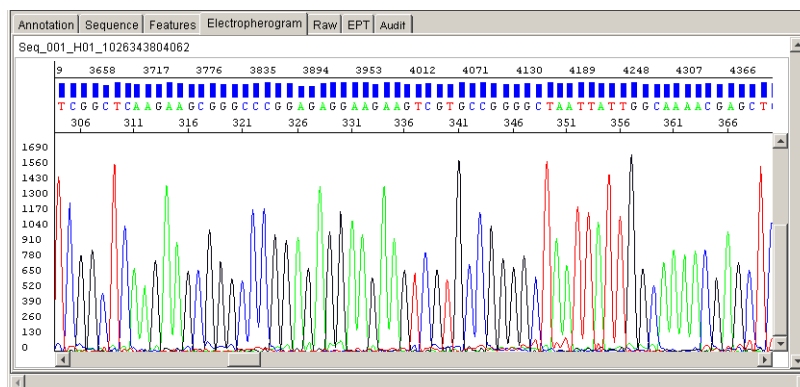
Figure 9   Example of electropherogram showing data analyzed with the KB™ basecaller

Software Products

Table 2 lists Applied Biosystems software products for analyzing sequencing data.

Table 2   Applied Biosystems analysis software

Product	Suggested Use
Sequence Scanner Software	Viewing or editing traces, evaluating trace quality, making trace QC reports
Sequencing Analysis Software	Viewing or editing traces, evaluating trace quality, making trace QC reports, Re-analyze traces
Variant Reporter™ Software	Mutation detection, SNP discovery and validation
SeqScape® Software	Mutation detection, SNP discovery and validation, sequence comparison, typing
MicroSeq® ID Analysis Software	With MicroSeq ID Kit, for bacterial and fungal identification

For more detailed information, see "Analysis Software" on page 159.

Data Analysis Options

Using Applied Biosystems Data Collection Software, you may analyze your sequencing files automatically, immediately after the electrophoresis run, or manually:

- **Autoanalysis** – With autoanalysis, the software applies analysis protocols to sequencing files immediately after Data Collection Software collects the data from the instrument. The analyzed data is saved in the sequencing file. You can review the analyzed data using Sequencing Analysis Software, SeqScape Software or MicroSeq ID Analysis Software.

- **Manual analysis** – With manual analysis, you obtain the sequencing files from the computer connected to the instrument, then move or copy the files to another computer that has any of the Applied Biosystems analysis software installed. To perform analysis, you manually apply the analysis protocols to the sequencing files, start analysis, and save the analyzed data.

Automated DNA Sequencing Workflow

DNA Template Preparation

(Chapter 3)

1. Prepare DNA template from plasmid, DNA fragment, BAC or YAC. Or prepare DNA template by PCR.
2. Design primers
3. Clean up templates.
4. Examine DNA quality.
5. Determine DNA quantity.

Examples of DNA Template Preparation Output:

- PCR products
- Plasmid DNA
- Genomic DNA
- Bacterial artificial chromosomes (BACs)
- Yeast artificial chromosomes (YACs)
- Cosmids

Cycle Sequencing

(Chapter 4)

1. Select the sequencing chemistry.
2. Prepare cycle sequencing reactions.
3. Run sequencing reactions in a thermal cycler.

Applied Biosystems Cycle Sequencing Kits:

- BigDye Terminator v1.1 and v3.1 kits
- dGTP BigDye Terminator v1.0 and v3.0 kits
- BigDye Primer kits
- dRhodamine Terminator kits

9700 thermal cycler

9800 thermal cycler

Veriti® thermal cycler

Cycle Sequencing Output:
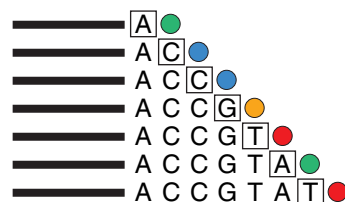
Dye terminator products or Dye primer products
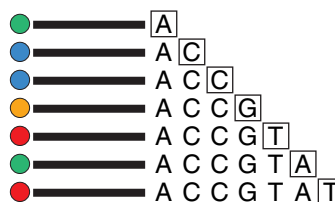
Extension Product Purification

(Chapter 5)

Purify extension products using one method:

- BigDye® XTerminator™ Purification
- Ethanol precipitation
- Spin-column purification
- Alternative cleanup procedures

After purification, prepare samples for electrophoresis

Extension Product Purification Output:

Purified dye terminator products or purified dye primer products

Capillary Electrophoresis

(Chapter 6)

1. Prepare the instrument.

2. Set up the plate record in Data Collection Software:
   – Results group
   – Instrument protocol
   – Analysis protocol

3. Load samples.

4. Perform the run.
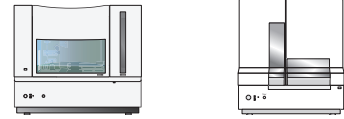
Capillary Electrophoresis Output:

 *.ab1 file

Applied Biosystems instruments:

3730/3730*xl* analyzer   3130/3130*xl* analyzer
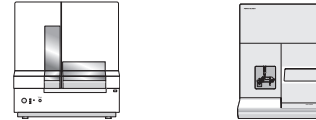
3100/3100-*Avant* analyzer   310 analyzer

Data Analysis

(Chapter 7)

1. Apply analysis protocols:
   – Basecaller
   – Mobility file

2. Run analysis.

3. Review the data.

Data Analysis Output:

Applied Biosystems software:

• Sequencing Analysis Software

• Variant Reporter™ Software

• SeqScape Software

• Sequence Scanner Software

• MicroSeq® ID Analysis Software
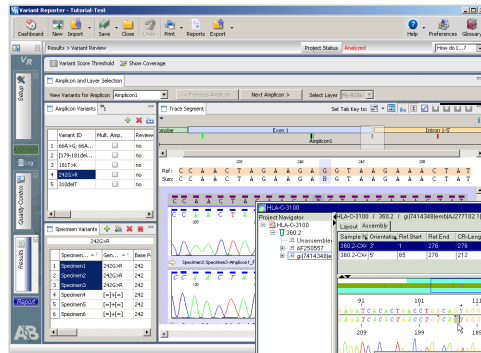
Analyzed project in Variant Reporter Software

Analyzed project in SeqScape Software

Analyzed sample file in Sequencing Analysis Software

Applications Overview

2
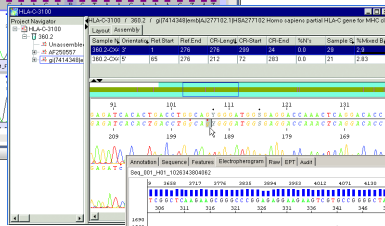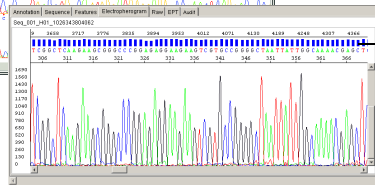
This chapter covers:

DNA Sequencing Applications and Approaches

DNA sequencing can be used for a variety of applications, including:

- *De novo* sequencing of genomes
- Detection of variants (SNPs) and mutations
- Biological identification
- Confirmation of clone constructs
- Detection of methylation events
- Gene expression studies
- Detection of copy number variation

Factors in Selecting an Approach

The approach used to sequence a target sample depends on several factors:

- **Length of the target** – Target length ranges from determining the sequence of a single base to a large genome.
- **Complexity of the sample** – Samples can range from a single homogenous sample source to a highly complex mixed sample that includes only a small amount of the target sequence mixed in a high background.
- **Number of samples** – The number of samples ranges from a single sample or thousands of samples.
- **Prior knowledge of the targeted sequencing region** – Knowledge of the region ranges from none (*de novo* sequencing technologies) to having a full reference (resequencing).

De Novo Sequencing of Genomes

De novo sequencing is used for the generation of the DNA sequence of a DNA molecule without any prior information about the sequence. For genome projects, an extremely high throughput level and high-end robotics are required in order to accommodate the sequencing workflow.

De novo whole or partial genome sequencing may be addressed through a variety of general approaches. Each of these broad strategies has been developed to include a number of specific techniques. The selection of an individual method relies on a number of different factors that include: genome size; whether the project involves an entire genome or targets some genome fraction (i.e. a specific chromosome); the desired coverage; and the resources available. The general approach selected will define most of a project's elements beginning with vector selection and ending with sequence data analysis and assembly.

This section includes a brief overview of the general approaches. Guidance for conducting individual protocols may be obtained from commonly available sources.

For *de novo* sequencing using capillary electrophoresis, the target DNA is fragmented and cloned into a viral or plasmid vector. Cloning provides amplification of the target DNA (by bacterial growth) and allows sequencing primers to bind to known sequence in the vector and extend the sequence into the unknown target DNA.

Genomic DNA fragments longer than 50 kb can be less efficient targets for PCR amplification than shorter genomic DNA fragments (*QIAGEN Genomic DNA Handbook,* 1999-2000). If you isolate long DNA fragments, you may need to shear the DNA by vortexing it for 3 to 5 minutes or by passing the preparation several times through an 18-gauge needle attached to a sterile syringe. See for more information.

Template Amplification Using Large Insert Vectors

Large-target DNA is cloned into Bacterial Artificial Chromosomes (BAC) with insert sizes ranging from 100 to 300kb. The BACs are then subcloned into smaller vectors that are more suitable for Sanger sequencing, with typical insert sizes of 1 to 10kb. Bacterial clones are isolated and grown in media, the plasmid or phage DNA is extracted, and the purified DNA template is used for forward and reverse sequencing reactions.

Isolate bacterial colonies

Prepare plasmid DNA

Run sequencing reactions

Perform cleanup

Perform capillary electrophoresis

Analyze data
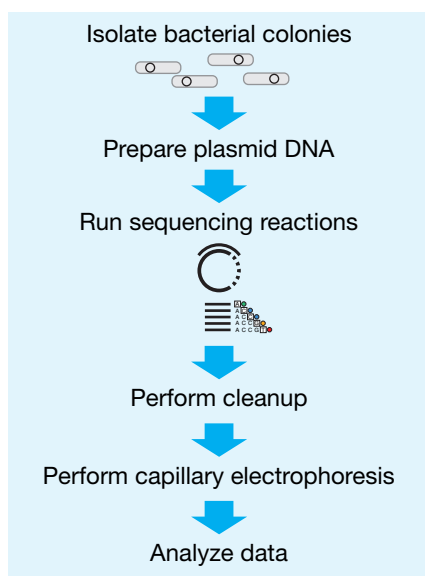
Figure 10   Proposed *de novo* sequencing workflow

Clones with small inserts can be completely sequenced using sequencing primers that hybridize to either end of the insert, then sequencing in the forward and reverse directions.

***De Novo* Sequencing Methods**

Various workflow strategies are used to perform *de novo* sequencing. They include but are not limited to:

- Shotgun sequencing
- Primer walking
- Using transposons to randomly prime sites for sequencing
- Nested deletions
- PCR amplification of template
- mRNA sequencing

• Expressed Sequence Tags (EST)

Shotgun Sequencing

For large target DNA, a time-efficient method of sequencing is to randomly shear the DNA into smaller pieces (0.5 to 1.5 kb) by enzymatic digestion or physical shearing. These shotgun fragments are subcloned into vectors, transformed into bacteria and isolated as colonies. The colonies are inoculated into media and grown overnight. The vector DNA is extracted and then sequenced from standard priming sites in the vector (Figure 11).

The shotgun method replaced directed sequencing, where a physical map of the clones and subclones was created before sequencing to serve as a guide to assemble the sequence traces. Shotgun sequencing does not require prior information about the sequence, and it can be used for DNA molecules as large as entire chromosomes.
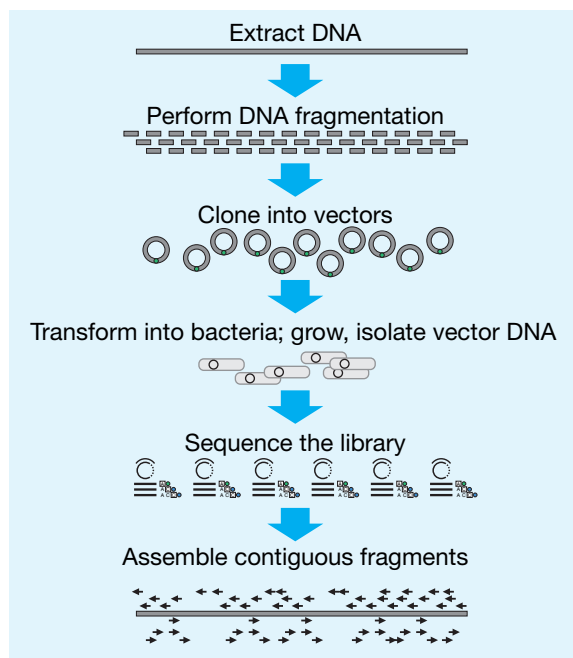
Figure 11   Shotgun sequencing

To ensure full coverage of double-stranded template DNA, it may be necessary to sequence a region 7 to10 times. A BAC with a 100 kb insert would require about 1500 subclones (500 to 1000 bp) for complete coverage. A cosmid (40 kb insert) may require about 250 to 500 subclones.

Primer Walking

An alternative to shotgun sequencing is primer walking. Following the initial sequencing determination, primed from a region of known sequence, subsequent primers are designed to hybridize to 3' regions, determined in previous steps (Figure 12). These primers then serve as sequencing start point to establish an additional >500 bp of sequence data. New primers are synthesized for the newly established sequence in the template DNA, and the process continues.
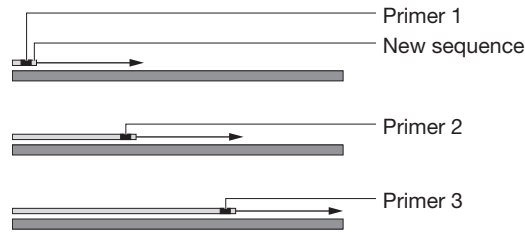
Figure 12   Primer walking

The primary advantage of primer walking is that extensive subcloning is not required. The amount of overlap or coverage required is also decreased because the direction and location of the new sequence is known, substantially decreasing the effort needed to assemble the final sequence. The primary disadvantage of primer walking is the amount of time required for each step in the primer walk and the need to design a robust primer for every step. Primer walking is often used to fill gaps in a sequence that has been determined by shotgun cloning.

Random Sequencing Priming Sites by Transposons

An alternative to subcloning is the random introduction of a jumping DNA element (transposon) into the target DNA. The target DNA is grown in a bacterial host with the appropriate element and a transposase gene. The vector DNA isolated from the transposase-positive strain and is used to transform the transposase-negative strain. The transposable element provides a known hybridization site for a sequencing primer, allowing sequencing and assembly of the target DNA from multiple internal locations (Figure 13).
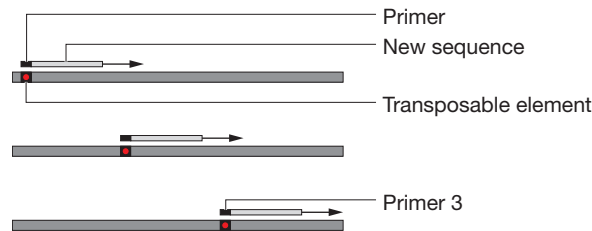
Figure 13   Transposon sequencing

Nested Deletions

Nested deletion strategies, with exonucleases or with restriction enzymes, help bring unknown DNA regions closer to the sequencing priming sites (Figure 14).
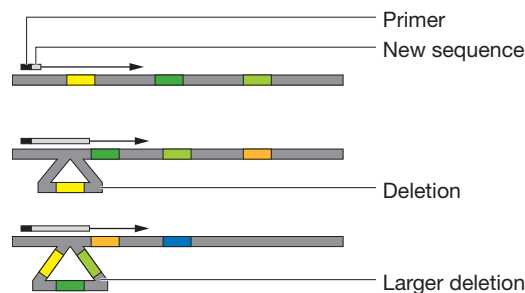
Figure 14   Nested-deletion sequencing

PCR Amplification of Template

All the above methods depend on template amplification by growth of bacteria. The advantage of cloning and bacterial growth is that it makes isolation and amplification of pure templates relatively easy. The primary disadvantage of bacterial growth is the time and effort required to produce a template. Typically, two days are required for transformation and isolation of colonies, bacterial growth and plasmid DNA preparation. It may be possible to shorten the overall time using single-molecule PCR. The fragmented genomic DNA is ligated to end-linkers and then diluted to a concentration of approximately one molecule per tube. The single molecule is then amplified by PCR and sequenced. Sequences that indicate mixed templates are discarded and "pure" sequences are assembled by standard procedures.

mRNA Sequencing

In addition to genomic DNA sequencing, sequencing of mRNA has been used to understand gene structure and to develop prediction rules for annotation of introns and exons annotations in genomic DNA sequence. Mature mRNA sequences are isolated from an organism, converted to cDNA sequences by reverse transcriptase, and cloned as libraries. The inserts in these libraries are then completely sequenced. Significant effort is expended to create clones that encompass the complete mRNA transcript and that also capture alternative forms of a transcript. To ensure that a complete mRNA is produced in the clone, cDNA is synthesized from the RNA using random primers in addition to the poly A primers. cDNA sequences are also extended to the 5′-end of a transcript by methods like rapid amplification of cDNA ends (5′-RACE) with mRNA specific primers. The overlapping sequences are then assembled to generate a super transcript that potentially includes all known transcripts of a specific gene.

Expressed Sequence Tags (EST)

The mRNA component of a biological sample may also be sequenced to aid in the identification of active genes in a tissue. The mRNA from a tissue is cloned into a vector. The vector is used to transform bacteria, followed by isolation of bacterial colonies, bacterial growth, DNA extraction, and sequencing. Typically, each clone is sequenced just once to produce a "single-pass" sequence tag of about 300 to 800 bases. These tags provide information about the gene content of a tissue under the conditions in which the tissue was harvested. The GenBank database, dbEST, contains sequence data and related information on sequences from a great many organisms.

Resequencing

Resequencing is defined as sequencing of DNA molecules followed by comparison to a known or reference sequence. Resequencing or directed sequencing is used for the discovery of sequence variants usually associated with a phenotypic change, for determining evolutionary changes, and/or for biological identification. Resequencing may be focused on coding regions of genes implicated in disease, or it may target the whole genome for the discovery of SNPs and other sequence variations between individuals. Comparative sequencing is usually defined as sequencing a specific region in different species or subspecies to identify highly conserved regions. Highly conserved regions are usually indicative of conserved function between the species, and they can be used to associate gene areas with conserved phenotype.

Resequencing is often carried out by amplifying a specific region of the genome by PCR and then sequencing the PCR fragment from both directions to generate a high-quality DNA sequence. Multiple DNA samples are processed simultaneously in micro-well plates, and the sequence traces are compared directly with each other to establish sequence variants.

A resequencing project may involve PCR amplifying and sequencing ten to hundreds of genes, with about 20 amplicons per gene for each individual genomic DNA sample.

High sensitivity, that is, a very high percentage of true positives and very low percentage of false negatives, is required to deliver complete mutation detection by revealing sequence variants in the sample, compared with a reference sequence. High sensitivity is also required to detect a small percentage of change in an overwhelmingly normal background (as in mixed samples such as cancer isolates or pooled DNA samples). For this reason, PCR fragments are sequenced bi-directionally to achieve greater than 99% accuracy.

Note: A mutation is a change in the sequence of the test sample when compared with the sequence of a reference. A polymorphism is a mutation that occurs in a substantial proportion of the population (typically greater than 1%).

Various strategies for resequencing include:

- Using the PCR primer as the sequencing primer
- Designing PCR primers with a sequencing tail
- Using nested (internal) sequencing primers
- Bisulfite sequencing for methlyation analysis

PCR Primer as the Sequencing Primer

Using the PCR primer as the sequencing primer (Figure 15) decreases the need for synthesizing specific sequencing primers, because an aliquot of the primers for PCR can be used for setting up the sequencing reaction. The disadvantage is that separate sequencing master mixes must be prepared for each sequencing direction and for each amplicon, increasing the number of pipetting steps and the possibility of error.
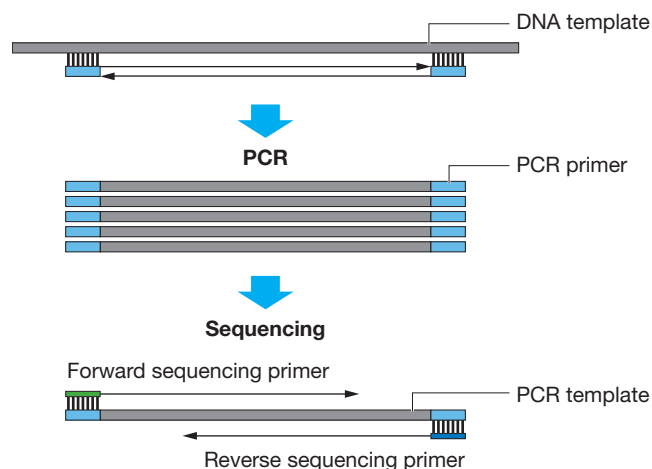
Figure 15   Sequencing with PCR primers

PCR Primers with a Sequencing Tail

For most large projects, it has become customary to include a standard primer tail on the PCR primers to simplify sequencing set-up (Figure 16). The most common tail is the sequence known as the M13 sequence because it was initially used for sequencing clones constructed in the single-stranded bacteriophage M13. Most sequence service providers provide the standard M13 primer at no additional cost.
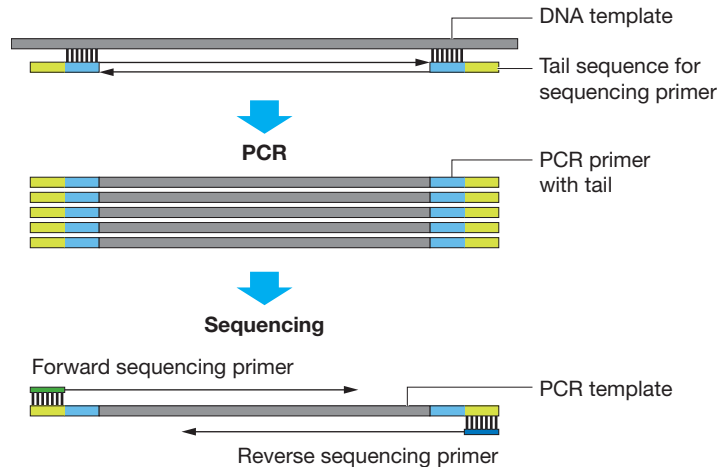
Figure 16   Sequencing with PCR primer and standard primer tail

Potential disadvantages of using tailed PCR primers are the greater challenge in designing primers with a tail and the need for higher quality oligonucleotides due to the increase in primer length.

Nested (Internal) Sequencing Primers

Designing PCR primers for amplification of closely related genes/pseudogenes can be challenging, because the PCR reaction may produce a mixture of PCR fragments. These fragments can be resolved by using an internal sequencing primer that is specific to only one of the PCR fragments.
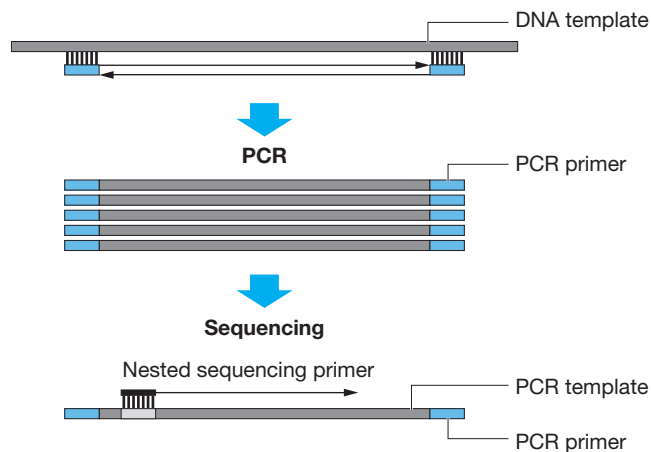
Figure 17   Sequencing with subtype-specific tail and PCR primer tail

Nested sequencing primers are used in the primer walking method discussed in the *de novo* sequencing section (page 18), and also for genotyping applications such as those served by the SNaPshot technology. The nested primer is designed to bind at the n-1 position of a suspected mutation. The primer is extended in the presence of the four fluorescent-labeled ddNTPs. Incorporation of a specific NTP (specific color) indicates the presence of that base on the template.

For more information, refer to the *ABI PRISM® SNaPshot®Multiplex Kit Protocol* (PN 4323357A).

Epigenetics

Methylation of DNA in vertebrate cells results in the regulation of gene expression and is responsible for normal (and abnormal) cellular differentiation pathways. This second code, the DNA methylation pattern, is an additional layer of information superimposed on the DNA code that determines many phenotypic attributes. Though the DNA code is largely unchanging, DNA methylation patterns do change in response to spatial, temporal, and environmental cues. To accurately describe the phenotype, the methylation pattern of DNA must be determined.

Selective gene inactivation has been shown to result from the DNA methylation of cytosine in the promoter regions.

A methylation-specific cystosine is often associated with a guanine residue as a CpG dinucleotide. Multiple CpG islands (that is, regions of < 500 bp with GC content of > 55%) have been identified around regulatory regions of genes.

Methylation of a CpG residue can be determined by treating genomic DNA with sodium bisulfite that converts non-methylated cytosine to uracil, while methylated cytosine is protected from bisulfite conversion (Figure 18). Comparing the sequence of bisulfite-converted DNA with untreated DNA clearly indicates the presence of methylated C residues, because they appear as C in bisulfite-converted DNA. Non-methylated C is converted to U (and to T in the sequencing reaction), so it appears as T.
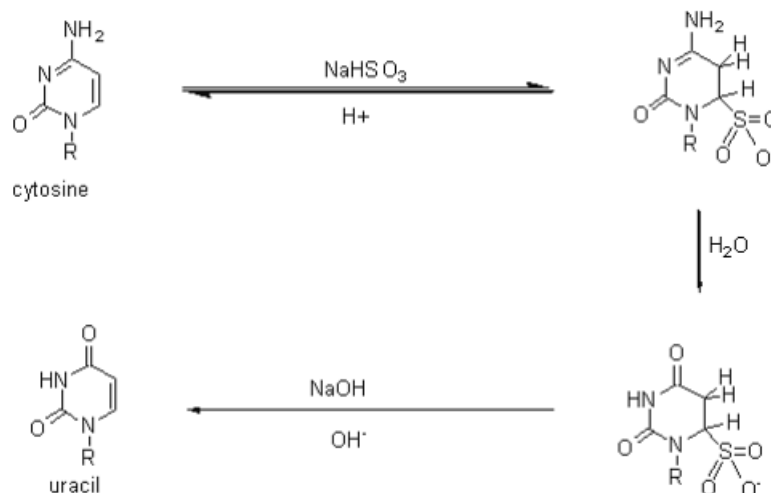
Figure 18   Conversion of methylcytosine to cytosine and non-methlylated cytosine to uracil

In principle, there are two approaches to methylation, depending upon the available information and the research goals: methylation-specific PCR or bisulfite-specific PCR. A researcher performs bisulfite treatment in order to transform an epigenetic event to a detectable, permanent genetic change *in vitro*, because the original methylation is lost during PCR.

Comparison of sodium-bisulfite treated DNA sequences with sequences obtained from untreated genomic DNA allows the precise identification of all methylated cytosines within a long stretch of DNA (Figures 19 and 20) (Boyd, 2004).
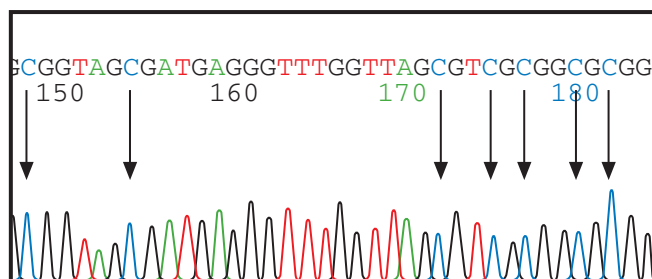
Figure 19   DNA sequence from untreated DNA. Arrows show location of unmethlyated cytosine located before guanine. After bisulfite treatment, unmethlyated cytosine is converted to T.

UTUUTAUTUATUAUUUTTTUUTTAUTUTTUTUUTUTUUU
        160   170   180   19
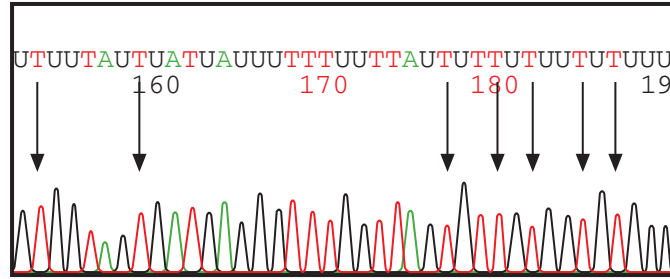
Figure 20   DNA sequence from bisulfite-treated DNA. Arrows show location of non-methylated cytosine converted to thymine after bisulfate sequencing.

Comparison of peaks from bisulfite sequencing and non-bisulfite sequencing is not quantitative. For more quantitative analysis of methlyation, perform fragment analysis.

Two options are available for collecting methylation sequencing data. Both options require bisulfite conversion and PCR amplification, but in one method, the PCR fragments are sequenced directly, while in the other method the fragments are cloned and then the clones are sequenced. The cloning method has been applied to genome wide sequence analysis of methylation (Meissner, 2005).

In the workflow shown in Figure 21, bisulfite sequencing without a cloning step (on the right) is compared with regular sequencing (on the left).
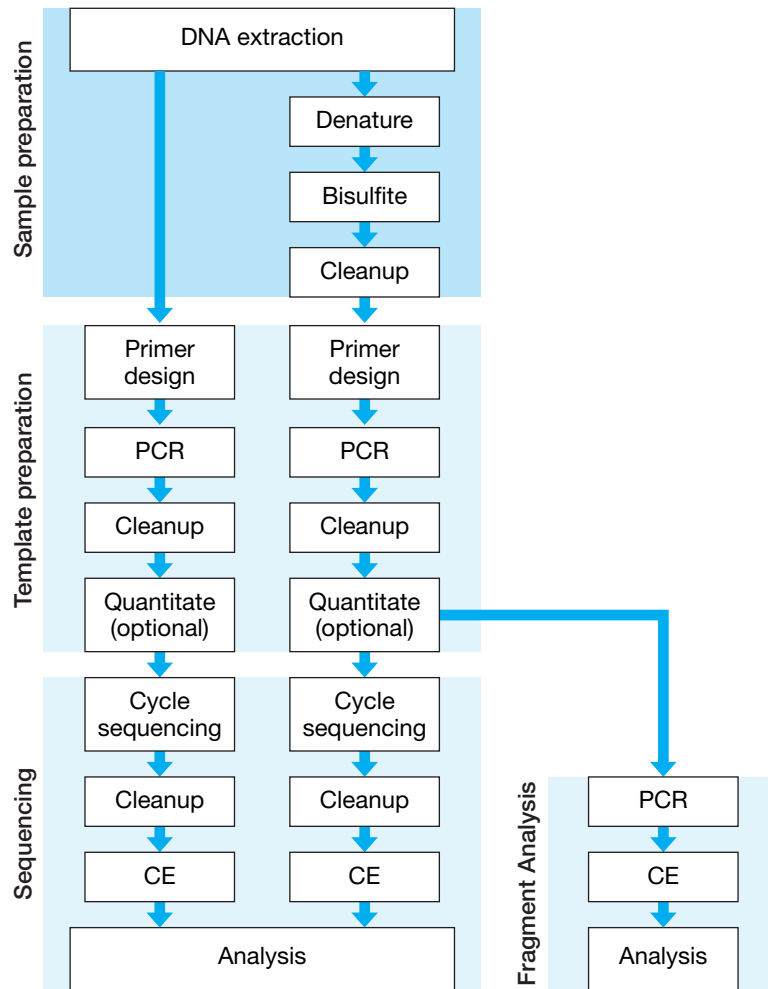
Figure 21   Steps for bisulfite sequencing compared with regular sequencing workflow

For More Information

The following Applied Biosystems documents provide more information about bisulfite sequencing:

- *Applied Biosystems methylSEQr™ Bisulfite Conversion Kit Protocol* (PN 4374710)
- *Advances in Capillary Electrophoresis-Based Methods for DNA Methylation Analysis Workflow Guide* (PN 106BR14-01)
- *Methylation Analysis Using FFPE Samples* (PN 137AP07-01) (Available from the Genetic Analysis section of the Applied Biosystems web site)

Microbial Analysis

Microbial analysis is used to identify and classify bacterial and fungal organisms at the species level. Molecular epidemiology, population structure studies and studies of pathogenic bacterial species use this information.

Strategies used to perform microbial analysis include:

- Multi Locus Sequence Typing (MLST) comparison of housekeeping genes to library standards
- MicroSeq® ID Analysis Software comparison of ribosomal sequences to library standards

Multi Locus Sequence Typing (MLST) Multi Locus Sequence Typing (MLST) is a nucleotide sequence-based approach for the unambiguous characterization and subspeciation of bacteria isolates and other organisms. The technique identifies alleles by direct DNA sequencing of fragments of housekeeping genes from known microorganisms. It is much more precise than indirect methods, which identify microorganisms on the basis of the electrophoretic mobility rates of large DNA fragments of gene products.

Isolate bacterial DNA samples

↓

Amplify housekeeping genes
with MLST primers using PCR

↓

Cycle sequence amplified genes
using BigDye Terminator v 1.1

↓

Obtain reference sequence from MLST web site
and import into SeqScape® software

↓

Collect sequencing data and
analyze with SeqScape software

↓

Obtain allelic profile from library match

↓

Identify sequence type using
allelic profile at MLST web site

Figure 22   Multi Locus Sequence Typing (MLST) workflow

The MLST technique characterizes isolates of bacterial species, using internal fragment sequences of approximately 450 to 500 bp from several housekeeping genes. Both strands of the fragments can be accurately sequenced by capillary electro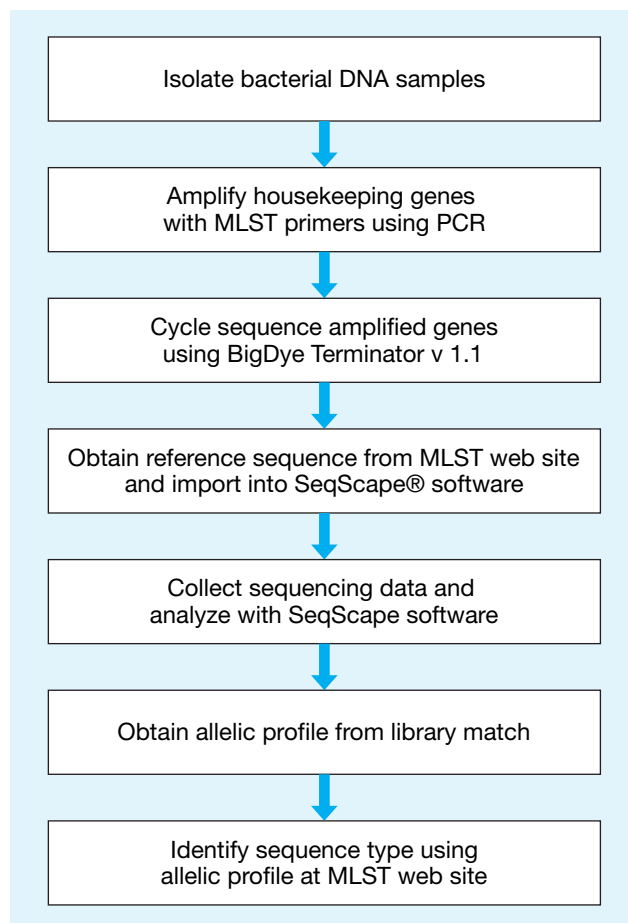phoresis. The various sequences present in a bacterial species for each housekeeping gene are specified as distinct alleles. For each isolate, the alleles at all loci define the allelic profile or sequence type (ST). This sequence type can be used to query the database in the MLST web site at **saureus.mlst.net/sql/allelicprofile_choice.asp**.

MicroSeq® ID Analysis Software

Microbial identification based on ribosomal RNA gene sequencing is used to identify microbial species including bacteria, yeasts, molds, and fungi. Bacteria are identified by sequencing the universal 16S ribosomal RNA (rRNA) gene, which forms the basis for bacterial taxonomic classification in Bergey's Manual. Fungi are identified by sequencing the D2 region of the 26S rRNA gene. These sequences are compared to validated sequences in the microbial libraries. MicroSeq ID Analysis Software automatically matches unknown samples to the selected percent match or the closest match in the library. The list of closest matches is ranked according to genetic distance from the sample, displayed with a phylogenetic tree.

Figure 23   MicroSeq® ID Analysis Software, showing the top match of an unknown sequence to the library

Libraries provided with the MicroSeq ID Analysis Software include entries for over 1,700 bacterial species, including gram-negative non-fermenters, Bacillus, Coryneforms, Mycobacteria, and Staphylococcus. The library for fungal species includes over 1,000 entries. Users can create libraries for species of interest and add sequences from new or proprietary strains.

Figure 24   MicroSeq® ID Analysis Software, showing the alignment of the forward and reverse sequences of a sample (with electropherograms) to the consensus sequence generated by the software (at the top). The consensus sequence is compared to the validated library.

Figure 25   For more information, see the *Applied Biosystems MicroSeq® ID Analysis Software Version 2.0 Getting Started Guide* (PN 4364623).

DNA Template Preparation

3

This chapter covers:

Overview

The DNA purification method used can affect the quality of the template. This chapter provides:

- Recommendations, considerations, and lists of commercial products for preparing the following types of DNA templates:
  - Vector-based templates (page 33)
  - Genomic DNA (page 34)
  - PCR DNA templates (page 37)
- Procedure for cleaning up templates (page 44)
- Guidelines for evaluating DNA quality (page 44)
- Guidelines for quantitating the DNA (page 45)
- Procedures for performing bisulfite sequencing (page 46)

Workflow
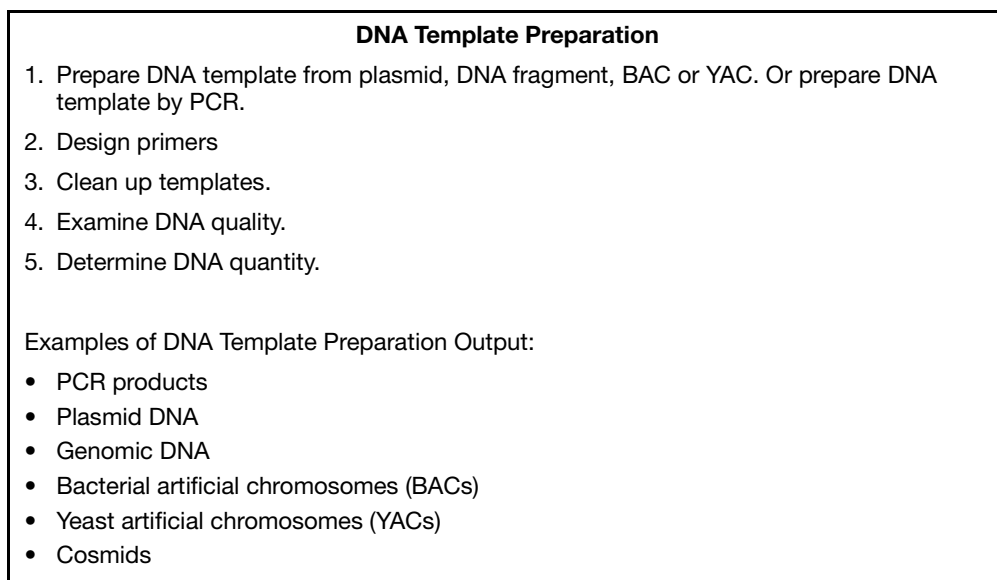
```
DNA Template Preparation

1. Prepare DNA template from plasmid, DNA fragment, BAC or YAC. Or prepare DNA
   template by PCR.

2. Design primers

3. Clean up templates.

4. Examine DNA quality.

5. Determine DNA quantity.


Examples of DNA Template Preparation Output:
• PCR products
• Plasmid DNA
• Genomic DNA
• Bacterial artificial chromosomes (BACs)
• Yeast artificial chromosomes (YACs)
• Cosmids
```

Cycle Sequencing (Chapter 4)

Extension Product Purification (Chapter 5)

Capillary Electrophoresis (Chapter 6)

Data Analysis (Chapter 7)

Preparing Vector-Based DNA Templates

Host Strain Variability

The host strain that you use to clone the template can impact template quality. If you plan to use a commercial template preparation kit, contact the vendor for information about host strains that work well with that kit.

For more information about host strain effects see the *QIAGEN Guide to Template Purification and DNA Sequencing (2nd edition)*. To obtain a copy of this guide, visit the QIAGEN web site (**www.qiagen.com**) or contact your local QIAGEN office.

Preparing Single-Stranded DNA

One method involves preparing single-stranded DNA templates from M13 phage by centrifugation and PEG precipitation of the phage particles. For more details, see Sambrook and Russell (2001).

For more information about preparing single-stranded DNA, see the *QIAprep M13 Handbook*.

Preparing Plasmid DNA

When purifying recombinant plasmids from bacteria, plate out the transformants to obtain isolated colonies. Select a single colony and streak it out on a plate. Select an isolated colony from the second plate to obtain plasmids with the desired insert.

The optimal method for preparing a particular plasmid depends on the particular bacterial strain and the yield of each construct. General methods:

- Alkaline lysis
- Cesium chloride (CsCl) purification (for low copy and high molecular weight plasmids)
- Simple boiling prep (not recommended for low copy number plasmids)

For more information about preparing plasmid DNA, see Sambrook and Russell (2001).

Preparing BAC DNA Templates

With larger DNA targets such as bacterial artificial chromosomes (BACs), the quality of DNA template is especially important to the success of the sequencing reaction. General methods that have given good sequencing results:

- Alkaline lysis, with extra phenol extraction and isopropanol precipitation if very clean DNA is desired (Marra *et al.*, 1996)
- Cesium chloride (CsCl) banding

For other BAC DNA preparation protocols, refer to the following:

- ABRF DNA Sequencing Research Group Electronic Posters. 1996-2008.
  www.abrf.org/index.cfm/group.show/DNASequencing.28.htm#R_2.
  Accessed 30 April 2008.
- Centre National de Séquençage (CNS, or Génoscope).
  www.cns.fr/externe/arabidopsis/protoBAC.html.
  Accessed 30 April 2008.
- University of Oklahoma Advanced Center for Genome Technology:
  www.genome.ou.edu/DblAcetateProcV3.html.
  Accessed 1 May 2008.
- Applied Biosystems Application Note: *A Workflow for Obtaining High Quality Sequencing Data from Bacterial Artificial Chromosome (BAC) DNA* (PN 107AP05-01)

Commercial Products

Table 3 lists commercial products that you can use to prepare vector-based DNA templates. This is not an exhaustive list. Applied Biosystems makes no specific recommendations on the use of these products. Follow the manufacturer's instructions to prepare the DNA templates for cycle sequencing.

Table 3   Commercial products for preparing vector-based DNA

Product	Source	Purpose
CosMCPrep® Kit	Agencourt Bioscience Corporation	To purify high-copy and low-copy plasmids, BACs, cosmids, and fosmids
NucleoBond Plasmid Purification Kits	Clontech Laboratories	To purify high-copy and low-copy plasmids, cosmids, BACs, PACs, and YACs
Aurum™ Plasmid Purification Kits and Quantum Prep Plasmid Purification Kit	Bio-Rad Laboratories	To purify plasmid DNA
ProPrep™ BAC 96	Biotech Support Group	To miniprep BAC DNA in 96-well format
SeqPrep™ Plasmid Prep Kit	Edge Biosystems	To purify plasmid DNA in 96-well or 384-well formats
illustra TempliPhiDNA Sequencing Template Preparation Kit	GE Healthcare	To amplify single- or double-stranded circular DNA templates from bacterial cells containing a plasmid, purified plasmids, or intact M13 phage
ChargeSwitch®-Pro Plasmid Miniprep Kit	Invitrogen	To purify small-scale plasmid DNA
Montage Plasmid Miniprep$_{HTS}$ 96 Kit	Millipore	To purify plasmid or BAC DNA in 96-well format
PSIΨClone BAC DNA Kits	Princeton Separations	To purify BAC DNA
Wizard® *Plus* Minipreps DNA Purification Systems	Promega	To purify plasmid DNA
Wizard SV 96 and SV 9600 Plasmid DNA Purification Systems	Promega	To purify plasmid DNA
QIAprep® Spin Miniprep Kits	QIAGEN	To purify plasmid DNA in 8-well or 96-well formats
GenElute™ Plasmid Miniprep Kit	Sigma-Aldrich	To purify small-scale plasmid DNA

Preparing Genomic DNA

Genomic DNA (gDNA) can be prepared from humans, animals, cell cultures, plants, or fosmids. There are several options for preparing genomic DNA. To obtain enough copies for fluorescent DNA sequencing, isolate the genomic DNA, then perform PCR amplification of the specific target sequence before proceeding with cycle sequencing.

Note: Applied Biosystems does not recommend direct sequencing of gDNA, with the exception of bacterial gDNA.

Source Material Considerations

Many sources of tissue can be used as starting points for the isolation of genomic DNA or RNA, including fresh venous blood, anticoagulated blood (either EDTA, citrate, or heparin), frozen blood, bone marrow, cultured cells, buccal scrapes, solid organ biopsies, and paraffin-embedded tissue.

Consider the following when planning to perform cycle sequencing using genomic DNA:

- **Tissue type and amount** – The type of source tissue and the amount available can influence the effectiveness or sensitivity of PCR amplification.
- **Heparin** – Heparin may weaken or completely inhibit amplification during PCR (Holodniy *et al.*, 1991). Only a few methods that reverse the effect of heparin have proven to be successful (Beutler *et al.*, 1990). The manufacturers of the Dynabeads DNA Direct Kit (Dynal, A.S.) and the QIAamp Blood Kit (QIAGEN, GmbH) report that the genomic DNA obtained from heparin blood using their products is ready for PCR amplification, because of the successful removal of heparin after the washing steps in these protocols.
- **Paraffin-embedded tissue** – Paraffin-embedded tissue provides numerous challenges to successful PCR and sequencing of the PCR products. Consider the following:
  - The fixative used
  - The length of time of fixing
  - The age of the block
  - The yield of DNA obtained
  - The amount of degradation of the DNA in the paraffin
  - The presence of any PCR inhibitors in the isolated DNA (Jackson *et al.*, 1991)

  These issues may significantly influence the size of the product that can be amplified and the reproducibility of amplification from one sample to another. Even if the fragment successfully amplifies, products contributed from the template preparation can result in decreased signal or increased background fluorescent noise from the sequencing reactions. Applied Biosystems recommends the RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE Tissues from Ambion (PN AM1975).
- **RNA as the starting material** – If you use RNA as your starting material via RT-PCR (for example, RNA viruses, RNA transcripts), apply special considerations. One allele might express differently from another or the gene may not be expressed at all in some tissues. Also, elimination of RNases is absolutely critical. For more information, see Sambrook and Russell (2001) or go to **www.ambion.com/techlib** and search for "RNA isolation".

Length of Genomic DNA Fragments

Genomic DNA fragments longer than 50 kbp can be less efficient targets for PCR amplification than shorter genomic DNA fragments (*QIAGEN Genomic DNA Handbook*). If you isolate long DNA fragments, you may need to shear the DNA by vortexing it for 3 to 5 minutes or by passing the preparation several times through an 18-gauge needle attached to a sterile syringe.

Methods Noncommercial methods typically involve lysing the cells with lysozyme, alkali, or detergents, then removing proteins and other contaminants by Proteinase K digestion or phenol/chloroform extraction. Using Proteinase K is advantageous because the enzyme destroys nucleases that can significantly reduce the molecular weight of the DNA. However, the Proteinase K activity must be eliminated before PCR (Sambrook and Russell, 2001).

Commercial Products Table 4 contains commercial products that you can use to prepare genomic DNA. This is not an exhaustive list, and you may have found other products to work. Applied Biosystems makes no specific recommendations on the use of these products. Follow the manufacturer's instructions to prepare the DNA templates for cycle sequencing.

Table 4 Commercial products for preparing genomic DNA

Product	Source	Purpose
MELT™ Total Nucleic Acid Isolation System	Ambion (PN AM1983)	To purify genomic DNA from tissue samples
RecoverALL™ Total Nucleic Acid Isolation for FFPE Tissues	Ambion (PN AM1975)	To purify genomic DNA from FFPE tissue
TRI Reagent®	Ambion (PN AM9738)	To isolate total RNA or RNA, DNA, and protein from a variety of fresh or frozen biological tissue or cells
NucPrep® DNA Isolation Chemistry Starter Kit	Applied Biosystems (PN 4340274)	To purify genomic DNA from cultured and fixed calls, gram-negative bacteria, plant and animal tissue, and whole blood and bone marrow cells
BloodPrep® DNA Starter Kit	Applied Biosystems (PN 4346860)	To purify genomic DNA directly from whole fresh and frozen blood or isolated blood cells of many animal species, and from tissue-cultured cells or buccal swab samples
Bacterial Genomic DNA Purification Kit	Edge Biosystems	To purify genomic DNA from bacterial cultures
PUREGENE® DNA Purification Kits	QIAGEN	To purify high molecular weight genomic DNA
QIAamp® DNA Blood Mini/Midi/Maxi Kits	QIAGEN	To purify genomic DNA from blood and blood-derived samples

Commercial Products for Preparing RNA or cDNA

Information on a multitude of DNA and RNA purifications products offered by Applied Biosystems is available at **www.appliedbiosystems.com**.

Browse from the home page by clicking **Products ▶ DNA/RNA Purification**.

For more information and to order, select the product line of interest.

Preparing PCR DNA Templates

This section provides information about preparing PCR products for sequencing, but it is not intended to be a detailed guide to PCR amplification. For general information on PCR amplification, refer to the product inserts included with Applied Biosystems PCR reagents.

PCR Strategies Because cycle sequencing involves many cycles of template denaturation and extension, adequate signal is produced in the sequencing reaction. In selecting the strategy for generating PCR DNA templates to be used for cycle sequencing, consider specificity and yield. Use Applied Biosystems PCR systems and reagents to perform PCR amplification.

Single Amplification

In the simplest PCR sequencing case, you use one set of primers to amplify the target DNA and to sequence the DNA. This method works well for many samples. If your samples do not work well with this method, you may need to minimize contaminants to increase the specificity of the PCR amplification and ensure adequate yield (see page 40).

A single PCR amplification is also compatible with the use of a sequencing primer which binds internally (semi-nested or nested) to one or both of the PCR primers. This nested primer approach can be helpful if primer-dimer (primer oligomerization) artifacts are a problem (see page 240).

Nested and Semi-Nested PCR

If you encounter difficulty with more complex samples, such as bacterial or eukaryotic genomic DNA, use a nested or semi-nested PCR. Nested and semi-nested PCR are useful when you have a small quantity of target because these methods increase specificity. Increased specificity provides superior sequencing data with reduced background signal. However, these methods may increase the likelihood of misincorporation because they require two amplifications:

1. Amplify with one set of PCR primers to convert a complex sample (such as bacterial or eukaryotic genomic DNA) into a non-complex sample consisting of the first PCR product and some side products.

2. Amplify 1% or less of the first PCR reaction product:
   - **Nested PCR** – Use a second set of PCR primers that hybridize at positions internal to the first set.
   - **Semi-nested PCR** – Use a second set of PCR primers with one primer that hybridizes internal to the first set and the other primer that is one of the original PCR primers.

Universal-Tailed PCR Primers

You can synthesize a universal-tailed PCR primer, which has a universal sequencing primer binding site added to the 5´ end (see page 263 in Appendix A for universal primer sequences). Uses for universal-tailed PCR primers:

- You can use this technique with dye terminator chemistries because universal sequencing primers have good annealing characteristics. However, longer PCR primers increase the reaction cost.

- You can use commercially available dye-labeled sequencing primers.
- You can use universal sequencing primers to sequence the resulting PCR product to simplify and standardize the sequencing step.

Fast PCR Versus Standard PCR

Fast PCR is a new technology that decreases run times by using combination of protocol changes, fast instruments, and fast reagents. Fast PCR technologies allow you to:

- Complete PCR runs in less time
- Use less sample and reagents
- Spend more time interpreting results and decision-making

For more information, see the Applied Biosystems application note *Advances in Fast PCR Contribute to a Fast Resequencing Workflow* (PN 104AP04-01).

Primer Design and Quantitation

When you perform dye terminator cycle sequencing reactions on PCR template, the primer sequence, primer synthesis method, and primer purification method can greatly affect the quality of the sequencing data.

The recommendations in this section are based on general knowledge or on the practical experience gained by Applied Biosystems scientists.

Optimizing Primer Design

Recommendations for optimizing primer design:

- Use Applied Biosystems Primer Express® Software (PN 4363991) for primer design:
  - To calculate melting temperature (T_m) accurately
  - To identify any secondary hybridization sites on the target DNA
  - To identify potential secondary structure problems
- Primers should be at least 18 bases long to ensure good hybridization and to minimize the probability of hybridizing to a second site on the target DNA.
- Use the recommended thermal cycling conditions for cycle sequencing, because primers with T_m>45 °C produce better results than primers with lower T_m.
- Avoid runs of an identical nucleotide, especially runs of four or more Gs.
- Avoid designing primers over a SNP. Consult SNP databases (dbSNP, SNP500, and/or SNPbrowser™) for SNP locations.
- Keep the G-C content in the range 30 to 80%, preferably 50 to 55%. For primers with G-C content less than 50%, you may need to increase the primer length beyond 18 bases to maintain a T_m>45 °C.
- Avoid primers that can hybridize to form dimers.
- Avoid palindromes because they can form secondary structures.
- The primer should be as pure as possible, preferably purified by HPLC.
- To design primers for sequencing bisulfite-converted DNA, use Applied Biosystems Methyl Primer Express® Software (PN 4376041). The software is available for free from the Applied Biosystems web site.

Formulas Calculate the melting temperature, primer concentration, and molecular weight for your primers.

Feature	Formula
Melting temperature (T_m)	T_m = (number of A + T residues) × 2 °C + (number of G + C residues) × 4 °C
Primer concentration (derived from Beer's Law)	C (pmol/µL or µM) = (A_{260} × 100)/(1.54n_A + 0.75n_C + 1.17n_G + 0.92n_T) where: C = concentration n_X = number of residues of base x in the oligonucleotide
Oligonucleotide molecular weight	Molecular weight of a DNA oligonucleotide (sodium salt, pH 7): MW = (N_A x 335.2) + (N_C x 311.2) + (N_G x 351.2)+ (N_T x 326.2) + P where: N_X = number of residues of base x in the oligonucleotide P = −101.0 for dephosphorylated oligonucleotides, 40.0 for phosphorylated oligonucleotides

VariantSEQr® Primer Sets

To simplify the identification of sequence variants, Applied Biosystems has pre-designed 420,000 PCR primer pairs for the resequencing of ~16,000 human genes. The VariantSEQr® primers, which include an M13 sequence tail, are designed for uniform PCR and sequencing conditions. These primer sequences are available for free download from the NCBI Probe database **www.ncbi.nih.gov/genome/probe**.

To download VariantSEQr® primer sequences:

1. Go to NCBI's online probe database: **www.ncbi.nlm.nih.gov/genome/probe**.

2. Enter "variantseqr and [*the symbol for your gene of interest*]" in the **for** field, then click **Go**.

3. Check the box next to the probe of interest then click **Download** to access all PCR primers for the gene.

4. Select specific primer sets:
   - In the graphical representation, click the horizontal lines corresponding to the primer set of interest.
   - In the list of probes, check the checkbox for the primer set of interest.

   Alternative transcripts are shown in blue and a union of all transcripts is shown in black. The RSS ID (Resequencing Set ID) references a set of RSAs (Resequencing Amplicons) that provide the most efficient coverage. To select primers for a specific transcript, click the button next to that transcript.

5. Click **Download** to export the primer sequences. Optionally, check **Hide M13 Primers** to omit the M13 tail sequences from the exported sequences.

   The sequences are saved in a tab-delimited text file (*.txt) on your computer.

Custom Primers

You can obtain custom primers from the Applied Biosystems Custom Oligonucleotide Synthesis Service.

To order custom primers:

1. Go to Applied Biosystems web site: **www.appliedbiosystems.com,** register and log in.

2. Click **Products and Services**, then **Primers and Probes**.

3. Click **Custom Unlabeled Primers ▸ Unlabeled Oligo**.

4. Choose a synthesis scale and click the appropriate **Configure** button.

5. Select the Formulation option: liquid or dry.

6. Follow the instructions for entering your primer names and sequences or uploading them from a file.

7. Order primers.

For more information, see *How to Order Primers and Probes* (PN 113920, Publication 132GU01-01).

PCR Contaminants That Affect Cycle Sequencing

Products carried over from the PCR amplification can affect cycle sequencing:

- **Excess PCR primers** – Compete with the sequencing primer for binding sites and reagents in the sequencing reaction. Additional primers in sequencing reactions using dye terminators result in the creation of multiple dye-labeled sequence ladders and noisy data.

- **Excess dNTPs** – Can affect the dNTP/ddNTP balance of the sequencing reaction, resulting in a decreased amount of short extension products.

- **Nonspecific PCR products** – Include primer-dimer artifacts and secondary PCR products. Nonspecific PCR products behave as templates in the sequencing reaction and cause the generation of multiple dye-labeled sequence ladders, which result in noisy data. Any significant quantity of nonspecific PCR products can result in poor quality sequencing data.

  Screen for nonspecific PCR products by running the PCR products on an agarose gel before sequencing. If you detect nonspecific PCR products, optimize and repeat the PCR amplification before sequencing. If you use a nested or semi-nested sequencing primer, you may obtain good sequence data. Alternatively, you can purify the desired PCR product directly from the agarose gel as long as the nonspecific PCR product is not the same size as the desired PCR product. However, significant contamination may remain because of incomplete separation.

Minimizing Contaminants

To minimize the contaminants listed above, use the following strategies to increase the specificity of the PCR amplification:

- PCR optimization strategies (Bartlett and Stirling, 2003; Innis and Gelfand, 1990):
  - Amount of starting DNA
  - Careful primer design
  - Primer concentration
  - Enzyme concentration
  - Magnesium ion (Mg^{2+}) concentration
  - Nucleotide concentration
  - Buffer composition

- – Number of cycles
- – pH
- • Manual hot-start method (if the enzyme does not have hot-start capability)
- • AmpliTaq Gold® DNA Polymerase as an automatic hot start
- • Use the following master mixes:
  - – AmpliTaq Gold® 360 PCR Master Mix
  - – AmpliTaq Gold® Fast PCR Master Mix , UP (for use with the Veriti® 96-Well Fast Thermal Cycler)

Purifying PCR Products for Sequencing

There are several methods for purifying PCR products. Select a method based on the amounts of components carried over from the PCR reaction and on the sequencing chemistry you plan to use:

- • Ultrafiltration
- • Ethanol precipitation
- • Gel purification
- • Enzymatic purification

IMPORTANT! If more than one PCR product is present, column purification, ethanol precipitation, or enzymatic purification will not isolate the desired product. Use gel purification to isolate the desired product or reoptimize the PCR to obtain a single product. Ultrafiltration may work if the contaminating PCR products are much smaller than the desired PCR product.

Commercial Products Table 5 contains many commercial products that you can use to prepare PCR DNA templates. This is not an exhaustive list. Applied Biosystems makes no specific recommendations on the use of these products. Follow the manufacturer's instructions to prepare the DNA templates for cycle sequencing.

Table 5   Commercial products for preparing PCR DNA templates

Product	Source	Description
AMPure® Kits PCR Purification System	Agencourt Bioscience Corporation	To remove unincorporated dNTPs, primers, and salts by magnetic separation of nucleic acids.
QuickStep™ 2 PCR Purification Kits	Edge Biosystems	To purify double-stranded DNA using a resin/gel filtration system in single, 96-well, or 384-well formats.
Montage® PCR Filter Units	Millipore	To remove primers and unincorporated dNTPs from PCR reactions. Recommended for PCR fragments > 300 bp.
PSIΨClone PCR ST Single Tube Kit	Princeton Separations	To remove primers, nucleotides, enzymes, and salts from PCR amplification reactions in single tube format.
GENECLEAN® Spin Kit	MP Biomedicals	To purify DNA fragments from agarose gels; remove proteins, primers, unincorporated nucleotides, and salts from enzymatic reactions; and isolate PCR product from genomic DNA and primers.

Table 5   Commercial products for preparing PCR DNA templates *(continued)*

Product	Source	Description
QIAquick® Gel Extraction Kits	QIAGEN	To purify PCR fragments from agarose gels. For DNA ranging from 70 bp to 10 kbp. Larger fragments should be extracted with the QIAEX II Gel Extraction Kits.
QIAquick® PCR Purification Kits	QIAGEN	To clean up DNA (up to 10 kbp). Removes primers, nucleotides, enzymes, salts, agarose, and ethidium bromide from DNA samples in individual spin-column, 8-well, or 96-well formats.
GenElute™ Minus EtBr Spin Columns	Sigma-Aldrich	To isolate DNA from agarose gels and remove ethidium bromide.
GenElute™ PCR Clean-Up Kit	Sigma-Aldrich	To purify single-stranded or double-stranded PCR amplification products from the reaction components (excess primers, nucleotides, DNA polymerase, and salts).
ExoSAP-IT®	USB	PCR cleanup to remove excess primers and unincorporated nucleotides.

Cleaning Up PCR Reactions With SAP/Exo I

An alternative to one of the more stringent purification methods listed above is treatment of PCR products with shrimp alkaline phosphatase (SAP) and Exonuclease I (Exo I) before sequencing. The SAP/Exo I method degrades nucleotides and single-stranded DNA (primers) remaining after PCR (Werle *et al.*, 1994).

IMPORTANT! This method does not remove primer-dimers or non-specific PCR amplification products.

To clean up PCR reactions with SAP/Exo I:

1.	For each sample, combine the following: • SAP (1 Unit/µL), 2.0 µL • Exo I (10 Units/µL), 0.2 µL • Deionized water, 6.0 µL **Note:** This method works well using 0.5 units of each enzyme per microliter of PCR products used. SAP buffer is excluded from this protocol because the method seems to work equally well with or without it.
2.	Add the SAP/Exo I mixture (8.2 µL) to the entire PCR product.
3.	Incubate at 37 °C for 1 hour.
4.	Incubate at 80 °C for 15 minutes to inactivate the enzymes.

You may need to dilute the PCR product before sequencing. Determine the dilution ratio empirically (start with 1:2 and 1:10 dilutions with deionized water).

Cleaning Up PCR Reactions with ExoSAP-IT

The ExoSAP-IT method removes excess PCR primers and unincorporated dNTPs. These carryover contaminants can present problems in dye terminator chemistry and cause noisy sequencing data.

To clean up the PCR reactions with ExoSAP-IT:

1.	Before opening the reaction plate, centrifuge it at $100 \times g$ for 1 minute.
2.	Add 2.0 µL of ExoSAP-IT to each amplified reaction.
3.	Seal the plate with MicroAmp® Clear Adhesive Film (PN 4306311).
4.	Vortex the plate briefly to mix the contents.
5.	Centrifuge the plate at $1600 \times g$ for 30 sec.
6.	Program the thermal cycler:

Stage	Description	Temp. °C	Time
1	Enzyme incubation	37	30 min
2	Enzyme inactivation	80	15 min
3	Hold	4	Indefinite hold

7.	Set the reaction volume to **12 µL**.
8.	Place the reaction plate in the thermal cycler, cover the plate with a MicroAmp® Optical Film Compression Pad (PN 4312639), then start the run.
9.	After the run is complete, centrifuge the plate at $100 \times g$ for 1 minute to bring the contents to the bottom of the wells.
10.	Store the plate according to when you are continuing the protocol: • Within 12 hours – store at 0 to 4 °C • After 12 hours – store at −15 to −25 °C for 2 to 3 days **Note:** Storage times are suggestions only and have not been rigorously tested.
11.	After storage and before opening the plate, centrifuge the plate at $100 \times g$ or higher for 1 minute.

DNA Template Quality

Poor template quality is the most common cause of sequencing problems. Follow recommended procedures to prepare templates.

Results characteristic of using poor quality templates:

- Noisy data or peaks under peaks (see page 237)
- No or low signal (see page 207 and page 211)
- Early loss or termination of extension (see page 219)

Contaminants That Affect Sequencing

Contaminants in cycle sequencing reactions negatively affect polymerase binding and amplification or extension. Resulting sequences produce poor quality data with low signal or high noise. Potential contaminants:

- Proteins
- RNA
- Chromosomal DNA
- Excess PCR primers, dNTPs, enzyme, and buffer components (from a PCR amplification used to generate the sequencing template)
- Residual salts
- Residual organic chemicals, such as phenol, chloroform, and ethanol
- Residual detergents
- Agarose gel, if DNA was extracted from a gel

Examining DNA Quality

Use both of the following methods to examine DNA quality:

- **Agarose gel electrophoresis** – Purified DNA should run as a single band on an agarose gel. Agarose gels reveal contaminating DNAs and RNAs, but not proteins.

  Note: Uncut plasmid DNA can run as three bands: supercoiled, nicked, and linear. RNA contamination up to 1 µg can be tolerated, but it affects DNA quantitation greatly.

- **Spectrophotometry** – The A_{260}/A_{280} ratio should be 1.8 to 2.0. Smaller ratios usually indicate contamination by protein or organic chemicals. Spectrophotometry can reveal protein contamination, but not DNA or RNA contamination.

Note: Neither agarose gel electrophoresis nor spectrophotometry can reveal contaminating salts. Salts can interfere with the sequencing reaction, capillary electrokinetic injection, or electrophoresis, resulting in noisy data.

Cleaning Up Dirty Templates

You can sometimes clean up a contaminated template with one of the following methods:

- **Ultrafiltration** (Microcon or Centricon filter units) – Most efficient method for salt removal. See Millipore's web site (**www.millipore.com**) for instructions on how to use the Microcon or Centricon filter units.
- **Spin columns** – May be used for salt removal. See Table 3 on page 34 for commercial products.

- **Phenol/chloroform extraction** – See Sambrook and Russell (2001) for detailed instructions.
- **Ethanol precipitation** – May be used for salt removal.

DNA Template Quantity

DNA template quantitation is critical for successful sequencing reactions. The most common way to determine DNA quantity is to measure the absorbance (optical density or O.D.) of a sample at 260 nm in a spectrophotometer.

Measuring UV Absorbance

One O.D. unit is the amount of a substance, dissolved in 1.0 mL, that gives an absorbance reading of 1.00 in a spectrophotometer with a 1-cm path length. For DNA quantitation, the wavelength is assumed to be 260 nm unless stated otherwise. A_{260} values can be converted into µg/µL using Beer´s Law:

Absorbance (260 nm) = sum of extinction coefficient contributions X cuvette pathlength X concentration

The following formulas are derived from Beer's Law (Ausubel *et al.*, 1998):

- Concentration of single-stranded DNA = A_{260} X 33 µg/µL.
- Concentration of double-stranded DNA = A_{260} X 50 µg/µL.

Note: Absorbance measurements of highly concentrated (O.D.>1.0) or very dilute (O.D.<0.05) DNA samples can be inaccurate. Dilute or concentrate the DNA as needed to obtain a reading within the acceptable range.

Other Methods

Applied Biosystems makes no specific recommendations on the use of these products for DNA quantitation:

- Fluorometric analysis using either Hoechst dye #33258 (Hoefer, Inc., 1993) or PicoGreen® dsDNA quantitation reagent (Molecular Probes)
- Fluorometric analysis using Quant-iT™ assays and the Qubit® fluorometer (Invitrogen)
- Measure UV-Vis absorbance using the NanoDrop ND-1000 spectrophotometer, which does not require dilution for many sample types (Nanodrop If you have a Real-Time PCR instrument, you can use Applied Biosystems TaqMan® RNase P Detection Reagents (PN 4316831) to measure DNA quantity.

Preparing Templates for Bisulfite Sequencing

DNA Extraction   The purity of the gDNA template is critical for the success of a complete bisulfite conversion. Proteins bound to the gDNA can interfere with the bisulfite conversion process, resulting in large sections of non-converted sequence. A proteinase K incubation step is recommended (Warnecke, 2002).

DNA for bisulfite sequencing can be isolated from various sample types including blood, cultured cells, and tissue [fresh/frozen and formalin-fixed-paraffin embedded (FFPE)]. Note that FFPE samples can be difficult to analyze due to variation in DNA quantity, quality and purity.

Depending on source of DNA, you can use any of the following products to prepare high-quality template for bisulfite sequencing:

- **MELT™ Total Nucleic Acid Isolation System** (PN AM1983) – For isolating nucleic acids from tissue without homogenizing tissue samples. After lysis in MELT solution, DNA is isolated using magnetic bead-based separation technology.
- **RecoverALL™ Total Nucleic Acid Isolation for FFPE Tissues** (PN AM1975) – Optimized to release a maximal amount of RNA and DNA fragments of all sizes with typical yields of >50% that of unfixed tissue from the same sample source.
- **NucPrep® DNA Isolation Kit** (PN 4340274) – For recovery of DNA from plant and animal tissue.
- **BloodPrep® DNA Isolation Kit** (PN 4340274) – For rapid isolation of DNA from fresh or frozen blood from any animal species, tissue-cultured cells, or buccal swabs.

Note: Include controls throughout the workflow to monitor incomplete bisulfite conversion.

Performing Bisulfite Conversion

The bisulfite conversion protocol for Applied Biosystems methylSEQr™ Bisulfite Conversion Kit (PN 4379580 consists of three procedures:

1. "Preparing gDNA" on page 46
2. "Performing Bisulfite Conversion" on page 47
3. "Purifying the Sample" on page 48

Preparing gDNA   1. Prepare your gDNA using standard procedures.

2. Determine the gDNA concentration spectrophotometrically at A260 or by fluorescence.

Performing Bisulfite Conversion

⚠️ **DANGER** **CHEMICAL HAZARD. MethylSeqr™ Conversion Reagent** causes eye, skin, and respiratory tract irritation. Exposure may cause allergic reactions.

⚠️ **DANGER** **MethylSeqr™ Denaturation Buffer** causes severe eye, skin, and respiratory tract burns. Read the MSDS and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.
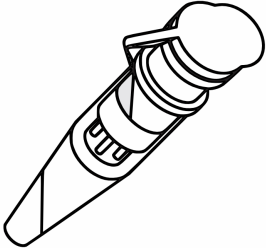
To convert gDNA using bisulfite:

1.	Combine in a labeled microcentrifuge tube: • 5 µL methylSEQr™ Denaturation Buffer • Up to 300 ng of gDNA in 45 µL deionized water The final volume is 50 µL. Seal the tube and thoroughly mix the contents. **IMPORTANT!** Do not exceed 300 ng of gDNA per conversion reaction. Excess gDNA may produce an incomplete bisulfite conversion.
2.	Incubate the tube at 37 °C for at least 15 minutes. Continue holding at 37 °C while preparing the conversion reagent in step 3.
3.	Prepare the methylSEQr™ Conversion Reagent. To one tube of methylSEQr Conversion Reagent, add: • 750 µL deionized water • 210 µL methylSEQr™ Denaturation Buffer **Note:** Each prepared tube of methylSEQr Conversion Reagent is enough for 10 samples.
4.	Seal the tube and vortex to mix as follows: • Vortex for 1 minute • Let rest for 2 minutes • Repeat 5 times **IMPORTANT!** Protect the prepared methylSEQr Conversion Reagent from light and use within 1 hour of preparation.
5.	Remove the denatured gDNA from 37 °C incubation and add 100 µL of the conversion reagent to each sample. The final volume is 150 µL.
6.	Incubate the sample(s) in the dark at 50 °C for 12 to 16 hours.

Purifying the Sample

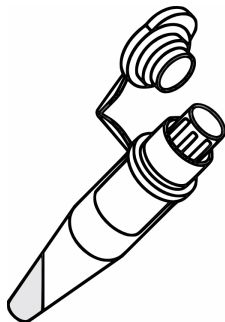⚠ **DANGER** **CHEMICAL HAZARD**. **Sodium hydroxide (NaOH)** causes severe eye, skin, and respiratory tract burns. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

To purify bisulfite-converted samples:

1.	Add 200 to 300 µL of deionized water to an assembled methylSEQr™ Purification Column.
2.	Add the 150-µL incubated sample and mix by pipetting up and down. Be careful not to puncture the column membrane.
3.	Centrifuge the sample at a maximum of 500 ✕ g for 20 minutes, then discard the filtrate.
4.	Add 350 µL of deionized water to the upper chamber of the purification column filter.
5.	Centrifuge the column at 500 ✕ g for 20 minutes, then discard the filtrate.
6.	Repeat steps 4 and 5.
7.	Add 350 µL of 0.1 M sodium hydroxide to the upper chamber of the purification column filter and allow the mixture to sit for 5 minutes.
8.	Centrifuge the column at 500 ✕ g for 20 minutes, then discard the filtrate.
9.	Add 350 µL of deionized water to the upper chamber, then centrifuge the column at a maximum of 500 ✕ g for 15 to 20 minutes, until the membrane is just damp, or a small amount of liquid remains in the upper chamber.
10.	Add 50 µL of 1✕ TE buffer to the upper chamber and mix gently by pipetting up and down. Let the column stand for 5 minutes.

To purify bisulfite-converted samples: *(continued)*

11.	Invert the column as shown and collect the bisulfite-treated gDNA in a clean microcentrifuge tube by centrifuging at 1000 $\times$ g for 30 seconds. The sample is now ready for PCR.
12.	Store any unused bisulfite-converted gDNA at 4 °C. The bisulfite-converted gDNA can be used in PCR for up to one year as long as there is no contamination. **Note:** Do not subject the bisulfite-converted gDNA to freeze-thawing.

Chapter 4

Cycle Sequencing

Cycle Sequencing

<div style="text-align: right; font-size: 3em;">4</div>

This chapter covers:

Overview

This chapter provides information on how to select the sequencing chemistry and cycle sequencing conditions for each chemistry.

Workflow

DNA Template Preparation (Chapter 3)

Cycle Sequencing

1. Select the sequencing chemistry.
2. Prepare cycle sequencing reactions.
3. Run sequencing reactions in a thermal cycler.

Applied Biosystems Cycle Sequencing Kits:

- BigDye Terminator v1.1 and v3.1 kits
- dGTP BigDye Terminator v1.0 and v3.0 kits
- BigDye Primer kits
- dRhodamine Terminator kits

9700 thermal cycler

9800 thermal cycler

Veriti® thermal cycler

Cycle Sequencing Output:

Dye terminator products   or   Dye primer products

A
A C
A C C
A C C G
A C C G T
A C C G T A
A C C G T A T

A
A C
A C C
A C C G
A C C G T
A C C G T A
A C C G T A T

Extension Product Purification (Chapter 5)

Capillary Electrophoresis (Chapter 6)

Data Analysis (Chapter 7)

Choosing a Sequencing Chemistry

Selection Criteria   When choosing a sequencing chemistry, consider the following:

- DNA sequencing application
- Sequence context
- DNA template type
- Length of desired read

Available Kits   Use Table 6 and Table 7 to select the cycle sequencing kit that meets your needs:

Table 6   Applied Biosystems cycle sequencing kits

Kit	Description	Page
BigDye® Terminator v3.1 Cycle Sequencing Kits	• Compatible with all automated genetic analyzers on Table 1 on page 10 • For comparative sequencing; *de novo* sequencing; resequencing; and sequencing PCR products, plasmids, cosmids, fosmids, and large templates, for example, bacterial artificial chromosome (BAC) clones • Can sequence difficult templates and dinucleotide repeats • Formulated with dITP in place of dGTP to reduce peak compressions	57
BigDye® Terminator v1.1 Cycle Sequencing Kits	• Compatible with automated genetic analyzers on Table 1 on page 10 • For optimal basecalling adjacent to the primer and sequencing short PCR product templates‡ • Can sequence difficult templates and dinucleotide repeats • Formulated with dITP in place of dGTP to reduce peak compressions	57
dGTP BigDye® Terminator Cycle Sequencing Kits	• Compatible with automated genetic analyzers on Table 1 on page 10 • Formulated with dGTP to produce higher-quality data when sequencing G-related motifs such as GT-, GA-, and GC-rich templates	58
dRhodamine Terminator Cycle Sequencing Kits	• Compatible with 3100/3100-*Avant* Genetic Analyzer and 310 Genetic Analyzer • For sequencing templates with homopolymers (AA, CC, GG, or TT) or T-rich motifs • Formulated with dITP in place of dGTP to reduce peak compressions	59
BigDye® Primer Cycle Sequencing Kits	• Compatible with 3100/3100-*Avant* Genetic Analyzer and 310 Genetic Analyzer • For sequencing single- and double-stranded DNA and PCR templates • Generates data with uniform peak heights and optimal basecalling adjacent to the primer	60

‡ In sequences obtained using BigDye Terminators v1.1 when run on rapid run modules and with a fast run polymer (POP-7 and POP-4), base mobility in beginning sequences is slightly worse than in sequences obtained using BigDye Terminators v3.1. However, when using BigDye Terminators v1.1, peak resolution of small fragments is better with POP-6 polymer.

Table 7 shows recommended chemistries, based on the sequencing application and/or characteristics of the DNA to be sequenced.

Ratings are:

- Recommended: ++
- Satisfactory: +
- Not recommended: –

Table 7   Chemistry recommendations for different sequencing applications and/or template characteristics

Application	BigDye Terminator v3.1	BigDye Terminator v1.1	dGTP BigDye Terminator	dRhodamine Terminator	BigDye Primer
DNA Sequencing Application					
Bisulfite sequencing	+	++	-	–	–
Comparative sequencing (germline mutations 50:50 heterozygotes)	++	++	–	+	++
Comparative sequencing (somatic mutations 10:90 heterozygotes)	–	–	–	–	+
Comparative sequencing (somatic mutations 30:70 heterozygotes)	+	+	–	–	++
De novo sequencing	++	++	+	+	+
Gap closure (custom primers)	++	++	++[‡]	+	–
Gene walking (custom primers)	++	++	+	+	–
Shotgun sequencing (universal primers, M13)	++	++	+	+	++
DNA Sequence Context					
AT-rich >65%	++	++	–	++	++
GC-rich >65%	++	++	+	+	+
GT-rich regions	+	+	++	++	++
Homopolymer A or T >25 bp[§]	–	–	–	++	–
Template Type					
BAC, cosmid, fosmid, lambda, large PCR product	++	++	–	+	+
Bacterial genomic DNA	++	++	–	–	–
Bisulfite-treated genomic DNA	+	++	–	–	–
PCR amplicon	++	++	+	++	++
PCR amplicon (heterozygous 10:90)	–	–	–	–	+

Table 7 Chemistry recommendations for different sequencing applications and/or template characteristics *(continued)*

Application	BigDye Terminator v3.1	BigDye Terminator v1.1	dGTP BigDye Terminator	dRhodamine Terminator	BigDye Primer
PCR amplicon (heterozygous 30:70)	+	+	–	–	++
PCR amplicon (heterozygous 50:50)	++	++	–	+	++
Plasmid (<15 kb)	++	++	+	++	++
Rolling circle amplified product	++	++	+	++	++
Single-stranded DNA	++	++	+	++	++

‡ Recommended for sequencing gaps with difficult GT- and GA-rich motifs.

§ All cycle sequencing chemistries can have difficulties with homopolymers >40 bp.

BigDye Terminator v1.1/3.1 Cycle Sequencing Kits

With BigDye Terminator v1.1 and v3.1 kits, you can sequence difficult templates and read through dinucleotide repeats and other challenging sequence motifs, using a wide range of template types and qualities. You can use these kits for a wide range of sequencing applications and obtain peak-height uniformity and optimized signal balance for longer, higher quality reads.

Note the peak uniformity and high quality basecalls across the GT repeats in Figure 26.
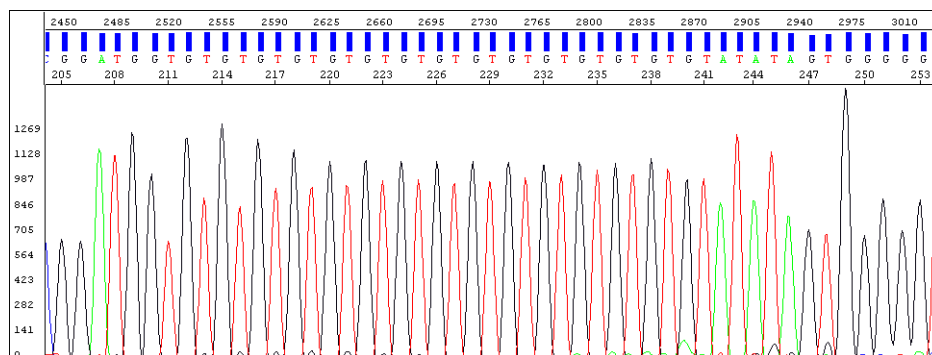
Figure 26 Peak uniformity and high quality basecalls using BigDye terminators

These kits provide the required reagent components for the sequencing reaction in a ready reaction, pre-mixed format. You need only to provide your template and the template-specific primer.

Note: These kits include BigDye® Terminator v1.1/v3.1 Sequencing Buffer (5✕), which has been specifically optimized for use with the v1.1 and v3.1 BigDye® Ready Reaction Mixes.
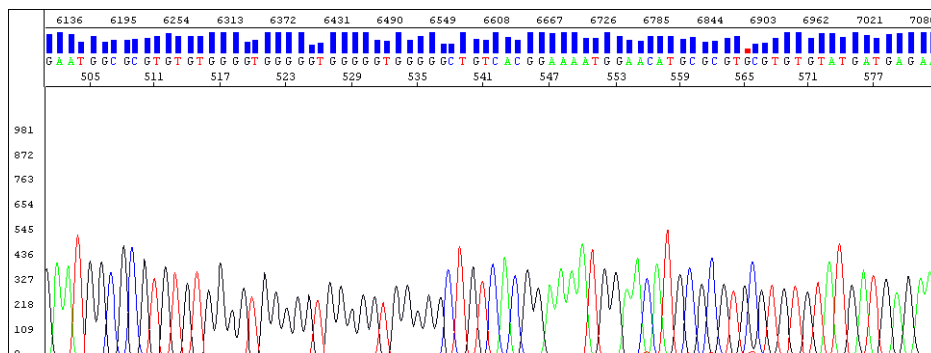
For more information, refer to the *BigDye® Terminator v3.1 Cycle Sequencing Kit Protocol* (PN 4337035) or the *BigDye® Terminator v1.1 Cycle Sequencing Kit Protocol* (PN 4337036).

dGTP BigDye Terminator Cycle Sequencing Kits

The dGTP BigDye® Terminator v1.0/v3.0 Cycle Sequencing Kits were developed for use with difficult templates where the standard terminator kits give data with early signal loss.

These kits use dGTP in the deoxynucleoside triphosphate mix instead of the dITP used in standard Applied Biosystems dye terminator cycle sequencing kits. The dITP is used in dye terminator kits to minimize peak compressions, but the substitution can lead to early signal loss in some sequence contexts.

The electropherogram in Figure 27 shows the sequence of a region of a plasmid that did not give satisfactory sequence results with other chemistry kits. Sequencing using dGTP BigDye terminators resulted in high quality basecalls through the difficult-to-sequence GT-rich region.

Figure 27   Sequence through a GT-rich region sequenced using dGTP BigDye terminators

IMPORTANT! Because of compressions, Applied Biosystems does not recommend using the dGTP BigDye Terminator v1.0/v3.0 Cycle Sequencing Kits for routine sequencing. They should be used only when you cannot obtain good data using standard terminator kits.

For more information, refer to:

- *dGTP BigDye® Terminator Ready Reaction Kit Protocol* (PN 4307169)
  or
- *dGTP BigDye® Terminator v3.0 Ready Reaction Cycle Sequencing Kit Protocol* (PN 4390038).

dRhodamine Terminator Cycle Sequencing Kits	dRhodamine Terminator Cycle Sequencing Kits can be used to perform fluorescence-based cycle sequencing reactions on single-stranded or double-stranded DNA templates, or on polymerase chain reaction (PCR) fragments. The dRhodamine kit is effective for sequencing through homopolymer regions and T-rich motifs.

The dRhodamine dyes have a narrow emission spectra, with small spectral overlap and therefore low noise (Figure 7 on page 8).

Figure 28 shows sequence through two homopolymer T regions in a plasmid sequenced using dRhodamine terminators. Note the minimal amount of slippage after each homopolymer region using dRhodamine terminators.
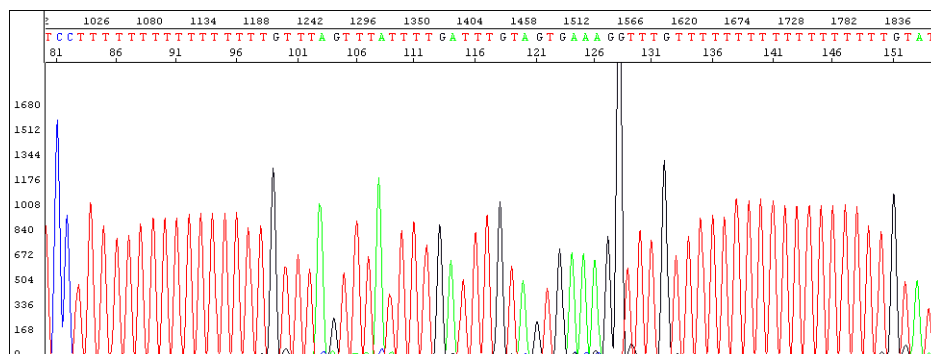
Figure 28   Sequence through two homopolymer T regions in a plasmid sequenced using dRhodamine terminators

These kits provide the required reagent components for the sequencing reaction in a ready reaction, pre-mixed format. You need only provide your template and the template-specific primer.

For more information, refer to the *dRhodamine Terminator Cycle Sequencing Ready Reaction Kit Protocol* (PN 403041).

BigDye Primer Cycle Sequencing Kits

BigDye Primer Cycle Sequencing Kits can be used to perform fluorescence-based cycle sequencing reactions on single-stranded or double-stranded DNA templates, on polymerase chain reaction (PCR) fragments, and on large templates, such as the ends of BAC clones. With these kits:

- The A, C, G, and T reactions are carried out in separate tubes or wells and in a 1:1:1:1 ratio.
- The deoxynucleotide/dideoxynucleotide mixes have been optimized to provide reads above 700 bases.
- Formulations contain 7-deaza-dGTP in place of dGTP to minimize peak compressions.
- Large templates, such as the ends of BAC clones, can be sequenced readily.

In Figure 29, note the peak uniformity throughout the general sequence using BigDye primers.
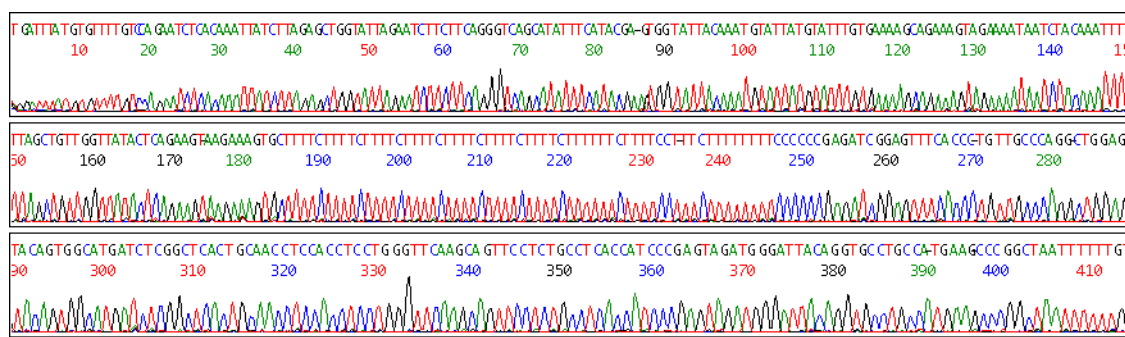
Figure 29   Peak uniformity throughout the general sequence using BigDye primers

In the Ready Reaction format, the dye-labeled primers, deoxynucleoside triphosphates, dideoxynucleoside triphosphates, DNA polymerase, pyrophosphatase, magnesium chloride, and buffer are premixed into A, C, G, and T Ready Reaction cocktails to eliminate time-consuming reagent preparation.

For more information, refer to the *BigDye® Primer Cycle Sequencing Ready Reaction Kit Protocol* (PN 403057).

Reagent and Equipment Considerations

Reagent Handling and Reaction Storage

For optimal performance using Applied Biosystems sequencing chemistry reagents, follow these recommendations. Handle and store other reagents according to manufacturer's recommendations.

- Store reagents in uncolored tubes or vials at –15 to –25 °C when not in use, and thaw completely at room temperature or in an ice bath (do not heat) before use.

  Note: Do not use a frost-free freezer. The automatic cycling of the temperature for defrosting can damage reagents, particularly enzymes.

- Avoid excessive freeze-thaw cycles. Aliquot reagents in smaller amounts if necessary.
- Protect reagents and sequencing reactions from light. Fluorescent dyes are susceptible to bleaching.
- To store sequencing reactions for future use, purify the extension products, then dry the reactions. Store the purified, dried reactions at –15 to –25 °C.

  Note: Do not store sequencing reactions at −70 to −80 °C.

- Sequencing reactions purified with the BigDye XTerminator Purification Kit can be stored as sealed reaction plates for up to 48 hours at room temperature or up to 10 days at 4 °C or −20 °C without having to dry down the reactions.

Preventing Dye Degradation

All fluorescent dyes are sensitive to degradation by a variety of chemical and physical agents. Degradation of the dyes can affect the sequencing analysis results ().

To prevent dye degradation:

- Protect fluorescently labeled DNA from light, heat, acidic conditions, and oxygen.

  IMPORTANT! Use the BigDye XTerminator Purification Kit to purify samples after cycle sequencing. After purification, samples are stable for up to 48 hours at room temperature or up to 10 days at 4 °C or −20 °C.

- Use fresh Hi-Di™ Formamide. Old Hi-Di Formamide or low quality formamide will contain formic acid, which can contribute to the degradation of fluorescent dyes.

  IMPORTANT! If the Hi-Di Formamide does not solidify at −20 °C, then it should be discarded.

  IMPORTANT! If the sequencing reactions are purified with the BigDye XTerminator Purification Kit, do not add Hi-Di Formamide.

- Heat-seal plates for the 3730/3730*xl* instruments if you are preparing multiple plates.
- After resuspending samples, run them on the instrument as quickly as possible.

Reaction Tubes and Plates

Use tubes or plates appropriate for the thermal cycler:

- GeneAmp® PCR Systems 9700 or Veriti® 96-Well Thermal Cycler
    - 0.2-mL MicroAmp® Reaction Tubes (PN N8010533)
    or
    - MicroAmp® Optical 96-Well Reaction Plate (PN N8010560)
- Applied Biosystems 9800 Fast PCR System or Veriti® FAST 96-Well Thermal Cycler
    - 96-Well Fast Thermal Cycling Plate (PN 4346907)

    Note: Fast plates require a 96-Well Fast (0.1mL) Plate Base (PN 4367470) and a 96-Well Fast (0.1mL) Plate Retainer (PN 4367471) for sequencing. For more information, see the *Introducing New 96-Well Fast Plate Adapters for Applied Biosystems Capillary Electrophoresis Systems User Bulletin* (PN 4370890).

For high-throughput demands, use:

- MicroAmp Optical 96-Well Reaction Plates with Barcode (PN 4306737)
    or
- MicroAmp Optical 384-Well Reaction Plates with Barcode (PN 4309849)

Other plates and tubes may be used. Refer to the user guide for your thermal cycler for part numbers.

Thermal Cyclers

The thermal cycling conditions in this chemistry guide were optimized using the GeneAmp PCR System 9700, the Applied Biosystems 9800 Fast PCR System, and the Veriti® 96-Well Thermal Cyclers. If you choose to use a thermal cycler not manufactured by Applied Biosystems, you may need to adjust the thermal cycling conditions due to differences in ramp rates and thermal accuracy. The ramp rate for thermal cyclers not manufactured by Applied Biosystems should be 1 °C/second. The type and performance of the thermal cycler can affect the quality of the reactions. Make sure that the thermal cycler is calibrated as recommended by the manufacturer.

DNA Quantity

DNA Template Quantities

The amount of DNA template used in a sequencing reaction can affect the quality of the data. Too much template makes data appear top heavy, with strong peaks at the beginning of the run that fade rapidly. Too little template or primer reduces the signal strength and peak height. In the worst case, the noise level increases so that bases cannot be called.

DNA sequencing reactions purified with the BigDye® XTerminator™ Purification Kit result in high signal strength when analyzed on a DNA sequencer. When you prepare sequencing samples for purification with the BigDye XTerminator reagents, you may need to decrease the amount of DNA template in the sequencing reactions to keep the fluorescence signals on scale during analysis.

Table 8 shows the recommended quantities of DNA template for each sequencing chemistry and for samples purified with the BigDye XTerminator Purification Kit.

Note: For information about preparing DNA templates for sequencing, see Chapter 3.

Table 8 Recommended DNA template quantities for cycle sequencing

Template	Cycle Sequencing Chemistry				
	BigDye Terminator v1.1 and v3.1	**BigDye XTerminator Purification Kit**	**dGTP BigDye Terminator v3.0**	**dRhodamine Terminator**	**BigDye Primer**
PCR product:					
100 to 200 bp	1 to 3 ng	0.5 to 3 ng	1 to 3 ng	1 to 3 ng	2 to 5 ng
200 to 500 bp	3 to 10 ng	1 to 10 ng	3 to 10 ng	3 to 10 ng	5 to 10 ng
500 to 1000 bp	5 to 20 ng	2 to 20 ng	5 to 20 ng	5 to 20 ng	10 to 20 ng
1000 to 2000 bp	10 to 40 ng	5 to 40 ng	10 to 40 ng	10 to 40 ng	20 to 50 ng
>2000 bp	40 to 100 ng	10 to 50 ng	40 to 100 ng	40 to 100 ng	50 to 150 ng
Bisulfite converted genomic DNA-PCR product	3 to 10 ng	3 to10 ng	not recommended	not recommended	not recommended
Single-stranded DNA	50 to 100 ng	10 to 50 ng	50 to 100 ng	50 to 100 ng	150 to 400 ng
Double-stranded DNA	200 to 500 ng	50 to 300 ng	200 to 500 ng	200 to 500 ng	200 to 800 ng
Cosmid, BAC	0.5 to 1.0 µg	200 to 1,000 ng	0.5 to 1.0 µg	not recommended	0.5 to 1.0 µg
Bacterial genomic DNA	2 to 3 µg	1,000 to 3,000 ng	2 to 3 µg	not recommended	not recommended

Using DNA Template Controls

Recommended Controls

Include a DNA template control in each set of sequencing reactions. The results from this control can help you determine whether failed reactions are caused by poor template quality or sequencing reaction failure.

Applied Biosystems recommends M13mp18 as a single-stranded control and pGEM®-3Zf(+) as a double-stranded control. Applied Biosystems DNA sequencing kits provide pGEM control DNA at 0.2 µg/µL. Applied Biosystems dye terminator cycle sequencing kits include a –21 M13 control primer at 0.8 pmol/µL. For control sequences, see .

Sequencing Control Reactions

Reagent	Quantity Per Reaction (µL)	
	96-Well	**384-Well‡**
Ready Reaction Mix	8.0	4.0
pGEM – 3Z(+) Control Template (0.2 µg/µL)	1.0	0.5
– 21 M13 forward primer (0.8 pmol/µL)	4.0	2.0
Deionized water	7.0	3.5
Total Volume	20.0	**10.0**

‡ Performing 10-µL reactions in 384-well reaction plates allows you to perform the post-reaction cleanup step in the same well.

Sequencing Control Reactions for Samples Prepared with BigDye XTerminator Purification Kit

Reagent	Quantity Per Reaction (µL)		
	96-Well (20µL reaction)	**96-Well (10µL reaction)**	**384-Well (5µL reaction)‡**
Ready Reaction Mix	8.0	4.0	2.0
pGEM – 3Z(+) Control Template (0.2 µg/µL)	0.5	0.25	0.125
– 21 M13 forward primer (0.8 pmol/µL)	4.0	2.0	1.0
Deionized water	7.5	3.75	1.875
Total Volume	20.0	**10.0**	**5.0**

‡ Performing 10-µL reactions in 384-well reaction plates allows you to perform the post-reaction cleanup step in the same well.

Thermal Cycling Conditions

Table 9   Thermal cycling conditions using DNA template controls

Mode	Thermal Cycling Conditions			
• 9600 emulation mode • 9700 standard mode • 9800 standard mode • Veriti® 96-Well Thermal Cycler	**Stage**	**Description**	**Temp. (°C)**	**Time**
	1	Denaturation	96	1 min
	2	Amplification: 25 cycles	96	10 sec
			50	5 sec
			60	4 min
	3	Hold	4	Indefinite hold
• 9800 Fast mode • Veriti® FAST 96-Well Thermal Cycler	**Stage**	**Description**	**Temp. (°C)**	**Time**
	1	Denaturation	96	1 min
	2	Amplification: 25 cycles	96	10 sec
			50	5 sec
			60	75 sec
	3	Hold	4	Indefinite hold

Using BigDye Terminators and dGTP BigDye Terminators

Sequencing Reaction Components

Reagent	Quantity Per Reaction		
	96-Well (20μL reaction)	**96-Well or 384-Well (10μL reaction)‡**	**384-Well (5μL reaction)**
Ready Reaction Mix	8.0 μL	4.0 μL	2.0 μL
Template	Quantity depends on template type and size.§		
Primer	3.2 pmol	3.2 pmol	3.2 pmol
Deionized water	q.s.	q.s.	q.s.
Total Volume	20 μL	10 μL‡	**5 μL**

‡ Performing 10-μL reactions in 384-well reaction plates allows you to perform post-reaction cleanup in the same well.

§ See Table 8, "Recommended DNA template quantities for cycle sequencing," on page 63.

Using BigDye Terminator v1.1/v3.1 Sequencing Buffer

Ready Reaction Mix contains BigDye Sequencing buffer and other components. If you are using less of the Ready Reaction Mix, add 5✕ BigDye Terminator Sequencing Buffer to bring the final buffer concentration to 1✕. The chemistry is optimized for a full strength reaction. Different instruments have different sensitivities and different detection systems.

Depending on the instrument you use, template quantity, and desired read length, you can make modifications using the following calculation.

For a given volume of Ready Reaction Mix:

0.5 (8 μL- Ready Reaction Mix Volume)   = Volume of BigDye Terminator Sequencing Buffer to add

IMPORTANT! BigDye Terminator v1.1/v3.1 Sequencing Buffer is intended for use only with BigDye Terminator v1.1/v3.1 Cycle Sequencing Kits.

Note: The use of the BigDye Terminator v1.1/v3.1 Sequencing Buffer without optimization may result in deterioration of sequencing quality.

Thermal Cycling Conditions

The thermal cycling conditions in Table 10 work for a variety of templates and primers. These thermal cycling conditions were optimized using the GeneAmp PCR System 9700, the Applied Biosystems 9800 Fast Thermal Cycler, the Veriti 96-Well Thermal Cycler, and the Veriti FAST 96-Well Thermal Cycler. If you choose to use other thermal cyclers, you may need to adjust the conditions because of differences in ramp rates and thermal accuracy.

Table 10   Thermal cycling conditions for BigDye terminators and dGTP BigDye terminators

DNA Template	Thermal Cycling Conditions			
BigDye Terminators v1.1 and v3.1 (9600 Emulation Mode, 9700 Standard Mode, 9800 Standard Mode, and Veriti 96-Well Thermal Cycler)				

	Stage	Description	Temp. (°C)	Time
• Double-stranded DNA • Single-stranded DNA • PCR product	1	Denaturation	96	1 min
	2	Amplification: 25 cycles	96	10 sec
			50	5 sec
			60	4 min
	3	Hold	4	Indefinite hold

	Stage	Description	Temp. (°C)	Time
BAC DNA	1	Denaturation	95	5 min
	2	Amplification: 50 cycles[‡]	95	30 sec
			50 to 55[§]	10 sec
			60	4 min
	3	Hold	4	Indefinite hold

‡ Some laboratories have found that increasing the number of cycles gives better results.
§ Set the annealing temperature according to the template.

Table 10   Thermal cycling conditions for BigDye terminators and dGTP BigDye terminators *(continued)*

DNA Template	Thermal Cycling Conditions			
Bacterial genomic DNA				

Stage	Description	Temp. (°C)	Time
1	Denaturation	95	5 min
2	Amplification: 45 cycles	95	30 sec
		50 to 55‡	20 sec
		60	4 min
3	Hold	4	Indefinite hold

‡ Set the annealing temperature according to the template.

Stage	Description	Temp. (°C)	Time
1	Denaturation	96	10 min
2	Amplification: 25 cycles	96	10 sec
		50	4 min
3	Hold	4	Indefinite hold

Bisulfite-treated template-PCR amplicon

Table 10   Thermal cycling conditions for BigDye terminators and dGTP BigDye terminators *(continued)*

DNA Template	Thermal Cycling Conditions			
dGTP BigDye Terminators v1.0 (9600 Emulation Mode, 9700 Standard Mode, 9800 Standard Mode, and Veriti 96-Well Thermal Cycler)				

DNA Template	Stage	Description	Temp. (°C)	Time
• Double-stranded DNA • Single-stranded DNA • PCR product	1	Amplification: 25 cycles	96	10 sec
			50	10 sec
			60	4 min
	2	Hold	4	Indefinite hold

dGTP BigDye Terminators v3.0 (9600 Emulation Mode, 9700 Standard Mode, 9800 Standard Mode, and Veriti 96-Well Thermal Cycler)				

DNA Template	Stage	Description	Temp. (°C)	Time
• Double-stranded DNA • Single-stranded DNA • PCR product	1	Amplification: 25 cycles	96	10 sec
			68	2 min
	2	Hold	4	Indefinite hold

Table 10   Thermal cycling conditions for BigDye terminators and dGTP BigDye terminators *(continued)*

DNA Template	Thermal Cycling Conditions			
BigDye Terminators v1.1/v3.1 and dGTP BigDye Terminators v1.0/v3.0 (9800 Fast Mode and Veriti FAST 96-Well Thermal Cycler)				
• Double-stranded DNA • Single-stranded DNA • PCR product	**Stage**	**Description**	**Temp. (°C)**	**Time**
	1	Denaturation	96	1 min
	2	Amplification: 25 cycles	96	10 sec
			50	5 sec
			60	75 sec
	3	Hold	4	Indefinite hold
BigDye Terminators v3.1 (9800 Fast Mode and Veriti FAST 96-Well Thermal Cycler)				
BAC DNA	**Stage**	**Description**	**Temp. (°C)**	**Time**
	1	Denaturation	96	2 min
	2	Amplification: 50 cycles	96	20 sec
			50	10 sec
			60	2 min
	3	Hold	4	Indefinite hold

Modifying Thermal Cycling Conditions

You may modify the thermal cycling conditions. Applied Biosystems makes the following suggestions for modifying these conditions. These suggestions have not been exhaustively tested at Applied Biosystems and they have not been found to work for all cases:

- For short PCR products, try reducing the extension time (for example, 2 minutes for a 300 bp or smaller fragment instead of 4 minutes) or reducing the number of cycles from 25 to 20.

  Note: For sequencing DNA with difficult contexts, decreasing extension times may result in reduced quality in the length of read and signal strength.

- If you observe high background signal and the T_m of a primer is >60 °C, try eliminating the annealing step.

- If you observe low signals and the T_m of a primer is <50 °C, increase the annealing time to 30 seconds or decrease the annealing temperature to 48 °C.

- For templates with high GC content (>70%), heat the tubes at 98 °C for 5 minutes before cycling to help denature the template.
- For sequencing large templates such as BACs and fosmids, increasing the number of cycles may help increase signal.

Using dRhodamine Terminators

Sequencing Reaction Components

Reagent	Quantity Per Reaction	
	96-Well	**384-Well**
Ready Reaction Mix	8.0 μL	4.0 μL
Template:	Quantity depends on template size.[‡]	
single-stranded DNA	50 to 100 ng	50 to 100 ng
double-stranded DNA	200 to 500 ng	200 to 500 ng
PCR product DNA	1 to 100 ng	1 to 100 ng
Primer	3.2 pmol	3.2 pmol
Deionized water	q.s.	q.s.
Total Volume	20 μL	**10 μL[§]**

[‡] See Table 8, "Recommended DNA template quantities for cycle sequencing," on page 63.

[§] Performing 10-μL reactions in 384-well reaction plates allows you to perform the post-reaction cleanup step in the same well.

Thermal Cycling Conditions

Table 11   Thermal cycling conditions using dRhodamine terminators

Mode	Thermal Cycling Conditions			
• 9600 emulation mode • 9700 standard mode • 9800 standard mode • Veriti 96-Well Thermal Cycler	**Stage**	**Description**	**Temp. (°C)**	**Time**
	1	Amplification: 25 cycles	96	10 sec
			50	5 sec
			60	4 min
	2	Hold	4	Indefinite hold
• 9800 Fast mode • Veriti FAST 96-Well Thermal Cycler	**Stage**	**Description**	**Temp. (°C)**	**Time**
	1	Denaturation	96	1 min
	2	Amplification: 25 cycles	96	10 sec
			50	5 sec
			60	75 sec
	3	Hold	4	Indefinite hold

Using BigDye Primers

The cycle sequencing protocols in this section are for using BigDye Primers in the 9600 emulation mode and the 9700 standard mode. Applied Biosystems has not validated cycle sequencing protocols for using BigDye Primers in 9800 Fast mode or for the Veriti thermal cyclers. If you want to use 9800 Fast mode or the Veriti thermal cycler, you may need to experiment to determine an effective protocol.

When sequencing using BigDye primers, select the reaction type and cycle type according to the DNA template:

Template	Reaction Type	Cycle Type
• PCR product • plasmid • M13	1X	standard
Large DNA templates: • Genomic DNA • Bacterial artificial chromosomes (BACs) • Yeast artificial chromosomes (YACs) • Cosmids • Long PCR products containing M13 forward and/or M13 reverse priming site(s)	High-sensitivity (2X)	modified

IMPORTANT! Prepare separate tubes for each of the four reactions (A, C, G, and T).

1X Reaction Components

Reagent	Quantity Per Reaction (μL)			
	A	**C**	**G**	**T**
Ready Reaction Premix	4.0	4.0	4.0	4.0
DNA template (see Table 8 on page 63 for quantity)	1.0	1.0	1.0	1.0
Total Volume	5.0	5.0	5.0	5.0

High-Sensitivity (2X) Reaction Components

Reagent	Quantity Per Reaction (μL)			
	A	**C**	**G**	**T**
Ready Reaction Premix	8.0	8.0	8.0	8.0
DNA template (see Table 8 on page 63 for quantity)	2.0	2.0	2.0	2.0
Total Volume	10.0	10.0	10.0	10.0

Thermal Cycling Conditions

The thermal cycling conditions are for 9600 emulation mode and 9700 standard mode. Select the conditions appropriate for the reaction type.

Table 12   Thermal cycling conditions using BigDye primers

Reaction Type	Thermal Cycling Conditions			

1✕

Stage	Description	Temp. (°C)	Time
1	Amplification: 15 cycles	96	10 sec
		55	5 sec
		70	1 min
2	Amplification: 15 cycles	96	10 sec
		70	1 min
3	Hold	4	Indefinite hold

High-sensitivity (2✕)

Stage	Description	Temp. (°C)	Time
1	Denaturation	95	5 min
2	Amplification: 20 cycles	95	30 sec
		50	15 sec
		70	1 min
3	Amplification: 15 cycles	95	30 sec
		70	1 min
4	Hold	4	Indefinite hold

Direct Sequencing From Cultures

Direct sequencing from cultures bypasses plasmid template preparation, reducing costs and work and allowing for faster screening of libraries for sequences of interest. However, previous protocols for direct sequencing produce a lower success rate and shorter read lengths. The protocols in this section can be used with BigDye® Terminator v1.1 and v3.1 Cycle Sequencing Kits to produce success rates and read lengths comparable to high throughput purified plasmid sequencing. The protocols are optimized for direct sequencing from cultures or colonies for various template types, in 96-well (below) or 384-well formats ().

Note: For purification of samples that are directly sequenced from a colony, use ethanol precipitation.

96-Well Direct Sequencing

To perform direct sequencing from cultures (96-well):

1.	Pick a colony or transfer glycerol backup to 200 µL TB/Amp (Costar 96-well cell culture cluster #3585) and incubate for 20 hours (no shaking).
2.	Prepare cell lysate: a. Transfer 50 µL of the culture to a 96-well reaction plate. b. Centrifuge the plate at 4000 rpm for 10 minutes. Remove the supernatant by inverting the plate, then centrifuge the plate again until the speed reaches 500 rpm. c. Resuspend the cell pellets in 12 µL of water by vortexing, then centrifuge the plate at 1000 rpm for 1 minute. d. Heat the cell lysate at 95 °C for 3 minutes, then cool it to 4 °C.
3.	Set up cycle sequencing reactions:

Reagent	Volume (µL)
Cell lysate	4.0
Sequencing primer (3.2 µM)	1.0
BigDye Terminator v3.1	4.0
Deionized water	1.0
Total	10.0

|

To perform direct sequencing from cultures (96-well): *(continued)*

4.	Perform cycle sequencing on the 9700 thermal cycler (9600 emulation mode):

Stage	Description	Temp. (°C)	Time
1	Denaturation	96	10 sec
2	Amplification: 50 cycles	50	5 sec
3		60	4 min
4	Hold	4	Indefinite hold

5.	Precipitate the DNA, performing the ethanol precipitation protocol for 96-well, 10-μL reaction volumes (page 97).

384-Well Direct Sequencing

To perform direct sequencing from cultures (384-well):

1.	Pick a colony or transfer glycerol backup to 50 µL TB/Amp (Nalgene 384-well plate, cell culture treated #164610) and incubate for 20 hours (no shaking).
2.	Prepare cell lysate: a. Transfer 5 µL of the culture to a 384-well reaction plate. b. Centrifuge the plate at 4000 rpm for 10 minutes. Remove the supernatant by inverting the plate, then centrifuge at 1000 rpm for 1 minute. c. Resuspend cell pellets in 2 µL of water by vortexing, then centrifuge the plate at 1000 rpm for 1 minute. d. Heat the cell lysate at 95 °C for 3 minutes, then cool to 4 °C.
3.	Set up cycle sequencing reactions:

Reagent	Volume (μL)
Cell lysate	2.0
Sequencing primer (3.2 µM)	1.0
BigDye Terminator v3.1	2.0
Total	5.0

To perform direct sequencing from cultures (384-well): *(continued)*

4.	Perform cycle sequencing on the 9700 thermal cycler (9600 emulation mode):			

Stage	Description	Temp. (°C)	Time
1	Denaturation	96	10 sec
2	Amplification: 50 cycles	50	5 sec
3		60	4 min
4	Hold	4	Indefinite hold

5.	Precipitate the DNA, performing the ethanol precipitation protocol for 384-well, 10-µL volumes (page 99).

Bisulfite Sequencing

PCR Amplification

Bisulfite-converted DNA can be a difficult template to amplify. After bisulfite conversion of gDNA, the double stranded nucleic acid is transformed into single stranded template, comprised of 5 different bases: A, G, T, U, and 5mC. Methylated promoter regions tend to be 5mCpG rich. C-G base pair-rich islands are known to be difficult to amplify.

Cloning

Amplification bias, slippage, and low primer specificity can make direct sequencing of PCR products difficult. Sequencing clones, rather than direct sequencing of amplicons derived from bisulfite-converted DNA template, can provide clean sequence, because clone sequencing produces a single amplicon insert per clone. Although sequencing clones can be time consuming, advantages include:

- No secondary sequence
- No PCR slippage
- No mixed bases
- Elimination of misaligned sequences due to mobility differences
- All 4 bases are represented for signal normalization due to sequence content from the cloning vector
- Ability to provide a semi-quantitative percentage of methylation

Bisulfite cloning followed by sequencing permits the assessment of methylation haplotypes for individual samples. Analyzing many clones can provide an estimate of the percentage of methylated targets at an individual site. However, like all quantitative methylation strategies, cloning is subject to biases, which limit the accuracy of quantitation.

Sources of bias can include:

- Purity of the sample extracted from biological sources
- Completeness of the bisulfite conversion
- Degree of bias in the bisulfite conversion purification step
- Number of clones sequenced

Polymerase Selection

After bisulfite conversion of gDNA, the double stranded nucleic acid is transformed into single stranded template, composed of 5 different bases: A, G, T, U, and 5mC. The polymerase used for PCR amplification must be capable of reading U and 5mC during the first round of synthesis in the reverse compliment strand. Master mixes containing uracil DNA glycosylase (UNG) should not be used, because the UNG cleaves U-containing DNA, degrading the template. High fidelity polymerase from archaebacteria such as Vent or Pfu DNA polymerase should not be used because they are strongly inhibited by uracil.

Use a hot-start polymerase in conjunction with relatively high temperature to avoid mismatch and amplification.

PCR Bias

The template derived from the unmethylated strand is frequently amplified more efficiently than the template derived from the methylated strand. The template derived from the unmethylated strand therefore dominates in a mixed sample.

Tips for reducing PCR bias when amplifying templates of mixed methylated states include:

- Use a hot-start PCR enzyme such as AmpliTaq Gold®DNA Polymerase or a PCR master mix containing this enzyme
- Use tailed primers with all 4 bases in design
- Use a PCR denaturant such as glycerol
- Set the annealing temperature approximately 2 to 5 °C above the calculated T_m (gene-specific portion)
- Increase the annealing temperature after the first few PCR cycles
- Perform Touchdown PCR
- Increase the extension time/temperature during PCR
- Decrease primer concentration (to reduce primer-dimer formation)

Cycle Sequencing for Bisulfite-Treated gDNA

The protocol below is optimized for bisulfite PCR using M13-tailed primers with a 5 °C T_m for the gene-specific portion of the primer. For fragment analysis, a dye-label (FAM) is included on the 5′ end of one of the primers. The tailed primer sequences are:

- **-21 M13 Forward Primer** – 5′ TGTAAAACGACGGCCAGT 3′
- **M13 Reverse Primer** – 5′ CAGGAAACAGCTATGACC 3′

To cycle sequence bisulfite-treated gDNA using M13 primers:

1. Prepare a solution containing 5 mg/mL BSA and 5% glycerol:

Reagent	Volume (μL)
GeneAmp® 10✕ Gold Buffer & MgCl$_2$	250
dNTP 2.5 mM each	700
Water	50
Total	**1000**

2. Set up cycle sequencing reactions. For each 5 µL reaction:

Reagent	Volume (μL)
GeneAmp® 10✕ Gold Buffer & MgCl$_2$	0.5
dNTP 2.5 mM each	0.4
MgCl$_2$ 25mM	0.4
AmpliTaq Gold® DNA polymerase (5U/μL)	0.1
Forward primer (5μM)	0.25
Reverse primer (5μM)	0.25
Bisulfite gDNA template (6ng/μL)	0.5
BSA-glycerol solution (from step 1 on page 79	0.5
Water	2.1
Total	**5.0**

To cycle sequence bisulfite-treated gDNA using M13 primers: *(continued)*

3. **If your sample is only for sequencing**, perform cycle sequencing on the 9700 thermal cycler (9600 emulation mode) as follows:

Stage	Description	Temp. (°C)	Time
1	Activate	95	5 min
2	Amplification: 40 cycles	95	30 sec
3		60	2 min
4		72	3 min
5	Hold	4	Indefinite hold

If you plan to perform fragment analysis, use the following conditions to perform cycle sequencing on the 9700 thermal cycler (9600 emulation mode):

Stage	Description	Temp. (°C)	Time
1	Activate	95	5 min
2	Amplification: 5 cycles	95	30 sec
3		60	2 min
4		72	3 min
5	Amplification: 35 cycles	95	30 sec
6		65	1 min
7		72	3 min
8	Hold	60	60
9	Hold	4	Indefinite hold

Note: The additional 60 minutes at 60 °C at the end of thermal cycling allows full conversion to the non-template A-addition amplicon product.

Chapter 5

Purification of Extension Products

Purification of Extension Products

<div style="text-align: right; font-size: 3em;">5</div>

This chapter covers:

Overview

This chapter presents procedures for purifying extension products and recommendations for preparing the purified samples for electrophoresis.

Workflow

DNA Template Preparation (Chapter 3)

⬇

Cycle Sequencing (Chapter 4)

⬇

Extension Product Purification

Purify extension products using one method:

- BigDye® XTerminator™ Purification
- Ethanol precipitation
- Spin-column purification
- Alternative cleanup procedures

After purification, prepare samples for electrophoresis

Extension Product Purification Output:

Purified dye terminator products or purified dye primer products

⬇

Capillary Electrophoresis (Chapter 6)

⬇

Data Analysis (Chapter 7)

Why Purification is Needed

The presence of both unlabeled and dye-labeled reaction components can interfere with electrokinetic injection, electrophoretic separation, and data analysis. For instance, fluorescent signals from unincorporated dye-labeled terminators that co-migrate with sequencing reaction extension products obscure the desired signal and interfere with basecalling. Purification of extension products can reduce or eliminate this interference. Methods for purifying extension products vary according to user preference and the cycle sequencing chemistry employed.

Dye Terminator Chemistries

Applied Biosystems supplies three dye terminator chemistries:

- BigDye® Terminators v1.1 and 3.1
- dGTP BigDye® Terminators v1.0 and v3.0
- dRhodamine Terminators

For each sequencing chemistry, there are different methods for removing excess dye terminators, using different reagents and processes. Applied Biosystems recommends performing controlled reactions with each method to determine the one that works best for you.

- Ethanol precipitation methods tend to be less expensive than commercially available products designed for this purpose, but these methods tend to be labor intensive and they are prone to variation in performance, depending upon a variety of factors. For more information, refer to the *Precipitation Methods to Remove Residual Dye Terminators from Sequencing Reactions User Bulletin* (PN 4304655).

- Methods using commercially available products such as the BigDye® XTerminator Purification Kit, spin columns, size-exclusion membranes, and magnetic beads efficiently remove terminators if performed correctly. Many of the commercial methods may be adapted to laboratory robotic systems. See page 119 for a list of currently available commercial products.

Dye Primer Chemistries   For dye primer chemistries (BigDye® Primers v3.0), the standard method for purifying extension products is ethanol precipitation, which concentrates the sample.

Purification with the BigDye® XTerminator™ Purification Kit

Overview   The BigDye® XTerminator™ Purification Kit sequesters cycle-sequencing reaction components such as salt ions, unincorporated dye terminators, and dNTPs, to prevent their co-injection with dye-labeled extension products into a capillary electrophoresis DNA analyzer.

The BigDye XTerminator reagents are compatible with BigDye® Terminators v1.1 and v3.1. Purification of cycle sequencing products with other dye chemistries has not been tested.

Samples purified with BigDye XTerminator can be directly injected into the capillary electrophoresis using BigDye XTerminator-specific run modules. The run modules are designed for the 3730/3730*xl* 3130/3130*xl*, and 3100/3100–*Avant* Analyzers, with Data Collection Software v2.0 or later.

To run samples purified with BigDye XTerminator on other instrument configurations, the purified sample must be transferred to a new plate.

DNA sequencing reactions purified with the BigDye XTerminator Purification Kit result in high signal strength. Follow the quantity guidelines in Table 8, "Recommended DNA template quantities for cycle sequencing," on page 63.

You can purify samples using two types of pipetting:

- **Premix pipetting** – Prepare a mixture of the two BigDye XTerminator reagents (referred to here as "premix") then pipette the premix into the reaction plate (see "Preparing the XTerminator Premix" and "Performing Purification Using the Premix" starting on page 90).
- **Sequential pipetting** – Add SAM™ Solution to the reaction plate first, followed by the XTerminator Solution (see "Performing Purification Using Sequential Pipetting" on page 93).

For more information see the *BigDye® XTerminator™ Purification Kit Protocol* (PN 4374408).

Advantages of Using the BigDye® XTerminator™ Purification Kit
- Simplifies workflow:
  - No need for spin column or ethanol precipitation
  - Fast
  - Little hands-on time needed
- Flexible throughput with purification in single tubes, 96-well or 384-well formats
- No bead removal prior to injection (depending upon the instrument used for sequencing)
- Removes dye "blobs" efficiently
- No danger of removing pellet (as can happen with ethanol precipitation)
- Higher signal intensity for the purified sample decreases quantity of DNA required

**BigDye®
XTerminator™
Purification Kit
Workflow**
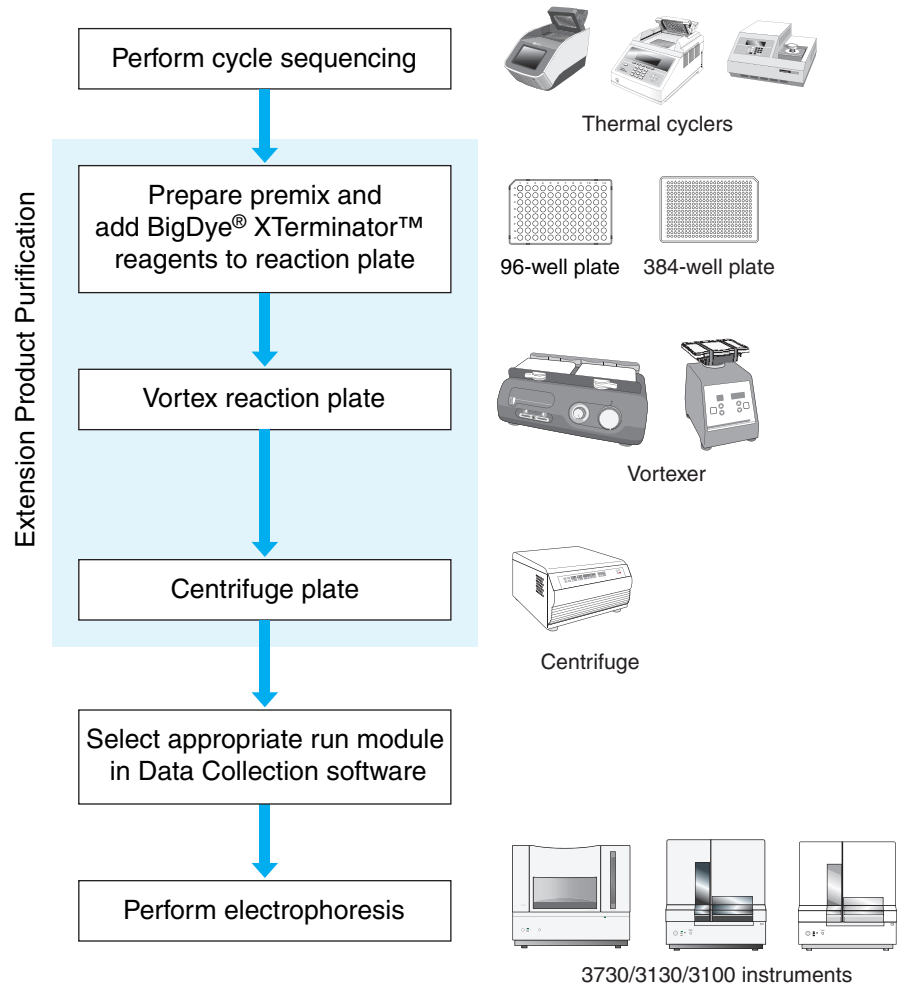
Figure 30   Sequencing workflow with extension product purification using the BigDye XTerminator Purification Kit

**Important Tips for
Using the BigDye
XTerminator
Purification Kit**

• When you pipette directly from the XTerminator Solution bottle:

  – Before pipetting, mix the XTerminator Solution until homogeneous.

  – *Use wide-bore pipette tips*.

  – Avoid pipetting near the surface of the liquid.

  – When you seal the reaction plate, verify that each well is sealed.

• To achieve optimum performance, use a recommended vortexer and follow the protocol when you vortex the reaction plate.

Preparing the XTerminator Premix

Guidelines for Preparing the Premix

These guidelines apply to single- and multi-dispense pipettes.

- *Use wide-bore pipette tips* (tips with an orifice >1.0 mm) for pipetting the XTerminator Solution.
- *Use conventional pipette tips* for pipetting the SAM Solution.
- Agitate the XTerminator Solution for at least 10 seconds using a standard laboratory vortexer at maximum speed before pipetting.

  IMPORTANT! XTerminator Solution that is allowed to stand for more than 2 minutes must be revortexed.

Note: If refrigerated, the premix is stable for no more than 5 days. Make only the volume of premix that you will use in 5 days.

Note: You may see fewer reactions per kit when using the premix method.

⚠ WARNING **CHEMICAL HAZARD. SAM Solution** is a flammable liquid and vapor. It may be harmful if absorbed through the skin, inhaled or swallowed. Exposure may cause eye, skin, and respiratory tract irritation, liver damage, and central nervous system depression. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

To prepare BigDye XTerminator premix:

1.	Based on your plate and reaction size, calculate the volumes of XTerminator Solution and SAM Solution needed.

Note: All volumes below include an additional 10% to account for dead volume.

Plate Type and Reaction Volume/Well	Volume/Well (µL)		Volume/Plate (µL)	
	XTerminator™ Solution	SAM™ Solution	XTerminator™ Solution	SAM™ Solution
384-well, 5 µL	5.5	24.75	2112	9504
96-well, 10 µL	11	49.5	1056	4752
96-well, 20 µL	22	99	2112	9504

To prepare BigDye XTerminator premix: *(continued)*

2.	Combine the SAM Solution and the XTerminator Solution to create the premix: a. Vortex the XTerminator Solution bulk container at maximum speed for at least 10 seconds, until it is homogeneous. b. *Using a wide-bore pipette tip* or a graduated cylinder, transfer the appropriate volume of XTerminator Solution to a clean container. **IMPORTANT!** Insert the pipette tip well below the surface of the liquid before aspirating. c. *Using a conventional pipette tip or graduated cylinder*, add the appropriate volume of SAM Solution to the container with the XTerminator Solution. Make sure that there are no particulates in the SAM Solution before pipetting. If particulates are present, heat the SAM Solution to 37 °C and mix to redissolve. Cool to room temperature before using. d. Mix the reagents until homogenous.
3.	Either store the premix up to 5 days for later use or continue to "Performing Purification Using the Premix" on page 91.

Performing Purification Using the Premix

Guidelines for Pipetting the Premix

- *Use conventional pipette tips* when pipetting the premix.
- When pipetting from a bottle, keep the premix agitated using a rocking motion.

  IMPORTANT! Using a stir bar on a stir plate does not keep the premix properly suspended.

- When pipetting from a trough, keep the premix agitated by:
  - Rocking the trough back and forth lengthwise to create a wave motion.
    or
  - Placing the pipette tips 1 to 2 mm above the trough bottom and moving them gently from side-to-side.
- Agitate the XTerminator premix before each aspiration.

To purify samples with BigDye XTerminator premix:

1.	Be sure the premix is well mixed, then transfer it to the trough or reservoir.
2.	After cycle sequencing is complete, centrifuge the reaction plate or tube, for 1 minute.

To purify samples with BigDye XTerminator premix: *(continued)*

3.	Into each well of the reaction plate, *use a conventional pipette tip* to add the volume of premix specified below:

Plate Type and Reaction Volume/Well	Volume of Premix/Well (µL)
384-well, 5-µL	27.5
96-well, 10-µL	55
96-well, 20-µL	110

Add more premix to the trough or reservoir as necessary.

IMPORTANT! For 384-well reactions with reaction volume less than 5 mL, add water to bring the volume to 5 mL before adding SAM Solution. For 96-well reactions with reaction volume less than 10 mL, add water to bring the volume to 10 mL before adding SAM Solution.

IMPORTANT! Dispense the premix within 1 minute of aspiration to avoid separation of the reagents in the pipette tip.

4.	Seal the plate using:

- A heat seal at 160 °C for 1.5 seconds.

  or

- MicroAmp® Clear Adhesive Films.

Verify that each well is sealed.

IMPORTANT! If you use a 3730/3730*xl* instrument and plan to use direct injection without a septa mat, only Applied Biosystems Heat Seal Film for Sequencing and Fragment Analysis Sample Plates (PN 4337570) is supported.

To purify samples with BigDye XTerminator premix: *(continued)*

5.	Vortex the reaction plate for 30 minutes, using the following conditions:

Vortexer	Plate Type	Speed
Digital Vortex-Genie 2	96-well	1800 rpm
	384-well	2000 rpm
Eppendorf MixMate	384-well	2600 rpm
IKA MS3 Digital	Either	2000 rpm[‡]
IKA Vortex 3	Either	Setting 5[§]
Taitec MicroMixer E-36	Either	Maximum
Union Scientific Vertical Shaker[#]	Either	Setting 100

‡ Set the vortexer to Mode B. See the BigDye® XTerminator™ *Purification Kit Protocol* for instructions.

§ Use the maximum setting that does not cause the vortexer to "walk" across the bench.

Add any additional plates to meet mass requirements. See the BigDye® XTerminator™ *Purification Kit Protocol* for information.

Note: Pause vortexing after 1 minute and examine the wells to verify that the contents are well mixed.

6.	In a swinging-bucket centrifuge, spin the plate at $1000 \times g$ for 2 minutes.
7.	Proceed with "Sample Preparation for Electrophoresis" on page 120.

Performing Purification Using Sequential Pipetting

⚠️ **WARNING**   **CHEMICAL HAZARD. SAM Solution** is a flammable liquid and vapor. It may be harmful if absorbed through the skin, inhaled or swallowed. Exposure may cause eye, skin, and respiratory tract irritation, liver damage, and central nervous system depression. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

To purify samples using sequential pipetting:

1.	After cycle sequencing is complete, centrifuge the reaction plate for 1 minute to spin down the plate contents.

To purify samples using sequential pipetting: *(continued)*

2.	To each well of the reaction plate, add the volume of SAM Solution specified below *using a conventional pipette tip*.

Make sure there are no particulates in the SAM Solution before pipetting. If particulates are present, heat the SAM Solution to 37 °C and mix to resuspend. Cool to room temperature before using.

Plate Type and Reaction Volume/Well	Volume of SAM™ Solution/Well (μL)
384-well, 5-μL	22.5
96-well, 10-μL	45
96-well, 20-μL	90

IMPORTANT! For 384-well reactions with reaction volume less than 5 μL, add water to bring the volume to 5 μL before adding SAM Solution. For 96-well reactions with reaction volume less than 10 μL, add water to bring the volume to 10 μL before adding SAM Solution.

3.	Add the XTerminator Solution:

a. Vortex the XTerminator Solution bulk container at maximum speed for at least 10 seconds, until it is homogeneous.

b. *Use a wide-bore pipette tip* to aspirate the XTerminator Solution.

   IMPORTANT! Avoid pipetting from the top of the liquid.

c. Into each well, add the volume of XTerminator Solution specified below:

Plate Type and Reaction Volume/Well	Volume of XTerminator™ Solution/Well (μL)
384-well, 5-μL	5
96-well, 10-μL	10
96-well, 20-μL	20

d. Discard the pipette tip.

4.	Seal the plate using:

• A heat seal at 160 °C for 1.5 seconds.

   or

• MicroAmp® Clear Adhesive Films.

Verify that each well is sealed.

IMPORTANT! If you use a 3730/3730*xl* instrument and plan to use direct injection without a septa mat, only Applied Biosystems Heat Seal Film for Sequencing and Fragment Analysis Sample Plates (PN 4337570) is supported.

To purify samples using sequential pipetting: *(continued)*

5.	Vortex the reaction plate for 30 minutes, using the following conditions:

Vortexer	Plate Type	Speed
Digital Vortex-Genie 2	96-well	1800 rpm
	384-well	2000 rpm
Eppendorf MixMate	384-well	2600 rpm
IKA MS3 Digital	Either	2000 rpm‡
IKA Vortex 3	Either	Setting 5§
Taitec MicroMixer E-36	Either	Maximum
Union Scientific Vertical Shaker#	Either	Setting 100

‡ Set the vortexer to Mode B. See the BigDye® XTerminator™ *Purification Kit Protocol* for instructions.

§ Use the maximum setting that does not cause the vortexer to "walk" across the bench.

Add any additional plates to meet mass requirements. See the BigDye® XTerminator™ *Purification Kit Protocol* for information.

It is recommended to pause vortexing after 1 minute and examine the wells to verify that the contents are well mixed.

6.	In a swinging-bucket centrifuge, spin the plate at $1000 \times g$ for 2 minutes.
7.	Proceed with "Sample Preparation for Electrophoresis" on page 120.

Purification by Ethanol Precipitation

Alcohol-based nucleic acid precipitation techniques include a wide variety of methods. Variations include: choice of alcohol (isopropanol, butanol or ethanol), choice and concentration of salt (NaOAc, NaCl, NH_4OAc, KCl or LiCl), temperature (–20 to 0 °C) and additives such as EDTA and Mg^{2+}. These variations are designed to yield slight differences in the precipitation products. Users are encouraged to experiment to obtain the specific technique best suited to their needs. The protocols below have been shown to obtain good performance across a broad variety of sample types.

Ethanol Concentrations

Inaccurate final ethanol concentrations can affect sequencing results. If ethanol concentrations are too low, you will not precipitate all of the extension products and you will lose them when you remove the supernatant. If ethanol concentrations are too high, you will precipitate unincorporated terminators with the extension products, producing large peaks (blobs) in the electropherogram (page 230).

- You may use 95% ethanol, but you must make sure to maintain the same final ethanol concentration for precipitation (65 to 71%).
- The ethanol concentration in absolute ethanol decreases gradually because absolute ethanol absorbs water from the atmosphere. Replace ethanol stocks frequently.

Methods

Select a method appropriate for the dye chemistry you use:

- BigDye® Terminators v1.1 and v3.1
  – Ethanol/EDTA precipitation: page 96
  – Ethanol/EDTA/sodium acetate precipitation: page 101
- dGTP BigDye® Terminators v1.0 and v3.0
  – Ethanol/sodium acetate precipitation: page 105
  – Ethanol precipitation: page 105
- dRhodamine Terminators
  – Ethanol/sodium acetate precipitation: page 109
  – Shrimp alkaline phosphatase digestion *(optional)*: page 110
  – Simplified ethanol precipitation: page 110
- BigDye® Primers: page 114

Ethanol Precipitation for BigDye® Terminators v1.1 and v3.1

Purification Methods

- Ethanol/EDTA precipitation:
  – Precipitating in 96-well reaction plates (below)
  – Precipitating in 384-well reaction plates (page 99)
- Ethanol/EDTA/Sodium acetate precipitation:
  – Precipitating in 96-well reaction plates (page 101)
  – Precipitating in 384-well reaction plates (page 103)

Ethanol/EDTA Precipitation

With the BigDye terminators v1.1 and v3.1, the ethanol/EDTA precipitation method produces consistent signal and is particularly good for removing unincorporated dye-labeled terminators.

Note: This method produces the cleanest signal, but it may cause loss of small fragments.

⚠ **WARNING** **CHEMICAL HAZARD. EDTA**. Exposure causes eye irritation. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

⚠ **WARNING** **CHEMICAL HAZARD**. **Ethanol** is a flammable liquid and vapor. Exposure causes eye, skin, and respiratory tract irritation and may cause central nervous system depression and liver damage. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

To precipitate 10-µL or 20-µL reactions in 96-well reaction plates:

1.	Remove the 96-well reaction plate from the thermal cycler and centrifuge the plate at $100 \times g$ for 1 minute, then remove the seal.
2.	Add the appropriate amount of 125 mM EDTA, pH 8.0 to each well. • **For 10 µL reactions** – 2.5 µL • **For 20 µL reactions** – 5 µL **IMPORTANT!** Make sure the EDTA reaches the bottom of the wells.
3.	Add the appropriate amount of ethanol to each well so that the final ethanol concentration is 67 to 71%. <table><tr><th>Sequencing Reaction Volume</th><th>Amount of 100% Ethanol to Add</th><th>Amount of 95% Ethanol to Add</th></tr><tr><td>10 µL</td><td>30 µL</td><td>36 µL</td></tr><tr><td>20 µL</td><td>60 µL</td><td>72 µL</td></tr></table>
4.	Seal the plate securely with self-adhesive film, then invert the plate 4 times to mix the contents. *or* Mix the contents well by pipetting up and down in a multichannel pipette 3 to 4 times, then seal the plate securely with self-adhesive film.

To precipitate 10-μL or 20-μL reactions in 96-well reaction plates: *(continued)*

5.	Incubate at room temperature for 15 minutes, then centrifuge the plate: <table><tr><td>**If you are using...**</td><td>**Then...**</td></tr><tr><td>a Beckman Allegra 6A centrifuge with a GH-3.8A rotor</td><td>set the centrifuge to 4 °C and centrifuge the plate at 1650 ✕ *g* for 45 minutes</td></tr><tr><td>any other centrifuge</td><td>use a plate adapter and centrifuge the plate at the maximum speed at 4 °C as follows: • 1400 to 2000 ✕ *g* for 45 minutes or • 2000 to 3000 ✕ *g* for 30 minutes</td></tr></table> **Note:** Using a centrifuge at 4 °C will yield better recovery of smaller molecular weight fragments, although a room temperature centrifuge can be used. **IMPORTANT!** Proceed to the next step immediately. If this is not possible, continue centrifuging the plate until you are ready to perform the next step.
6.	Remove the self-adhesive film, invert the plate onto a paper towel, centrifuge at 185 ✕ *g* for 1 minute, then remove the plate from the centrifuge. **Note:** Start timing the spin when the rotor starts moving.
7.	Add the appropriate amount of 70% ethanol to each well. • **For 10 μL reactions** – 30 μL • **For 20 μL reactions** – 60 μL
8.	Seal the plate with self-adhesive film, then centrifuge the plate at 1650 ✕ *g* for 15 minutes.
9.	Remove the self-adhesive film, invert the plate onto a paper towel, centrifuge up to 185 ✕ *g* for 1 minute, then remove the plate from the centrifuge. **Note:** Start timing when the rotor starts moving.
10.	Make sure the wells are dry. You can use a vacuum centrifuge for 5 minutes to dry the plate. **IMPORTANT!** Protect the samples from light while they are drying. **IMPORTANT!** Do not heat the samples to speed up ethanol evaporation.
11.	*(Optional)* If you plan to store the plate before proceeding with electrophoresis, seal the plate tightly with aluminum tape and store it at −20 °C protected from light.
12.	Proceed with .

Precipitating in 384-Well Reaction Plates

Note: When precipitating sequencing reactions less than 10 µL, add enough deionized water to the reactions to bring the volume to 10 µL. Then follow the protocol below for precipitation.

⚠ WARNING **CHEMICAL HAZARD. EDTA**. Exposure causes eye irritation. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

⚠ WARNING **CHEMICAL HAZARD**. **Ethanol** is a flammable liquid and vapor. Exposure causes eye, skin, and respiratory tract irritation and may cause central nervous system depression and liver damage. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves

To precipitate 10-µL sequencing reactions in 384-well reaction plates:

1.	Remove the 384-well reaction plate from the thermal cycler and centrifuge the plate at $100 \times g$ for 1 minute, then remove the seal.
2.	Add 2.5 µL of 125 mM EDTA, pH 8.0 to each well. **IMPORTANT!** Make sure the EDTA reaches the bottom of the wells.
3.	Add 25 µL of 100% ethanol to each well. **Note:** The final concentration of ethanol should be 67 to 71%. The use of 95% ethanol is not recommended because the final volume would exceed 38 µL (the maximum final volume when using 384-well plates).
4.	Seal the plate securely with self-adhesive film, then invert the plate 4 times to mix the contents. *or* Mix the contents well by pipetting up and down in a multichannel pipette 3 to 4 times, then seal the plate securely with self-adhesive film.

To precipitate 10-μL sequencing reactions in 384-well reaction plates: *(continued)*

5.	Incubate the plate at room temperature for 15 minutes, then centrifuge the plate:

If you are using...	Then...
a Beckman Allegra 6A centrifuge with a GH-3.8A rotor	set the centrifuge to 4 °C and centrifuge the plate at 1650 × *g* for 45 minutes
any other centrifuge	use a plate adapter and centrifuge the plate at the maximum speed at 4 °C as follows: • 1400 to 2000 × *g* for 45 minutes or • 2000 to 3000 × *g* for 30 minutes

Note: If you use a room temperature centrifuge, you may not get complete recovery of small fragments.

IMPORTANT! Proceed to the next step immediately. If this is not possible, then spin the tubes for an additional 2 minutes immediately before performing the next step.

6.	Remove the self-adhesive film, invert the plate onto a paper towel, centrifuge at 185 × *g* for 1 minute, then remove the plate from the centrifuge. **Note:** Start timing the spin when the rotor starts moving.
7.	Add 30 μL of 70% ethanol to each well, seal the plate with self-adhesive film, then centrifuge the plate at 1600 to 2000 × *g g* for 15 minutes.
8.	Remove the self-adhesive film, invert the plate onto a paper towel, centrifuge at 185 × *g* for 1 minute, then remove the plate from the centrifuge. **Note:** Start timing the spin when the rotor starts moving.
9.	Make sure the wells are dry. You can use a vacuum centrifuge for 5 minutes to dry the plate. **IMPORTANT!** Protect the samples from light while they are drying. **IMPORTANT!** Do not heat the samples to speed up ethanol evaporation.
10.	*(Optional)* If you plan to store the plate before proceeding with electrophoresis, seal the plate tightly with aluminum tape and store it at −20 °C protected from light.
11.	Proceed with .

Ethanol/EDTA/ Sodium Acetate Precipitation

Ethanol/EDTA/sodium acetate precipitation is recommended when you require good signal for sequences close to the end of the primer. However, for reactions containing high concentrations of unincorporated terminators, some residual terminators may be carried through the precipitation. To completely remove excess terminators in these cases, ethanol/EDTA precipitation is recommended (see page 97).

⚠ **WARNING** **CHEMICAL HAZARD. EDTA**. Exposure causes eye irritation. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

⚠ **CAUTION** **CHEMICAL HAZARD**. **3 M sodium acetate** may cause eye, skin, and respiratory tract irritation. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

⚠ **WARNING** **CHEMICAL HAZARD**. **Ethanol** is a flammable liquid and vapor. Exposure causes eye, skin, and respiratory tract irritation and may cause central nervous system depression and liver damage. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

To precipitate sequencing reactions in 96-well reaction plates:

1.	Remove the 96-well reaction plate from the thermal cycler and centrifuge the plate at 100 $\times$ g for 1 minute, then remove the seal.
2.	Add the appropriate amount of 125 mM EDTA, pH 8.0 to each well. • **For 10 µL reactions** – 1 µL • **For 20 µL reactions** – 2 µL **IMPORTANT!** Make sure the EDTA reaches the bottom of the wells.
3.	Add the appropriate amount of 3 M sodium acetate to each well. • **For 10 µL reactions** – 1 µL • **For 20 µL reactions** – 2 µL **Note:** Make sure the sodium acetate reaches the bottom of the wells.
4.	Add the appropriate amount of ethanol to each well.

Sequencing Reaction Volume	Amount of 100% Ethanol to Add	Amount of 95% Ethanol to Add
10 µL	25 µL	30 µL
20 µL	50 µL	60 µL

To precipitate sequencing reactions in 96-well reaction plates: *(continued)*

5.	Seal the plate securely with self-adhesive film, then mix by inverting the plate 4 times. *or* Mix well by pipetting up and down in a multichannel pipette 3 to 4 times, then seal the plate securely with self-adhesive film.
6.	Incubate the plate at room temperature for 15 minutes, then centrifuge the plate: <table><tr><td>**If you are using...**</td><td>**Then...**</td></tr><tr><td>a Beckman Allegra 6A centrifuge with a GH-3.8A rotor</td><td>set the centrifuge to 4 °C and centrifuge the plate at 1650 × g for 45 minutes</td></tr><tr><td>any other centrifuge</td><td>use a plate adapter and centrifuge the plate at the maximum speed at 4 °C as follows: • 1400 to 2000 × g for 45 minutes or • 2000 to 3000 × g for 30 minutes</td></tr></table> **IMPORTANT!** Proceed to the next step immediately. If this is not possible, then spin the tubes for an additional 2 minutes immediately before performing the next step.
7.	Remove the self-adhesive film, invert the plate onto a paper towel, centrifuge at 185 × g for 1 minute, then remove the plate from the centrifuge. **Note:** Start timing the spin when the rotor starts moving.
8.	Add the appropriate amount of 70% ethanol to each well. <table><tr><td>**Sequencing Reaction Volume**</td><td>**Amount of 70% Ethanol to Add**</td></tr><tr><td>10 µL</td><td>35 µL</td></tr><tr><td>20 µL</td><td>70 µL</td></tr></table>
9.	Seal the plate with self-adhesive film, then with the centrifuge set to 4 °C, spin the plate at 1650 × g for 15 minutes.
10.	Remove the self-adhesive film, invert the plate onto a paper towel, centrifuge up to 185 × g for 1 minute, then remove the plate from the centrifuge. **Note:** Start timing when the rotor starts moving.

To precipitate sequencing reactions in 96-well reaction plates: *(continued)*

11.	Make sure the wells are dry. You may use a vacuum centrifuge for 5 minutes to dry the plate. **IMPORTANT!** Protect the samples from light while they are drying. **IMPORTANT!** Do not heat the samples to speed up ethanol evaporation.
12.	*(Optional)* If you plan to store the plate before proceeding with electrophoresis, seal the plate tightly with aluminum tape and store it at −20 °C, protected from light.
13.	Proceed with "Minimum Sample Volume" on page 122.

Precipitating in 384-Well Reaction Plates

When precipitating sequencing reactions that are less than 10 μL, add enough deionized water to the reactions to bring the volume up to 10 μL. Then follow the protocol below for precipitation.

⚠️ **WARNING**   **CHEMICAL HAZARD. EDTA**. Exposure causes eye irritation. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

⚠️ **CAUTION**   **CHEMICAL HAZARD**. **3 M sodium acetate** may cause eye, skin, and respiratory tract irritation. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

⚠️ **WARNING**   **CHEMICAL HAZARD**. **Ethanol** is a flammable liquid and vapor. Exposure causes eye, skin, and respiratory tract irritation and may cause central nervous system depression and liver damage. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

To precipitate 10-μL sequencing reactions in 384-well reaction plates:

1.	Remove the 384-well reaction plate from the thermal cycler, centrifuge the plate at 100 $\times$ *g* for 1 minute, then remove the seal.
2.	Add 1 μL of 125 mM EDTA, pH 8.0 to each well. **IMPORTANT!** Make sure that the EDTA reaches the bottom of the wells.
3.	Add 1 μL of 3 M sodium acetate to each well. **Note:** Make sure that the sodium acetate reaches the bottom of the wells.

To precipitate 10-μL sequencing reactions in 384-well reaction plates: *(continued)*

4.	Add 25 μL of 100% ethanol to each well. **Note:** The final volume in the 384-well plate should not exceed 38 μL. **Note:** The final ethanol concentration should be 67 to 71%. **Note:** Use of 95% ethanol is not recommended because the final volume would exceed 38 μL.
5.	Seal the plate securely with self-adhesive film, then mix by inverting the plate 4 times. *or* Mix well by pipetting up and down in a multichannel pipette 3 to 4 times, then seal the plate securely with self-adhesive film.
6.	Incubate at room temperature for 15 minutes and then centrifuge the plate: {{TABLE_6}} **IMPORTANT!** Proceed to the next step immediately. If this is not possible, then spin the tubes for an additional 2 minutes immediately before performing the next step.
7.	Remove the self-adhesive film, invert the plate onto a paper towel, centrifuge at 185 $\times$ *g* for 1 minute, then remove the plate from the centrifuge.
8.	Add 30 μL of 70% ethanol to each well, seal the plate with self-adhesive film, then with the centrifuge set to 4 °C, spin at 1650 $\times$ *g* for 15 minutes.
9.	Remove the self-adhesive film, invert the plate onto a paper towel, centrifuge at 185 $\times$ *g* for 1 minute, then remove the plate from the centrifuge. **Note:** Start timing when the rotor starts moving.
10.	Make sure the wells are dry. You may use a vacuum centrifuge for 5 minutes to dry the plate. **IMPORTANT!** Protect the samples from light while they are drying. **IMPORTANT!** Do not heat the samples to speed up ethanol evaporation.

Table within step 6:

If you are using...	Then...
a Beckman Allegra 6A centrifuge with a GH-3.8A rotor	set the centrifuge to 4 °C and centrifuge the plate at 1650 $\times$ *g* for 45 minutes
any other centrifuge	use a plate adapter and centrifuge the plate at the maximum speed at 4 °C as follows: • 1400 to 2000 $\times$ *g* for 45 minutes or • 2000 to 3000 $\times$ *g* for 30 minutes

To precipitate 10-μL sequencing reactions in 384-well reaction plates: *(continued)*

11.	*(Optional)* If you plan to store the plate before proceeding with electrophoresis, seal the plate tightly with aluminum tape and store at −20 °C protected from light.
12.	Proceed to "Minimum Sample Volume" on page 122.

Ethanol Precipitation for dGTP Terminators v1.0 and v3.0

Purification Methods

- Ethanol/sodium acetate precipitation (below)
  - dGTP terminators v3.0 (below)
  - dGTP terminators v1.0 (below)
- Ethanol precipitation (below)
  - Precipitating in 96-well reaction plates (page 106)
  - Precipitating in microcentrifuge tubes (page 107)

Ethanol/Sodium Acetate Precipitation

dGTP Terminators v3.0 Only

Ethanol/sodium acetate precipitation produces consistent signal, and minimizes unincorporated dyes. A final 70% ethanol wash is required.

Note: This method produces the cleanest signal, but it may cause loss of small fragments.

For procedures, refer to the appropriate protocol:

- **96-well reaction plates or microcentrifuge tubes method** – Refer to the *dGTP BigDye® Terminator v3.0 Ready Reaction Cycle Sequencing Kit Protocol* (PN 4390038).
- **384-well reaction plates method** – Refer to the *BigDye® Terminator v3.1 Cycle Sequencing Kit Protocol* (PN 4337035).

dGTP Terminators v1.0 Only

To perform ethanol/sodium acetate precipitation with dGTP terminators v1.0 kits, refer to the *dGTP BigDye® Terminator Ready Reaction Kit Protocol* (PN 4307169).

Ethanol Precipitation

⚠ **WARNING** **CHEMICAL HAZARD**. **Ethanol** is a flammable liquid and vapor. Exposure causes eye, skin, and respiratory tract irritation and may cause central nervous system depression and liver damage. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

Ethanol precipitation improves the recovery of small fragments, but you may observe residual terminator peaks resulting from unincorporated terminators precipitating with the small fragments.

To precipitate 20-µL reactions in 96-well reaction plates:

1.	Remove the 96-well MicroAmp® plate from the thermal cycler. Remove the caps from each tube.
2.	Add the following for each sample: • 16 µL of deionized water • 64 µL of non-denatured 95% ethanol The final ethanol concentration should be 60 ± 3%.
3.	Seal the tubes with strip caps or by applying a piece of self-adhesive film. Press the film onto the tubes to prevent any leakage.
4.	Invert the plate a few times to mix.
5.	Leave the plate at room temperature for 15 minutes to precipitate the extension products. **Note:** Precipitation times <15 minutes result in the loss of very short extension products. Precipitation times >24 hours increase the precipitation of unincorporated dye terminators.
6.	Place the plate in a tabletop centrifuge with a tube-tray adapter and spin it at the maximum speed, which must be ≥1400 × g but <3000 × g: • 1400 to 2000 × g: 45 minutes • 2000 to 3000 × g: 30 minutes **Note:** A MicroAmp tube in a MicroAmp plate can withstand 3000 × g for 30 minutes. **IMPORTANT!** Proceed to the next step immediately. If this is not possible, then spin the tubes for an additional 2 minutes immediately before performing the next step.
7.	Without disturbing the precipitates, remove the adhesive tape and discard the supernatant by inverting the plate onto a paper towel folded to the size of the plate.
8.	Place the inverted plate with the paper towel into the table-top centrifuge and spin at 50 × g for 1 minute.
9.	Add 150 µL of 70% ethanol to each pellet.
10.	Spin the plate for 10 minutes at maximum speed. See step 6 above.
11.	Without disturbing the precipitates, remove the adhesive tape and discard the supernatant by inverting the plate onto a paper towel folded to the size of the plate.

To precipitate 20-μL reactions in 96-well reaction plates: *(continued)*

12.	Place the inverted plate with the paper towel into the table-top centrifuge and spin at 50 ✕ *g* for 1 minute.
13.	Remove the plate and discard the paper towel. **Note:** Pellets may or may not be visible. Vacuum drying of the samples is not necessary.
14.	Proceed with "Minimum Sample Volume" on page 122.

Ethanol Precipitation in Microcentrifuge Tubes

With ethanol precipitation, residual terminator peaks may be seen. However, the recovery of small fragments is improved using this precipitation method because of the higher g forces generated in a microcentrifuge, compared with a tabletop centrifuge.

⚠ **WARNING**   **CHEMICAL HAZARD**. **Ethanol** is a flammable liquid and vapor. Exposure causes eye, skin, and respiratory tract irritation and may cause central nervous system depression and liver damage. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

To precipitate 20-μL reactions in microcentrifuge tubes:

1.	Pipette the entire contents of each extension reaction into a 1.5-mL microcentrifuge tube.
2.	Add the following for each sample: • 16 μL of deionized water • 64 μL of non-denatured 95% ethanol The final ethanol concentration should be 60 ± 3%.
3.	Close the tubes and vortex them briefly.
4.	Leave the tubes at room temperature for 15 minutes to precipitate the extension products. **Note:** Precipitation times <15 minutes result in the loss of very short extension products. Precipitation times >24 hours increase the precipitation of unincorporated dye terminators.
5.	Place the tubes in a microcentrifuge and mark their orientations. Spin the tubes for 20 minutes at maximum speed. **IMPORTANT!** Proceed to the next step immediately. If this is not possible, then spin the tubes for an additional 2 minutes immediately before performing the next step.

To precipitate 20-μL reactions in microcentrifuge tubes: *(continued)*

6.	Carefully aspirate the supernatants with a separate pipette tip for each sample and discard. Pellets may or may not be visible.
	IMPORTANT! The supernatants contain unincorporated dye terminators. You must remove the supernatants completely to remove unincorporated dye terminators.
7.	Add 250 μL of 70% ethanol to the tubes and vortex them briefly.
8.	Place the tubes in the microcentrifuge in the same orientation as in step 5 and spin at maximum speed for 10 minutes.
9.	Aspirate the supernatants carefully, as in step 6.
10.	Dry the samples in a vacuum centrifuge for 10 to 15 minutes or to dryness. Do not over-dry.
11.	Proceed with "Minimum Sample Volume" on page 122.

Ethanol Precipitation for dRhodamine Terminators

Purification Methods

- Ethanol/sodium acetate precipitation (below)
- Shrimp alkaline phosphatase digestion (page 110)
- Simplified ethanol precipitation:
  - Precipitating in microcentrifuge tubes (page 111)
  - Precipitating in MicroAmp trays (page 112)

Ethanol/Sodium Acetate Precipitation

Because sequencing reactions using dRhodamine terminators produces less signal than reactions using BigDye terminators, take care when performing ethanol precipitation of sequencing reactions using dRhodamine terminators. With ethanol precipitation, you may observe minimal traces of unincorporated terminators at the beginning of the sequence data up to base 40. You may also observe loss in the recovery of the smallest fragments.

⚠ **CAUTION** **CHEMICAL HAZARD. 3 M sodium acetate** may cause eye, skin, and respiratory tract irritation. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

⚠ **WARNING** **CHEMICAL HAZARD. Ethanol** is a flammable liquid and vapor. Exposure causes eye, skin, and respiratory tract irritation and may cause central nervous system depression and liver damage. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

To perform ethanol/sodium acetate precipitation (20-μL reactions):

1.	For each sequencing reaction, prepare a 1.5-mL microcentrifuge tube containing: • 2.0 μL of 3 M sodium acetate (NaOAc), pH 4.6 • 50 μL of 95% ethanol (EtOH) **Note:** The final ethanol concentration should be ~66%.
2.	Precipitate the extension products: a. Pipette the entire contents of each extension reaction into each tube of sodium acetate/ethanol mixture. Mix each tube thoroughly. b. Vortex the tubes and place them on ice for 10 minutes.
3.	Pellet the precipitated extension products: a. Centrifuge the tubes at maximum speed for 15 to 30 minutes. b. Carefully aspirate the supernatant with a pipette, then discard the supernatant.

To perform ethanol/sodium acetate precipitation (20-μL reactions): *(continued)*

4.	Perform a 70% ethanol rinse: a. Add 250 μL of 70% ethanol to the pellet. b. Centrifuge the pellet at maximum speed for 5 minutes. c. Carefully aspirate the supernatant with a pipette, then discard the supernatant.
5.	Dry the pellet in a vacuum centrifuge for 10 to 15 minutes, or until dry. Do not over-dry the pellet.
6.	Proceed with "Minimum Sample Volume" on page 122.

Shrimp Alkaline Phosphatase Digestion *(Optional)*

The following shrimp alkaline phosphatase (SAP) digestion procedure can be used for more efficient removal of unincorporated dye terminators, but is optional. If you use this procedure for dye removal, then you must perform a simplified ethanol precipitation (below) afterwards.

To perform shrimp alkaline phosphatase (SAP) digestion:

1.	At the end of thermal cycling, add 2 μL of SAP (1 U/μL) and 18 μL of 1 ✕ SAP buffer to each tube. Seal each tube and incubate them at 37 °C for 30 minutes.
2.	Proceed with simplified ethanol precipitation: • To precipitate in microcentrifuge tubes, see the procedure below. • To precipitate in MicroAmp trays, go to page 112.

Simplified Ethanol Precipitation

These protocols require 70% ethanol (EtOH) containing 0.5 mM $MgCl_2$. This reagent can be prepared *in situ* or as a stock solution. Two protocols are given below:

• Precipitating in microcentrifuge tubes (below)
• Precipitating in MicroAmp trays (page 112)

IMPORTANT! Do **not** perform this protocol when purifying extension products with any of the BigDye terminators, because magnesium interferes with the removal of unincorporated BigDye terminators.

⚠️ **WARNING** **CHEMICAL HAZARD**. **Ethanol** is a flammable liquid and vapor. Exposure causes eye, skin, and respiratory tract irritation and may cause central nervous system depression and liver damage. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

⚠️ **WARNING** **CHEMICAL HAZARD**. **Magnesium chloride** may cause eye, skin, and respiratory tract irritation, central nervous system depression, and kidney damage. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

To precipitate in microcentrifuge tubes:

1.	Pipette the entire contents of each extension reaction into a 1.5-mL microcentrifuge tube.

2.	Select one method for adding the 70% ethanol (EtOH)/0.5 mM MgCl$_2$ solution.
	
	Add the reagents directly:
	• **For reactions after SAP digestion** – Add 40 μL of 2 mM MgCl$_2$ and then 110 μL of 95% ethanol.
	• **For reactions that do not use SAP digestion** – Add 20 μL of 2 mM MgCl$_2$ and then 55 μL of 95% ethanol.
	
	Create an EtOH/MgCl$_2$ stock solution and add it:
	a. Combine the following in a 1.5-mL microcentrifuge tube:
	   – 1 mL 70% EtOH
	   – 1 μL 0.5 M MgCl$_2$
	b. Vortex briefly.
	c. Add the stock solution:
	   – **For reactions after SAP digestion** – Add 150 μL of the EtOH/MgCl$_2$ stock solution to each tube.
	   – **For reactions that do not use SAP digestion** – Add 74 μL of the EtOH/MgCl$_2$ stock solution to each tube.

3.	Close the tubes and vortex them briefly.
4.	Leave the tubes at room temperature for 10 to 15 minutes to precipitate the extension products. **Note:** Precipitation times less than 5 minutes result in the loss of very short extension products. Precipitation times greater than 24 hours increase the precipitation of unincorporated dye terminators.
5.	Place the tubes in a microcentrifuge and mark their orientations. Centrifuge the tubes at maximum speed for 10 to 20 minutes. **IMPORTANT!** Proceed to the next step immediately.
6.	Carefully aspirate and discard the supernatants with a separate pipette for each sample. Pellets may or may not be visible. **IMPORTANT!** The supernatants must be removed completely because unincorporated dye terminators are dissolved in them. The more residual supernatant left in the tubes, the more unincorporated dye terminators remain in the samples.
7.	Visually inspect the sample tubes for residual supernatants. If there are any residual supernatants: a. Place the tubes in the microcentrifuge in the same orientation as in step 5 and spin for 5 to 10 seconds. b. Aspirate the supernatants carefully as in step 6.

To precipitate in microcentrifuge tubes: *(continued)*

8.	Dry the samples in a vacuum centrifuge for 10 to 15 minutes or to dryness. (Or, place the tubes with the lids open in a heat block or thermal cycler at 90 °C for 1 minute.)

To precipitate in MicroAmp® trays:

1.	Remove the MicroAmp Tray from the thermal cycler. Remove the caps from each tube.
2.	Select one method for adding the 70% ethanol (EtOH)/0.5 mM $MgCl_2$ solution. **Add the reagents directly:** • **For reactions after SAP digestion** – Add 40 μL of 2 mM $MgCl_2$ and then 110 μL of 95% ethanol. • **For reactions that do not use SAP digestion** – Add 20 μL of 2 mM $MgCl_2$ and then 55 μL of 95% ethanol. **Create an EtOH/$MgCl_2$ stock solution and add it:** a. Combine the following in a 1.5-mL microcentrifuge tube: – 1 mL 70% EtOH – 1 μL 0.5 M $MgCl_2$ b. Vortex briefly. c. Add the stock solution: – **For reactions after SAP digestion –** Add 150 μL of the EtOH/$MgCl_2$ stock solution to each tube. – **For reactions that do not use SAP digestion** – Add 74 μL of the EtOH/$MgCl_2$ stock solution to each tube.
3.	Seal the tubes by applying a piece of 3M Scotch Tape 425 adhesive-backed aluminum foil tape. Press the foil onto the tubes to prevent any leakage.
4.	Invert the tray a few times to mix.
5.	Leave the tray at room temperature for 10 to 15 minutes to precipitate the extension products. **Note:** Precipitation times less than 5 minutes result in the loss of very short extension products. Precipitation times greater than 24 hours increase the precipitation of unincorporated dye terminators.
6.	Place the tray in a table-top centrifuge with tube-tray adaptor for 20 to 30 minutes at the maximum speed (at least 1400 ✕ g but less than 3000 ✕ g). **Note:** A MicroAmp tube in a MicroAmp Tray can withstand 3000 ✕ g for 30 minutes. **IMPORTANT!** Proceed to the next step immediately.

To precipitate in MicroAmp® trays: *(continued)*

7.	Without disturbing the precipitates, remove the adhesive tape and discard the supernatant by inverting the tray onto a paper towel folded to the size of the tray.
8.	Place the inverted tray with the towel into the table-top centrifuge and spin at 500 to 1000 ✕ *g* for 1 minute.
9.	Remove the tray and discard the paper towel.
	Note: Pellets may or may not be visible. Vacuum drying of the samples is not necessary.
10.	Proceed with "Minimum Sample Volume" on page 122.

Ethanol Precipitation for BigDye Primers

Required Materials

Reagents and equipment required:

- 1.5-mL microcentrifuge tubes
- Benchtop microcentrifuge, capable of reaching at least $14000 \times g$
- Vacuum centrifuge
- 95% Ethanol (ACS reagent grade), non-denatured

Note: This method does not use salt.

Ethanol Precipitation

⚠️ WARNING **CHEMICAL HAZARD**. **Ethanol** is a flammable liquid and vapor. Exposure causes eye, skin, and respiratory tract irritation and may cause central nervous system depression and liver damage. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

1.	Select one method for adding the 95% ethanol and combining the reactions into one tube. **Transfer the extension reactions to a tube containing 95% ethanol:** a. Add 53 μL of 95% ethanol (100 μL if sequencing BAC DNA or other high-sensitivity reactions) to a clean microcentrifuge tube. **Note:** Sodium acetate is not necessary for precipitation. b. Pipette the extension reactions from the bottom of each of the four tubes into the ethanol mixture. Mix thoroughly. *or* **Add 95% ethanol to one reaction, then transfer the mixture to another reaction**: a. Add 53 μL of 95% ethanol (100 μL if sequencing BAC DNA or other high-sensitivity reactions) to the "A" reaction tube. b. Transfer the contents of the "A" reaction tube into the "C" reaction tube. c. Pipette the mixture from the "C" reaction tube into the "G" reaction tube and so on until the contents of all four reaction tubes are combined in one 1.5-mL microcentrifuge tube.
2.	Place the tube on wet ice or leave it at room temperature for 10 to 15 minutes to precipitate the extension products.
3.	Centrifuge the tube at maximum speed for 10 to 20 minutes. Carefully aspirate the supernatant and discard. At this point, a pellet may or may not be visible.
4.	*(Optional)* Rinse the pellet with 250 μL of 70% ethanol and centrifuge for 5 minutes. Carefully aspirate the supernatant and discard. This rinse step may remove some of the salts from the pellet but is often not necessary. **Note:** If you use sodium acetate, rinse the pellet to reduce the carryover of salt.

5.	Dry the pellet in a vacuum centrifuge for 1 to 3 minutes, or until dry. Do not over-dry.
6.	Proceed with "Minimum Sample Volume" on page 122.

Purification with Spin Columns and Spin Plates

Overview

1. Perform SDS/heat treatment of extension products (required for BigDye terminators v3.1 and dGTP BigDye terminators v3.0) (below).

2. Prepare the spin column (page 117) or the spin plate (see manufacturer's instructions).

3. Perform purification with the spin column (page 118) or with the spin plate (page 118).

Performing SDS/Heat Treatment

Perform this step to prepare extension products for both spin column and 96-well spin plate purification. This SDS/heat treatment effectively eliminates unincorporated dye terminators from cycle sequencing reactions.

IMPORTANT! This step **is required** for BigDye terminators v3.1 and dGTP BigDye terminators v3.0 but **is not required** for BigDye terminators v1.1 or dGTP BigDye terminators v1.0.

⚠ **DANGER** **CHEMICAL HAZARD**. **Sodium dodecyl sulfate (SDS).** Exposure causes eye, skin, and respiratory tract irritation. Exposure may cause severe allergic respiratory and skin reaction. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

To prepare extension products (20-μL reactions):

1.	Prepare 2.2% SDS (sodium dodecyl sulfate) in deionized water. This SDS solution is stable at room temperature.
2.	Add an appropriate amount of the 2.2% SDS solution to each sample to bring the final SDS concentration to 0.2%. For example: Add 2 μL of 2.2% SDS to each 20-μL completed cycle sequencing reaction.
3.	Seal the tubes and mix thoroughly, then centrifuge the tubes at 1000 rpm for 1 minute to spin down the contents.
4.	Heat the tubes to 98 °C for 5 min, then allow the tubes to cool to ambient temperature before proceeding to the next step. **Note:** A convenient way to perform this heating/cooling cycle is to place the tubes in a thermal cycler and set it as follows: • 98 °C for 5 min • 25 °C for 10 min
5.	Centrifuge the tubes briefly to spin down the contents.
6.	Continue with spin-column or 96-well spin plate purification.

Recommended Spin Columns

We recommend Centri-Sep™ spin columns (Within the US: Princeton Separations; Outside the US only: Applied Biosystems, PN 401763 for 32 columns and PN 401762 for 100 columns).

Optimizing Spin Column Purification

Directions below apply only when using Centri-Sep individual spin columns.

IMPORTANT! When using the BigDye terminators v3.1, hydrate the column for 2 hours (see step 3 below).

Tips for optimizing spin column purification when using individual columns:

- Do not process more columns than you can handle conveniently at one time.
- Load the sample in the center of the column bed slowly. Make sure that the sample does not touch the sides of the column and that the pipette tip does not touch the gel surface.

Note: If you do not load the samples properly, you may not remove sufficient unincorporated dye terminators and you may observe peaks from them in the electropherogram. Failure to remove all of the SDS can result in damaged capillaries.

- Spin the column at 325 to 730 $\times$ *g* for best results. Use the following formula to calculate the best speed for your centrifuge:

$$g = 1.12 \times r \times (rpm/1000)^2$$

where:

g = relative centrifugal force

r = radius of the rotor in mm

rpm = revolutions per minute

- Do not spin for more than 2 minutes.
- Perform the entire procedure without interruption to ensure optimal results. Do not allow the column to dry out.

Preparing the Spin Column

To prepare the spin column:

1.	Prepare the extension products according to "Performing SDS/Heat Treatment" on page 116.
2.	Tap the column gently to cause the gel material to settle to the bottom of the column.
3.	Hydrate the column: a. Remove the upper end cap and add 0.8 mL of deionized water. b. Replace the upper end cap and vortex or invert the column a few times to mix the water and gel material. c. Allow the gel to hydrate at room temperature for at least 2 hours. **Note:** You can store hydrated columns for a few days at 2 to 6 °C. Longer storage in water is not recommended. Allow columns stored at 2 to 6 °C to warm to room temperature before use.
4.	Remove any air bubbles by inverting or tapping the column and allowing the gel to settle.
5.	Remove the upper end cap first, then remove the bottom cap. Allow the column to drain completely by gravity. **Note:** If flow does not begin immediately, apply gentle pressure to the column with a pipette bulb.

To prepare the spin column: *(continued)*

6.	Insert the column into the wash tube provided.
7.	Centrifuge the column at 730 × *g* for 2 minutes to remove the interstitial fluid.
8.	Remove the column from the wash tube and insert it into a sample collection tube (for example, a 1.5-mL microcentrifuge tube).

Purifying with the Spin Column

To perform purification with the spin column:

1.	Remove the extension reaction/SDS mixture from its tube and load it carefully onto the center of the gel material.
2.	Centrifuge the column at 730 × *g* for 2 minutes. **Note:** If you use a centrifuge with a fixed-angle rotor, the surface of the gel will be at an angle in the column after the first spin. After the first spin, return the column to its original orientation.
3.	Discard the column. The sample is in the sample collection tube.
4.	Dry the sample in a vacuum centrifuge without heat or at low heat for 10 to 15 minutes or until dry. Do not over-dry.

Performing Spin Plate Purification

For large-scale procedures, you can use spin plates, such as DTR kits from Edge Biosystems, Montage Filter Plates from Millipore, or Centri•Sep Multi-Well Filter Plates from Princeton Separations.

Note: You may use other spin plate systems to remove unincorporated dye terminators. However, because of the large number of variables associated with using spin plate systems, optimize the performance of your system in your own laboratory.

Purifying with a Spin Plate

To perform purification with the spin plate:

1.	Prepare the extension products according to "Performing SDS/Heat Treatment" on page 116.
2.	Prepare the spin plate per the manufacturer's instructions.
3.	Perform the purification per the manufacturer's instructions.

Alternative Purification Procedures

Other manufacturers provide products for removing unincorporated dye terminator nucleotides, primers, excess salts, and other contaminants from sequencing reactions. Table 13 lists some commercially available alternatives. Applied Biosystems has no specific recommendations on the use of these products. Refer to the manufacturer's documentation for instructions.

Table 13   Alternative purification methods for extension products

Product	Source
Size Exclusion Products	
Performa DTR Ultra 96-Well Plates	Edge Biosystems
Sephadex G-50 or Sephadex G-75 columns	Major laboratory suppliers
Montage SEQ$_{96}$ and SEQ$_{384}$ Filter Plates	Millipore
MultiScreen SEQ$_{384}$ Filter Plates	Millipore
Centri•Sep Columns	Princeton Separations
Centri•Sep 96 Multi-Well Filter Plates	Princeton Separations
DyeEx 2.0 Spin Kit or DyeEx 96 Kit	QIAGEN
SigmaSpin 96-Well Post-Reaction Clean-Up Plates	Sigma-Aldrich
SigmaSpin Post-Reaction Clean-Up Columns	Sigma-Aldrich
Magnetic Bead Adsorption/Elution	
CleanSEQ	Agencourt
MagDTR Magnetic Resin	Edge Biosystems
Wizard MagneSil Sequencing Reaction Clean-Up System	Promega

Sample Preparation for Electrophoresis

This section has instructions for preparing purified samples for electrophoresis. Choose the instructions based on how your sample was purified:

- BigDye XTerminator Purification kit, see below
- Other purification methods, see

Samples Purified with the BigDye XTerminator Purification Kit

Storage of Purified Samples

Sequencing reactions cleaned with BigDye XTerminator can be stored under the following conditions:

- **Room temperature** – Plates sealed with heat seal film, adhesive film, or septa can be stored for up to 48 hours at room temperature (20 to 25 °C).
- **Refrigerated storage** – Plates sealed with heat seal film or adhesive film can be stored for up to 10 days at 4 °C (recommended).
- **Frozen storage** – Plates sealed with heat seal film or adhesive film can be stored for up to 1 month at –20 °C.

Minimum Sample Volume

Because there is a high concentration of dye-labeled sequencing fragments, one sample can be injected several times because very little sample volume is used for each injection. Make sure that you obtain the minimum sample volume so that the ends of the capillaries remain submerged in liquid during injection. The minimum sample volume is 10 µL for 96-well plates and 5 µL for 384-well plates.

Note: Because injection is electrokinetic, the resuspension volume does not affect the signal as it does for slab gel electrophoresis.

Resuspension Solutions

Sequencing reaction products purified with BigDye XTerminator do not require any additional resuspension solutions.

Note: Do not heat samples or add Hi-Di™ Formamide when using BigDye XTerminator to purify samples.

Sample Preparation Procedure

To prepare the plate after BigDye XTerminator purification:

1. Place the reaction plate in the genetic analyzer:

Plate Type	Instrument	Seal Used for Vortexing	Instructions
96-well	3730/3730*xl*	Heat seal	Place the plate directly in the instrument.
		MicroAmp® Clear Adhesive Film	Remove the seal, replace it with a septa mat, then place the plate in the instrument.
	3100/3100-Avant‡, 3130/3130*xl*	Either	Remove the seal, replace it with a septa mat, then place the plate in the instrument.
	310 Genetic Analyzer	Either	Transfer 10 µL of supernatant to a clean plate, cover the plate with a septa mat, then place the plate in the instrument.
384-well	3730/3730*xl*	Heat seal	Place the plate directly in the instrument.
		MicroAmp Clear Adhesive Film	• Remove the clear adhesive film, replace it with a heat seal, then place the plate in the instrument. *or* • Transfer 10 µL of supernatant to a clean plate, cover with a septa mat, then place the plate in the instrument.
	3100/3100-Avant‡, 3130/3130*xl*, 310 Genetic Analyzer	Either	Transfer 10 µL of supernatant to a clean plate, cover with a septa mat, then place in the instrument.

‡ The instrument must be using Data Collection Software v2.0 or later. If not, transfer 10 µL of supernatant to a clean plate, cover the plate with a septa mat, then place the plate in the instrument.

2. Select the appropriate BigDye XTerminator run module for your instrument and plate type (see "Selecting a Run Module" on page 137).

   Note: Use standard run modules if you transferred the supernatant to a clean plate after centrifuging.

Samples Purified with Other Purification Methods

Storing Sequencing Reactions

You can store dried, cleaned up, and sealed sequencing reactions at –20 °C for many days.

IMPORTANT! Protect fluorescently labeled DNA from light, heat, acidic conditions, and oxygen to prevent dye degradation.

Minimum Sample Volume	Minimum sample volume is the volume needed so that the ends of the capillaries remain submerged in liquid during injection. The minimum sample volume is 10 µL for 96-well plates and 5 µL for 384-well plates. Because the sample is transferred to the capillary by electrokinetic injection, the sample volume does not change and you can inject each sample multiple times.
Resuspension Solutions	Applied Biosystems recommends using Hi-Di Formamide to resuspend your purified sequencing products. For purification methods that result in extension products in water, Applied Biosystems recommends drying the sample in a speed vacuum and then resuspending the dried sample in Hi-Di Formamide.

If you choose to resuspend your samples in formamide not purchased from Applied Biosystems, make sure that you use only high-quality formamide. Also, Applied Biosystems recommends that you eliminate the denaturation step. Heating samples that are resuspended in formamide may result in dye degradation and shoulders on all peaks (page 234).

Resuspension in water is not recommended because oxidative effects on terminator dyes lead to earlier dye breakdown of sequencing extension products, affecting basecalling.

Sample Preparation Procedure

To prepare samples for capillary electrophoresis on a 310 instrument, see the *BigDye® Terminator v3.1 Sequencing Standard Product Insert* (PN 4336878).

⚠ **WARNING** **CHEMICAL HAZARD**. **Formamide**. Exposure causes eye, skin, and respiratory tract irritation. It is a possible developmental and birth defect hazard. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

To prepare samples for capillary electrophoresis on 31XX and 37XX instruments:

1.	Add 10 µL Hi-Di™ Formamide to each sample pellet: **IMPORTANT!** To prevent dye degradation: • Use fresh Hi-Di Formamide. Old Hi-Di Formamide or low quality formamide can have formic acid that can contribute to the degradation of fluorescent dyes. • Run samples on the instrument as soon as possible after resuspending them.
2.	Seal the wells with aluminum sealing tape.
3.	Vortex thoroughly, then centrifuge briefly.
4.	Peel off the aluminum sealing tape and replace the tape with either septa or a heat seal. **Note:** You must use tube septa or a heat seal to prevent exposure to air and evaporation of samples, especially if you place the samples in the autosampler more than 6 hours before starting electrophoresis. **Note:** Heat seals are available for 3730/3730*xl* instruments only.

Capillary Electrophoresis

<div style="text-align: right; font-size: 3em;">6</div>

This chapter covers:

Overview

This chapter provides an overview of capillary electrophoresis on Applied Biosystems instruments for automated DNA sequencing. For more information, refer to your instrument user guide.

Workflow

DNA Template Preparation (Chapter 3)

⬇

Cycle Sequencing (Chapter 4)

⬇

Extension Product Purification (Chapter 5)

⬇

Capillary Electrophoresis

1. Prepare the instrument
2. Calibrate the instrument
3. Set up the plate record in Data Collection Software:
   – Results group
   – Instrument protocol
   – Analysis protocol
4. Load samples.
5. Perform the run.

Capillary Electrophoresis Output:

 *.ab1 file

Applied Biosystems instruments:

3730/3730*xl* analyzer

3130/3130*xl* analyzer

3100/3100-*Avant* analyzer

310 analyzer

⬇

Data Analysis (Chapter 7)

Electrophoresis Workflow

Follow the general flow below to perform a run on Applied Biosystems genetic analyzers. For more information and procedures, refer to your instrument user guide.

1.	Prepare the instrument: a. Start the computer, the instrument, and the Data Collection Software. b. Select the capillary length, number of capillaries, and polymer type (page 130). c. Check the polymer delivery system for sufficient polymer and absence of bubbles. d. Prepare the buffer and water/waste reservoirs.
2.	Perform spatial calibration, if necessary (page 131).
3.	Perform spectral calibration, if necessary (page 131).
4.	Load the prepared samples: a. (Multicapillary instruments only) Load the prepared samples onto 96-well or 384-well plates. b. Place the tubes or the plate assembly into the instrument.
5.	Set up the run using Data Collection Software: a. Create a plate record (page 136). b. Create a results group, with file name and folder preferences (page 136). c. Create an instrument protocol, including run module and dye set configurations (page 137). d. Create an analysis protocol (page 143). e. Determine the run parameters (page 151). f. (Multicapillary instruments only) Link the plate.
6.	Start the run.

Instrument Consumables

Sequencing Standards

The cycle sequencing standards provide an additional control for troubleshooting electrophoresis runs because they can help you distinguish between chemistry problems and instrument problems. These standards contain lyophilized prelabeled sequencing reactions that require only resuspension before use. Standards available:

- BigDye® Terminator v3.1 Sequencing Standard (3730, 3730*xl*) (PN 4336743)
- BigDye® Terminator v3.1 Sequencing Standard (310, 3100-*Avant*, 3100, 3130, 3130*xl*) (PN 4336935)
- BigDye® Terminator v1.1 Sequencing Standard (3730, 3730*xl*) (PN 4336799)

- BigDye® Terminator v1.1 Sequencing Standard (310, 3100-*Avant*, 3100, 3130, 3130*xl*) (PN 4336791)

Note: For preparation of these standards, refer to the product inserts.

POP™ Polymer

Applied Biosystems genetic analyzers use POP™ Performance Optimized Polymer to separate DNA fragments. Use only the polymer recommended for your instrument.

IMPORTANT! Do not leave polymer on the instrument more than 7 days.

Applied Biosystems recommends that you minimize actions that could introduce bubbles or particles into the polymer. Introduction of dust into the polymer can cause spikes in the data. To minimize bubbles and particles:

- Make sure that the polymer cap is closed to minimize exposure of the polymer to air during storage.
- Clean the polymer delivery system with deionized water.
- Discard capillaries that are exposed to dust or are dried out.
- Change the buffer and water and discard the waste daily or before each set of runs.

Capillaries

In all Applied Biosystems capillary instruments, the capillary has an opaque, polyamide external coating except in the detection window area. During electrophoresis, the laser and detector read samples through the window in the coating. Capillaries are very fragile in the uncoated window area.

If treated properly, capillaries are guaranteed for 300 runs for 3730/3730*xl* instruments, for 150 runs for 3130 and 3100-*Avant* instruments, and for 100 runs for 3130*xl,* 3100, and 310 instruments. You may be able to get more injections from a capillary, depending on your template preparation methods and run conditions.

- Store the capillary ends in buffer or deionized water when not in use to prevent the capillary ends from drying out.
- Store unused capillaries in a dust-free environment.
- Do not touch capillary windows. If you accidentally touch a window, clean it according to the procedure in your instrument user guide.

Possible signs of capillary failure:

- Gradual loss of resolution (see)
- High baseline
- Noisy data or peaks under peaks throughout the sequence (see)
- Shoulders on peaks or irregular peaks (see)

Genetic Analyzer Running Buffer

Use only Applied Biosystems genetic analyzer running buffer for electrophoresis. Other running buffers may not perform well and using them may void warranty or service contracts. Applied Biosystems genetic analyzer running buffers are supplied at 10✕ concentrations. Dilute to 1✕ with distilled water before use:

- **3730/3730*xl* instruments –** Applied Biosystems 3730/3730*xl* 10✕ Running Buffer with EDTA (PN 4335613)
- **3130/3130*xl*, 3100/3100-*Avant* instruments, and 310 instruments –** 10✕ Genetic Analyzer Buffer with EDTA (PN 402824)

Calibrating the Instrument

Multiple capillary instruments require calibration before performing a sample run to maximize data quality and accuracy.

Spatial Calibration

The Data Collection Software uses images collected during the spatial calibration to map each signal detected by the CCD camera to a position in the capillary array. Spatial calibration is required for all multi-capillary instruments.

When to Perform

You are required to perform a spatial calibration when you:

- Install or replace a capillary array
- Temporarily remove the capillary array from the detection block
- Move the instrument
- Open the detection block

Spectral Calibration

Performing a spectral calibration is similar to performing a sample run. When you perform a spectral calibration, you run spectral calibration standards as samples and use a spectral calibration module as a run module. The results from a spectral calibration run are used to create a matrix of spectral values that define the spectral overlap between the different dyes.

Note: If you are using the 310 instrument, create a matrix file manually after performing the matrix run.

The values in the matrix are unique for each instrument, for each dye set, and for each specific set of run conditions. Data Collection Software applies the values in the matrix to the sample data to perform multicomponent analysis: the reduction of raw data from the instrument to the 4-dye data stored in sequencing files.

When to Perform

You must perform a spectral calibration run:

- When you use a new dye set on the instrument
- When you change the capillary array or polymer
- For each combination of capillary array length and each sequencing dye set that you use
- After the laser or CCD camera has been realigned/replaced by a service engineer
- If you begin to observe a decrease in the quality of raw or analyzed data (for example, pull-up and/or pull-down peaks with a distinct pattern)

Spectral Calibration Standard Types

There are two types of spectral calibration standards:

- **Matrix standards for sequencing** – A tube that contains four different fragments, each labeled with a different single dye.

  Note: Matrix standards are not designed for use on 3730 instruments. Use sequencing standards for spectral calibration of 3730 instruments.

- **Sequencing standards** – A tube of a standard chemistry reaction that contains multiple fragments labeled with the four dyes.

Parameter Files for Spectral Calibration

The Data Collection Software uses a parameter file to process the data to create a matrix. Each parameter file contains an algorithm to process the source data and information to evaluate the final matrix.

Table 14 describes when to use each type of parameter file.

Table 14   Spectral calibration criteria

Dye	Parameter File	For Each Dye…	Q Value	Condition Bounds
Matrix standard set DS-01	MtxStd{Sequencing SetE}.par	Single peak	0.92	3.0 to 5.0
BigDye Terminator standard or sequencing reaction	SeqStd{Sequencing-SetE}.par	Multiple peaks	0.95	3.0 to 5.0
Matrix standard set for BigDye v1.1	MtxStd{Sequencing-SetE}.par	Single peak	0.95	3.0 to 5.0
BigDye Terminator v3.1 standard or sequencing reaction	SeqStd{Sequencing-SetZ}.par	Multiple peaks	0.95	3.0 to 5.0
Matrix standard-unknown dye set	MtxStd{AnyDyeSet}.par	Single peak	0.95	None specified
A sequencing reaction that uses a 4-color dye set other than dye set E/Z	SeqStd{AnyDyeSet}.par	Multiple peaks	0.95	None specified

During the Calibration

Matrix standards are run in all capillaries. The Data Collection Software collects the data and stores it into separate temporary files. The Data Collection Software then analyzes the data, then generates and stores a mathematical description of the spectral overlap for each capillary.

Dye Set and Spectral Calibration Standards

Use Table 15 to determine the correct dye set and spectral calibration standard for the cycle sequencing kit you use. Note that the standards are instrument-specific.

A dye set is a place holder for spectral information for a given set of four dyes.

Note: Because different calibration algorithms are used for Big Dye Terminator v3.1 and Big Dye Terminator v1.1, the matrix standards are not interchangeable.

Table 15   Dye sets and spectral calibration standards

Cycle Sequencing Kit	Dye Set	Spectral Calibration Standard‡
• BigDye® Terminator v3.1 Cycle Sequencing Kits • dGTP BigDye® Terminator v3.0 Cycle Sequencing Kits	Z	Matrix standards: • BigDye® Terminator v3.1 Matrix Standard (3100-*Avant*, 3100, 3130, 3130*xl*) (PN 4336974) • BigDye® Terminator v3.1 Matrix Standards (310) (PN 4336948)
		Sequencing standards: • BigDye® Terminator v3.1 Sequencing Standard (3730, 3730*xl*) (PN 4336943) • BigDye® Terminator v3.1 Sequencing Standard (3130, 3130*xl,* 3100-*Avant,* 3100, 310) (PN 4336935)
• BigDye® Terminator v1.1 Cycle Sequencing Kits • BigDye® Primer Cycle Sequencing Kits • dGTP BigDye® Terminator Cycle Sequencing Kits	E	Matrix standards: • BigDye® Terminator v1.1 Matrix Standard (3100-*Avant*, 3100) (PN 4336824) • BigDye® Terminator v1.1 Matrix Standards (310) (PN 4336805)
		Sequencing standards: • BigDye® Terminator v1.1 Sequencing Standard (3730, 3730*xl*) (PN 4336799) • BigDye® Terminator v1.1 Sequencing Standard (3130, 3130*xl,* 3100-*Avant,* 3100, 310) (PN 4336791)
dRhodamine Terminator Cycle Sequencing Kits	E	dRhodamine Matrix Standards Kit (310) (PN 403046, 4 tubes; PN 403047, 10 reactions)
		Long Read dRhodamine Terminator Cycle Sequencing Standard (PN 4303120)

‡ Matrix standards are not designed for use on 3730 instruments. Use sequencing standards for spectral calibration of 3730 instruments.

Evaluating the Calibration Results

Evaluate the Data

Use the following criteria to evaluate the data:

- **Q Value** – A value which measures the consistency between the final matrix and the data from which it was computed. When the Q value is 1.0, the fit is perfect. The matrix is ideal and there were no detected pull-up or pull-down peaks.
- **Condition Number** – The condition number is a value that represents the amount of overlap between the dye peaks in the emission spectra of the dyes in a dye set.
- **Spectral profile** – The spectral profile shows the emission spectra of the four dyes.
- **Raw data –** The emission image exhibits four distinct fluorescence emission maxima, one for each dye, if matrix standards are used (Figure 31). If sequencing standards are used, there are multiple peaks (Figure 32).
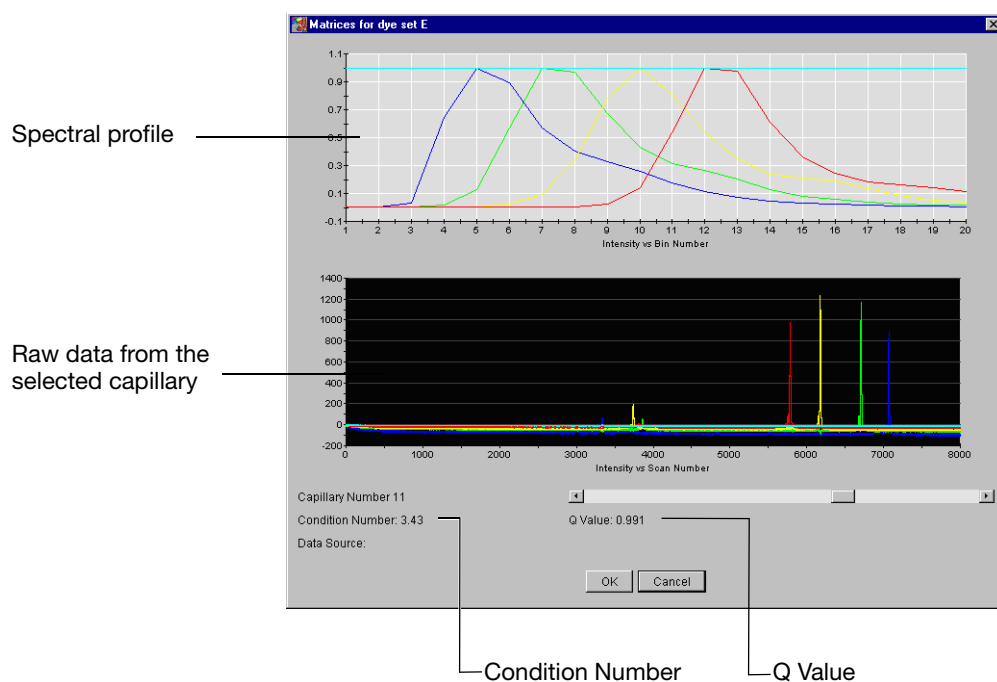
Figure 31   Example output from a spectral calibration using matrix standards

Spectral profile

Raw data from the
selected capillary

Condition Number   Q Value
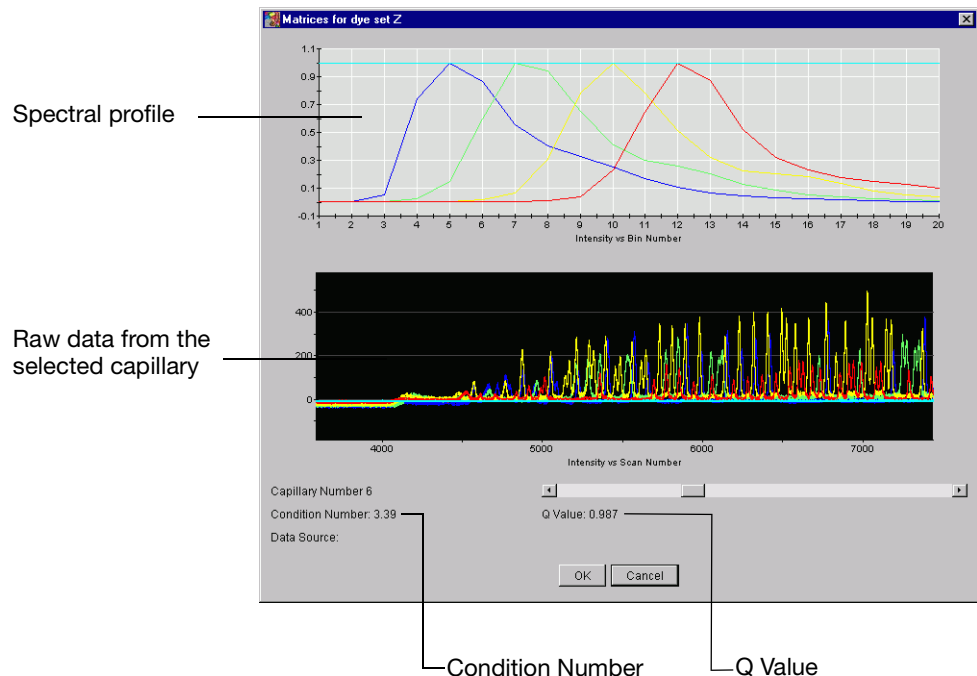
Figure 32   Example output from a spectral calibration using sequence standards

Corrective Actions and Troubleshooting

If the spectral calibration failed, or if you did not like the appearance of a calibration that passed, try one or more of the following:

- Check instrument status for any run errors**.**
- Verify the correct parameter file and run module were selected (see Table 14 on page 132). Correct and repeat run.
- Check the freshness and preparation of reagents.
- Check for possible contamination of matrix standards.
- Make sure that there are no bubbles in the sample wells**.**
- Verify that all peaks were detected.

  A slow running system can cut off the blue peak partially or entirely. Add time to the run or change reagents (if they are suspect) and repeat the run.
- Use the Override Spectral Calibration command to replace a matrix with a neighboring one.

Note: There may be instances when the spectral calibration passes in the software, yet the quality of the calibration is not acceptable. In these instances, you can override the calibration.

For troubleshooting, the spectral calibration log file is located in: E:\
AppliedBiosystems\UDC\DataCollection\data*instrument_model*\
SpectralCalTmpFiles\Z, where *instrument_model* is the model of your instrument.

If you are creating a matrix on the 310 instrument, please refer to the *ABI PRISM*® *310 Genetic Analyzer User Guide* (PN 4317588).

Creating the Plate Record in Data Collection Software

The Plate Record   Create a plate record for each plate to be run. A plate records consists of the following information about the plate and all samples on the plate.

For detailed information about creating the plate record, see the appropriate user guide for your instrument.

- Plate information:
  - ID or barcode number (for 3730/3730*xl* instruments only)
  - Plate name
  - Plate type
  - Application type
  - Owner name
  - Operator name
- For each sample:
  - Sample name
  - Comment *(optional)*
  - Results group – Contains settings for file names and locations, the sequencing analysis software in use, and the autoanalysis setting (see page 136).
  - Analysis protocol – Contains settings necessary for analysis and post-processing of the sequence (see page 143).
  - Instrument protocol – Contains settings for running the instrument (see page 151).

Note: For 310 instruments, the injection list contains information about the samples, not the plate record. Use the 310 Data Collections preferences to specify file names.

Defining the Results Group

Results Group Contents   The results group defines:

- Results group name
- Analysis type
- Location for the *.ab1 sample files and any additional files generated by the analysis
- Naming conventions to be used for sample files and the run folder
- AutoAnalysis setting

Only the Autoanalysis setting is described here.

Autoanalysis Setting   In the Data Collection Software Results Group settings, choose whether to analyze your sequencing files automatically, immediately after the electrophoresis run, or manually.

- **Autoanalysis** – With autoanalysis, the software applies analysis protocols to sequencing files immediately after Data Collection Software collects the data from the instrument.

  Note: Because autoanalysis is performed on the same computer that collects the data, you must register the analysis software with the Data Collection Software.

- **Manual analysis** – With manual analysis, you obtain the sequencing files from the computer connected to the instrument, then move or copy the files to another computer that has Sequencing Analysis, Variant Reporter, or SeqScape Software installed. To perform analysis, you apply the analysis protocols to the sequencing files.

Defining the Instrument Protocol

Instrument Protocol Contents

The instrument protocol contains settings for running the instrument:

- Protocol name and type
- Run module
- Dye set
- Polymer
- Capillary array length
- Chemistry

Only the run module is discussed in detail in this section.

Selecting a Run Module

Run Module Contents

The run module contains settings required for electrophoresis, including:

- Oven temperature
- Prerun voltage and time
- Injection voltage and time
- Run voltage settings
- Current settings
- Run time

Available Run Modules

Use the tables in this section to select a run module based on the desired read length and the run time for your sequencing chemistry. Choosing the incorrect run module can result in data starting earlier or later than expected (see).

- Table 16, "3730/3730xl instrument run modules," on page 138
- Table 17, "3130/3130xl instrument run modules," on page 138
- Table 18, "3100 instrument run modules," on page 139
- Table 19, "3100-Avant instrument run modules," on page 139

Note: In Tables 16 through 20, read lengths are based on BigDye Terminator v3.1 sequencing standards. These read lengths are minimum specifications for BigDye Terminator v3.1 sequencing standards. Average base spacing values for the KB basecaller and the ABI basecaller range from 10 to 24.

Table 16   3730/3730*xl* instrument run modules

Run Module	Array Length (cm)	Run Time (minutes)	Throughput (samples/day)		Read Length (bases)	
			3730 Analyzer	3730*xl* Analyzer	ABI Basecaller[‡]	KB Basecaller[§]
TargetSeq™ Resequencing System	36	20	3456[#]	6912[#]	NA	400
Rapid sequencing	36	35	1920	3840	500	550
Standard sequencing	36	60	1152	2304	650	700
Fast sequencing	50	60	1152	2304	NA	700
Long sequencing	50	120	576	1152	800	850
Extra long sequencing	50	180	384	768	NA	900

‡ Read length using the ABI basecaller with 98.5% basecalling accuracy.

§ Read length using the KB basecaller with QVs of 20 (99% basecalling accuracy).

Throughput numbers for 400bp reads (Long read standards—HSP69 template). The run module can be customized to run 200-400 bases.

Table 17   3130/3130*xl* instrument run modules

Run Module	Array Length (cm)	Polymer	Run Time (minutes)	Throughput (samples/day)		Read Length[‡] (bases)
				3130 Analyzer	3130*xl* Analyzer	
Ultra rapid sequencing	36	POP-4	40	144	576	400
Rapid sequencing	36	POP-6	60	96	384	500
Ultra rapid sequencing	36	POP-7	35	164	656	500
Rapid sequencing	36	POP-7	60	96	384	600
Standard sequencing	50	POP-4	100	56	224	600
Standard sequencing	50	POP-6	150	36	144	600
Fast sequencing	50	POP-7	60	96	384	700
Standard sequencing	50	POP-7	120	48	192	850
Long sequencing	80	POP-4	210	24	96	700

Table 17   3130/3130*xl* **instrument run modules** *(continued)*

Run Module	Array Length (cm)	Polymer	Run Time (minutes)	Throughput (samples/day)		Read Length‡ (bases)
				3130 Analyzer	3130*xl* Analyzer	
Long sequencing	80	POP-7	170	32	128	950

‡ Read length using the KB basecaller with QVs of 20 (99% basecalling accuracy).

Table 18   3100 instrument run modules

Run Module	Array Length (cm)	Polymer	Run Time (minutes)	Throughput (samples /day)	Read Length (bases)	
					ABI Basecaller ‡	KB Basecaller§
Ultra rapid sequencing	36	POP-4	40	576	500	400
Rapid sequencing	36	POP-6	60	384	500	500
Standard sequencing	50	POP-4	100	224	NA	600
Standard sequencing	50	POP-6	150	144	650	600
Long read sequencing	80	POP-4	210	96	950	700

‡ Read length using the ABI basecaller with 98.5% basecalling accuracy.
§ Read length using the KB basecaller with QVs of 20 (99% basecalling accuracy).

Table 19   3100-*Avant* **instrument run modules**

Run Module	Array Length (cm)	Polymer	Run Time (minutes)	Throughput (samples /day)	Read Length (bases)	
					ABI Basecaller ‡	KB Basecaller§
Ultra rapid sequencing	36	POP-4	40	144	500	400
Rapid sequencing	36	POP-6	60	96	500	500
Standard sequencing	50	POP-4	100	53	NA	600
Standard sequencing	50	POP-6	150	36	650	600
Long read sequencing	80	POP-4	210	24	950	700

‡ Read length using the ABI basecaller with 98.5% basecalling accuracy.
§ Read length using the KB basecaller with QVs of 20 (99% basecalling accuracy).

Table 20   310 instrument run modules

Run Module	Capillary Length (cm)	Polymer	Run Time (minutes)	Throughput (samples /day)	Read Length (bases)	
					ABI Basecaller ‡	KB Basecaller§
Standard sequencing	47	POP-4	50	27	525	650
Rapid sequencing	47	POP-4	38	37	425	600
Rapid sequencing	47	POP-6	60	24	400	500
Standard sequencing	61	POP-6	180	8	600	700

‡ Read length using the ABI basecaller with 98.5% basecalling accuracy.

§ Read length using the KB basecaller with QVs of 20 (99% basecalling accuracy).

Run Modules for BigDye® XTerminator™ Purified Samples

Applied Biosystems provides BigDye XTerminator Purification Kit run modules for the 3730/3730*xl,* 3130/3130*xl*, and 3100/3100-*Avant* Data Collection Software v2.0 or greater. Use these run modules when you run a plate containing BigDye XTerminator reagents. The modules adjust the sample injection height to prevent injection failures

These run modules can be downloaded from the Applied Biosystems web site. Refer to Appendix A in the *BigDye® XTerminator Purification Kit Protocol* (PN 4374408) for instructions on installing these run modules.

Note: If you transfer the supernatant to a clean plate after centrifuging, refer to Tables 16 through 20 for the appropriate run modules for your instrument.

Table 21   BigDye® XTerminator™ Purification Kit run modules

Run Module	Capillary Length (cm)	Polymer‡	Read Length§ (bases)
3730/3730*xl* DNA Analyzer			
BDx_XLRSeq50_POP7	50	POP-7™	900
BDx_LongSeq50_POP7			850
BDx_FastSeq50_POP7			700
BDx_StdSeq36_POP7	36	POP-7™	700
BDx_RapidSeq36_POP7			550
Applied Biosystems 3100/3100-*Avant*, 3130/3130*xl* Genetic Analyzer			
BDx_LongSeq80_POP7	80	POP-7™	950
BDx_LongSeq80_POP4		POP-4™	700

Table 21   BigDye® XTerminator™ Purification Kit run modules *(continued)*

Run Module	Capillary Length (cm)	Polymer‡	Read Length§ (bases)
BDx_StdSeq50_POP7	50	POP-7™	850
BDx_StdSeq50_POP6		POP-6™	600
BDx_StdSeq50_POP4		POP-4™	600
BDx_FastSeq50_POP7		POP-7™	700
BDx_RapidSeq36_POP7	36	POP-7™	600
BDx_RapidSeq36_POP6		POP-6™	500
BDx_UltraSeq36_POP7		POP-7™	500
BDx_UltraSeq36_POP4		POP-4™	400

‡ POP-7 can only be used on 3730/3730*xl* and 3130/3130*xl* instruments.

§ Read length using the KB basecaller with QVs of 20 (99% basecalling accuracy).

Modifying Run Modules

The default run modules provided by Applied Biosystems are optimized for many applications. If you choose to modify run modules, you can use this section for guidelines on modifying electrokinetic injection and electrophoresis conditions.

For More Information

For more information on modifying run modules, refer to your instrument user guide:

- *Applied Biosystems 3730/3730xl DNA Analyzer Getting Started Guide* (PN 4359476)
- *Applied Biosystems 3130/3130xl Genetic Analyzers Getting Started Guide* (PN 4352715)
- *ABI PRISM® 3100/3100-Avant Genetic Analyzers User Guide* (PN 4347102)
- *ABI PRISM® 310 Genetic Analyzer User Guide* (PN 4317588)

Modifying Electrokinetic Injection

The best way to control the amount of fluorescence signal generated during a run is to optimize the amount of DNA template that you use in your sequencing reactions (see page 63). However, if you consistently observe that the signal is too strong or too weak or that the resolution is poor, you may modify the injection parameters. The goal is to inject sufficient DNA to yield peaks of adequate height (that is, data with a good signal-to-noise ratio) while maintaining resolution and read length.

Modifying the injection parameters should be viewed as a temporary fix. You should perform further troubleshooting to determine the root cause of the problem, in order to extend the lifetime of the capillary array and other system components.

IMPORTANT! Modifying electrokinetic injection can greatly affect data quality and run-to-run reproducibility.

Note: For information on setting electrokinetic injection values, refer to your instrument user guide.

Reducing Signal Strength

If the signal is too strong:

- Decrease the concentration of DNA fragments in the sample.
- Decrease the injection time.
- Decrease the injection voltage.

Note: Strong signal intensity can also be a symptom of a late start. If you have a late start, check the DNA concentration and adjust as necessary.

Increasing Signal Strength

If the signal is too weak:

- Increase the concentration of the DNA extension products.
- Increase the injection time to increase the total electrokinetic injection (EKI) product. However, increased template loads may result in decreased resolution.
- Reduce the amount of salt in the sample.

  IMPORTANT! Negative ions (for example, EDTA and acetate) compete with DNA for injection. To reduce the amount of salt in a sequencing reaction, use column purification.

Note: Applied Biosystems does not recommend increasing the voltage to increase signal for runs on multicapillary instruments. Increasing the voltage may reduce resolution across the capillary array. It is better to adjust the injection time to increase signal.

Improving Resolution

If the resolution needs to be improved:

- Decrease the injection time. Decreasing the injection time decreases the signal strength. To compensate for the loss in signal, lower the salt concentration in the sample or increase the DNA extension product concentration.
- Decrease the run voltage. The rapid protocol is especially sensitive to changes in the running voltage. A 10% decrease in running voltage (along with a 20% increase in collection time) results in a marked improvement in resolution. The standard protocol is much less responsive to decreases in running voltage.

Modifying Electrophoresis Conditions

Modifying electrophoresis conditions (run time, run voltage, and run temperature) can greatly improve data quality, run-to-run reproducibility, and/or throughput. When selecting values for these parameters, consider the following factors:

- Desired read length

• Required degree of resolution

Determining the Required Run Time

To ensure that you collect sufficient data to perform analysis, set the electrophoresis run time approximately 5 to 10 minutes longer than the migration time of the longest fragment to detect. You can change the data collection time for special requirements. For example, you can shorten the data collection time if you only need information about short extension products (for example, in PCR sequencing).

Run Temperature and Run Voltage

Decreased run voltage or temperature decreases the migration rate of fragments. Longer run times are required to collect the same size fragments as in standard conditions. However, the basecallers provided with the Sequencing Analysis Software have not been optimized to analyze under some modified run conditions; therefore, the basecallers may not estimate the spacing values successfully.

Increased run voltage or temperature increases migration rates, allowing for shorter run times, but decreased resolution.

Defining the Analysis Protocol

Analysis protocol are shared between Data Collection Software and any of the following: Sequencing Analysis Software, SeqScape Software, MicroSeq ID Analysis Software. (Variant Reporter Software also contains analysis protocols but it does not interact with Data Collection Software.)

Note: On 310 instruments, create the analysis protocol in Sequencing Analysis Software using the Analysis Protocol Manager.

Analysis Protocol Contents

An analysis protocol contains the settings necessary to determine the sequence and to perform post-processing of the sequence:

• **General settings** – The name, description of the analysis protocol, and the sequence file formats to be generated (page 143).
• **Basecalling settings** – The basecaller, mobility file, analysis stop point, and other options used by the basecaller (page 144).
• **Mixed bases settings** *(optional)* – Setting for identifying mixed bases when two peaks are found at the same position. Definition for the percent value of the second highest to the highest peak (page 149).
• **Clear range setting** – Setting for trimming the low quality sequences that are typically at the 5′ and 3′ ends. The trimming can be based on base positions, sample quality values, and/or number of ambiguous bases (Ns) (page 150).

General Settings

Protocol Name

When you name your analysis protocol, assign a name that can help you distinguish between your analysis protocols.

Sequence File Formats

Select a format for the sequence files generated when analysis is completed. You save these files in the Sample Manager. The files are exported in the same run folder as the *.ab1 files.

Table 22   Sequence file format settings

Setting	Function
Write .Seq File	Select to create a *.seq file for printing the sequence as a text file or for use in other software. • Select **ABI** format to use with Applied Biosystems software. • Select **FASTA** for use with other software.
Write Clear Range in .Seq File	When Write .Seq File is selected, select to include only the clear range sequence (see page 150) in the *.seq file.
Write Standard Chromatogram Format (.scf)	Select to create a *.scf file to use with other software. **Note:** When created, the .scf extension is not appended to the file name.
Write Phred (.phd.1) File	Select to create *.phd.1 file to use with other software if you use the KB basecaller. Contains the bases with quality values for each base.

Basecalling Settings

Basecallers   Sequencing Analysis Software v5.x, SeqScape Software v2.x, and MicroSeq ID Analysis Software include two basecallers: the KB basecaller and the ABI basecaller. Both basecallers determine the bases of a sequence, but they differ in how they process the data.

Note: Variant Reporter Software v1.0 includes only the KB basecaller.

IMPORTANT! For bisulfite sequencing samples, you must use the KB basecaller.

Select a basecaller based on your sequencing chemistry and instrument from the tables in "Run Parameters for Instrument and Analysis Protocols" on page 151.

Table 23   Comparison of the KB and ABI basecallers

Attribute	KB Basecaller	ABI Basecaller
Functions	• Processes raw traces • Provides processed traces • Provides pure bases only *or* Provides pure and mixed calls • Provides quality values (QVs) • Generates *.phd.1 and *.scf files • Provides a sample score	• Processes raw traces • Provides processed traces • Provides ACGTN calls

Table 23   Comparison of the KB and ABI basecallers *(continued)*

Attribute	KB Basecaller	ABI Basecaller
Base assignment options	Four options available: • Assigns ACGT and QV to each peak • Assigns ACGT and QV to each peak; reassigns Ns to peaks with QVs below a defined threshold • Assigns ACGT or a mixed base and QV to each peak • Assigns ACGT or a mixed base and QV to each peak; reassigns Ns to peaks with QVs below a defined threshold	Assigns Ns to mixed bases **Note:** Further processing, either manually or with additional software, is required to assign IUB codes to the Ns or pure bases.
Base assignments for failed samples‡	Assigns 5 Ns to the entire sample Analysis report flags the files containing failed samples	Attempts to call bases For failed samples, many Ns are assigned
Baseline appearance in processed data	Less smooth	Smoother
Data processing	Calls bases and estimates QVs on Windows operating systems	Calls bases on Windows operating systems
Data and future support	Applied Biosystems 3730/3730*xl*, 3130/3100*xl,* 3100/3100-*Avant, and* 310 instruments Development ongoing	Applied Biosystems 3730/3730*xl*, 3130/3100*xl,* 3100/3100-*Avant, and* 310 instruments Development ceased 2003

‡ Failed samples provide no signals and indicate chemistry failure.

For more information on the KB basecaller, refer to the *KB™ Basecaller v1.4 FAQ: KB Basecaller Software v1.4* (PN 4362968),

Mobility File   A mobility file contains mobility shift information. The different dyes affect the electrophoretic mobility of cycle sequencing extension products, especially for shorter fragments. However, these mobility shifts are consistent for each dye set, and they can be corrected during analysis. The analysis software compensates for these mobility differences by applying mobility shifts to the data so that evenly spaced peaks are presented in the analyzed data. The mobility file also corrects for the differences between the dye-to-nucleotide relationships in the raw data and the analyzed data.

Selecting a Mobility File

Select a mobility file that matches the chemistry and basecaller that you are using. Mobility shifts and mobility file names for the dRhodamine Terminators are similar to those for the BigDye Terminators, but the dye-set information differs because the terminators are labeled with different dyes. Make sure that you use the correct mobility file to prevent miscalling the C and T bases.

Note: If you select the wrong mobility file, you can change analysis settings and reanalyze the data with the correct mobility file.

Tables 25 through 29, starting on page 151, list the appropriate mobility files for each instrument based on the dye chemistry, polymer, and capillary array to be used. Refer to your instrument user guide for more specific information about choosing a mobility file.

Mobility File Names

Mobility file names use a combination of characters to indicate the basecaller, instrument, chemistry, and polymer type.

Table 24   Naming conventions for mobility files

Abbreviation	For Runs Using...
Basecaller	
KB	KB basecaller
DP	Dye primer chemistry and the ABI basecaller
DT	Dye terminator chemistry and the ABI basecaller
Type of Polymer	
POP4	POP-4™ polymer
POP5	POP-5™ polymer
POP6	POP-6™ polymer
POP7	POP-7™ polymer
Chemistry	
BDTv3 {BDv3}	BigDye® Terminators v3.1 and dGTP BigDye® Terminators v3.0
{BDv1} {BD}	BigDye® Terminators v1.1 and dGTP BigDye® Terminators
{dRhod}	dRhodamine Terminator Cycle Sequencing Kits
{-21M13}	Dye primer chemistry with the 21 M13 forward primer labeled
{M13Rev}	Dye primer chemistry with the M13 reverse primer labeled

Figure 32 shows an example of a mobility file name.

KB_3730_POP7_BDTv3.mob
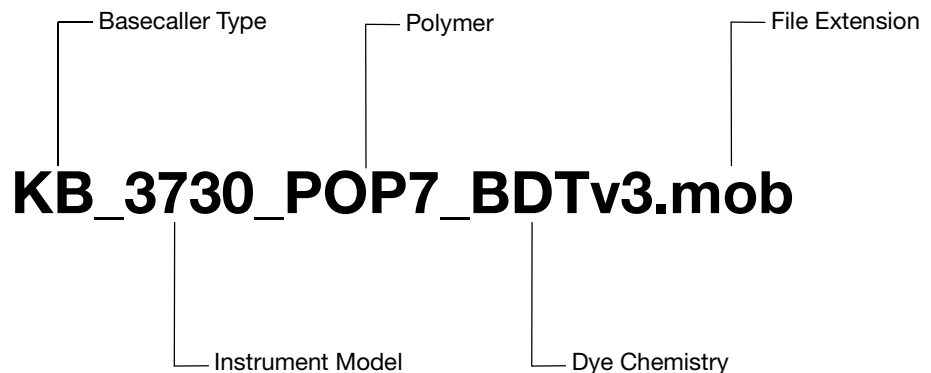
Figure 33   Mobility file name, beginning with the basecaller abbreviation

Profile

The profile selection scales the processed traces for display in the electropherogram or the analyzed data view.

Setting	Function
True Profile	Select to scale the processed traces uniformly. The average height of peaks in the region of strongest signal is about equal to a fixed value. The profile of the processed traces is very similar to that of the raw traces.
Flat Profile	Select to scale the processed traces semi-locally. The average height of peaks in any region is about equal to a fixed value. The profile of the processed traces is flat on an intermediate scale (greater than about 40 bases). **Note:** This option can be applied only to data analyzed with the KB basecaller. If you use the ABI basecaller, the profile option reverts to the True Profile.

Ending Base

With reasonable data quality without sequencing artifacts at the 3′ end, the basecaller can identify the correct stop point.

The ending base options in the analysis protocol allow you to define an ending base for the basecaller. You may designate one or more endpoints for data analysis. If you select all options, the ending base is determined based on the value you enter in the **After __ Bases** check box.

Setting	Function
At PCR Stop check box	When the check box is selected, the software determines the analysis endpoint by locating the signal drop that occurs at the end of a short PCR fragment (the PCR endpoint). If the endpoint peak is not sufficiently large, the software may not recognize the PCR endpoint. **Note:** Noise after the PCR data is considered by the software as signal, and the software incorrectly calculates the endpoint after the noise. **IMPORTANT!** Applied Biosystems recommends selecting this check box when analyzing short PCR fragments with the KB basecaller.
After __ Ns in __ bases check box[‡]	Sets the analysis endpoint after a certain number of Ns occur within a certain number of bases (for example, after 5 Ns are detected within a range of 10 bases). The window described by **in __ bases** differs depending on the basecaller selected: • KB basecaller − the window begins at the 51st base. • ABI basecaller − the window begins at the 1st base.
After __ Ns check box[‡]	Sets the analysis endpoint after a certain number of Ns occur (for example, after 20 Ns are detected). The basecaller starts calling bases from the 5′ end of the sequence and stops when the specified number of Ns is reached.
After __ Bases check box	Sets the analysis endpoint after a certain number of bases (for example, after 800 bases are detected).

‡ To select this option, you must also select Assign 'N' for bases with QV < 15 in the Quality Threshold section (see below). This setting applies only to the KB basecaller.

Quality Threshold (KB Basecaller Only)

The quality threshold settings determine how base assignments are made with the KB basecaller.

Setting	Function
Call all bases and assign QV	With the KB basecaller, assigns a base and QV to every position.
	When the resolution of the data is poor, the KB basecaller provides the best estimate of the basecall and assigns an appropriate QV to help the user interpret the accuracy of the basecall.
Assign 'N' for bases with QV < X	With the KB basecaller, assigns Ns for bases with QVs less than the set point. Useful if the downstream software application that you use to view the electropherograms cannot display the QV bars assigned by the KB basecaller.
	IMPORTANT! Use this option with caution if you expect the data to contain mixed bases. Because mixed bases are typically assigned QVs lower than pure bases (see "Mixed Bases" on page 149), high quality mixed base calls may be substituted with Ns.

Note the difference in how the KB basecaller and the ABI basecaller make N calls:

- **KB basecaller** – Assigns Ns for bases below a certain user-defined QV.
- **ABI basecaller** – Assigns Ns when the basecaller cannot determine whether the base is an A, C, G, T, or a mixed base.

Mixed Bases

The mixed bases settings define whether to identify the presence of mixed bases and the threshold for the identification of mixed bases. Mixed bases settings are active only with the KB basecaller.

Mixed Bases Settings (KB Basecaller Only)

Setting	Function
Use Mixed Base Identification‡	Select to assign mixed bases. This setting is required but not sufficient for the KB basecaller to assign mixed bases.
2nd highest peak threshold	Enter a threshold for secondary peaks:
	• Positions with secondary peaks below the set value are not called as mixed bases.
	• Positions with secondary peaks above the set value may be called as mixed bases, depending on the KB basecaller evaluation of other settings, such as noise level in the sample and peak width.
	Note: Do not enter a value less than 15%.

‡ When Use Mixed Base Identification is selected, you must unselect N in the Basecalling tab.

Clear Range

The clear range is the region of sequence that remains after excluding the low-quality or error-prone sequence at the 5′ and 3′ ends. In the Electropherogram and Sequence views, the excluded data is displayed in gray. The bases outside the clear range cannot be edited.

Clear Range Settings

You can choose one or more methods to set the clear range. If you apply multiple methods, the methods are applied in order to create a sequence with the smallest clear range.

Setting	Function
Use clear range minimum and maximum	Select to set the clear range based on specified starting and ending base. Ending base can be specified as a numeric value (for example 500 bases after the start) or as a number of bases to trim from the 3′ end.
Use quality values	Select to set the clear range based on the number of bases in a window with a specified quality value. For example, remove bases from the ends until fewer than 4 bases out of 20 have QVs <20.
Use identification of N calls	Select to set the clear range based on the number of bases in a window with a specified number of Ns. For example, remove bases from the ends until there are fewer than 4 Ns out of 20 bases.

Run Parameters for Instrument and Analysis Protocols

Use the tables in this section to identify the appropriate Data Collection Software parameters for the instrument and analysis protocols.

Table 25   3730/3730*xl* instrument run parameters

DNA Sequencing Chemistry	Dye Set	Polymer Type	Capillary Array Length (cm)	Run Module	Basecaller File (.bcp)	Mobility File (.mob)‡
KB Basecaller						
BigDye Terminator v1.1 and dGTP BigDye Terminator	E	POP-7	all lengths	all types	KB	KB_3730_POP7_BDTv1
BigDye Terminator v3.1 and dGTP BigDye Terminator v3.0	Z	POP-7	all lengths	all types	KB	KB_3730_POP7_BDTv3
ABI Basecaller						
BigDye Terminator v1.1 and dGTP BigDye Terminator v3.0	E	POP-7	36	Rapid	Basecaller-3730POP7RR	DT3730POP7{BD}
			36	Standard	Basecaller-3730POP7SR	
			50	Long	Basecaller-3730POP7LR	
BigDye Terminator v3.1 and dGTP BigDye Terminator v3.0	Z	POP-7	36	Rapid	Basecaller-3730POP7RR	DT3730POP7{BDv3}
			36	Standard	Basecaller-3730POP7SR	
			50	Long	Basecaller-3730POP7LR	

‡ Mobility files are not available for running dRhodamine terminator chemistry or BigDye primer chemistry on 3730/3730*xl* instruments.

Table 26   3130/3130*xl* instrument run parameters

DNA Sequencing Chemistry	Dye Set	Polymer Type	Capillary Array Length (cm)	Run Module	Basecaller File (.bcp)	Mobility File (.mob)
KB Basecaller						
BigDye® Terminator v1.1 and dGTP BigDye Terminator	E	POP-4	36	Ultra rapid	KB	KB_3130_POP4_BDTv1
			50	Standard		
			80	Long		
		POP-6	36	Rapid	KB	KB_3130_POP6_BDTv1
			50	Standard		
		POP-7	36	Ultra rapid	KB	KB_3130_POP7_BDTv1
			50	Fast		
			80	Long		
			36	Rapid		
			50	Standard		
BigDye® Terminator v3.1 and dGTP BigDye Terminator v3.0	Z	POP-4	36	Ultra rapid	KB	KB_3130_POP4_BDTv3
			50	Standard		
			80	Long		
		POP-6	36	Rapid	KB	KB_3130_POP6_BDTv3
			50	Standard		
		POP-7	36	Ultra rapid	KB	KB_3130_POP7_BDTv3
			50	Fast		
			80	Long		
			36	Rapid		
			50	Standard		
ABI Basecaller						
BigDye Terminator v1.1 and dGTP BigDye Terminator	E	POP-4	36	Ultra rapid	Basecaller-3130POP4UR	DT3100POP4LR{BD}v1
			36	Rapid	Basecaller-3130POP4UR	DT3100POP6{BD}v1
		POP-6	50	Standard	Basecaller-3130POP6SR	
BigDye Terminator v3.1 and dGTP BigDye Terminator v3.0	Z	POP-4	36	Ultra rapid	Basecaller-3130POP4UR	DT3100POP4{BDv3}v1
			80	Long	Basecaller-3130POP4_80cmv3	
		POP-6	36	Rapid	Basecaller-3130POP6RRv2	DT3100POP6{BDv3}v1

Table 26   3130/3130*xl* instrument run parameters *(continued)*

DNA Sequencing Chemistry	Dye Set	Polymer Type	Capillary Array Length (cm)	Run Module	Basecaller File (.bcp)	Mobility File (.mob)
dRhodamine Terminator	E	POP-4	36	Ultra rapid	Basecaller-3130POP4UR	DT3100POP4{dRhod}v2
		POP-6	36	Rapid	Basecaller-3130POP6RRv2	DT3100POP6{dRhod}v2
			50	Standard	Basecaller-3130POP6SR	
BigDye Primer	E	POP-6	36	Rapid	Basecaller-3130POP6RRv2	DP3130POP6{BD-21M13}v1 DP3130POP6{BD-21M13Rev}v1
			50	Standard	Basecaller-3130POP6SR	

Table 27   3100 instrument run parameters

DNA Sequencing Chemistry	Dye Set	Polymer Type	Capillary Array Length (cm)	Run Module	Basecaller File (.bcp)	Mobility File (.mob)
KB Basecaller						
BigDye® Terminator v1.1 and dGTP BigDye Terminator	E	POP-4	36	Ultra rapid	KB	KB_3100_POP4_BDTv1
			50	Standard		
			80	Long		
		POP-6	36	Rapid	KB	KB_3100_POP6_BDTv1
			50	Standard		
BigDye Terminator v3.1 and dGTP BigDye Terminator v3.0	Z	POP-4	36	Ultra rapid	KB	KB_3100_POP4_BDTv3
			50	Standard		
			80	Long		
		POP-6	36	Rapid	KB	KB_3100_POP6_BDTv3
			50	Standard		
ABI Basecaller						
BigDye Terminator v1.1 and GTP BigDye Terminator	E	POP-4	36	Ultra rapid	Basecaller-3100POP4UR	DT3100POP4LR{BD}v1
		POP-6	36	Rapid	Basecaller-3100POP6RRv2	DT3100POP6{BD}v2
			50	Standard	Basecaller-3100POP6SR	
BigDye Terminator v3.1 and GTP BIgDye Terminator v3.0	Z	POP-4	36	Ultra rapid	Basecaller-3100POP4UR	DT3100POP4{BDv3}v1
			80	Long	Basecaller-3100POP4_80cmv3	
		POP-6	36	Rapid	Basecaller-3100POP6RRv2	DT3100POP6{BDv3}v1
			50	Standard	Basecaller-3100POP6SR	

Table 27   3100 instrument run parameters *(continued)*

DNA Sequencing Chemistry	Dye Set	Polymer Type	Capillary Array Length (cm)	Run Module	Basecaller File (.bcp)	Mobility File (.mob)
dRhodamine Terminator	E	POP-4	36	Ultra rapid	Basecaller-3100POP4UR	DT3100POP4{dRhod}v2
		POP-6	36	Rapid	Basecaller-3100POP6RRv2	DT3100POP6{dRhod}v2
			50	Standard	Basecaller-3100POP6SR	
BigDye Primer	E	POP-6	36	Rapid	Basecaller-3100POP6RRv2	DP3100POP6{BD-21M13}v1 DP3100POP6{BD-M13Rev}v1
			50	Standard	Basecaller-3100POP6SR	

Table 28   3100-*Avant* instrument run parameters

DNA Sequencing Chemistry	Dye Set	Polymer Type	Capillary Array Length (cm)	Run Module	Basecaller File (.bcp)	Mobility File (.mob)
KB Basecaller						
BigDye® Terminator v1.1 and dGTP BigDye Terminator	E	POP-4	36	Ultra rapid	KB	KB_3100_POP4_BDTv1
			50	Standard		
			80	Long		
		POP-6	36	Rapid	KB	KB_3100_POP6_BDTv1
			50	Standard		
BigDye® Terminator v3.1 and dGTP BigDye Terminator v3.0	Z	POP-4	36	Ultra rapid	KB	KB_3100_POP4_BDTv3
			50	Standard		
			80	Long		
		POP-6	36	Rapid	KB	KB_3100_POP6_BDTv3
			50	Standard		
ABI Basecaller						
BigDye Terminator v1.1 and dGTP BigDye Terminator	E	POP-4	36	Ultra rapid	Basecaller-3100APOP4UR	DT3100POP4LR{BD}v1
		POP-6	36	Rapid	Basecaller-3100APOP6RRv2	DT3100POP6{BD}v2
			50	Standard	Basecaller-3100APOP6SR	
BigDye Terminator v3.1 and dGTP BigDye Terminator v3.0	Z	POP-4	36	Ultra rapid	Basecaller-3100APOP4UR	DT3100POP4{BDv3}v1
			80	Long	Basecaller-3100APOP4_80cmv3	
		POP-6	36	Rapid	Basecaller-3100APOP6RRv2	DT3100POP6{BDv3}v1

Table 28   3100-*Avant* instrument run parameters *(continued)*

DNA Sequencing Chemistry	Dye Set	Polymer Type	Capillary Array Length (cm)	Run Module	Basecaller File (.bcp)	Mobility File (.mob)
dRhodamine Terminator	E	POP-4	36	Ultra rapid	Basecaller-3100APOP4UR	DT3100POP4{dRhod}v2
		POP-6	36	Rapid	Basecaller-3100APOP6RRv2	DT3100POP6{dRhod}v2
			50	Standard	Basecaller-3100APOP6SR	
BigDye Primer	E	POP-6	36	Rapid	Basecaller-3100APOP6RRv2	DP3100POP6{BD-21M13}v1 DP3100POP6{BD-M13Rev}v1
			50	Standard	Basecaller-3100APOP6SR	

Table 29   310 instrument run parameters

DNA Sequencing Chemistry	Dye Set	Polymer Type	Capillary Array Length (cm)	Basecaller File (.bcp)	Mobility File (.mob)
KB Basecaller					
BigDye® Terminator v1.1 and dGTP BigDye® Terminator	E	POP-4	47	KB	KB_310_POP4_BDTv1_36Rapid KB_310_POP4_BDTv1_36Std
		POP-6	47	KB	KB_310_POP6_BDTv1_36Rapid
		POP-6	61	KB	KB_310_POP6_BDTv1_50Std
BigDye® Terminator v3.1 and dGTP BigDye Terminator v3.0‡	E	POP-4	47	KB	KB_310_POP4_BDTv3_36Rapid KB_310_POP4_BDTv3_36Std
		POP-6	47	KB	KB_310_POP6_BDTv3_36Rapid
		POP-6	61	KB	KB_310_POP6_BDTv3_50Std
ABI Basecaller					
BigDye® Terminator v1.1 and dGTP BigDye® Terminator	E	POP-4	47	Basecaller-310POP4	DT310POP4{BD}v2
		POP-6	47	Basecaller-310POP6	DT310POP6{BD}
		POP-6	61	Basecaller-310POP6	DT310POP6{BD-LR}v3
BigDye® Terminator v3.1 and dGTP BigDye Terminator v3.0	Z	POP-4	47	Basecaller-310POP4	DT310POP4{BDv3}v2
		POP-6	47	Basecaller-310POP6	DT310POP6{BDv3}v2 DT310POP6{BDv3}v2
		POP-6	61		
dRhodamine Terminator	E	POP-4	47	Basecaller-310POP4	DT310POP4{dRhod}v1
		POP-6	47	Basecaller-310POP6	DT310POP6{dRhod}v2

Table 29   310 instrument run parameters *(continued)*

DNA Sequencing Chemistry	Dye Set	Polymer Type	Capillary Array Length (cm)	Basecaller File (.bcp)	Mobility File (.mob)
BigDye® Primer	E	POP-4	47	Basecaller-310POP4	DP310POP4{BD-21M13}v1 DP310POP4{M13Rev}v1
		POP-6	47	Basecaller-310POP6	DP310POP6{BD-21M13}v1 DP310POP6{M13Rev}v1
		POP-6	61		

‡ For instructions on running BigDye terminators v3.1 and dGTP BigDye terminators v3.0 on 310 instruments, refer to the 310 instrument user guide.

Data Analysis

<div style="text-align: right">7</div>

This chapter covers:

Overview

This chapter provides an overview of data analysis using Applied Biosystems software for DNA sequencing. The information in this chapter is a high-level view of the software and does not contain detailed instructions for using the software. For detailed instructions, please refer to the appropriate user guide and/or online help.

Workflow

DNA Template Preparation (Chapter 3)

Cycle Sequencing (Chapter 4)

Extension Product Purification (Chapter 5)
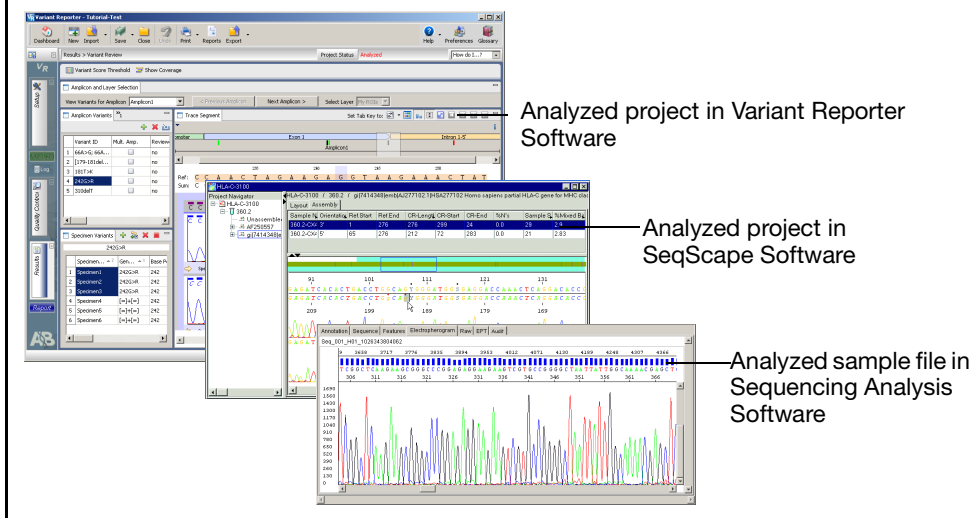
Capillary Electrophoresis (Chapter 6)

Data Analysis

1. Choose software for your application.

2. Apply analysis protocols, including:
   – Basecaller
   – Mobility file

3. Run analysis. ▶

4. Review the data.

Applied Biosystems software:

• Sequence Scanner Software
• Sequencing Analysis Software
• Variant Reporter™ Software
• SeqScape® Software
• MicroSeq® ID Analysis Software

Data Analysis Output:

Analyzed project in Variant Reporter Software

Analyzed project in SeqScape Software

Analyzed sample file in Sequencing Analysis Software

Analysis Software   The following analysis software is available from Applied Biosystems at
www.appliedbiosytems.com:

Table 30   Analysis software available from Applied Biosystems

Product	Suggested Use
Sequence Scanner Software	Viewing or editing traces, evaluating trace quality, making trace QC reports
Sequencing Analysis Software	Viewing or editing traces, evaluating trace quality, making trace QC reports, reanalyzing traces
Variant Reporter™ Software	Detecting mutations, discovering and validating SNPs
SeqScape® Software	Detecting mutations, discovering and validating SNPs, comparing sequences, typing
MicroSeq® ID Analysis Software	With the MicroSeq ID Kit, identifying bacteria and fungi

Choosing Analysis Software by Application

Answer the following questions to help you to choose the most appropriate package
for your lab or review "Choosing Analysis Software by Available Features" on
page 162.

1. What applications are performed in your lab with your genetic analyzer?

    Use Table 31 below to select an analysis software package.

    If the table recommends more than one type of software, go to 2.

Table 31   Analysis software recommendations, by application

Sequencing Application	Sequencing Analysis Software	Variant Reporter™ Software	SeqScape® Software
Sequence quality control	Yes	Yes	No
BAC end sequencing	Yes	No	No
Checking clone constructs	Not Recommended	Yes	Yes
De novo sequencing	Yes	No	No
Multi Locus Sequence Typing (MLST)	Not Recommended	No	Yes
HLA typing	Not Recommended	No	Yes
Methylation analysis using bisulfite DNA conversion	Not Recommended	Yes	Yes
Mitochondrial DNA (mtDNA) sequencing	No	Yes	Yes

Table 31   Analysis software recommendations, by application *(continued)*

Sequencing Application	Sequencing Analysis Software	Variant Reporter™ Software	SeqScape® Software
Resequencing – substitutions (SNPs), heterozygous mutations, insertions and deletions	Not Recommended	Yes	Yes
Virus subtyping	Not Recommended	No	Yes

2. Are 21CFR part 11 compliance features required in your lab?

    and/or

    Do you use BigDye Primer Chemistry?

    • If the answer is yes to either question, choose SeqScape Software.

       SeqScape Software includes 21CFR part 11 compliance features such as User Access Protection, Audit Trail and Electronic Signature and is also the only analysis software that can analyze data generated using BigDye Primer Chemistry.

    • If no, continue to 3.

3. Will you be analyzing projects without a reference sequence?

    and/or

    Do you need to analyze large projects (>500 traces)?

    • If the answer is yes to either question, choose Variant Reporter Software.

       This package can use higher quality traces to build its own reference, whereas SeqScape Software requires a reference file.

       Variant Reporter Software is specifically designed to handle up to 5000 traces in only one project. Basecalling, the most time-consuming part of the analysis, can be skipped if data are pre-basecalled by Sequencing Analysis Software.

    • If no, continue to 4.

4. If none of the above questions have helped you decide, you can:

    • Examine Table 32 for more information.

    • Download a demo version of either SeqScape Software or Variant Reporter Software from **www.appliedbiosystems.com**.

       To learn more, you can register for a web-based training course at **https://learn.appliedbiosystems.com/sequencing**.

    • Contact your Applied Biosystems sales representative or field application specialist.

Table 32 Features in Applied Biosystems analysis software

Software	Features and Functions

Sequencing Analysis Software	With Sequencing Analysis Software you can:
	
	• Perform basecalling and trim low quality bases at the 5′ and 3′ ends. With the KB™ basecaller, you can also:
	  – Identify pure and mixed bases
	  – Assign a quality value for each base
	  – Replace Ns for bases with quality values below a set threshold
	  – Detect failed sequence samples
	  – Calculate quality values (QV) for each base
	• Reanalyze the data by modifying analysis settings
	• Review data for troubleshooting using metrics provided in the Analysis Report
	• Export analyzed sequences to formats including *.seq, *.phd1 and *.scf to integrate with downstream workflows
	• Create an audit trail to track all changes to bases and analysis settings

Variant Reporter™ Software	With Variant Reporter Software, you can: • Analyze sequence data and generate an accurate consensus sequence for each specimen using bidirectional sequencing coverage • Call and edit bases using the improved algorithms and additional quality metrics in KB™ Basecaller v1.4 • Align the consensus sequence to identify variants • Import a reference sequence from a public database such as the National Center for Biotechnology Information (NCBI) • Customize the reference sequence by creating unique sequence layers composed of different features, such as exons, introns, and untranslated regions (UTRs) • Set quality control metrics to filter out low quality data, ensuring higher confidence results and reducing manual review time • Archive projects with sample files and all associated reference data (analysis settings, reference, etc.) for data sharing purposes
SeqScape® Software	With SeqScape Software, you can: • Analyze sequence data and generate an accurate consensus sequence for each specimen using bidirectional sequencing coverage • Call bases using the improved algorithms and additional quality metrics in KB™ Basecaller v1.4 • Import a reference sequence from a public database such as the National Center for Biotechnology Information (NCBI) • Compare sequence data to the known reference sequence to identify variants or to a library of sequences to identify the perfect match • Display amino acid variants in the consensus sequence
MicroSeq® ID Analysis Software	With MicroSeq ID Analysis software, you can: • Compare sequence data from unknown bacterial or fungal species to sequences of known bacteria or fungi stored in a library • Generate a final identification list of organisms that are the closest matches to the unknown, based on a percent similarity that reflects how closely the unknown isolate matches the library sequence

Table 32   Features in Applied Biosystems analysis software *(continued)*

Software	Features and Functions
Sequence Scanner Software	With Sequence Scanner Software, you can: • View, edit, print, and export sequence data • Align analyzed peaks to raw data peaks • Map traces to plate well positions to visualize quality patterns • Generate and export graphical reports • Export sequence data to file formats including *.fsta, *.seq, *.phd1 and *.scf

Choosing Analysis Software by Available Features

This section describes the different analysis tasks that can be performed by Applied Biosystems analysis software for capillary electrophoresis sequencing instruments.

- **Basecalling** – Translates the collected color-data images into the corresponding nucleotide basecalls.
- **Autoanalysis** – Enables automatic analysis after data collection from the Genetic Analyzer.
- **Sequence alignment with reference** – Aligns sample files (traces) to a user-supplied reference DNA sequence.
- **Sequence alignment, no reference** – Aligns sample files (traces) to each other to create a reference DNA sequence.
- **Mutation detection** – Compares the sample files (traces) to a reference DNA and detects nucleotide substitutions, insertions, deletions, and heterozygous insertion/deletions.
- **Typing** – Compares the sample files (traces) to a group of known reference DNA sequences and determines the best match.
- **Audit and e-signature features** – Assists with 21 CFR Part 11 compliance, providing audit trail, access control, and e-signature features.

Note: Table 33 lists the tasks that each analysis software performs.

Table 33   Tasks performed by Applied Biosystems analysis software

Feature	Software Package				
	Sequence Scanner Software	Sequencing Analysis Software	Variant Reporter™ Software	SeqScape® Software	MicroSeq® ID Analysis Software
Basecalling	No	Yes	Yes[‡]	Yes[‡]	Yes
Autoanalysis	No	Yes	No	Yes	Yes
Sequence alignment with reference	No	No	Yes	Yes	No
Sequence alignment, no reference	No	No	Yes	No	No
Mutation detection	No	No	Yes	Yes	No

Table 33   Tasks performed by Applied Biosystems analysis software *(continued)*

Feature	Software Package				
	Sequence Scanner Software	**Sequencing Analysis Software**	**Variant Reporter™ Software**	**SeqScape® Software**	**MicroSeq® ID Analysis Software**
Typing§	No	No	No	Yes	Yes
Auditing and electronic signature features (21 CFR Part 11)	No	Yes	No	Yes	Yes

‡ For reanalysis

§ With sequence library

Data Quality   Signal-to-noise ratios and variation in peak heights heavily influence the accuracy of heterozygote detection. If the data noise level is high, heterozygote detection algorithms are likely to detect many false positives that you will need to review manually.

The degree of variation in peak heights depends on the sequencing chemistry used. Because BigDye® Terminator chemistry produces very even signal intensities, the number of false negatives is reduced. Sequencing using alternative dye terminator chemistries may produce data with uneven peak heights, which may result in the failure to detect many true heterozygote locations.

Well-defined peak resolution, uniform peak spacing, and high signal-to-noise ratios characterize good quality sequencing data. These characteristics enable more accurate automated mixed-base identification, which saves time that might otherwise be required for manual sequence review and editing.

Reviewing Data with Sequence Scanner Software

Overview and Applications

Sequence Scanner Software allows you to view, edit, print, and export data generated using Applied Biosystems genetic analyzer instruments after data has been processed by Sequencing Analysis Software.

Software Workflow

1. Perform basecalling using Sequencing Analysis Software.

2. Import data into Sequence Scanner Software.

3. Review the results.

4. Export and print results and reports.

For More Information

See Chapter 8 for information on troubleshooting sequencing data.

Tips and Hints

- Sequence Scanner Software can be downloaded for free from the Applied Biosystems website at **www.appliedbiosystems.com/sequencescanner**.
- Exporting results of quality analysis as an Excel file or importing into a custom database is a good way to monitor system variables over time.

This section contains some frequently asked questions about Sequence Scanner Software.

- "How do I review and edit bases?" on page 164
- "How can I use the reports to review the data?" on page 166
- "How do I export traces or sequences?" on page 169

How do I review and edit bases?

1. Use the Sequence View to identify bases with low quality values.

   In the figure below:
   - Base 71 is red and displays a mixed base call of Y with a low quality value (QV) of 2 (shown in the tool tip).
   - Base 75 is also red, with a mixed base call of Y.
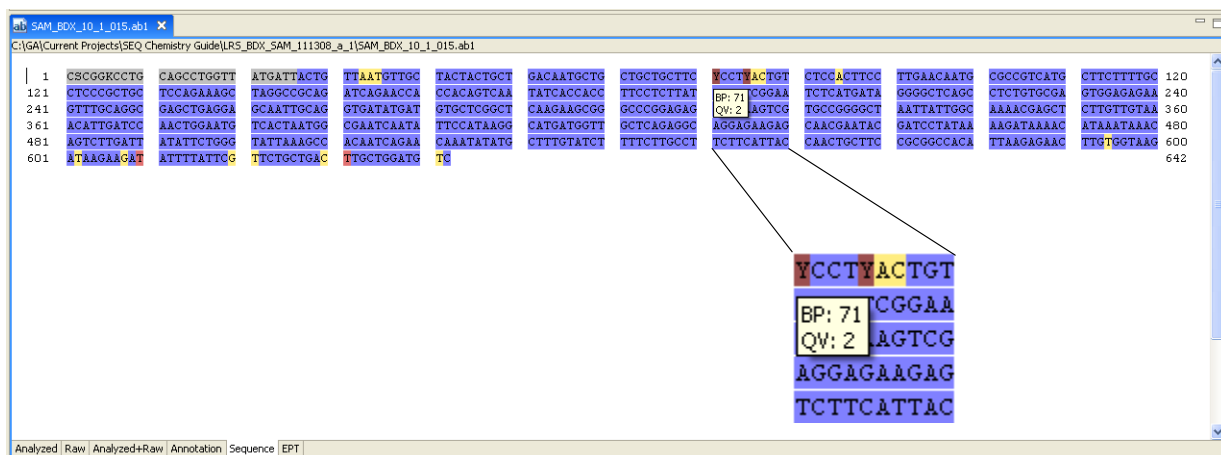
2. Click the base to select it, then click the **Analyzed+Raw** tab.
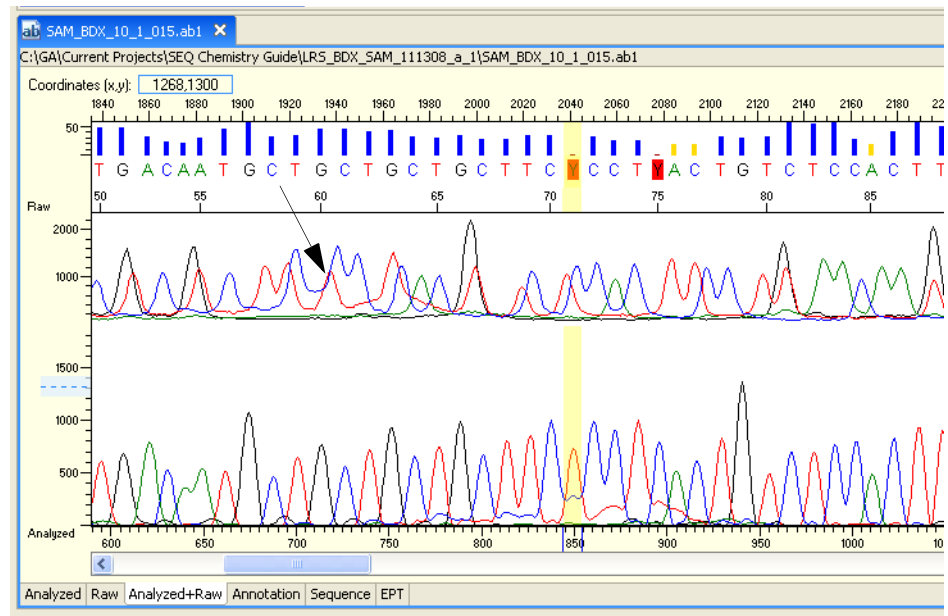
The yellow highlight indicates the selected base in the sequence and in the Analyzed data pane (at the bottom of the window).

The arrow shows the corresponding base in the raw data. (The raw data has different mobility than the analyzed data). In the Analyzed data pane it is apparent that there is an unambiguous T base at position 71 and a C base peak at position 75.

The mixed base call is caused by an elevated baseline due to poor resolution of the CTCC sequence.

3. Type over the nucleotide sequence to change the mixed base calls:

   - At base 71, change the mixed base call to T.
   - At base 75, change the mixed base call to C.
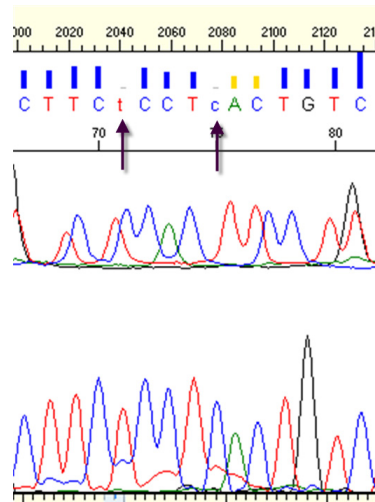
How can I use the reports to review the data?

1. Click the Reports tab to view the View Reports menu.

2. Click **Plate Report** to view the Plate Report.

    The Plate Report gives an at-a-glance overview of the data quality of the plate that was processed for sequencing. The prominent peak seen in some wells on the right side of the plate indicates the presence of a strong dye blob; these samples were not purified with sufficient BigDye Exterminator reagent prior to electrophoresis.

    Note that sample E4 (capillary 24) shows a red header bar; this indicates a failed result.

    Double-click any of the wells to view the corresponding trace.

3. Click **Signal Strength Report**.

The Signal Strength Report shows the average intensity of the raw signal for the four bases in each sample.

Note that there is considerable variation in signal strength from sample to sample. This is typical in most electrokinetic injections. The signal strength is not a suitable metric for overall data quality. However, if signal strength is below 200 rfu for a number of samples the data quality of the traces may appear poor and the validity of the data should be carefully examined. Re-injecting the samples using longer injection times may boost the signal strength.
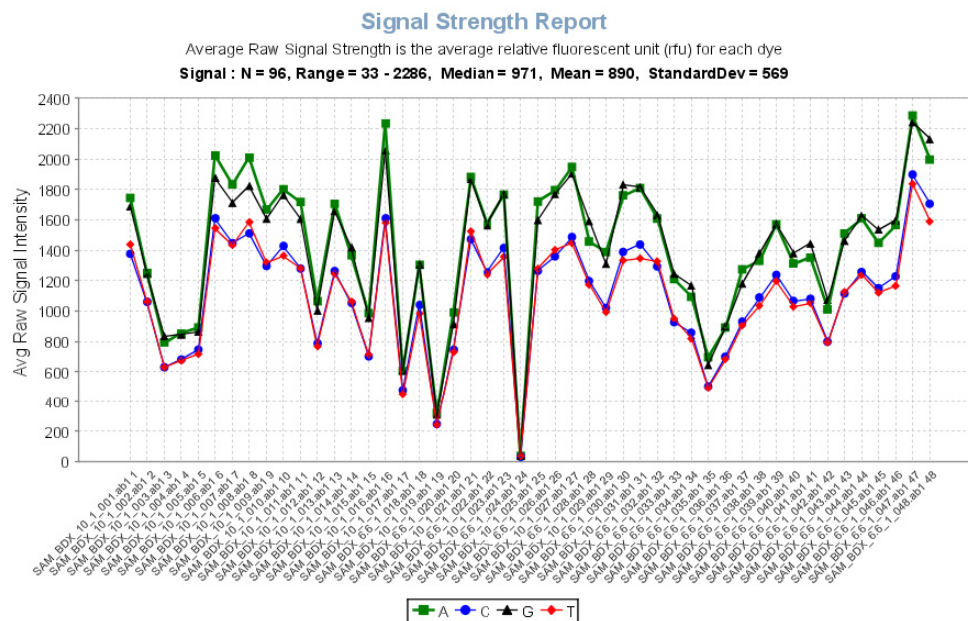
Click any of the data points in the graph to open the corresponding trace.

4. Use the Contiguous Read Length Report (CRL) to easily identify the longest uninterrupted stretch of bases with a set quality value (e.g. QV20).

In the report below, note the drop-out in capillary 24; this indicates a failed sample, probably due to a clogged capillary.

A similar report is the QV20 report that scores the longest sequence read with a given quality value.

Contiguous Read Length Report

The Contiguous Read Length (CRL) is the longest uninterrupted stretch of bases with quality higher than a specified limit.   In the evaluation of the quality of each base, not only the quality value of that base is used, but also those of adjacent bases within a specified window size.

Contiguous Read Length : N = 96, Range = 0 - 651, Median = 591, Mean = 585, StandardDev = 88
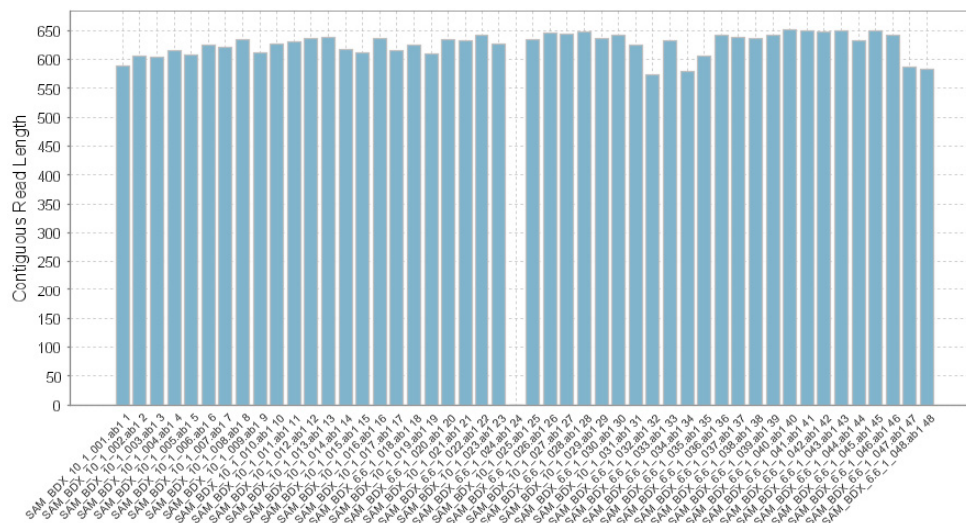
Click any of the columns in the graph to open the corresponding trace.

5. Use the Thumbnail view to display a graphical view of the traces.

   In the figure below, the peak in sample 32 indicates a dye blob. Sample 24 is the sample with the clogged capillary.
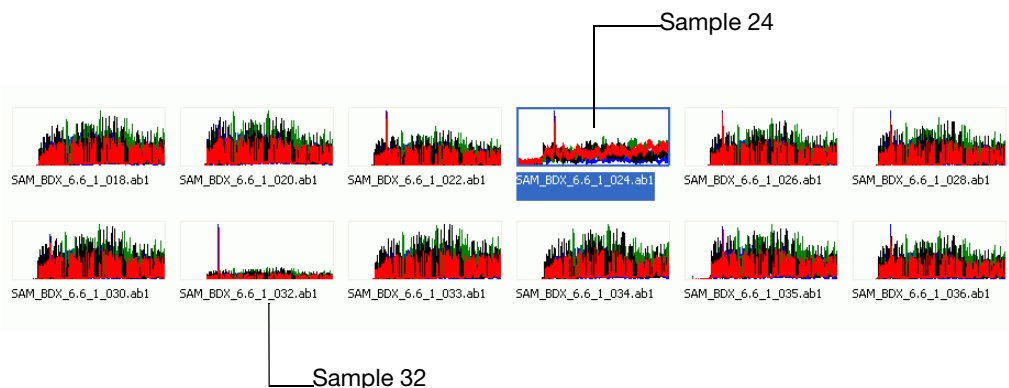
Double-click any of the thumbnails to open the corresponding trace.

How do I export traces or sequences?

1. In the Trace Manager tab, click **Export Traces** to open the Export Traces dialog box.
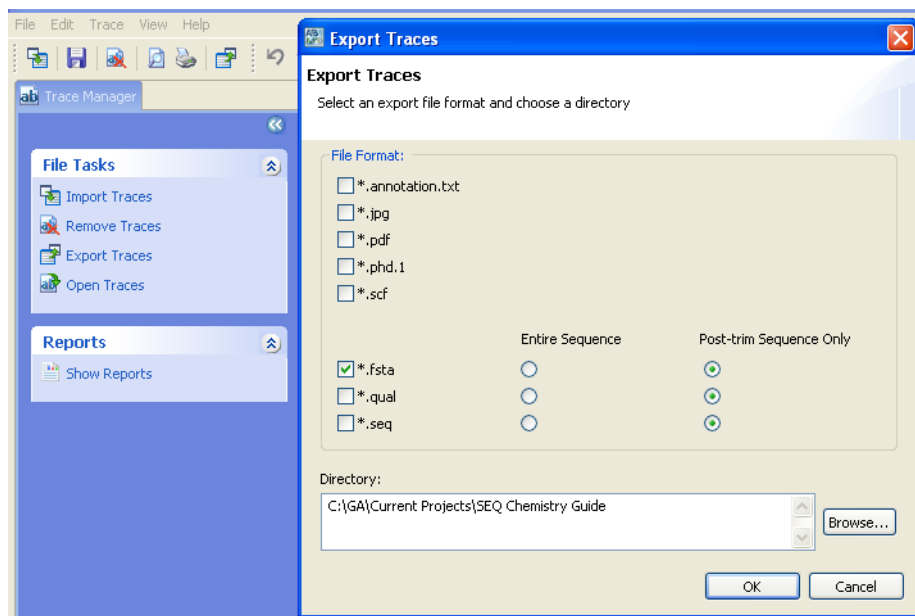
2. Check the options for the sequence and the file format, then click **OK**.

3. *(Optional)* For sequences in FASTA format, you can use the BLAST database search to identify the sequence.

   A BLAST server is located at: **http://blast.ncbi.nlm.nih.gov/Blast.cgi**.

Analyzing Data with Sequencing Analysis Software

Overview and Applications

Sequencing Analysis Software is a data review tool designed to basecall, trim, display, edit, and print data from all Applied Biosystems capillary electrophoresis DNA sequencing instruments.

Software Workflow

1. Import sample files.

2. Define the analysis protocol.

3. Run the analysis.

4. Review the results.

5. If needed, edit the bases.

6. If needed, reanalyze the data.

7. Generate the analysis report and/or print traces.

For More Information

See Chapter 8 for information on troubleshooting sequencing data.

Refer to the following documents for more information about Sequencing Analysis Software:

- *Sequencing Analysis Software v5.2 User Bulletin* (PN 4358355)
- *Applied Biosystems DNA Sequencing Analysis Software Version 5.1 for Windows® XP and 2000 Platforms User Guide* (PN 4346366)

To obtain the latest software updates and patches, go to **www.appliedbiosystems.com**, then click the link for **Support**.

Tips and Hints

Performing analysis with Sequencing Analysis Software can reduce processing time in Variant Reporter Software.

This section contains some frequently asked questions about Sequencing Analysis Software.

- "Why do I see Ns in samples analyzed by the KB™ Basecaller Software?" on page 171
- "Why are the quality value bars displayed in gray?" on page 171
- "How can I adjust the y-axis so that all traces have the same scale?" on page 171
- "What are the differences between quality values of mixed bases and pure bases?" on page 172
- "How do I find the QV chart?" on page 172
- "How do I select what I want to print?" on page 173
- "How can I increase the number of traces displayed in the Sample View window?" on page 174
- "Why does the spacing value sometimes appear in red and have a negative value?" on page 174

Why do I see Ns in samples analyzed by the KB™ Basecaller Software?

When using the KB Basecaller, you see the sequence "NNNNN" when the sample failed analysis. Omit this file from further analysis. The Analysis Report in Sequencing Analysis Software also flags these files.

In addition to pure and mixed bases with QV bars, you may also see N's and gray QV bars when you choose to reassign Ns to all bases with less than the user-specified QV threshold. This option is present to allow those who analyze data with the KB Basecaller but share data with others who do not have software to display the quality values. This allows you to take advantage of the longer read length and more accurate basecalling provided by the KB Basecaller while still viewing data with software that does not display QVs.
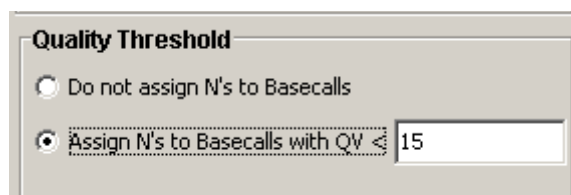
Why are the quality value bars displayed in gray?

A quality value is assigned to a specific basecall. If you alter the basecall, or reassign the bases as Ns for bases below a certain QV, the QV bar is not applicable to the new basecall and it is displayed as a gray bar. The base is shown in lower-case text indicating the basecall has been altered.
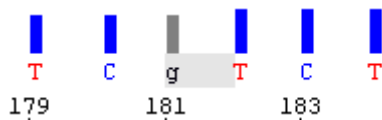
How can I adjust the y-axis so that all traces have the same scale?

1. Select **View ▸ Y Axis Scale ▸ Scale To**… to open the Set Y Scale for all Electropherograms and Raw Data dialog box.

2. Enter the values for the data range for each view.

3. Click **Apply** to view the data with the new data range. If the range is acceptable click **OK** to save the settings.
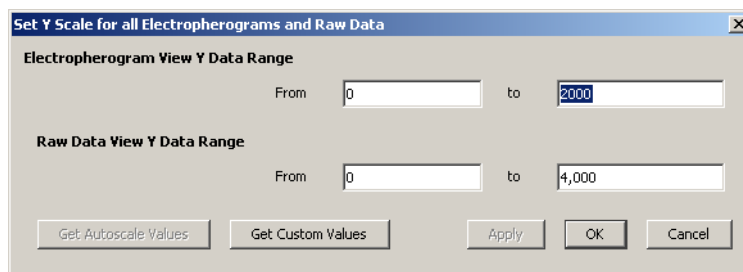
What are the differences between quality values of mixed bases and pure bases?

The definition of quality values is the same for pure and mixed bases. In both cases the probability of error for the associated basecall is 10-q/10. The distribution of quality values assigned to mixed bases, however, differs dramatically from that for pure bases. Typically, high quality pure bases will be assigned QVs of 20 or higher. Good mixed bases, on the other hand, may be assigned quality values as low as 10. The reason that a high quality mixed base may receive such low QVs is that the probability of error with more complex signals is higher. Do not discard mixed bases with QV between 10 and 20. It is a good idea to review them. For mixed bases, quality values greater than 30 are rare.

How do I find the QV chart?

Select **Help ▸ Quality Values Chart**.

Quality Values Chart

Quality Value = $-10\log_{10}{}_{(Pe)}$		
Pe is probability of error		
KB basecaller generates QVs from 1 to 99		
Typical high quality pure bases will have QV 20- 50		
Typical high quality mixed bases will have QV 10-50		
Size and color of QVs bars are identical for QVs 50-99		

QV	Pe	QV	Pe	QV	Pe
1	79%	21	0.790%	41	0.0079%
2	63%	22	0.630%	42	0.0063%
3	50%	23	0.500%	43	0.0050%
4	39%	24	0.390%	44	0.0039%
5	31%	25	0.310%	45	0.0031%
6	25%	26	0.250%	46	0.0025%
7	20%	27	0.200%	47	0.0020%
8	15%	28	0.150%	48	0.0015%
9	12%	29	0.120%	49	0.0012%
10	10%	30	0.100%	50	0.0010%
11	7.9%	31	0.079%	60	0.0001%
12	6.3%	32	0.063%	70	0.00001%
13	5.0%	33	0.050%	80	0.000001%
14	4.0%	34	0.040%	90	0.0000001%
15	3.2%	35	0.032%	99	0.00000012%
16	2.5%	36	0.025%		
17	2.0%	37	0.020%		
18	1.6%	38	0.016%		
19	1.3%	39	0.013%		
20	1.0%	40	0.010%		

More information available in Chapter 6 of Sequencing Analysis User Guide and Chapter 10 of SeqScape User Guide

How do I select what I want to print?

1. Select **Tools** ▶ **Options** and click the **Printing** tab.

2. In the Options dialog box, select the print options and the data tabs to print, then click **OK**.
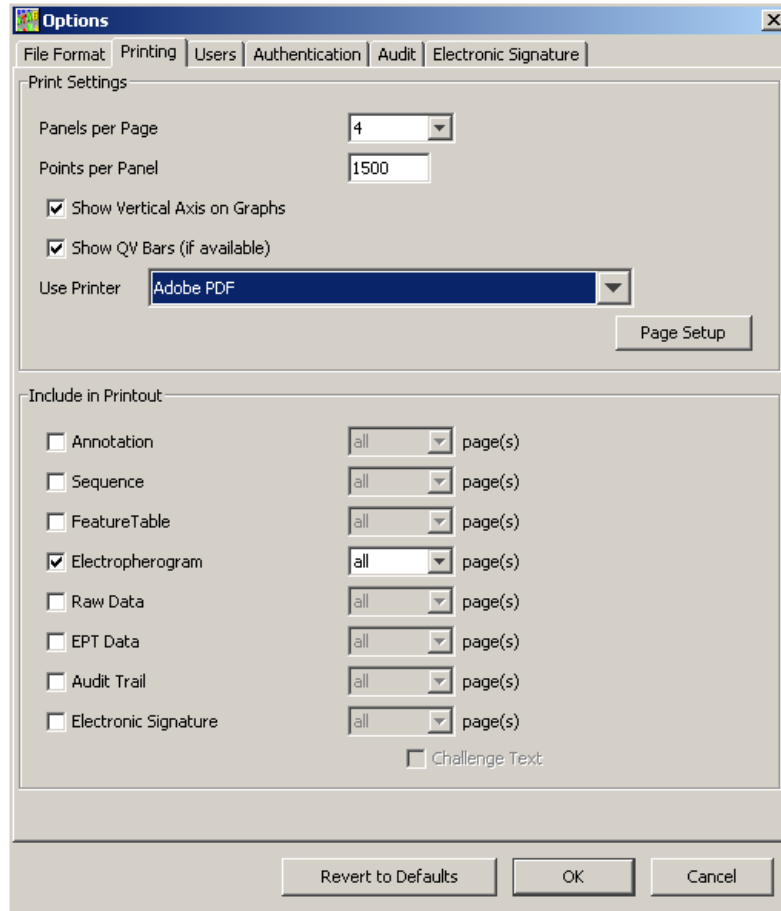
How can I increase the number of traces displayed in the Sample View window?

1. Select **Analysis** ▸ **Display Settings** and click the **Data** tab.

2. Change the values in the Pane Height, Multiple Samples row for the different views, then click **OK**.

    • To increase the number of traces displayed, enter smaller values.

    • To decrease the number of traces displayed, enter larger values.
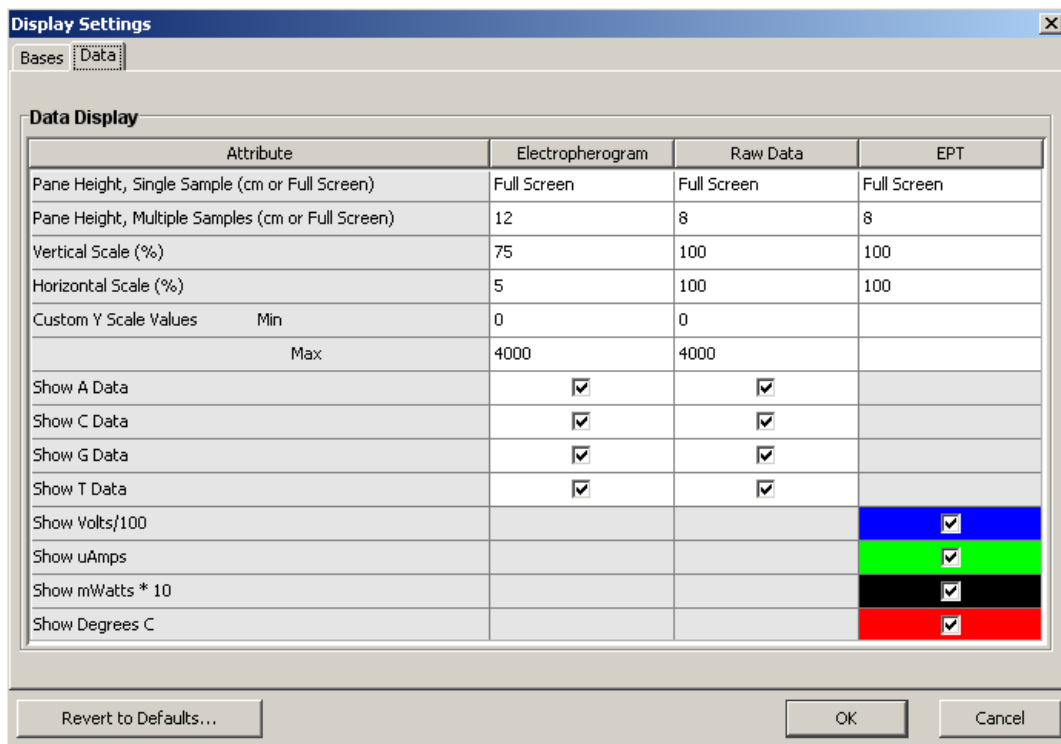
Why does the spacing value sometimes appear in red and have a negative value?

• **For the ABI Basecaller** – When the ABI Basecaller fails to determine a spacing value for a sample file, it uses a default value of 12.00 for all run conditions. This number appears in red in the Sample Manager and the Annotation view displays "–12.00".

• **For the KB Basecaller** – When the KB Basecaller fails to determine a spacing value for a sample file, it uses a default value specific to the particular instrument/polymer/chemistry/run condition used to generate the sample file. This number appears in red in the Sample Manager and the Annotation view displays –1 times this value.

Analyzing Data with Variant Reporter™ Software

Overview and Applications

Variant Reporter™ Software is an intuitive resequencing software designed to accurately discover and validate mutations and SNPs. Variant Reporter Software provides a task-based guided workflow and analyzes large-volume data faster than other software.

Variant Reporter Software, with a more intuitive workflow, is designed for:

- Large numbers of samples
- SNP discovery and validation
- Mutation analysis and heterozygote identification
- Sequence confirmation for mutagenesis or clone-construct confirmation studies

Software Workflow

1. Import and assign traces into amplicons.

2. Define the analysis protocol.

3. *(Optional, but recommended)* Specify a reference sequence (either a text or trace file) for the project.

4. Set up the amplicons.

5. Analyze the project.

6. Review variants.

7. Export and print results and/or reports.

For More Information

See Chapter 8 for information on interpreting and troubleshooting results.

Refer to the *Applied Biosystems Variant Reporter™ Software v1.0 User Guide* (PN 4376590) and Variant Reporter Software online help for more information.

To obtain the latest software updates and patches, go to **www.appliedbiosystems.com**, then click the link for **Support**.

Tips and Hints

This section contains some frequently asked questions about Variant Reporter Software.

- "How do I align my amplicons to the reference? " on page 175
- "Why don't my amplicons align with the reference?" on page 176
- "How do I edit bases?" on page 177
- "How can I save time during the Variant Reporter Software analysis?" on page 178
- "How do I change the folder where my project data is stored?" on page 178
- "How do I remove or exclude traces from my project? " on page 180

How do I align my amplicons to the reference?

1. In the Define Reference page, click **Amplicon Primers/Known Variants**, then select **Add/Edit Primer Sequences** to open the Add/Edit Primer Sequences dialog box.

2. Click **Add**, place your cursor in the Amplicon column, then double-click to enter an amplicon name. (Double-clicking the table activates the edit mode, allowing you to enter a name manually).

3. To enter a value for the forward and reverse primer sequences from outside Variant Reporter Software, copy the value from your source and right-click and select **Paste** (or Ctrl+V) into the appropriate column.
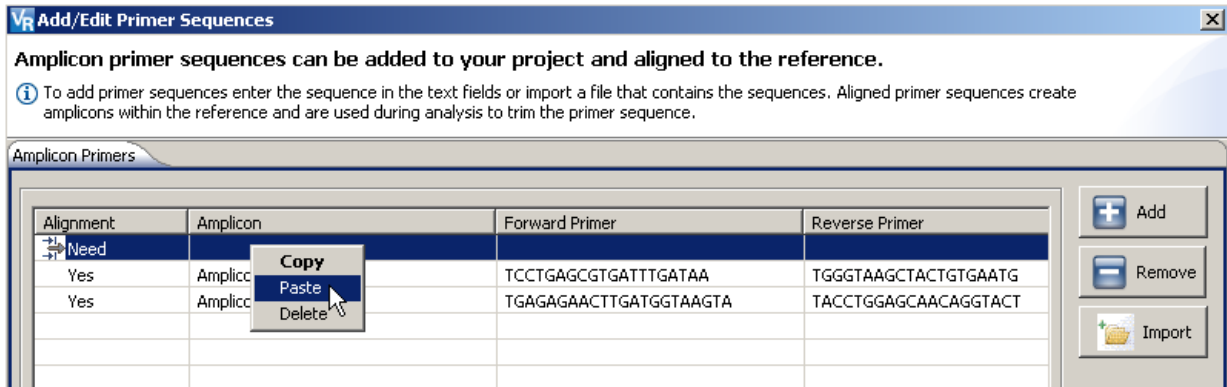
4. To align your primer sequences automatically, click **Align**, then click **OK**.

Why don't my amplicons align with the reference?

Amplicon names in the grid must match the amplicon names in the reference.

Reference sequence   Aligned amplicon   Unaligned amplicon
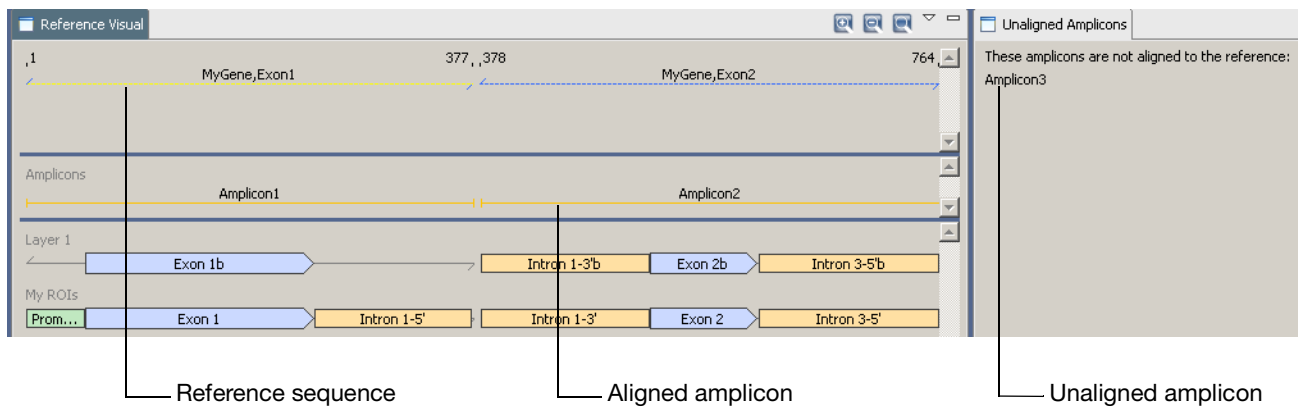
How do I edit bases?

1. Open the Variant Review page.

2. In the Trace Segment tab, select a base in the consensus sequence.
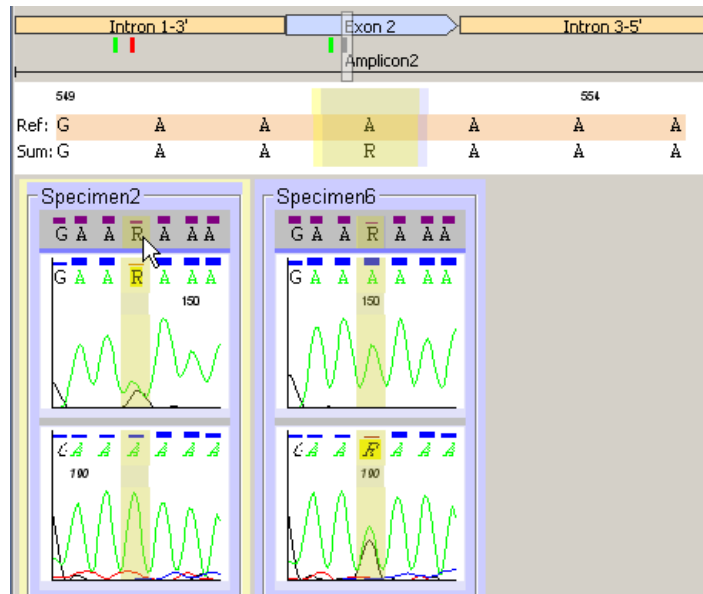
3. Enter the new letter (IUB code) that you want to change the base to, then click **OK** at the prompt.
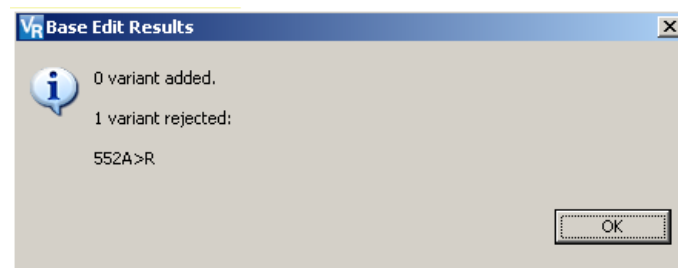
4. To remove a base, select it in the consensus sequence, click **Delete**, then click **OK**.

How can I save time during the Variant Reporter Software analysis?

You can save time if you import pre-basecalled sample files into Variant Reporter Software.

1. Use Sequencing Analysis protocol templates specific to your instrument, or edit your existing analysis protocol to match the following:

   a. Click the **Basecalling** tab and select **Do not assign Ns to Basecalls**.
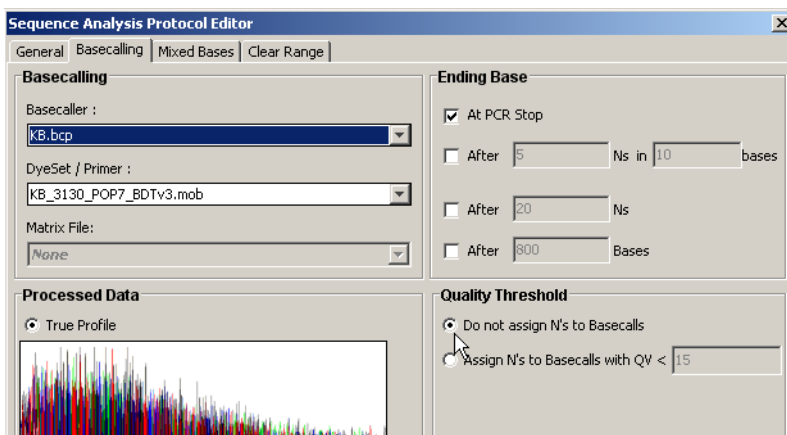
   b. Click the **Mixed Bases** tab and select **Use Mixed Base Identification**.
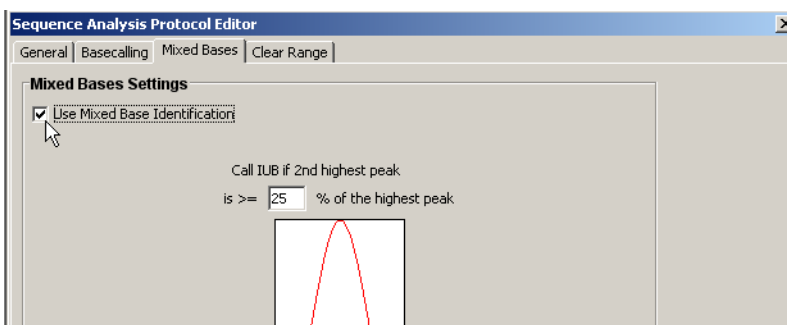
How do I change the folder where my project data is stored?

1. Open the Dashboard, then in the Data Store section, click **Manage**.
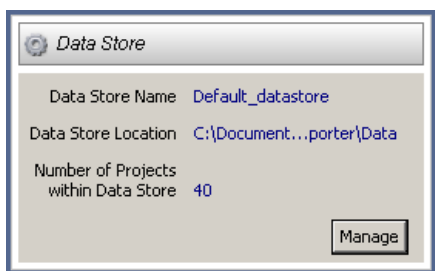
2. Click **Create** to select a new location.
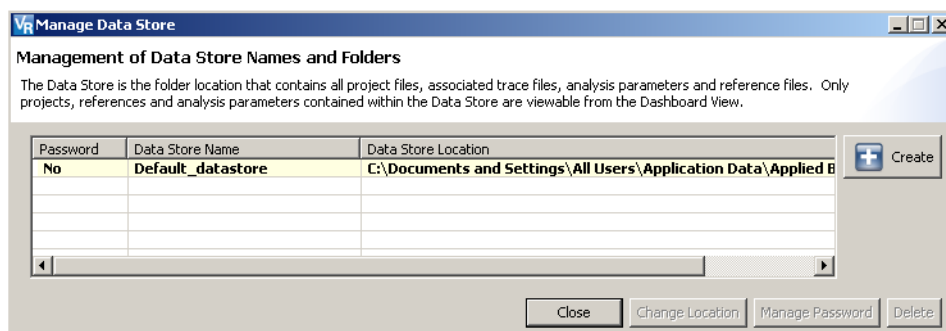
3. In the Create Data Store dialog box, enter a name for the Data Store, browse to where you want the Data Store to reside (the target folder must be empty), then click **OK**.

   Note: If you want password protection, select **Enable Password for Access** before clicking **OK**.
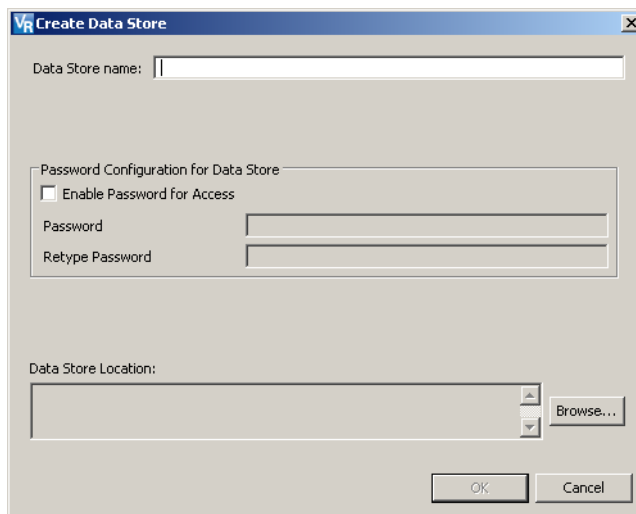
4. In the Manage Data Store dialog box, the current location of your Data Store is indicated by bold font. Select the new Data Store location, then click **Change Location**.

How do I remove or exclude traces from my project?

1. Open the Import and Manage Traces page.

2. To remove a trace permanently from a project:

   a. Select a cell in the Amplicon and Specimen Trace grid. All the traces represented by the cell fill the Traces in Selected Cell pane.

   b. Select the trace(s) you want to remove, right-click the selected trace(s), then select **Remove Selected Traces**.

   c. Click **Yes** when prompted to verify the trace removal. The changes affect the Amplicon and Specimen Trace Grid and the Amplicon Specimen Quality Grid.
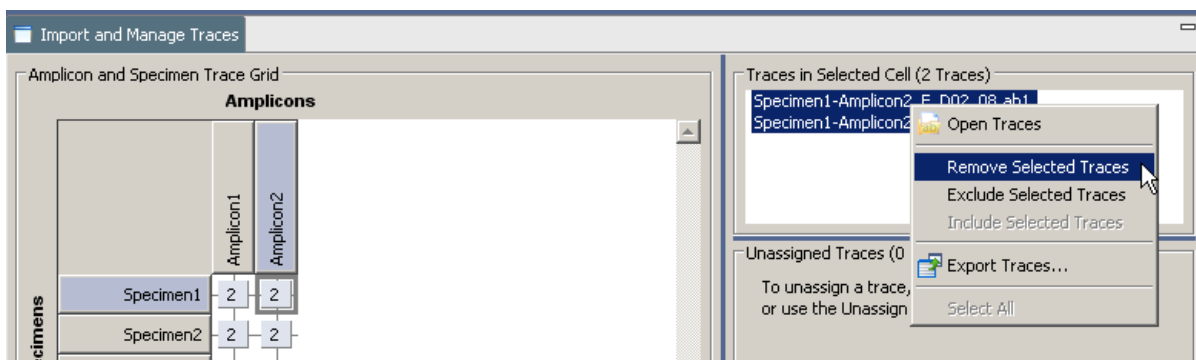
3. To exclude a trace from a project:

   a. Select a cell in the Amplicon and Specimen Trace Grid. All the traces represented by the cell fill the Traces in Selected Cell pane.

   b. Select the traces you want to exclude, right-click the traces, then select **Exclude Selected Traces**. (Excluding a trace prevents analysis of the trace with the project data).

Tips for Verifying Heterozygotes

Two useful ways to verify a heterozygote in a sample are:

- Run the selected chemistry on a reference or a wild-type sample.
- Sequence the opposite strand of the potentially heterozygous sample.

If your application requires close to 100% heterozygote detection, you must run both samples and compare the sequences and peak heights of both orientations of the homozygous reference to both orientations of the sample.

In a resequencing study, you obtain the consensus sequence for each donor by assembling one or more traces for each sequenced region. The consensus-calling algorithm in Variant Reporter Software improves the accuracy of the consensus sequence for each donor. It is particularly designed to identify heterozygous bases in the electropherogram traces. The algorithm also assigns calibrated quality values for each consensus base to help you review data quality.

Analyzing Data with SeqScape® Software

Overview and Applications

SeqScape Software is a sequence comparison tool designed for nucleotide and amino acid variant identification and allele library searching. It is used in resequencing applications, where the DNA sequence from specific genes or regions from one or more individuals is compared to a known reference sequence to determine if any genetic variations are present.

Common goals for resequencing include:

- SNP discovery and validation
- Mutation analysis and heterozygote identification
- Sequence confirmation for mutagenesis or clone-construct confirmation studies
- Identification of genotype, allele, and haplotype from a library of known sequences
- Pathogen subtyping
- Allele identification
- Sequence confirmation

Software Workflow

1. Create a project template.

2. Add sequence files to the project.

3. Analyze the data (manually or automatically).

4. Review the data using quality values and the Analysis Report.

5. Review the Mutations Report using the Project view and the QC and Mutations reports.

6. If needed, modify settings or edit data.

7. Export and print results and/or reports.

For More Information

See Chapter 8 for information on interpreting and troubleshooting results.

Refer to the *Applied Biosystems SeqScape® Software v2.5 User Guide* (PN 4359442) and online help for more information.

To obtain the latest software updates and patches, go to **www.appliedbiosystems.com**, then click the link for **Support**.

Tips and Hints

This section contains the following tips and hints:

- "How do I set analysis settings?" on page 182
- "How do I set preferences for viewing data for all my projects?" on page 183
- "What is a Reference Data Group?" on page 184
- "How do I create a Reference Data Group?" on page 184
- "Tips for Reviewing Results in the Project View" on page 188
- "Tips for Reviewing Results in the Report View" on page 188
- "Tips for Verifying Heterozygotes" on page 188

How do I set analysis settings?

You set analysis settings in the Edit Analysis Settings dialog box.

1. Select **Tools ▸ SeqScape Manager**. In the SeqScape Manager dialog box, click the **Analysis Defaults** tab.

2. Click a row in the table for the default settings to change, then click **Properties**.
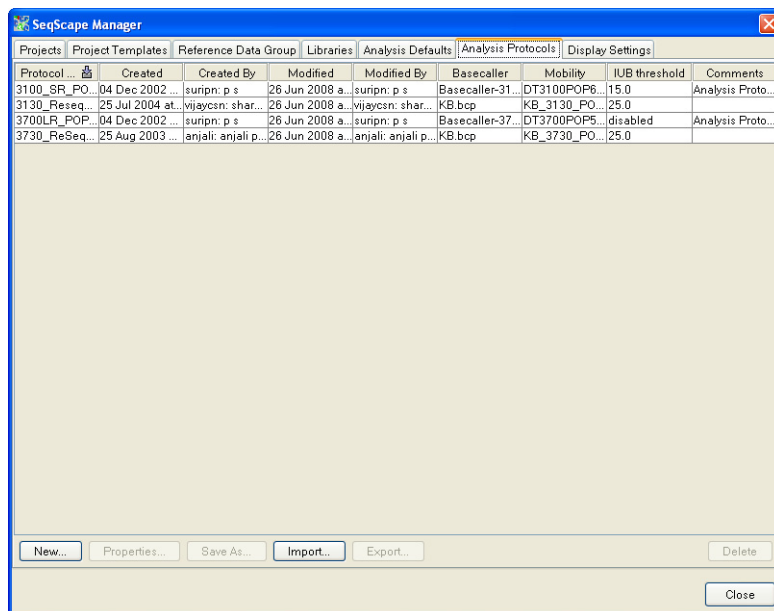
The Edit Analysis Settings dialog box opens.

3. Click **General** to set naming conventions and how modifications made by other users are monitored.
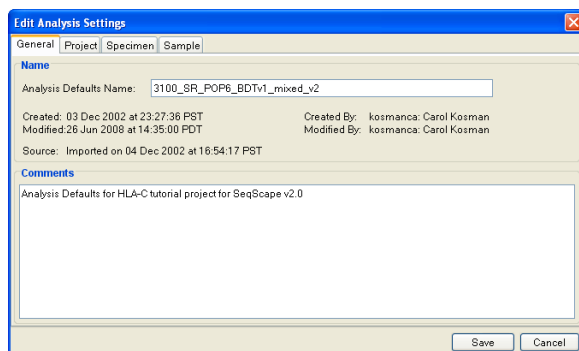
4. Click **Specimen** and:
   - Set the penalties for the alignment of samples to the reference sequence.
   - If you have called the bases in Sequencing Analysis Software, uncheck **Basecall Samples** so that SeqScape Software performs only sample filtering and assembly.
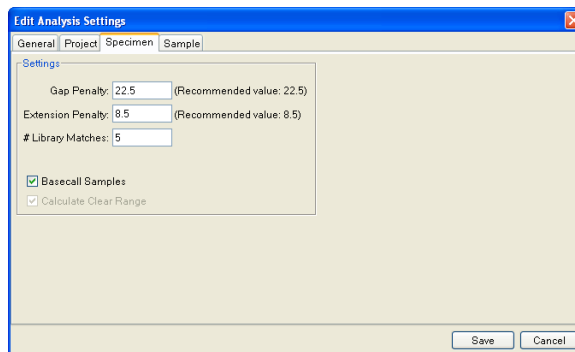
5. Click **Save** to close the dialog box and then **Close** to close the SeqScape Manager dialog box.

How do I set preferences for viewing data for all my projects?

Set preferences for viewing data in the Edit Display Settings dialog box.

1. Select **Tools ▸ SeqScape Manager**. In the SeqScape Manager dialog box, click the **Display Settings** tab.

2. Click a row in the table for the display settings you want to change, then click **Properties**.

    The Edit Display Settings dialog box opens.

3. Click **Views** and make changes to a variety of basic preferences.

    In the General View Setting group, click the buttons for the data to display in a projects.
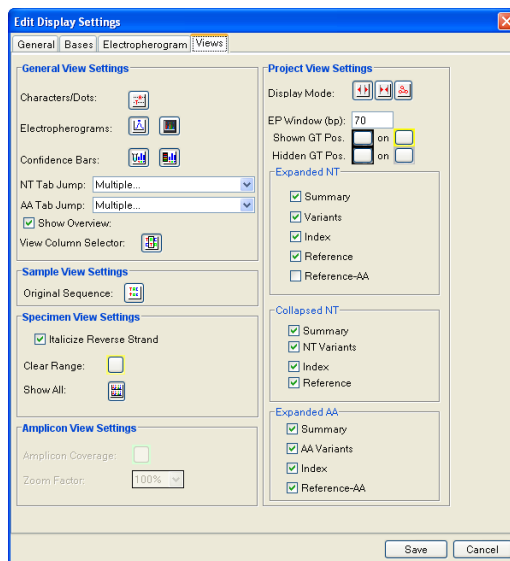
4. Click **Save** to close the dialog box and then **Close** to close the SeqScape Manager dialog box.

What is a Reference Data Group?

SeqScape Software compares the consensus segments to the Reference Data Group (RDG). The Reference Data Group contains the reference sequence and reference-associated data. The reference sequence is the "backbone" sequence for the project, consisting of one or more reference segments separated by reference breaks.

The RDG contains gene- and analysis-specific information including:

- Layers, which are units of analysis in any project, and regions of interest (ROIs) grouped together into layers for display and translation:
    - Associated allele libraries
    - User-defined styles for identification of variants in the project
- A reference sequence, made up of one or more reference segments, which are continuous or discontinuous sequences made up of one or more reference segments.
- One or more reference segments. A reference segment is a portion of the reference sequence corresponding to a single contiguous DNA sequence. It is also a region of interest. The reference segment can consist of any of the following:
    - An analyzed sample file
    - A text-only, FASTA, or .seq file
    - A GenBank format file

How do I create a Reference Data Group?

Create a Reference Data Group in one of two ways.

- Use the RDG Wizard.

    or

- Use the SeqScape Software Manager dialog box to open a new RDG.

1. For either method, select **Tools ▸ SeqScape Manager**. In the SeqScape Manager dialog box, click the **Reference Data Group** tab.

2. Click **Wizard**.

3. Enter a name for the reference data group, select the codon table and click **Next**.

4. Add reference segments:

    a. Click **Add Ref Segment**.

    b. In the Open File dialog box, locate the file containing the reference segment, and then click **Import**.

    You can select files in *.txt, *.fsta, *.seq, or GenBank format. You can also select analyzed sample files or an aligned consensus sequence in FASTA formats.
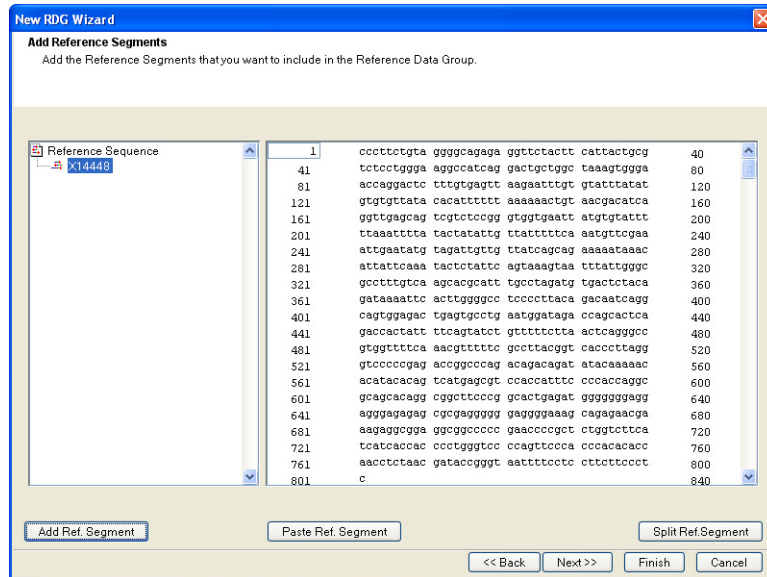
c. Click **Next** to open the Create ROIs page.

5. In the Create ROIs page, define a region of interest:

- Click and drag to highlight the ROI in the sequence, then click **Add ROI**.

   or

- Enter the first base pair in the **Starting with** field, the last base pair in the **Ending with** field, then click **Find**. If the ROI is correct, click **Add ROI**.

   Regions of Interest (ROI) define exons, introns, splice junctions, etc.

   The ROI (region of interest) can be generated from several different sources. Depending upon the difficulty of the gene and structure the ROI can consist of many different layers to define all the areas of interest.
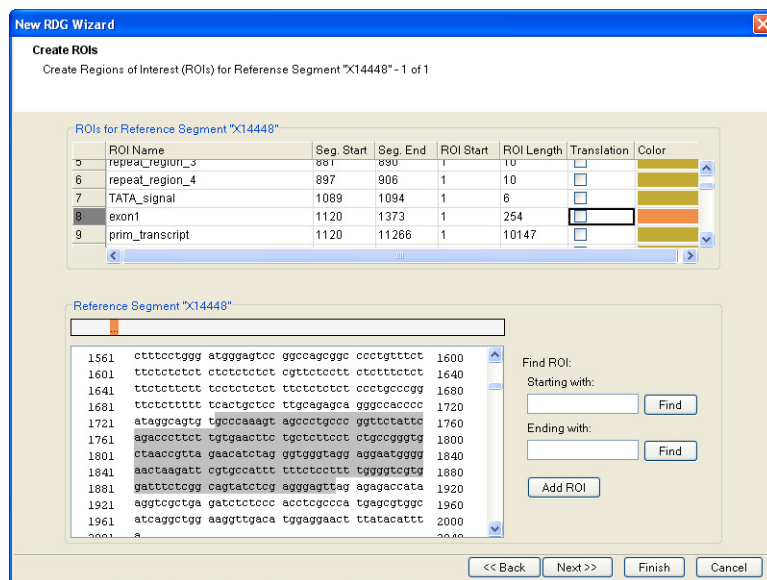
6. Click **Next** to add layers. For each layer:

a. Enter the name of the layer in the **Layer Name** field.

b. Check the check box to the left of each ROI to be included in this layer.

c. Click **New Layer**.

Create layers to indicate any smaller portions of interest within the main sequence by highlighting parts of the sequence. These can be color-coded for quick identification.
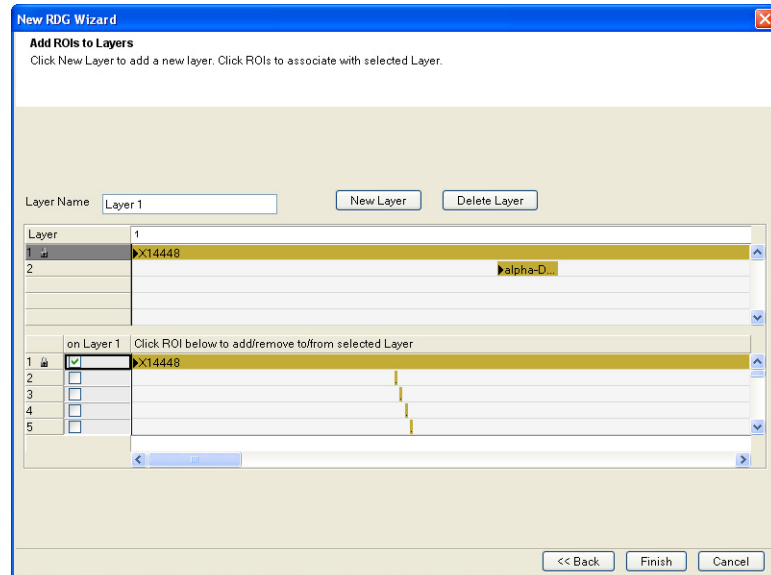
7. Click **OK**.

The new RDG appears in the Reference Data Group list in the SeqScape Manager dialog box.

8. To view the RDG, in the SeqScape Manager dialog box, click the row for the RDG in the Reference Data Group tab then click **Properties**.
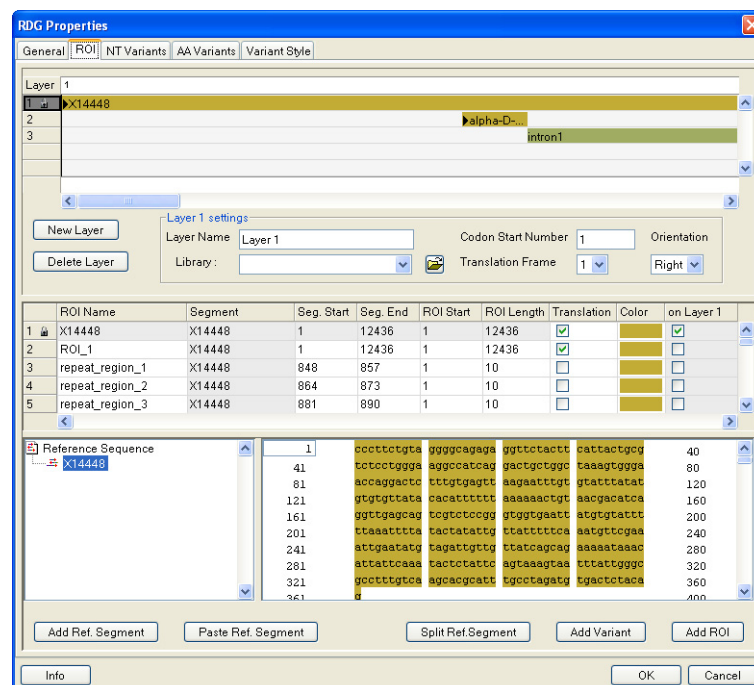
Tips for Reviewing Results in the Project View

- Use the Character/Dots view to easily scan for constant position errors.
- Use tab-jumps to easily scan low quality bases.
- Review the quality values displayed above every basecall.

Tips for Reviewing Results in the Report View

- Use the hyperlinks in the report to navigate between the report and the data.
- Tile the windows to view the report and the data at the same time.
- In the Mutations report, sort the mutations or genotypes according to the QV by double-clicking the column header.
- In the report, hide or show columns by right-clicking the column header.

IMPORTANT! Make sure you save your project after analyzing and reviewing your data.

Tips for Verifying Heterozygotes

Two useful ways to verify a heterozygote in a sample are:

- Run the selected chemistry on a reference or a wild-type sample.
- Sequence the opposite strand of the potentially heterozygous sample.

If your application requires close to 100% heterozygote detection, you must run both samples and compare the sequences and peak heights of both orientations of the homozygous reference to both orientations of the sample.

In a resequencing study, you obtain the consensus sequence for each donor by assembling one or more traces for each sequenced region. The consensus-calling algorithm in SeqScape Software improves the accuracy of the consensus sequence for each donor. It is particularly designed to identify heterozygous bases in the electropherogram traces. The algorithm also assigns calibrated quality values for each consensus base to help you review data quality.

Analyzing Data with MicroSeq® ID Analysis Software

Note: MicroSeq® ID Analysis Software is specialized for bacterial and fungal identification and is not suitable for generalized sequence analysis. Ask your Applied Biosystems field application specialist for more information.

Overview and Applications

MicroSeq ID Analysis Software is a tool for identification of bacteria and fungi. The software analyzes data generated using a MicroSeq® chemistry kit and an Applied Biosystems capillary-based genetic analyzer.

The MicroSeq ID Analysis Software:

1. Compares the DNA sequence from an informative region of the ribosomal RNA to the sequences of known reference strains of bacteria or fungi stored in a library.

2. Generates a final identification list of organisms that are the closest matches to the unknown sequence from the ribosomal RNA.

3. Reports the percent similarity that reflects how closely the unknown isolate matches the library sequence

Software Workflow

1. Create a project.

2. Analyze the project.

3. Evaluate the results.

4. If needed, edit the data in the analyzed project.

5. Export and print reports.

For More Information

See Chapter 8 for information on troubleshooting sequencing data.

Refer to the *Applied Biosystems MicroSeq® ID Analysis Software v2.0 Getting Started Guide* (PN 4364623) and the online help for instructions.

To obtain the latest software updates and patches, go to **www.appliedbiosystems.com**, then click the link for **Support**.

Chapter 8

Troubleshooting

Troubleshooting

<div style="text-align: right; font-size: 3em;">8</div>

This chapter covers:

Troubleshooting Overview

This chapter provides information for troubleshooting automated DNA sequencing results from capillary electrophoresis runs.

Assumptions   Troubleshooting suggestions listed in this chapter assume the following:

- The instrument completed the run(s) and data are visible in Data Collection Software
- Sample files were extracted successfully
- The run folder was created and saved on the instrument computer
- The correct number of *.ab1 sample files were created within the run folder
- *.ab1 sample files can be opened and viewed in an Applied Biosystems analysis software program, such as Sequence Scanner or Sequencing Analysis Software

If these conditions are not met, you may have an instrument or Data Collection Software problem. You may need to repeat data extraction and/or data analysis. Refer to your instrument user guide to continue troubleshooting.

Using Controls   To simplify troubleshooting, Applied Biosystems recommends that you run controls with every run for multicapillary instruments or each set of runs on 310 instruments:

- **DNA template control (pGEM®-3Zf(+) or M13mp18)** (page 64) – Results can help you determine whether failed reactions are caused by poor template quality or sequencing reaction failure.
- **Sequencing standards** (page 129) – Results can help you distinguish between chemistry problems and instrument problems.

Troubleshooting Workflow

When troubleshooting, follow this workflow to identify the problem. In general, check for the errors that can be resolved most easily. The figures in this section show Sequencing Analysis Software examples, however you can use Sequence Scanner Software. For more information, see "Analyzing Data with Sequencing Analysis Software" on page 170.

1. Review the electropherogram (page 195).

2. Review data analysis settings (page 195).

3. Review run and data analysis information (page 196).

4. Review experimental setup (page 199).

5. Note any patterns in the occurrence of the problem. For example, does the problem occur in specific capillaries, specific regions of the plate, an entire run, or multiple runs?

6. If you have not resolved your problem, identify the symptom in "Table of Troubleshooting Symptoms" on page 201. Then, determine the cause and perform the actions to resolve the problem.

7. If the problem persists, contact Applied Biosystems Technical Support.

Reviewing the Electropherogram

1. Open the sample file in Sequencing Analysis Software and select the Electropherogram tab for the analyzed view. The analyzed view is rescaled. Then go to the raw view.

2. Review the current, voltage, temperature, and power throughout the electrophoresis run to determine whether an electrical problem occurred during the run. Large fluctuations in the values can result in poor quality data.

   Note: Sequencing Analysis 5.X rescales the raw data to improve peak visibility in the Electropherogram view. Peak height in the Electropherogram view should not be used as the only indicator of data quality.
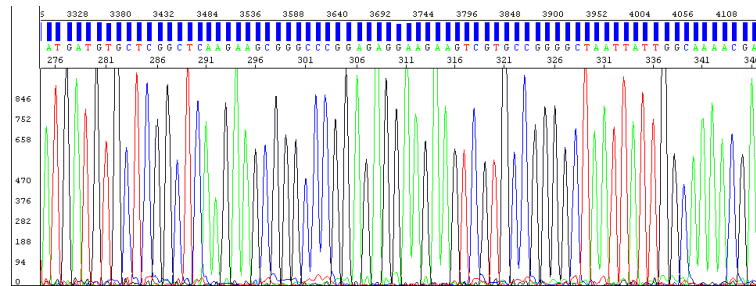
Figure 34   Example of electropherogram with high quality data

Reviewing Data Analysis Settings

Review data analysis settings using Sequencing Analysis Software.

1. In the Sample Manager, verify that the appropriate basecaller, mobility file (dyeset/primer), and matrix file (310 instruments only) are used:

   Verify that the mobility file (DyeSet/Primer) is appropriate for the basecaller

Bold, italic text indicates the file is not in the appropriate location

Matrix files are required for 310 instruments only

2. A change in nomenclature occurred between software versions. If the analysis file is in bold, italic text, verify that the analysis files are in the appropriate location:

   • **Basecaller file** – In the same folder as the Sequencing Analysis Software (for example: X:\Applied Biosystems\SeqA5.X\AppSeqA\bin\Basecaller\ Params)

   • **Mobility file** – In the Mobility folder (for example: X:\Applied Biosystems\SeqA5.X\AppSeqA\bin\Basecaller\Mobility)

   • **Matrix file** – In the Matrix folder (for analysis of 310 instrument data) (for example: X:\Applied Biosystems\SeqA5.X\AppSeqA\bin\Basecaller\ Matrix)

   See Appendix C in the *Applied Biosystems DNA Sequencing Analysis Software User Guide* for more information on troubleshooting these files.

3. In the analysis protocol and settings, verify the basecaller settings.

Reviewing Run and Analysis Information

Review run and analysis information using Sequencing Analysis Software.

1. Click **Show** next to the sample you want to display.

2. Select the Raw tab and review the raw, unprocessed fluorescence data for the sample to assess the signal quality. Check for the following:

    *   **Artifacts** – Are there any artifacts, such as four-color spikes? For an example of spikes, see page 226.
    *   **Peak heights** – Are peaks well-resolved, with reasonable heights (Figure 35)? For examples of low or no signal, see pages 207 through 211; for examples of top-heavy data, see pages 222 through 225.
    *   **Data start points** – Do any data start points deviate from others in the run? For examples of start point deviation, see pages 213 and 214.
    *   **Length of read** – Was the expected length of read obtained? Does the signal stop suddenly? For examples of sudden, premature drops in signal, see pages 219 through 221.
    *   **Baseline** – Is there background noise for all the peaks? Zoom in horizontally and vertically to verify the baseline noise.
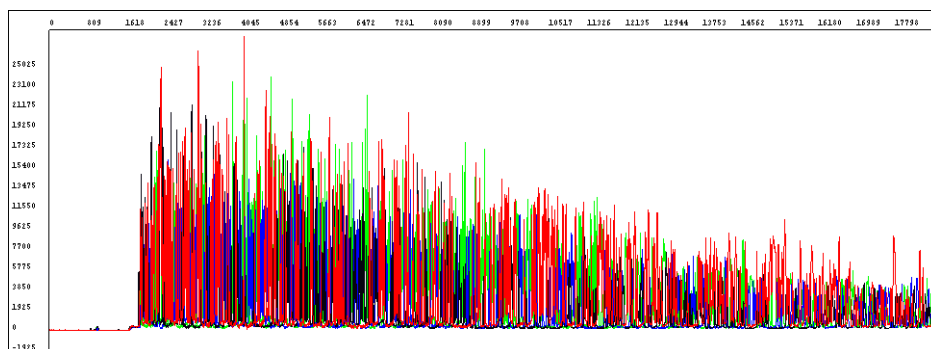
Figure 35   Example of high quality raw data with tightly resolved peaks from the 3730 Genetic Analyzer

3. Select the Annotation tab and review the data collection and data analysis settings and values for the sample file (Table 34).

Table 34   Annotation tab information

Setting	Comments
Data Collection Settings	
Note: Incorrect Data Collection settings can result in basecalling errors during data analysis.	
Instrument Model	Make sure that the run parameters were appropriate for the instrument model (for more information, see page 151).
Length to Detector	Capillary length. If the incorrect length was set, peaks can begin later than expected (for an example, see page 213).
	Note: For 310 instruments, the length to detector value does not affect data analysis.
Run Module Name	If the incorrect run module was used, peaks can begin later than expected (for an example, see page 213) or basecalling may be affected.
Data Analysis Settings	
Basecaller Name	For more information about the basecallers, see page 144.
DyeSet/Primer or Mobility File	If the incorrect mobility file was applied in the analysis, peaks are not evenly spaced, especially peaks in the first 100 to 150 bases (for an example, see page 229) and/or base assignments may be incorrect.
Ave Signal Intensity	Low or high values can produce low quality data (for examples, see pages 231, 233, page 239, and page 242).
	Generally acceptable values:
	• 3730/3730*xl* instruments: 500 to 10,000 rfus
	• 310 and 31XX instruments: 50 to 1000 rfus
	Note: The values listed above are not specifications.
Signal:Noise	Average relative fluorescent units (rfu) divided by the noise level for each dye. High quality data normally yields a signal to noise ratio >100, although accurate basecalling can be achieved with values as low as 25.
Base Spacing Used	A negative number indicates abnormal peak spacing values. Basecalling may not be accurate for the sample.

4. Select the EPT tab and review the current, voltage, temperature, and power throughout the electrophoresis run to determine whether a gross electrical problem occurred during the run. Large fluctuations in the values can result in poor quality data.
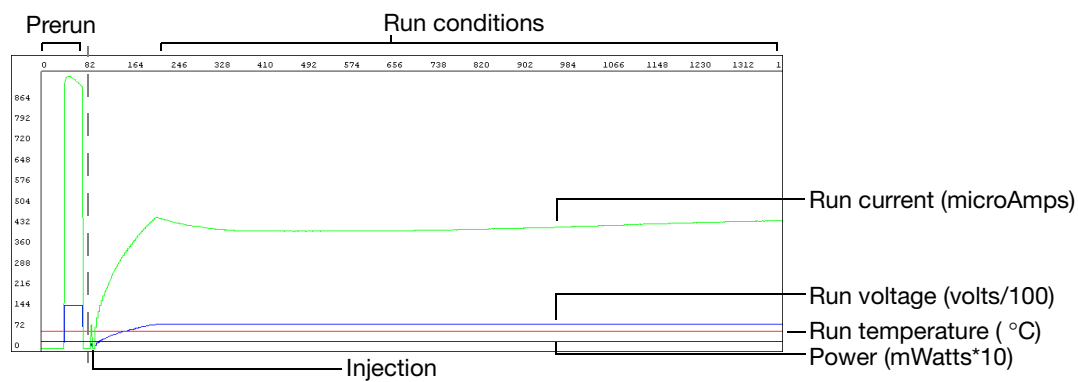
Figure 8-36   Example of EPT tab information for high quality data

Reviewing Experimental Setup

1. Confirm that you used the optimal quality and quantity of DNA using Table 35.

Table 35   Reviewing DNA quality and quantity checklist

✓	Recommendation	Comments
	Run an agarose gel to detect any contaminating DNA or RNA.	Purified DNA runs as a single band on an agarose gel. **Note:** Uncut plasmid DNA can run as three bands: supercoiled, nicked, and linear. **Note:** RNA contamination up to 1 µg can be tolerated in the sequencing reaction, but it affects DNA quantitation greatly.
	Measure the A_{260}/A_{280} ratio of your samples.	For pure preparations of DNA (in TE), the A_{260}/A_{280} ratio is 1.8. For pure preparations of RNA (in TE), the ratio is 2.0. Very clean samples in pure water can give a ratio of 1.5 to 1.6. (Sambrook *et al.*, 1989 Smaller ratios may indicate the presence of protein or organic contaminants. Ratios less than 1.8 may still produce high quality results.
	Quantitate the DNA template using the absorbance at 260 nm (A_{260}).	Quantitation by agarose gel electrophoresis may not be accurate because ethidium bromide incorporation is not consistent and the method of comparing the standard and sample brightness is subjective.
	Dilute or concentrate the DNA as needed to obtain an A_{260} reading between 0.05 and 1.00.	A_{260} values below 0.05 or above 1.00 are not accurate because Beer's law generally applies only within a certain concentration range. Outside of this concentration range, the relationship between absorbance and concentration is nonlinear.
	Use the amount of DNA template in Table 8, "Recommended DNA template quantities for cycle sequencing," on page 63. Calculate the template concentration using the formulas on page 45.	Too little template can result in no or low signal. Too much template can result in top heavy data (page 222 through 225).
	Use the primer concentrations recommended in Chapter 4: 3.2 pmol in a 20-µL reaction (dye terminator chemistry). Calculate the primer concentrations using the formula on page 39.	Too little primer can result in no or low signal (pages 207 through pages 211). Too much primer can lead to overamplification of the 5′ end of the template, resulting in top heavy data (page 222 and 224).

2. Confirm that the primer design and quality are optimal using Table 36.

Table 36   Reviewing primer design checklist

✓	Recommendation	Comments
	Ensure that the primer has T_m >45 °C.	If the T_m is too low, it may result in poor priming and low or no signal (pages 207 through pages 211).
	Ensure that primers are at least 18 bases long.	Primers that are too short may have T_ms that are too low.
	Ensure that there are no known secondary hybridization sites on the target DNA.	Secondary hybridization sites on the target DNA can result in double peaks throughout the sequence (page 237).
	Choose primers that do not have runs of identical nucleotides, especially 4 or more Gs.	Runs of identical nucleotides in primers can cause n+1 or n-1 effects (page 244). Also, these primers may be more difficult to synthesize.
	Choose primers with G-C content in the range of 30 to 80%, preferably 50 to 55%.	If the G-C content is too low, the T_m may be too low. If so, increase the primer length beyond 18 bases to obtain a T_m>45 °C.
	Design primers to minimize the potential for secondary structure and/or hybridization (see page 38).	Primer-dimer formation from hybridization can result in mixed sequence at the beginning of the sequence (page 240). Secondary structure in the primer, particularly at the 3′ end can result in poor priming and low or no signal (pages 207 through pages 211).
	Purify primers by HPLC to reduce the quantity of n-1 primers.	Primers containing contaminants or synthesized primers of the wrong length can cause problems in sequencing reactions, such as failed reactions, noisy data, or poor sequencing results. If the primer is a short oligo that contains n-1 primers, HPLC cannot always remove the n-1 contaminants.

Table of Troubleshooting Symptoms

The table below lists troubleshooting symptoms and a page reference for an example of the symptom and possible causes and actions to take to resolve the problem. If there are two or more possible causes for the symptom, the causes are grouped and listed in the following order: data analysis issues, electrophoresis issues, then sequencing reaction issues.

Table 37   Table of troubleshooting symptoms

Table 37   Table of troubleshooting symptoms *(continued)*

Troubleshooting Examples

Spacing value is red in Sequence Analysis or Sequence Scanner Software

Row	Show	Sample File Name	Sample Name	BC	PP	P	BaseCaller	DyeSet/Primer	Matrix File	Spacing	Peak 1	Start	Stop
1	☐	BDT7946_P21_m9	6463	☑	☐	☐	KB.bcp	KB_3730_POP7_B...	*None*	(16.16)	2798	2799	18436

Red spacing value in
Sample Manager

Possible Cause(s)	**Recommended Action**
Data analysis issue: The red color indicates that the basecaller applied a default value for spacing. The basecaller determined that the sample cannot be analyzed because the spacing estimation algorithm failed. This error may occur if the data has been collected using modified run modules or if data are poor.	Verify that analysis settings are appropriate for the run setup.
	Manually set a spacing value and reanalyze the data. To estimate a spacing value: 1. Refer to the raw data after 1000 scan points. 2. Measure the distance between the crests of two adjacent peaks with the same color. For more information, see the appropriate Sequencing Analysis Software user guide.

Mixed base not called correctly
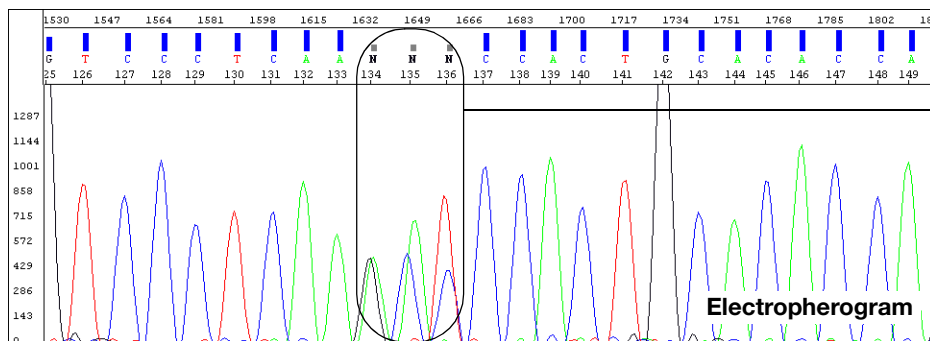
Ns or low QVs for pure bases are assigned instead of mixed bases (analysis using the KB basecaller only)

Electropherogram

Possible Cause(s)	Recommended Action
Data analysis issue: The quality threshold setting and the mixed bases settings are not correctly defined in the analysis protocol.	1. Review the quality threshold setting (page 149) and the mixed bases settings (page 149) in the analysis protocol that you used for the analysis.
	2. Correct the settings if necessary, then reanalyze the data.
	Note: Significant improvements in mixed basecalling have been made with later versions of Sequencing Analysis Software and the KB basecaller. Please check the Applied Biosystems web site for the latest updates.

Too many mixed bases called

Electropherogram
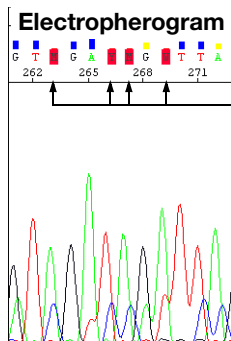
Too many mixed bases are called (analysis using the KB basecaller only)

Possible Cause(s)	Recommended Action
Data analysis issues:	
2nd highest peak threshold for mixed base identification is set too low. The recommended range is 15 to 25%.	Review the Mixed Bases settings in the analysis protocol that you used for the analysis (page 149). Change the settings if necessary, then reanalyze.
Electrophoresis issues (likely in multiple lanes and/or runs):	
Carryover from contaminated septa.	Replace septas and change buffer, water, and waste.
Electrical noise.	Check the uninterruptible power supply (UPS).
Contaminated water or buffer because of dirty containers, microbial growth, or use of tap water for cleaning.	Clean all reservoirs, upper and lower polymer block, and septa with deionized water.
Poor or incorrect spectral calibration (spectral pullup).	Perform the spectral calibration again.
Shifted spatial calibration.	Perform the spatial calibration again.
Poor CCD alignment.	Contact Applied Biosystems to arrange a service engineer visit.
Sequencing reaction issues (in individual samples or multiple samples):	
Secondary primer site in the template was sequenced.	Design a new sequencing primer (page 38).
Secondary amplification product in the PCR product used as a sequencing template.	Use gel purification to isolate the desired product. For more information, see "Purifying PCR Products for Sequencing" on page 41.
	Design new PCR primers or optimize amplification parameters to obtain a single product. For more information, see "Preparing PCR DNA Templates" on page 37.
PCR primers were not completely removed from the PCR product used as a sequencing template.	Remove PCR primers completely before using PCR products as sequencing templates. For more information, see "Purifying PCR Products for Sequencing" on page 41.
Mixed templates.	Review the DNA quality.

Possible Cause(s)	**Recommended Action**
Pull-up caused by overloading the capillaries with too much product.	Review DNA quantity.
	Use standard run modules
	Click the **Annotation** tab and examine the Ave Signal Intensity. Excessive signal: • 3730/3730*xl* instruments: >10,000 rfus • 310 and 31XX instruments: >1000 rfus Load less labeled sample by performing one of the following: • Remove some of the sample and replace with Hi-Di™ Formamide • Inject sample for less time • Resequence the samples, using less template in the sequencing reaction, especially if you use the BigDye® XTerminator™ Purification Kit (see Table 8, "Recommended DNA template quantities for cycle sequencing," on page 63).
Stutter during either PCR amplification and/or cycle sequencing. Stutter is most common in any homopolymeric region greater than 2 bases. It can also be seen with simple repeated DNA sequences. The results are worse when the stutter occurs during PCR amplification. It is thought that stutter occurs when a partially extended primer and template dissociate, then reanneal improperly before extension continues. Partially extended primers and templates commonly dissociate during the reaction, but if they reanneal with complete fidelity, the reaction produces only one product. Improper annealing results in one or more products that are represented in the sequencing results.	If stutter occurs during PCR amplification, little can be done to correct the problem, except using anchored sequencing primers. If stutter occurs during cycle sequencing: • Try using dRhodamine terminators. They have been shown to be less prone to produce stutters, specifically with poly-T regions. • Some customers have found that they can get past poly(A) regions using a mixture of oligo dT_{18} primers with either a C, A, or G as the 3′ terminal dinucleotide or 2-base anchors.

No signal

Raw Data

Unincorporated dyes

Flat signal profile

Electropherogram

KB basecaller generated 5 Ns to indicate a possible failed sequencing sample

Possible Cause(s)	Recommended Action
Sequencing reaction issues (likely with multiple or all samples):	
Loss of labeled product during purification of extension products.	See Chapter 5 for suggestions on retaining labeled product during purification.
Thermal cycler malfunction.	Determine with the manufacturer how to test your thermal cycler for proper performance.
One of the components of the sequencing reaction (template, primer, or Ready Reaction Mix) was either omitted, was the wrong material, or was of poor quality.	Review the entire experiment carefully. 1. Check the quantitation and quality of the sequencing reaction components. 2. For each component, replace the component, perform a sequencing run, then evaluate the results until you have identified the problem or replaced all of the reaction components. 3. Run a DNA template control to determine whether the sequencing reaction failed or the template quality is low (page 64).
Insufficient template added to sequencing reactions, leading to too few sequencing products generated during PCR.	Check DNA quantitation and quality (page 44 and 45).
Template contains sequencing inhibitors such as phenol (page 44).	Follow recommended procedures to prepare templates. Check DNA quality (page 44). If necessary, clean up dirty templates.

Possible Cause(s)	Recommended Action
No enzyme activity because Ready Reaction Mix was stored improperly or it separated upon storage.	Check the color of the Ready Reaction Mix. If the color is not uniform, the Ready Reaction Mix separated upon storage. Mix the Ready Reaction Mix gently before using it.
	Run a DNA template control to test enzyme function (page 64).
Weak priming because of poor primer design.	Review primer design (page 38). Make new primers, then repeat the sequencing experiment.

Low signal
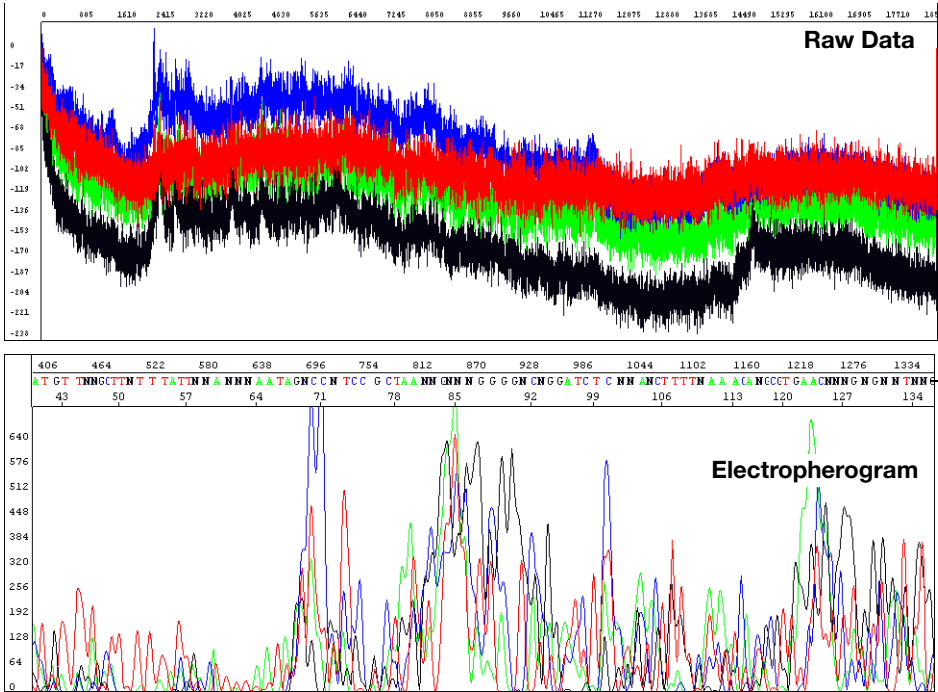
Electropherogram shows Ns (with ABI or KB basecaller) or low quality bases (with KB basecaller)

Possible Cause(s)	Recommended Action
Electrophoresis issues:	
One or more broken or blocked capillaries.	Visually check the capillaries. If any are broken or blocked, replace the entire array. If subsequent runs show failure in the same capillary, replace the entire array.
	Check the results using the long read sequencing standard.
Optical path is obstructed (3100/3100-*Avant* instruments only).	Check the laser power, using the EPT in Data Collection Software. Perform the spatial calibration again.
	Check whether you can hear the shutter clicking during data collection. If you cannot hear it click, contact Applied Biosystems for a service engineer visit.
	If all capillaries show no signal or low signal, contact Applied Biosystems for a service engineer visit.

Possible Cause(s)	Recommended Action
Sample evaporated because water was used as the injection solution.	Use Hi-Di™ Formamide to resuspend your samples (see page 122). For future experiments, consider using the BigDye® XTerminator™ Purification Kit to purify samples (see page 88).
	Use a heat sealer to seal the plates (3730/3730*xl* instruments only).
	Add more resuspension solution to the samples before loading them.
Sample volume is too low.	Resuspend samples using sufficient volumes (at least 10 µL) (see page 122).
Autosampler alignment is off and the tips did not enter the sample.	1. Verify the correct run module was used. 2. If you are using samples purified with BigDye® XTerminator™ Purification Kit and your autosampler was recently calibrated, run the BDX Update utility. Select **Start ▸ All Programs ▸ Applied Biosystems ▸ BDX Updater**. (The utility is installed with the BigDye XTerminator run modules.) 3. Contact Applied Biosystems to arrange a service engineer visit.
Slightly unstable current and voltage during electrophoresis.	Check the current and voltage.
Buffer is old.	Replace the buffer according to the procedures in your instrument user guide.
Too much template or sample temporarily clogging the capillary.	Reinject the sample.
Injection failed.	• Verify correct run module was used. • Verify correct volume in well. • Verify capillaries are not broken or blocked.

Low signal throughout

Raw Data
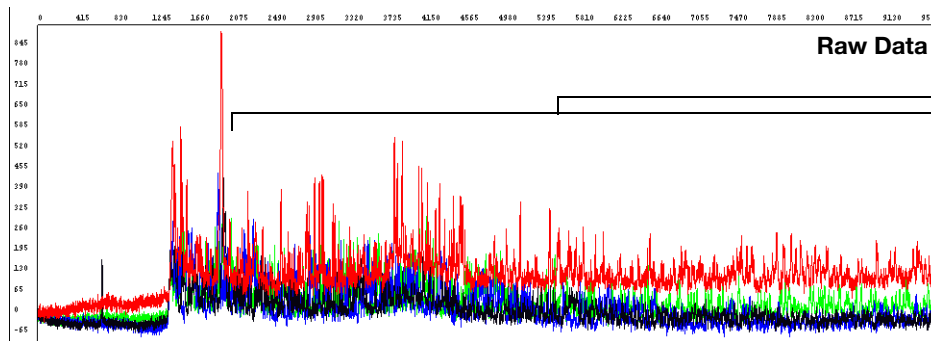
Low signal throughout the entire sequence

Electropherogram
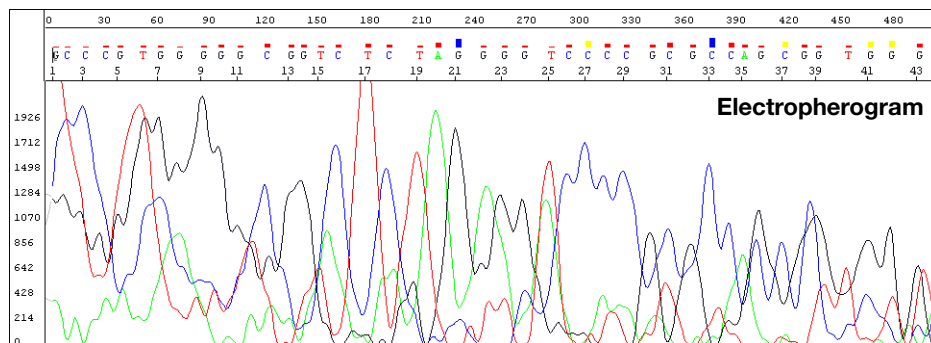
Ave Signal Intensity:	G (93), A (120), T (143), C (127)
Noise:	G (9), A (10), T (12), C (10)
Signal:Noise:	G (10), A (12), T (12), C (12)

Annotation tab shows low average signal intensity values for data from 3730 instrument

Possible Cause(s)	Recommended Action
Sequencing reaction issues:	
Sequencing reaction failed.	Check the control template and primer.
Partial loss of labeled products during purification of extension products.	See Chapter 5 for suggestions on retaining labeled product during purification.
Sample contains salts from insufficient purification of templates, PCR products, or sequencing reactions with ethanol precipitation. Salts in the sample interfere with proper electrokinetic injection.	Review DNA quality, PCR purification, and sequencing reaction purification steps.
The amount of Ready Reaction Mix in the reactions was insufficient, usually because the sequencing chemistry was diluted.	Follow recommended procedures to prepare sequencing reactions with Ready Reaction Mixes. See page 66 for recommended procedures. Applied Biosystems does not support diluted reactions or guarantee the performance of diluted BigDye chemistry.
Not enough primer or template in the cycle sequencing reaction.	Review DNA quantity (page 199). Use the amounts recommended on page 63. Run a DNA template control to check sequencing reaction quality (page 64).

Possible Cause(s)	**Recommended Action**
Poor template quality.	Follow recommended procedures to prepare templates. Check DNA quality (page 44). If necessary, clean up dirty templates. Run a DNA template control to check sequencing reaction quality (page 64).
Failure caused by difficult template sequence.	Use Table 7 on page 56 to select a chemistry kit for certain difficult templates.

Signal starts later than expected: no resolution loss

Data starts later than expected

Possible Cause(s)	Recommended Action
Electrophoresis issues:	
Incorrect capillary length (Length to Detector) or run module was selected.	1. Review run information in the Annotation tab using Sequencing Analysis Software (see page 197): – Length to Detector – Run module 2. If an incorrect selection was made, run the samples again using the correct settings.
Variation in lab temperature leads to faster or slower runs.	Stabilize the lab temperature.
Sample heated during vortexing step of BigDye® XTerminator™ purification.	1. Repeat the sequencing reactions. 2. Perform BigDye XTerminator purification using recommended vortexer and plate adapter. 3. Run the samples again.
Too much template used.	Run the samples again, using less template.

Signal starts later than expected: with resolution loss

Data starts later than expected

Raw Data

Loss of resolution

Electropherogram

Peaks are broad in the region of resolution loss marked above

Possible Cause(s)	Recommended Action
Electrophoresis issues:	
Capillaries overloaded with sequencing product, possibly unlabeled DNA or RNA.	Click the **Annotation** tab and examine the Ave Signal Intensity. Excessive signal: • 3730/3730*xl* instruments: >10,000 rfus • 310 and 31XX instruments: >1000 rfus Re-inject the samples using decreased injection time and/or lower voltage. Load less labeled sample by using less template in the sequencing reaction (see Table 8, "Recommended DNA template quantities for cycle sequencing," on page 63).
Temperature in room and/or oven fluctuating.	Review the EPT tab using Sequencing Analysis Software (see page 198). If the oven temperature is fluctuating, the oven may be leaking because of a poor seal. Contact Applied Biosystems to arrange a service engineer visit.
Contaminant migrated through the capillary during electrophoresis.	Run the sample again.
Capillary not filling.	Check the pin valve in the polymer block, amount of polymer in the bottle, leaks in the check valves, and polymer pump function. Contact Applied Biosystems to arrange a service engineer visit.
Temperature in the array heater fluctuating more than ±0.5 °C (3730/3730*xl* and 3130/3130*xl* instruments and POP-7 only).	Using Data Collection Software, check the array heater temperature. If it fluctuates more than ±0.5 °C, contact Applied Biosystems to arrange a service engineer visit.

Possible Cause(s)	Recommended Action
Water in polymer system caused by insufficient flushing after water wash maintenance.	Flush the polymer, using the wizard if possible.
Extension products purified using bead-based kits were injected without removing the magnetic beads. The beads may interfere with the extension products during injection and cause overloading or other injection anomalies.	Remove magnetic beads before loading the sample.
Variables that affect current set incorrectly.	• Replace buffer in system with fresh 1✕ running buffer. • Inspect system for leaks (wet or dry polymer around fitting indicates a leak) and tighten fittings as needed. • Look for discoloration in the block channels or tubing. If present, perform a water wash on the system using the wizard in Data Collection Software.

Negative baseline: one color
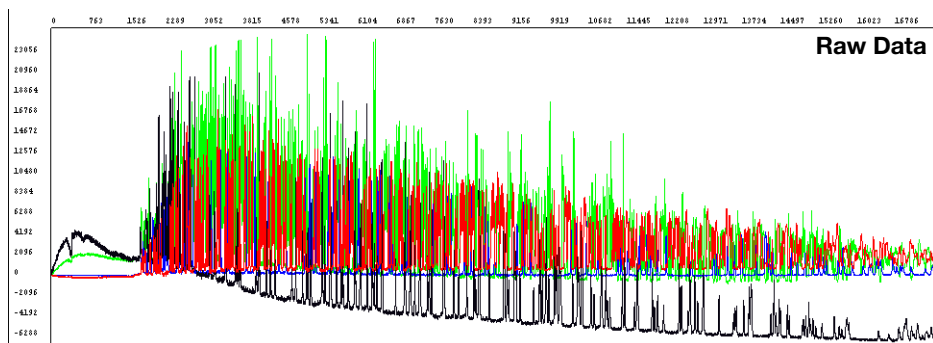
Baseline fluorescence for one color is below 0 rfus
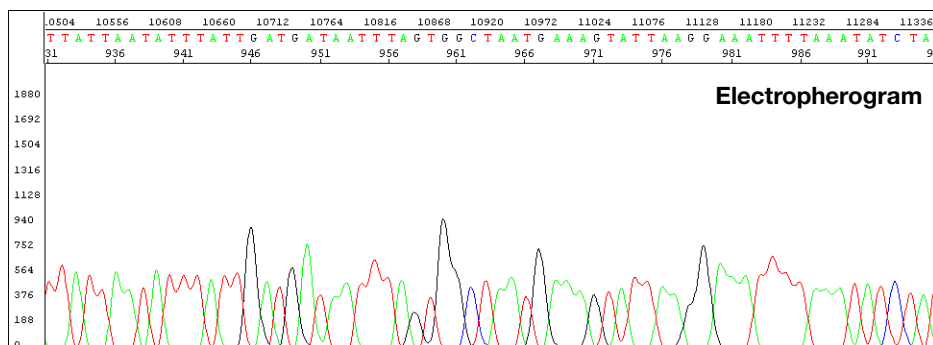
Software corrects the baseline fluorescence during data analysis

Possible Cause(s)	Recommended Action
You are using an early version of Sequencing Analysis Software. This error, found in versions earlier that v5.2, was corrected in Basecaller updater v2.0.	Upgrade Sequencing Analysis Software v5.2 or later. To obtain the latest software updates and patches, go to **www.appliedbiosystems.com**, then click the link for **Support**.

Negative baseline: all four bases

Raw Data
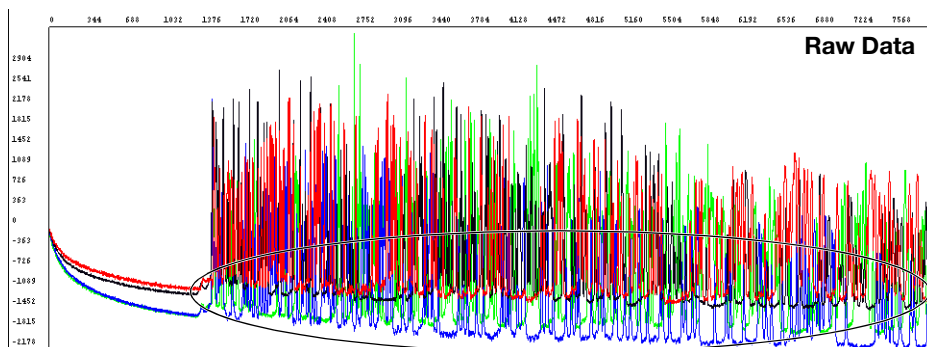
Baseline fluorescence for all four colors is below 0 rfus

Possible Cause(s)	Recommended Action
Electrophoresis issue: Excessive fluorescent contamination in the detection area that bleaches out over the duration of the run (3730/3730*xl* instruments only).	Use manual control to turn on the laser before starting the run to negate the effects of excessive fluorescent contaminant. Contact Applied Biosystems technical support or a field applications specialist.
	Perform a water wash on all components of the system using the wizard in Data Collection Software, then replace the capillary array.

Waterfall baseline

Raw Data

Raised baseline in all colors

Baseline for all colors falls in a "waterfall" pattern

Signal dropout at point of waterfall
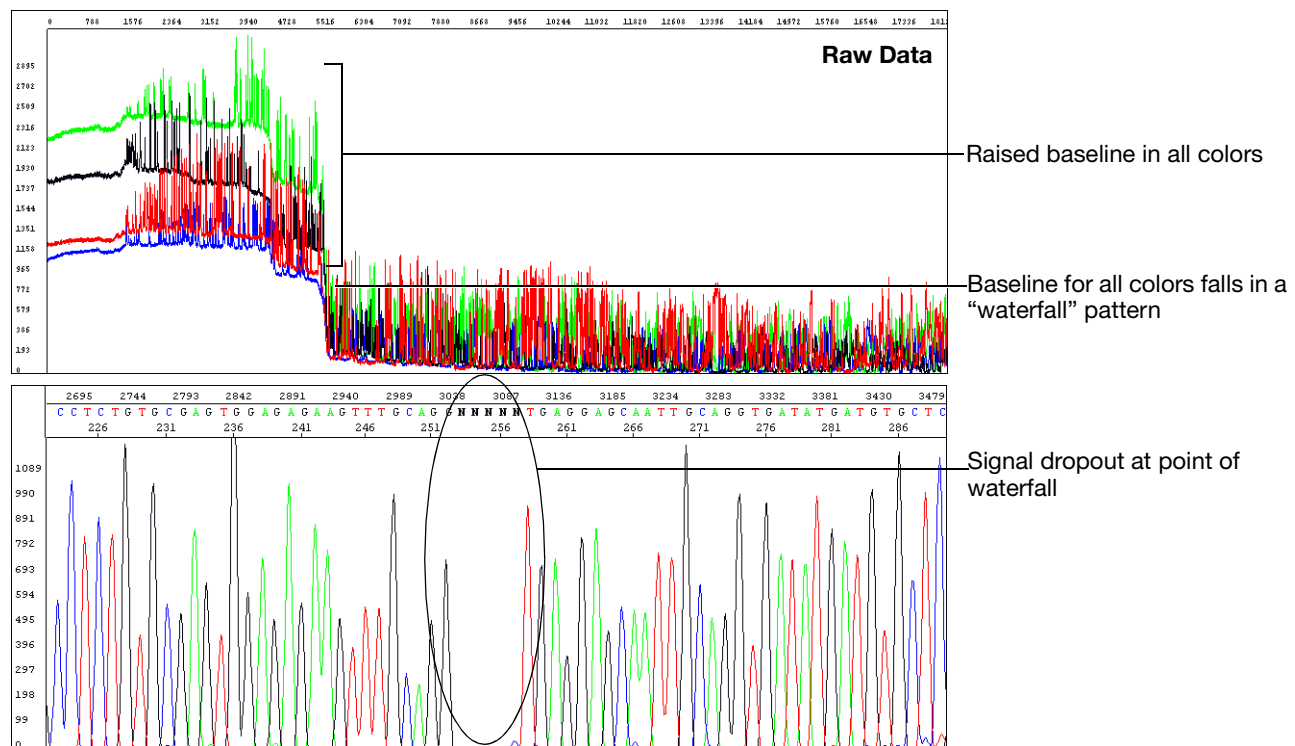
Possible Cause(s)	Recommended Action
Residue from cleansers used on glassware. **Note:** Primarily observed in syringe-based instruments.	Rinse all components with deionized water. Check gasket on syringe(s), replace if necessary. Replace syringe(s).

Sudden drop in signal: corresponds to basecalling stop when sequencing short template

Sudden drop in signal

Basecalling stops when the signal drops

Possible Cause(s)	Recommended Action
Data analysis issue: The drop in signal identifies a PCR stop point and the basecaller stops calling bases beyond this point. With the ABI basecaller, you observe Ns beyond the PCR stop. With the KB basecaller, the analyzed trace is displayed until the last basecall.	Select the **At PCR Stop** check box in the analysis protocol using Sequencing Analysis Software (see page 148).

Sudden drop in signal: early sudden drop with sequence termination

Early sudden drop in signal

Early sudden termination of sequence

Possible Cause(s)	Recommended Action
Sequencing reaction issue:	
DNA polymerase enzyme stopped at a region of the template that was difficult to sequence.	• Depending on sequence contexts, you can try sequencing some template with dGTP kits (if the problem sequence is G-rich), dRhodamine kits, or BigDye primer kits. • If termination of sequencing was caused by hairpins or secondary structure, redesign primers around the problem region. Some customers report that certain additives can help, but Applied Biosystems cannot recommend any specific protocols.
Not enough Ready Reaction Mix was used in the sequencing reaction.	Follow recommended procedures to prepare sequencing reactions with Ready Reaction Mixes. See page 66 for recommended procedures. Applied Biosystems does not support diluted reactions or guarantee the performance of diluted BigDye chemistry.

Sudden drop in signal: sudden drop with continued basecalling

Raw Data

Sudden drop in signal

Electropherogram

Basecalling continues beyond the drop in signal

Possible Cause(s)	Recommended Action
Sequencing reaction issues:	
DNA polymerase had difficulty processing through a particular sequence context.	Depending on sequence contexts, you can try sequencing some template with dGTP kits, dRhodamine kits, or BigDye® primer kits.
Not enough Ready Reaction Mix was used in the sequencing reaction.	Follow recommended procedures to prepare sequencing reactions with Ready Reaction Mixes. See page 66 for recommended procedures. Applied Biosystems does not support diluted reactions or guarantee the performance of diluted BigDye chemistry. If the problem persists, try sequencing using the dGTP kits.

Top-heavy data: gradual loss of signal

Early peaks are taller

Raw Data

Peak height decreases

Peak heights fall to zero before the end of the sequence

Large number of small molecular weight peaks in the sequencing reaction

Possible Cause(s)	Recommended Action
Sequencing reaction issues:	
Improper cycling conditions for extension. The extension time is too short or the extension temperature is too high.	Increase the extension time or decrease the extension temperature.
Improper ratio of primer to template in the sequencing reaction.	Set up a matrix of reactions with varying ratios of primer:template to determine which ratio produces the best peak profile.
Sequencing template contains a contaminant that inhibits DNA polymerase activity.	Review how templates are prepared. Try a different method or clean up dirty templates (page 44).
Not enough Ready Reaction Mix was used in the sequencing reaction.	Follow recommended procedures to prepare sequencing reactions with Ready Reaction Mixes. See page 66 for recommended procedures. Applied Biosystems does not support diluted reactions or guarantee the performance of diluted BigDye chemistry.
Template or extension products are degraded. With degraded extension products, the data are noisy, with a higher baseline at the start of peaks.	Review how templates are prepared and stored. Try a different method (Chapter 3) and store at – 20 °C.

Top-heavy data: ski slope profile
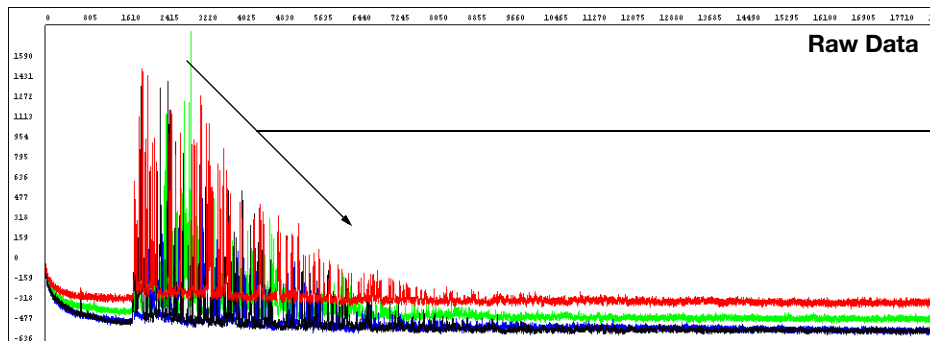
Peak profile is similar to a ski slope: peak heights decrease as the fragment lengths increase

Possible Cause(s)	Recommended Action
Sequencing reaction issues:	
Not enough or too much template was used in the sequencing reaction.	Review the DNA quantity (page 199).
Not enough or too much primer was used in the sequencing reaction.	
Not enough Ready Reaction Mix was used in the sequencing reaction.	Follow recommended procedures to prepare sequencing reactions with Ready Reaction Mixes. See page 66 for recommended procedures. Applied Biosystems does not support diluted reactions or guarantee the performance of diluted BigDye chemistry.
Template is degraded.	Review how templates are prepared and stored. Try a different method (Chapter 3) and store at – 20 °C.

Top-heavy data: preferential amplification of short sequence
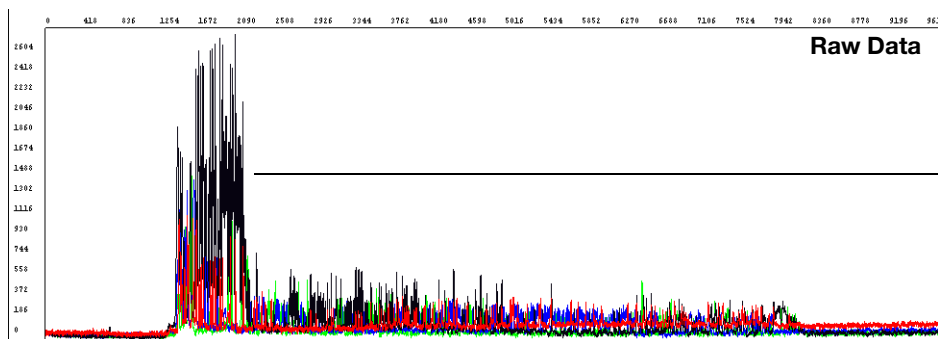
Short 5′ sequence, instead of the entire template, is amplified preferentially, (see also page 240)

Possible Cause(s)	Recommended Action
Sequencing reaction issue: Primer-dimer formation during the PCR reaction.	Redesign the PCR primers to eliminate the sequences that allow primer-dimer formation.
	Use a "hot start" PCR enzyme to inhibit primer-dimer formation.

Top-heavy data: split peaks with excessive signal

Peaks with excessive signal

Raw Data
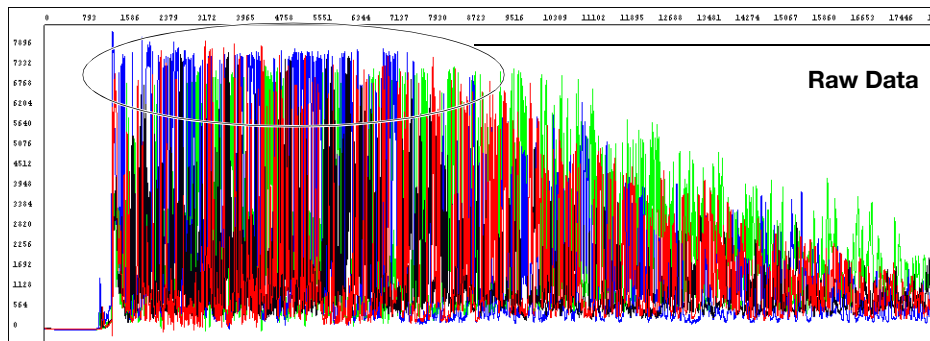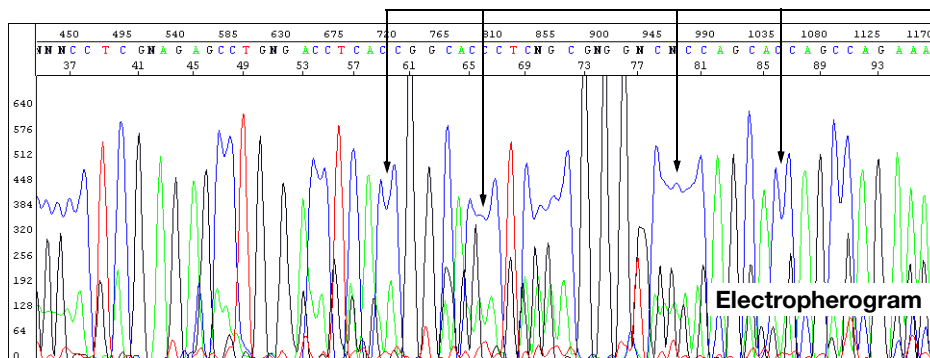
Split peaks and pullup at the beginning of the sequence in the region of peaks with excessive signal (circled above)

Electropherogram

Possible Cause(s)	Recommended Action
Sequencing reaction issues:	
Signal is too high because too much template was used in the sequencing reaction and too much sequencing product was created.	You do not have to repeat the reaction. Click the **Annotation** tab and check the Ave Signal Intensity. Excessive signal: • 3730/3730*xl* instruments: >10,000 rfus • 310 and 31XX instruments: >1000 rfus Especially if your samples were purified using the BigDye® XTerminator™ Purification Kit, load less labeled sample by performing one of the following: • Remove some of the sample and replace with Hi-Di™ Formamide • Inject sample for less time • Resequence the samples, using less template in the sequencing reaction, especially if you use the BigDye® XTerminator™ Purification Kit (see Table 8, "Recommended DNA template quantities for cycle sequencing," on page 63).
	For future reactions, reduce the amount of template in the sequencing reaction.
Injection height incorrect due to incorrect run module.	Use correct run module, especially for samples purified with the BigDye XTerminator Purification Kit (see "Selecting a Run Module" on page 137).

Four-color spikes

Raw Data

Four-color spikes

Electropherogram

Four-color spikes

Electropherogram (zoomed in)

Zoomed in electropherogram shows spikes contain all four colors

Possible Cause(s)	Recommended Action
Electrophoresis issues:	
Dust, bubbles, or crystals in polymer passed through the path of the laser beam.	1. Eliminate large amounts of dust in the environment.
	2. Inspect the upper gel block for bubbles. If present, flush all bubbles out of the system and out of the array manually.
	3. Check the polymer bottle for crystals. If present, warm the polymer gently to 30 °C with gentle mixing, then refill the syringes and array with the polymer.
	4. Replace polymer if the condition persists.
Polymer is expired or was stored at room temperature for more than 7 days.	Replace the polymer.

One-color spikes
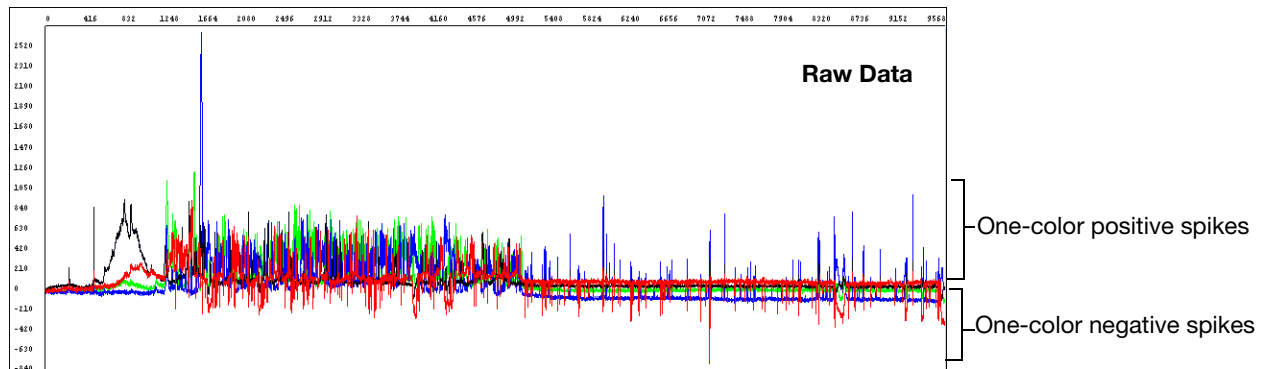
Possible Cause(s)	Recommended Action
Electrical noise or power fluctuations.	Verity the power source, use uninterruptible power supply.
Polymer temperature is too high.	• Verify the shipping temperature of the polymer. • Verify lab temperature is below 26 °C.
Well volume is too low.	• Verify volume is ≥10µL for 96-well plates and ≥15µL for 384-well plates. • If using septa, verify septa are fresh to minimize evaporation.

Large spike at the end of the run

Raw Data

Large spike at end of run

Possible Cause(s)	Recommended Action
The large spike at the end of the run, called a reptation peak, occurs with almost all electrophoretic separations of DNA on capillary instruments. With typical run conditions, data collection stops well before the spike occurs. There is no useful sequencing information in the spike or just before the spike. Because some run modules are designed for the longest possible read lengths, data collection stops just before the spike occurs. Normal run variation within a lab may result in the spike appearing in some electropherograms.	None needed. Shorten the data collection time a few minutes to remove a persistent spike from your data.

Improperly spaced peaks, especially peaks in the first 100 to 150 bases

Peaks are improperly spaced, especially peaks in the first 100 to 150 bases
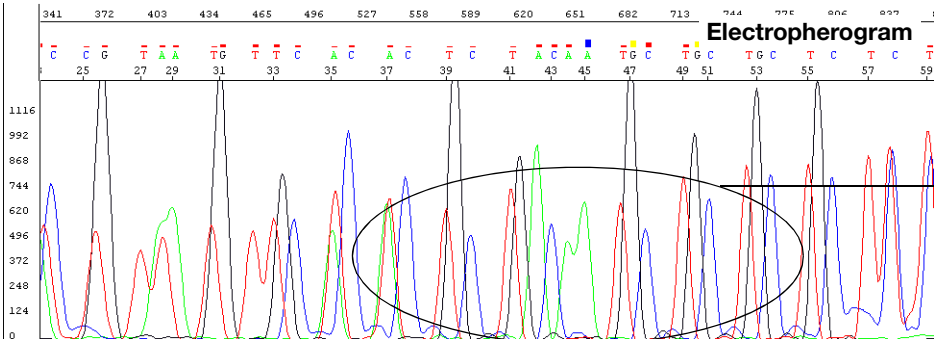
Possible Cause(s)	Recommended Action
Data analysis issue: Wrong mobility file applied to the sequence data.	Reanalyze the data using the correct mobility file to observe proper spacing of all peaks. For more information about mobility files, see page 145.

Large peaks (blobs) in the first 120 bases

Electropherogram
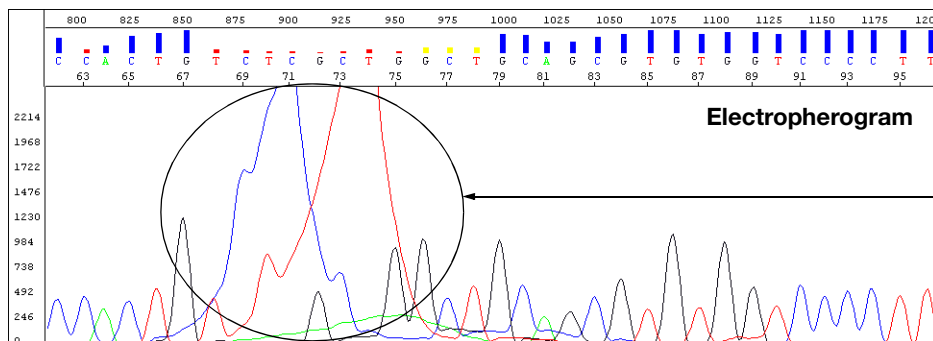
Blobs in the first 120 bases

Precise location of the blobs varies according to the dye used and the specific configuration

Possible Cause(s)	Recommended Action
Sequencing reaction issues:	
Incomplete removal of dye-labeled terminators after the cycle sequencing reaction.	Review the methods described in Chapter 5, "Purification of Extension Products." If you are using a third-party product for purifying extension products, contact the manufacturer for troubleshooting help. For future experiments, consider using the BigDye® XTerminator™ Purification Kit to purify samples (see page 88).
Poor incorporation of terminators, leaving excess unincorporated terminators.	Review the entire experiment carefully. • Check the quantitation (page 45). • Check the quality of the sequencing components. Replace each component, one at a time. • Run a DNA template control to determine whether the sequencing reaction failed or the template quality is low (page 64). • Check expiration dates on all reagents and replace any that have expired.
If using BigDye XTerminator Purification Kit, insufficient mixing during vortexing step.	• Verify plate is firmly attached to vortexer. • Follow protocol for vortexing.
If using BigDye XTerminator Purification Kit, incorrect ratio of BigDye XTerminator reagents.	• Vortex the XTerminator Solution bulk container at maximum speed for at least 10 seconds before dispensing. • Use wide-bore pipette tips to dispense the XTerminator Solution.

Irregular C peaks using BigDye® Terminators v3.1

C peaks have shoulders

C peaks are small and rough

No C peaks

Ave Signal Intensity for C is lower than for G, A, or T

Possible Cause(s)	Recommended Action
Sequencing reaction issues:	
The dye labels attached to the ddC terminators are degraded. Initial degradation results in shoulders on all C peaks. With further degradation, the C peaks appear very small or rough or disappear completely.	Protect the fluorescently labeled DNA from light, heat, acidic conditions, and oxygen (see "Storing Sequencing Reactions" on page 121). If no C peaks are visible, repeat the sequencing reactions with fresh reagents.
The Hi-Di™ Formamide is degraded.	Resuspend the samples using a newer lot of Hi-Di Formamide.
Sequencing reactions were exposed to light, heat, acidic conditions, and/or oxygen before they were loaded onto the instrument.	Use tube septa or a heat seal to prevent exposure to air and evaporation of samples, especially if you place the samples in the autosampler more than 6 hours before starting electrophoresis.
	Verify that the primer and template pHs are not acidic.

Possible Cause(s)	Recommended Action
Electrophoresis issue:	
The buffer heater is powered on (3730/3730*xl* instruments only).	Verify that the buffer heater is not powered on.
Severe arcing events can mask the C signal.	• Perform several water washes using the wizard in Data Collection Software. • Disassemble the system and clean out all components with warm water (<42 °C).

Irregular G peaks using BigDye® Terminators v1.1 and 3.1

Irregular G peaks

Possible Cause(s)	Recommended Action
Electrophoresis issue: The buffer heater is powered on (3730/3730*xl* instruments only).	If you are using the 3730 instrument, confirm that the buffer heater is not powered on.
Sequencing reaction issues:	
The Hi-Di™ Formamide is degraded.	Resuspend the samples using a newer lot of Hi-Di Formamide.
Sequencing reactions were exposed to light, heat, acidic conditions, and/or oxygen before they were loaded onto the instrument.	Use tube septa or a heat seal to prevent exposure to air and evaporation of samples, especially if you place the samples in the autosampler more than 6 hours before starting electrophoresis.
	Verify that the primer pH and the template pH are not acidic.
The dye labels attached to the ddG terminators are degraded. As shown in the figure above, the pattern for degradation of dye labels on ddG terminators is different than for ddC terminators. The G peak patterns are very irregular, and the complexity increases as degradation progresses. This problem can occur with BigDye Terminators v1.1 and less frequently with BigDye Terminators v3.1.	Protect the fluorescently labeled DNA from light, heat, acidic conditions, and oxygen (see "Storing Sequencing Reactions" on page 121).
Water used as Injection solution. **Note:** Resuspending samples in water leads to breakdown of C and/or G-labeled fragments.	Degradation of the dye labels attached to the ddG terminators is less likely to occur in Hi-Di Formamide or 0.1 mM EDTA.

Shoulders on all peaks

Electropherogram

All peaks show shoulders

Possible Cause(s)	Recommended Action
Electrophoresis issues:	
Capillary array needs to be replaced.	Replace the capillary array.
Overloaded sample.	Shorten the injection time.
	Amplify less DNA.
Sequencing reaction issues:	
Contamination of the sequencing primer with n+1 or n-1 sequencing primer.	Use the primers with a different template. If the problem persists, resynthesize the primers before repeating the experiment.
Stutter during either PCR amplification and/or cycle sequencing. Stutter is most common in any homopolymeric region greater than 2 bases. It can also be seen with simple repeated DNA sequences. The results are worse when the stutter occurs during PCR amplification. It is thought that stutter occurs when a partially extended primer and template dissociate, then reanneal improperly before extension continues. Partially extended primers and templates commonly dissociate during the reaction, but if they reanneal with complete fidelity, the reaction produces only one product. Improper annealing results in one or more products that are represented in the sequencing results.	If stutter occurs during PCR amplification, little can be done to correct the problem, except using anchored sequencing primers. If stutter occurs during cycle sequencing: • Try using dRhodamine terminators. They have been shown to be less prone to produce stutters, specifically with poly-T regions. • Some customers have found that they can get past poly(A) regions using a mixture of oligo dT$_{18}$ primers with either a C, A, or G as the 3′ terminal dinucleotide or 2-base anchors.
Blending Ready Reaction Mixes from dGTP BigDye terminator kits with BigDye terminator vx.1 kits.	Do not use blended Ready Reaction Mixes of dGTP BigDye terminator kits and BigDye Terminator vx.1 kits.

Peak compressions
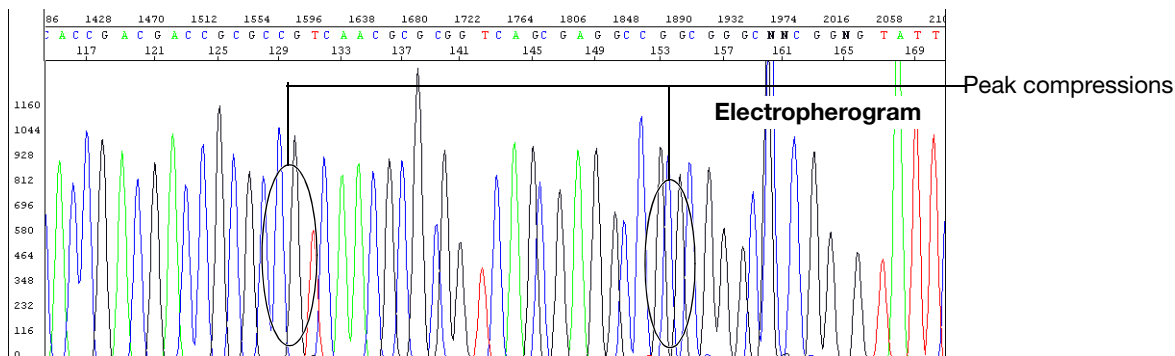
Possible Cause(s)	**Recommended Action**
Sequencing reaction issue: Observed when sequencing GC-rich regions using dGTP sequencing chemistry. Thought to result from incomplete denaturation of the synthesized DNA.	No corrective action is known at this time. Some customers report that using BigDye primers corrects this problem.

Broad peaks for bisulfite-converted sequences

Broad peaks in sequence with partial bisulfite conversion

Same sequence before bisulfite conversion

Possible Cause(s)	Recommended Action
Sequencing reaction issue: Mobility of fragments is uneven because the sample contains both Cs (from methylated samples) and no Cs (from unmethlyated samples).	• Repeat bisulfite conversion • Ensure amplicon is 250 to 400 bp for cloning and 100 to 250 bp for direct sequencing. • Include and extra incubation at the end of thermal cycling run for non-templated A addition.

Double peaks: peaks under peaks throughout

Possible Cause(s)	Recommended Action
Electrophoresis issues:	
Carryover from contaminated septa.	Replace septas, then change buffer, water, and waste.
Electrical noise.	Check the uninterruptible power supply (UPS).
Dirty containers and/or tap water were used to clean instrument components, resulting in contaminated water or buffer.	Clean the containers to be used for cleaning instrument components, then rinse the containers thoroughly with deionized water. It is preferable to use deionized water to clean the instrument components.
Shifted spatial calibration.	Redo the spatial calibration.
Poor CCD alignment.	Contact Applied Biosystems to arrange a service engineer visit.
Poor or incorrect spectral calibration (spectral pull-up).	Redo the spectral calibration.
Sequencing reaction issues:	
Secondary primer site in the template was sequenced.	Design a new sequencing primer (page 38).
Secondary amplification product in the PCR product used as a sequencing template.	Use gel purification to isolate the desired product or design new PCR primers to obtain a single product. For more information, see "Preparing PCR DNA Templates" on page 37.
PCR primers were not completely removed from the PCR product used as a sequencing template.	Remove PCR primers completely before using PCR products as sequencing templates. For more information, see "Preparing PCR DNA Templates" on page 37.
Mixed or contaminated templates or primers.	Review the DNA quality.

Possible Cause(s)	**Recommended Action**
Stutter during either PCR amplification and/or cycle sequencing. Stutter is most common in any homopolymeric region greater than 2 bases. It can also be seen with simple repeated DNA sequences. The results are worse when the stutter occurs during PCR amplification. It is thought that stutter occurs when a partially extended primer and template dissociate, then reanneal improperly before extension continues. Partially extended primers and templates commonly dissociate during the reaction, but if they reanneal with complete fidelity, the reaction produces only one product. Improper annealing results in one or more products that are represented in the sequencing results.	If stutter occurs during PCR amplification, little can be done to correct the problem, except using anchored sequencing primers. If stutter occurs during cycle sequencing: • Try using dRhodamine terminators. They have been shown to be less prone to produce stutters, specifically with poly-T regions. • Some customers have found that they can get past poly(A) regions using a mixture of oligo dT_{18} primers with either a C, A, or G as the 3′ terminal dinucleotide or 2-base anchors.
Very strong or offscale data	Reduce the signal: • Adjust the injection time and/or lower the voltage • Reduce the template concentration or use less sample

Double peaks: with high average signal intensity values

Electropherogram
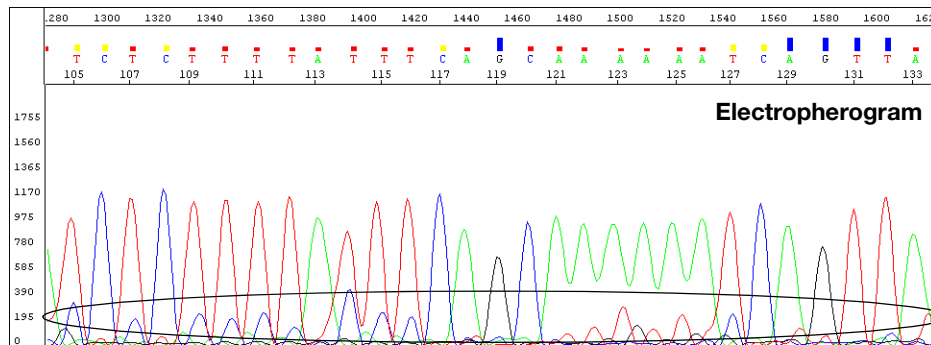
Secondary peaks under each peak

Number of Bases Detected:	565
Sample Score (from whole sequence):	21
Base Call Start (Scan #):	2117
Base Call End (Scan #):	14593
Peak 1 Location (Scan #):	2117
Ave Signal Intensity:	G (3062), A (4426), T (4530), C (3217)
Noise:	G (4), A (6), T (6), C (5)
Signal:Noise:	G (714), A (798), T (817), C (704)

Annotation

Ave Signal Intensity values are too high for data from a 3100 instrument

Possible Cause(s)	Recommended Action
Sequencing reaction issue: Signal is too high for data from the instrument. See page 197 for valid ranges.	Click the **Annotation** tab and examine the Ave Signal Intensity. Excessive signal: • 3730/3730*xl* instruments: >10,000 rfus • 310 and 31XX instruments: >1000 rfus Load less labeled sample by performing one of the following: • Dilute the resuspended product with Hi-Di™ Formamide before loading onto the instrument • Inject sample for less time • Resequence the samples, using less template in the sequencing reaction, especially if you use the BigDye® XTerminator™ Purification Kit (see Table 8, "Recommended DNA template quantities for cycle sequencing," on page 63).
Electrophoresis issue: Modified run module with increased injection time was used.	Use an unmodified standard run module.

Double peaks: at the beginning of the sequence

See also page 224 for the raw data view

Double peaks at the beginning of the sequence

Single peaks for the remaining sequence

Possible Cause(s)	Recommended Action
Sequencing reaction issue: Observed when a PCR product is used as a sequencing template. Caused by the formation of primer-dimers during the PCR reaction. The primer-dimers anneal and are filled in to create short, non-template PCR products.	If the sequence within the region affected by the primer-dimer sequence is important, either: • Redesign the PCR primers to eliminate the sequences that allow primer-dimer formation or • Use a "hot start" PCR enzyme to inhibit primer-dimer formation
More than 1 PCR product is present in the PCR reaction.	Re-examine the sequence for primer site homology. Redesign as necessary
More than 1 priming site (either upstream or downstream) on the sequencing template.	

Double peaks: at the beginning of the sequence (bisulfite conversion)

Possible Cause(s)	Recommended Action
Sequencing reaction issue: Observed when a PCR product is used as a sequencing template. Caused by the formation of primer-dimers during the PCR reaction. The primer-dimers anneal and are filled in to create short, non-template PCR products.	If the sequence within the region affected by the primer-dimer sequence is important, use M13 tails with both forward and reverse primers and either: • Redesign the PCR primers to eliminate the sequences that allow primer-dimer formation or • Use a "hot start" PCR enzyme to inhibit primer-dimer formation

Double peaks: specific peaks under specific bases

Electropherogram

C peaks under every peak

Possible Cause(s)	Recommended Action
Electrophoresis issues:	
Poor or incorrect instrument spectral calibration. Inspection of the raw data shows all secondary peaks directly under primary peaks.	Perform a new spectral calibration run. Follow the procedures in your instrument user guide. Then run your samples again.
Poor quality matrix (310 instruments only).	Create a new matrix file.
Sequencing reaction issue: Signals of the sample exceed the range used for spectral calibration because too much template was used.	Click the **Annotation** tab and examine the Ave Signal Intensity. Excessive signal: • 3730/3730*xl* instruments: >10,000 rfus • 310 and 31XX instruments: >1000 rfus Load less labeled sample by performing one of the following: • Dilute the resuspended product with Hi-Di™ Formamide before loading onto the instrument • Inject sample for less time • Resequence the samples, using less template in the sequencing reaction, especially if you use the BigDye® XTerminator™ Purification Kit (see Table 8, "Recommended DNA template quantities for cycle sequencing," on page 63).

Double peaks: specific peaks under specific bases

Electropherogram
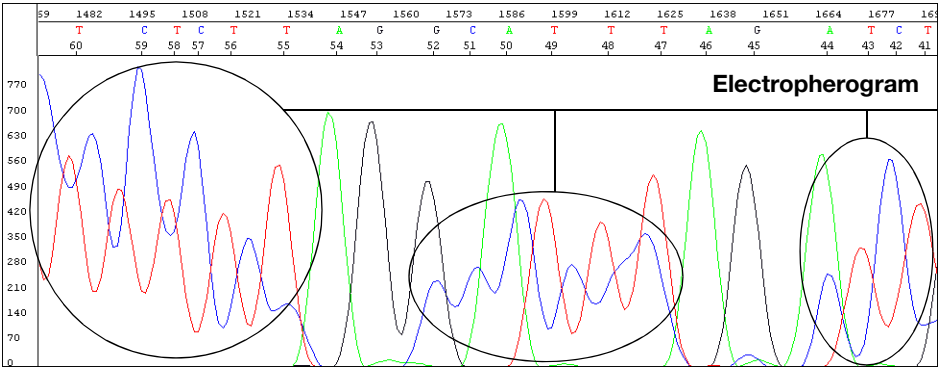
C peaks under many peaks using the ABI basecaller

Electropherogram
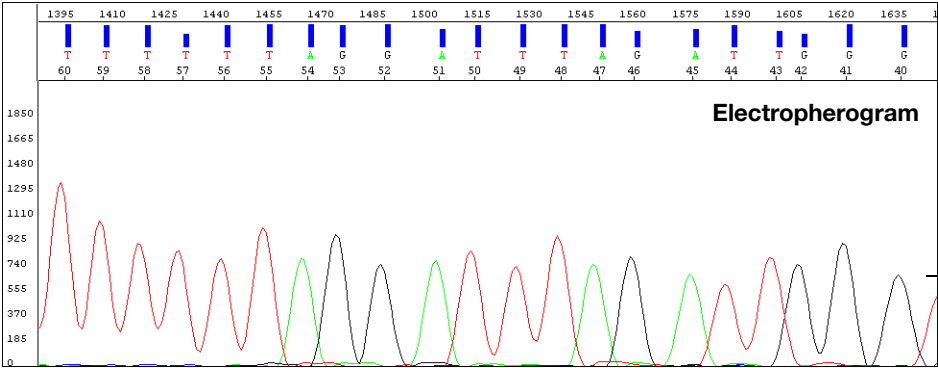
Clean sequence using the KB basecaller

Possible Cause(s)	Recommended Action
Data analysis issue: Using the ABI basecaller when analyzing sequences for bisulfite-treated DNA. Bisulfite treatment of DNA for methylation studies should convert all unmethylated Cs to uracil, so the sequence should contain very few C peaks. However, during sequence analysis, the analysis software overcalibrates for the absence of C peaks.	Use the KB basecaller to analyze sequences for bisulfite-treated DNA.

Double peaks: peaks under peaks throughout (bisulfite conversion)

Possible Cause(s)	Recommended Action
Sequencing reaction issue: Incomplete bisulfite conversion, indicated by the presence of Cs (blue) not adjacent to Gs (black). A C at a non-CpG position serves as an internal control for complete bisulfite conversion. Incomplete bisulfite conversion may be due to: • Impure gDNA • Too much gDNA • Inadequate denaturation of gDNA prior to bisulfite conversion	1. Check DNA quantitation and quality (page 44 and 45). 2. Repeat the bisulfite conversion 3. Repeat the sequencing.

Double peaks: double sequence (n+1 or n-1) throughout

n– 1 sequence
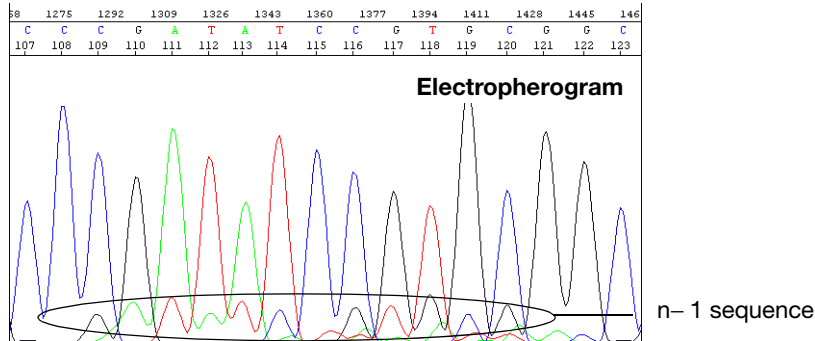
Possible Cause(s)	Recommended Action
Sequencing reaction issues:	
Contamination of the PCR primer with n+1 or n-1 primer.	Use the primers with a different template. If the problem persists, resynthesize the primers before repeating the experiment.
Contamination of the sequencing primer with n+1 or n-1 sequencing primer.	
Sequencing primer contains a run of identical nucleotides, especially 4 or more Gs.	Design new sequencing primers, avoiding runs of identical nucleotides, especially 4 or more Gs.
Homopolymer at the beginning of the sequence.	See page 246.

Double peaks: after a homopolymer or repeated sequence

Electropherogram

Double peaks after a homopolymeric region

Possible Cause(s)	Recommended Action
Sequencing reaction issue: Stutter during either PCR amplification and/or cycle sequencing. Stutter is most common in any homopolymeric region greater than 2 bases. It can also be seen with simple repeated DNA sequences. The results are worse when the stutter occurs during PCR amplification. It is thought that stutter occurs when a partially extended primer and template dissociate, then reanneal improperly before extension continues. Partially extended primers and templates commonly dissociate during the reaction, but if they reanneal with complete fidelity, the reaction produces only one product. Improper annealing results in one or more products that are represented in the sequencing results.	If stutter occurs during PCR amplification, little can be done to correct the problem, except using anchored sequencing primers. If stutter occurs during cycle sequencing: • Try using dRhodamine terminators. They have been shown to be less prone to produce stutters, specifically with poly-T regions. • Some customers have found that they can get past poly(A) regions using a mixture of oligo dT_{18} primers with either a C, A, or G as the 3′ terminal dinucleotide or 2-base anchors.

Double peaks: after a homopolymer or repeated sequence (bisulfite sequencing)

Region with repeating As

Electropherogram

Double sequence after a homopolymer region

Possible Cause(s)	Recommended Action
Sequencing reaction issue: Stutter during either PCR amplification and/or cycle sequencing. Stutter is most common in any homopolymeric region greater than 2 bases. It can also be seen with simple repeated DNA sequences. The results are worse when the stutter occurs during PCR amplification. It is thought that stutter occurs when a partially extended primer and template dissociate, then reanneal improperly before extension continues. Partially extended primers and templates commonly dissociate during the reaction, but if they reanneal with complete fidelity, the reaction produces only one product. Improper annealing results in one or more products that are each represented in the sequencing results.	If stutter occurs during PCR amplification, little can be done to correct the problem, except using anchored sequencing primers. If stutter occurs during cycle sequencing: • Try using dRhodamine terminators. They have been shown to be less prone to produce stutters, specifically with poly(T) regions. • Some customers have found that they can get past poly(A) regions using a mixture of oligo dT_{18} primers with either a C, A, or G as the 3′ terminal dinucleotide or 2-base anchors. • Avoid stretches with > 8 As or Ts. • Use BigDye® Terminator Ready Reaction Mix at full strength. • Use AmpliTaq Gold polymerase.

Double peaks: double sequence after clean sequence

Electropherogram
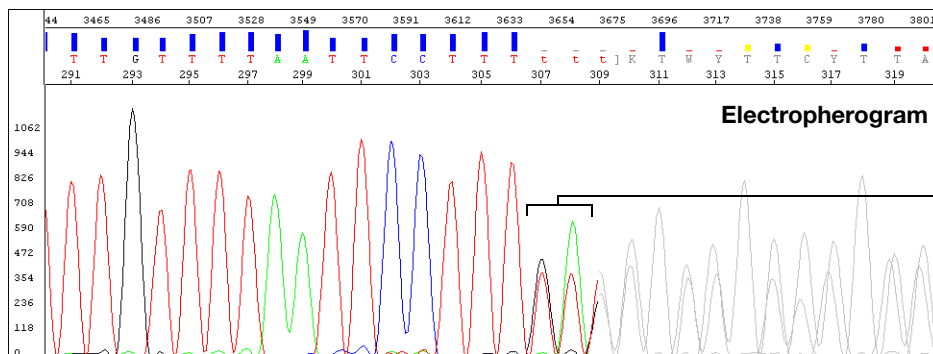
Double sequence after clean sequence

SeqScape Software Project View
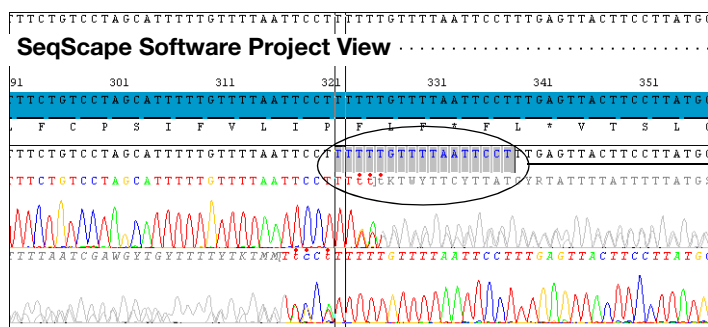
SeqScape Software detects HIM in the forward and reverse strands

Possible Cause(s)	Recommended Action
Heterozygous indel mutation (HIM).	Obtain forward and reverse sequence data and assemble using SeqScape or Variant Reporter™ Software. • SeqScape Software lists HIMs in the Mutations Report. Review the mutation by clicking the Base Change in the Mutations Report to view the mutation in the Project view. • Variant Reporter Software lists HIMs in the Project Summary Report.

Resolution loss: at beginning of run

Possible Cause(s)	Recommended Action
Sequencing reaction issues:	
XTerminator™ Solution or premix exposed to temperature over 25 °C.	• Use appropriate adapter for vortexer • Make sure plate does not heat up during vortexing step
BigDye XTerminator Purification Kit reagents past their expiration date.	• Verify expiration dates on reagents and discard if expired • Store XTerminator Solution at 4 °C • Store SAM Solution at room temperature

Resolution loss: in the middle of the run
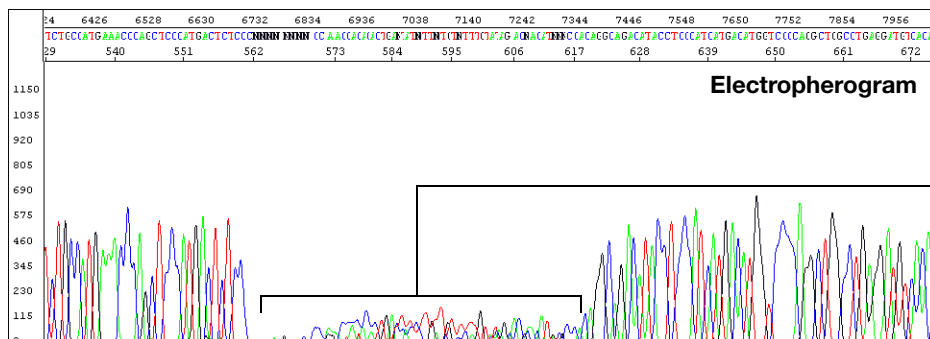
Loss of peak resolution in the middle of the run

Possible Cause(s)	Recommended Action
Electrophoresis issues:	
Migration of a contaminant or microbubbles through the capillary during electrophoresis.	Run the sample again.
Syringes, polymer block, or septa contaminated with chemicals during cleaning.	1. Perform a water wash through the polymer delivery system, using the Data Collection Software wizard. 2. Replace polymer, buffer, and water/waste with fresh materials. 3. Run the sample again.
Incomplete replacement of polymer between runs.	Check the polymer delivery system for leaks, looking for residue in and around the polymer block area; check the pin valve for signs of arcing on the tip; check for polymer in the anode buffer jar. If you see evidence of a leak, retighten, then run the sample again. If the leaking persists, contact Applied Biosystems to arrange a service engineer visit.

Resolution loss: gradual early loss

Raw Data

Resolution loss marked by broad peaks

Raw Data (zoomed in)

Zooming in on raw data (circled above) shows broad peaks

Electropherogram

Possible Cause(s)	Recommended Action
Electrophoresis issues:	
Capillary array degrading.	1. Perform a water wash through the polymer delivery system, using the Data Collection Software wizard. 2. Replace the capillary/array. 3. Run a sequencing standard. 4. If the problem persists, replace reagents, then run your samples again.
Samples degraded because they sat in the instrument too long, >48 hours.	Prepare additional sample for electrophoresis, referring to "Minimum Sample Volume" on page 122. Then, run the samples again.
Expired or old reagents: polymer, Hi-Di™ Formamide, buffer, or water.	Replace the reagent, then run your samples again.

Possible Cause(s)	Recommended Action
Electrophoresis source is faulty, resulting in unstable current.	Contact Applied Biosystems to arrange a service engineer visit.
Extension products purified using bead-based kits were injected without removing the magnetic beads. The beads may interfere with the extension products during injection and cause overloading or other injection anomalies.	Remove magnetic beads before loading the sample.
Capillaries overloaded with sequencing product.	Click the **Annotation** tab and examine the Ave Signal Intensity. Excessive signal: • 3730/3730*xl* instruments: >10,000 rfus • 310 and 31XX instruments: >1000 rfus Load less labeled sample by performing one of the following: • Dilute the resuspended product with Hi-Di Formamide before loading onto the instrument • Inject sample for less time • Resequence the samples, using less template in the sequencing reaction (especially if you use the BigDye® XTerminator™ Purification Kit) (see Table 8, "Recommended DNA template quantities for cycle sequencing," on page 63).
Blending Ready Reaction Mixes from dGTP BigDye terminator kits with BigDye terminator vx.1 kits.	Do not use blended Ready Reaction Mixes of dGTP BigDye Terminator kits and BigDye Terminator vx.1 kits in these cases.
Use of non-Applied Biosystems reagents.	1. Perform a water wash on all components of the system using the wizard in Data Collection Software. 2. Replace reagents with Applied Biosystems products. **Note:** the performance of non-Applied Biosystems reagents cannot be guaranteed.

High quality sequence in unassembled category in SeqScape Software

Gray sequence is in the unassembled category

High quality data
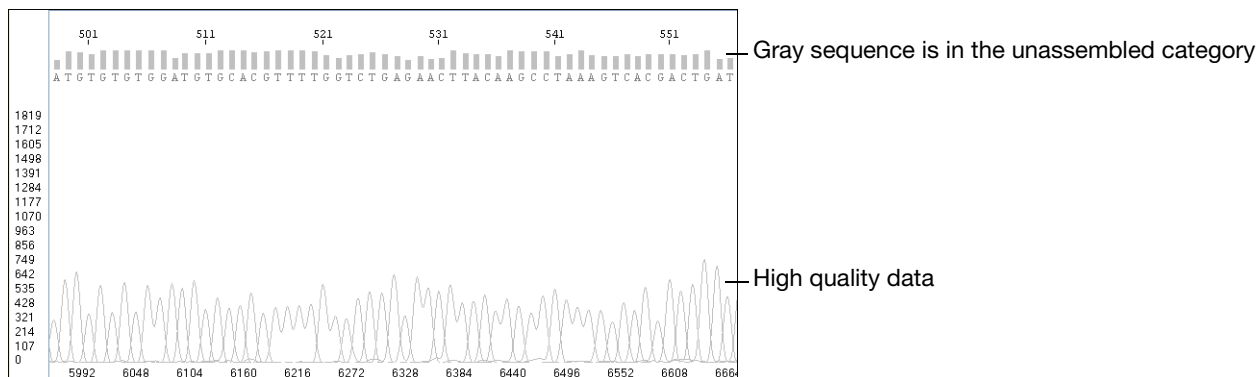
Possible Cause(s)	Recommended Action
SeqScape or Variant Reporter™ Software detects no similarity between the sample sequence and the reference sequence. The gray sequence indicates that the trimming of the data to the reference sequence failed.	Make sure that the sample is included in the right project.
Incorrect sample identification when a sample belonging to another project was imported.	

Troubleshooting Index

Product Information

A

This chapter covers:

Peak Color/Base Relationships

Peak Color in Raw Data

Because sequencing chemistries are developed to produce the most uniform signal in the analyzed data, the different chemistries may have different color (dye)/base relationships.

Table 38   Color/base relationships in raw data

Base	Peak Color in Raw Data			
	BigDye® Terminators	**dGTP BigDye Terminators**	**BigDye Primers**	**dRhodamine Terminators**
A	green	green	green	green
C	red	red	blue	black
G	blue	blue	black	blue
T	black	black	red	red

Peak Color in Electropherograms

All Applied Biosystems sequencing analysis software compensate for the different color/base relationships when the correct mobility file is used (see "Run Parameters for Instrument and Analysis Protocols" on page 151). The software always displays analyzed data in the electropherogram as shown in Table 39.

Table 39   Color/base relationships in electropherograms

Base	Peak Color in Electropherogram
A	green
C	blue
G	black
T	red

Control Sequences

M13 Control Primers   All Applied Biosystems dye terminator cycle sequencing kits include M13 control primers at 0.8 pmol/μL.

M13 Forward Primer Sequence

```
5´TGTAAAACGACGGCCAGT 3´
```

M13 Reverse Primer Sequence

```
5´CAGGAAACAGCTATGACC 3´
```

pGEM-3Zf(+) Sequence   All Applied Biosystems DNA sequencing kits provide pGEM control DNA at 0.2 μg/μL. The pGEM-3Zf(+) sequence below is the sequence of the 1000 bases that follow the M13 forward primer (GenBank accession number X65306).

```
                   20                           40                           60
GAATTGTAAT  ACGACTCACT  ATAGGGCGAA  TTCGAGCTCG  GTACCCGGGG  ATCCTCTAGA
                   80                          100                          120
GTCGACCTGC  AGGCATGCAA  GCTTGAGTAT  TCTATAGTGT  CACCTAAATA  GCTTGGCGTA
                  140                          160                          180
ATCATGGTCA  TAGCTGTTTC  CTGTGTGAAA  TTGTTATCCG  CTCACAATTC  CACACAACAT
                  200                          220                          240
ACGAGCCGGA  AGCATAAAGT  GTAAAGCCTG  GGGTGCCTAA  TGAGTGAGCT  AACTCACATT
                  260                          280                          300
AATTGCGTTG  CGCTCACTGC  CCGCTTTCCA  GTCGGGAAAC  CTGTCGTGCC  AGCTGCATTA
                  320                          340                          360
ATGAATCGGC  CAACGCGCGG  GGAGAGGCGG  TTTGCGTATT  GGGCGCTCTT  CCGCTTCCTC
                  380                          400                          420
GCTCACTGAC  TCGCTGCGCT  CGGTCGTTCG  GCTGCGGCGA  GCGGTATCAG  CTCACTCAAA
                  440                          460                          480
GGCGGTAATA  CGGTTATCCA  CAGAATCAGG  GGATAACGCA  GGAAAGAACA  TGTGAGCAAA
                  500                          520                          540
AGGCCAGCAA  AAGGCCAGGA  ACCGTAAAAA  GGCCGCGTTG  CTGGCGTTTT  TCCATAGGCT
                  560                          580                          600
CCGCCCCCCT  GACGAGCATC  ACAAAAATCG  ACGCTCAAGT  CAGAGGTGGC  GAAACCCGAC
                  620                          640                          660
AGGACTATAA  AGATACCAGG  CGTTTCCCCC  TGGAAGCTCC  CTCGTGCGCT  CTCCTGTTCC
                  680                          700                          720
GACCCTGCCG  CTTACCGGAT  ACCTGTCCGC  CTTTCTCCCT  TCGGGAAGCG  TGGCGCTTTC
                  740                          760                          780
TCATAGCTCA  CGCTGTAGGT  ATCTCAGTTC  GGTGTAGGTC  GTTCGCTCCA  AGCTGGGCTG
                  800                          820                          840
TGTGCACGAA  CCCCCCGTTC  AGCCCGACCG  CTGCGCCTTA  TCCGGTAACT  ATCGTCTTGA
                  860                          880                          900
GTCCAACCCG  GTAAGACACG  ACTTATCGCC  ACTGGCAGCA  GCCACTGGTA  ACAGGATTAG
                  920                          940                          960
CAGAGCGAGG  TATGTAGGCG  GTGCTACAGA  GTTCTTGAAG  TGGTGGCCTA  ACTACGGCTA
                  980                         1000
CACTAGAAGG  ACAGTATTTG  GTATCTGCGC  TCTGCTGAAG
```

Sequencing Standard Sequence

This section contains the sequence for the following sequencing standards:

- BigDye® Terminator v1.1 Sequencing Standard
- BigDye® Terminator v3.1 Sequencing Standard
- dRhodamine Terminator Cycle Sequencing Standard

You can obtain the sequence of the first 1000 bases using GenBank accession number AY390769.

			20		40	60
AATTCCCTGC	AGGCGTGGCT	GCAGCCTGGT	TATGATTACT	GTTAATGTTG	CTACTACTGC
	80		100		120
TGACAATGCT	GCTGCTGCTT	CTCCTCACTG	TCTCCACTTC	CTTGAACAAT	GCGCCGTCAT
	140		160		180
GCTTCTTTTG	CCTCCCGCTG	CTCCAGAAAG	CTAGGCCGCA	GATCAGAACC	ACCACAGTCA
	200		220		240
ATATCACCAC	CTTCCTCTTA	TAGATTCGGA	ATCTCATGAT	AGGGGCTCAG	CCTCTGTGCG
	260		280		300
AGTGGAGAGA	AGTTTGCAGG	CGAGCTGAGG	AGCAATTGCA	GGTGATATGA	TGTGCTCGGC
	320		340		360
TCAAGAAGCG	GGCCCGGAGA	GGAAGAAGTC	GTGCCGGGGC	TAATTATTGG	CAAAACGAGC
	380		400		420
TCTTGTTGTA	AACATTGATC	CAACTGGAAT	GTCACTAATG	GCGAATCAAT	ATTCCATAAG
	440		460		480
GCATGATGGT	TGCTCAGAGG	CAGGAGAAGA	GCAACGAATA	CGATCCTATA	AAAGATAAAA
	500		520		540
CATAAATAAA	CAGTCTTGAT	TATATTCTGG	GTATTAAAGC	CACAATCAGA	ACAAATATAT
	560		580		600
GCTTTGTATC	TTTTCTTGCC	TTCTTCATTA	CCAACTGCTT	CCGCGGCCAC	ATTAAGAGAA
	620		640		660
CTTGTGGTAA	GATAAGAAGA	TATTTTATTC	GTTCTGCTGA	CTTGCTGGAT	GTCGGGAAAT
	680		700		720
ATTCTGCATT	TGATAAGAGG	CGGTTAATTG	CAGATATAAT	TGGTAGTGAA	AAGGGTCGTT
	740		760		780
GCTATGGTCA	CCGTGAAGCG	AGTACAGCAG	CACAAGAATG	TGTGCCGTTC	TCAGTTAATA
	800		820		840
TTGTTTGAAT	ATGGTAACCT	GTTTTAGTCG	GTTTAAAGGT	AAGAAGATCT	AACCAAAAAC
	860		880		900
AACACTGCAG	TGACTGATTG	TAGTATTTAT	TTTTTTACTT	AATCTTAATT	TTGGTGTAAA
	920		940		960
CATCAACGGC	GCACTTCAAC	CAATACTCCA	ATGTTTTATC	CATCGACATG	ACGTTCGAGA
	980		1000		1020
TAGGGTTGAG	TGTTGTTCCA	GTTTGGAACA	AGAGTCCACT	ATTAAAGAAC	GTGGACTCCA
	1040		1060		1080
ACGTCAAAGG	GCGAAAAACC	GTCTATCAGG	GCGATGGCCC	ACTACGTGAA	CCATCACCCA
	1100		1120		1140
AATCAAGTTT	TTTGGGGTCG	AGGTGCCGTA	AAGCACTAAA	TCGGAACCCT	AAAGGGAGCC
	1160		1180		1200
CCCGATTTAG	AGCTTGACGG	GGAAAGCCGG	CGAACGTGGC	GAGAAAGGAA	GGGAAGAAAG

Parts List

B

This chapter covers:

Kits and Reagents

**BigDye®
XTerminator™
Purification Kits**

Product	Approximate Number of 20-μL Reactions	Part Number
BigDye® XTerminator™ Purification Kit (2-mL)	100	4376486
BigDye XTerminator Purification Kit (20-mL)	1,000	4376487
BigDye XTerminator Purification Kit (50-mL)	2,500	4376484
BigDye XTerminator Purification Kit (800-mL)	40,000	4376485

**BigDye®
Terminator v1.1
Cycle
Sequencing Kits**

Product	Part Number
BigDye® Terminator v1.1 Cycle Sequencing Kit (24 rxns)	4337449
BigDye® Terminator v1.1 Cycle Sequencing Kit (100 rxns)	4337450
BigDye® Terminator v1.1 Cycle Sequencing Kit (1000 rxns)	4337451
BigDye® Terminator v1.1 Cycle Sequencing Kit (5000 rxns)	4337452
BigDye® Terminator v1.1/v3.1 Sequencing Buffer (5 ✕) (1 mL)	4336697
BigDye® Terminator v1.1/v3.1 Sequencing Buffer (5 ✕) (25 mL)	4336699
BigDye® Terminator v1.1/v3.1 Sequencing Buffer (5 ✕) (230 mL)	4336701
BigDye® Terminator v1.1 Matrix Standard (3100-*Avant, 3100*)	4336824
BigDye® Terminator v1.1 Matrix Standards (310)	4336805
BigDye® Terminator v1.1 Sequencing Standard (3730, 3730*xl*)	4336799
BigDye® Terminator v1.1 Sequencing Standard (310, 3100-*Avant*), 3100	4336791

**BigDye
Terminator v3.1
Cycle
Sequencing Kits**

Product	Part Number
BigDye® Terminator v3.1 Cycle Sequencing Kit (24 rxns)	4337454
BigDye® Terminator v3.1 Cycle Sequencing Kit (100 rxns)	4337455
BigDye® Terminator v3.1 Cycle Sequencing Kit (1000 rxns)	4337456
BigDye® Terminator v3.1 Cycle Sequencing Kit (5000 rxns)	4337457
BigDye® Terminator v3.1 Cycle Sequencing Kit (25000 rxns)	4337458
BigDye® Terminator v1.1/v3.1 Sequencing Buffer (5 ✕) (1 mL)	4336697
BigDye® Terminator v1.1/v3.1 Sequencing Buffer (5 ✕) (25 mL)	4336699

Product	Part Number
BigDye® Terminator v1.1/v3.1 Sequencing Buffer (5 ✕) (230 mL)	4336701
BigDye® Terminator v3.1 Matrix Standards (310)	4336948
BigDye® Terminator v3.1 Matrix Standard (3100-*Avant*, 3100, 3130, 3130*xl*)	4336974
BigDye® Terminator v3.1 Sequencing Standard (310, 3100-*Avant*, 3100, 3130, 3130*xl*)	4336935
BigDye® Terminator v3.1 Sequencing Standard (3730, 3730*xl*)	4336943

BigDye Terminator dGTP Cycle Sequencing Kits

Note: 5 ✕ Sequencing Buffer for the BigDye Terminator dGTP Cycle Sequencing Kit is not the same as BigDye® Terminator v1.1/v3.1 Sequencing Buffer. Be sure to use the correct buffer.

Product	Part Number
5 ✕ Sequencing Buffer (1 tube)	4305605
5 ✕ Sequencing Buffer (9 tubes)	4305603
dGTP BigDye® Terminator v1.0 Cycle Sequencing Ready Reaction Kit (100 rxns)	4307175
dGTP BigDye® Terminator v3.0 Cycle Sequencing Ready Reaction Kit (100 rxns)	4390229

dRhodamine Terminator Cycle Sequencing Kits

Note: 5 ✕ Sequencing Buffer for the dRhodamine Terminator Cycle Sequencing Kit is not the same as BigDye® Terminator v1.1/v3.1 Sequencing Buffer. Be sure to use the correct buffer.

Product	Part Number
5 ✕ Sequencing Buffer (1 tube)	4305605
5 ✕ Sequencing Buffer (9 tubes)	4305603
dRhodamine Dye Terminator Cycle Sequencing Ready Reaction Kit (1000 rxns)	403045
dRhodamine Dye Terminator Cycle Sequencing Ready Reaction Kit (5000 rxns)	4303143
dRhodamine Matrix Standards Kit (310) (10 rxns)	403047
dRhodamine Matrix Standards Kit (310) (4 tubes)	403046
Long Read dRhodamine Terminator Cycle Sequencing Standard (1 tube)	4303120

BigDye Primer Cycle Sequencing Kits

Product	Part Number
BigDye® Primer Cycle Sequencing Ready Reaction Kit, – 21 M13 (100 rxns and protocol)	403051
BigDye® Primer Cycle Sequencing Ready Reaction Kit, – 21 M13 (5000 rxns)	403049
BigDye® Primer Cycle Sequencing Ready Reaction Kit, M13 REV (100 rxns and protocol)	403052
BigDye® Primer Cycle Sequencing Ready Reaction Kit, M13 REV (5000 rxns)	403050

Custom Primers

You can obtain custom primers from the Applied Biosystems Custom Oligonucleotide Synthesis Service. See page 40 for instructions.

Matrix Standards

Standard	Part Number
BigDye® Terminator v1.1 Matrix Standard (3100-*Avant*, 3100)	4336824
BigDye® Terminator v1.1 Matrix Standards (310)	4336805
BigDye® Terminator v3.1 Matrix Standard (3100-*Avant*, 3100, 3130, 3130*xl*)	4336974
BigDye® Terminator v3.1 Matrix Standards (310)	4336948
dRhodamine Matrix Standards Kit (310) (4 tubes)	403046
dRhodamine Matrix Standards Kit (310) (10 rxns)	403047
Matrix Standard Set DS-01, dROX, dTAMRA, dR6G, dR110	4305609

Sequencing Standards

Standard	Part Number
BigDye® Terminator v1.1 Sequencing Standard (3730, 3730*xl*)	4336799
BigDye® Terminator v1.1 Sequencing Standard (310, 3100-*Avant*, 3100	4336791
BigDye® Terminator v3.1 Sequencing Standard (3730, 3730*xl*)	4336943
BigDye® Terminator v3.1 Sequencing Standard (310, 3100-*Avant*, 3100, 3130, 3130*xl*)	4336935
Long Read dRhodamine Terminator Cycle Sequencing Standard (1 tube)	4303120

Reagent Kit Protocols

Protocol	Part Number
BigDye® Terminator v1.1 Cycle Sequencing Kit Protocol	4337036
BigDye® Terminator v3.1 Cycle Sequencing Kit Protocol	4337035
dGTP BigDye® Terminator Ready Reaction Kit With AmpliTaq® DNA Polymerase, FS Protocol	4339924
dGTP BigDye® Terminator v3.0 Ready Reaction Cycle Sequencing Kit Protocol	4307169
dRhodamine Terminator Cycle Sequencing Ready Reaction Kit With AmpliTaq® DNA Polymerase, FS Protocol	4339921
BigDye® Primer Cycle Sequencing Ready Reaction Kit With AmpliTaq® DNA Polymerase, FS Protocol	4339922

Plastics

Product	Part Number
MicroAmp® Fast 96-Well Reaction Plate, 0.1 mL	4346907
MicroAmp® Optical Film Compression Pad (5 per pack)	4312639
MicroAmp® Optical 384-Well Reaction Plate	4343370
MicroAmp® Clear Adhesive Films	4306311
MicroAmp® Optical 96-Well Reaction Plate	N8010560
MicroAmp® Reaction Tube without Cap, 0.2-mL	N8010533
Genetic Analyzer Sample Tubes, 0.5 mL	401957

Instrument Parts

Capillary
Consumables

Product	Part Number
10 ✕ Genetic Analyzer Buffer With EDTA (25 mL)	402824
Hi-Di™ Formamide (25 mL)	4311320
3730/3730*xl* **Capillary Consumables**	
10 ✕ Genetic Analyzer Buffer With EDTA (25 mL)	402824
Hi-Di Formamide (25 mL)	4311320
3730 36-cm capillary array	4331247
3730 50-cm capillary array	4331250
3730*xl* 36-cm capillary array	4331244
3730*xl* 50-cm capillary array	4331246
Applied Biosystems 3730/3730*xl* 10 ✕ Running Buffer With EDTA (500 mL)	4335613
POP-7™ Polymer (one 28-mL bottle)	4363929
POP-7™ Polymer (ten 28-mL bottles)	4363935
POP-7™ Polymer (thirty 28-mL bottles)	4335611
96-well sample plates w/barcode	4306737
96-well sample plates, no barcode	N8010560
96-well plate septa	4315933
384-well sample plates w/barcode	4309849
384-well plate septa	4315934
Heat Seal Film For Sequencing and Fragment Analysis Sample Plates	4337570
3130/3130*xl* **Capillary Consumables**	
10 ✕ Genetic Analyzer Buffer With EDTA (25 mL)	402824
Hi-Di Formamide (25 mL)	4311320
3130 22-cm capillary array	4333463
3130 36-cm capillary array	4333464
3130 50-cm capillary array	4333466
3130 80-cm capillary array	4333465
3130*xl* 22-cm capillary array	4319898

Product	Part Number
3130*xl* 36-cm capillary array	4315931
3130*xl* 50-cm capillary array	4315930
3130*xl* 80-cm capillary array	4319899
POP-4™ Polymer (3.5 mL)	4363752
POP-4™ Polymer (7 mL)	4352755
POP-6™ Polymer (3.5 mL)	4363783
POP-6™ Polymer (7 mL)	4352757
POP-7™ Polymer (3.5 mL)	4363785
POP-7™ Polymer (7 mL)	4352759
3100-*Avant* Capillary Consumables	
10 ✕ Genetic Analyzer Buffer With EDTA (25 mL)	402824
Hi-Di Formamide (25 mL)	4311320
Autosampler 96-well plate kit	4316471
Autosampler 384-well plate kit	4316472
3100 POP-6™ Polymer (7 mL)	4316357
3100 POP-4™ Polymer (7 mL)	4316355
96-well plate septa (20)	4315933
384-well plate septa (20)	4315934
3100 Instrument Consumable: Reservoir Septa (20)	4315932
3100-*Avant* 22-cm capillary array	4333463
3100-*Avant* 36-cm capillary array	4333464
3100-*Avant* 50-cm capillary array	4333466
3100-*Avant* 80-cm capillary array	4333465
3100 Capillary Consumables	
10 ✕ Genetic Analyzer Buffer With EDTA (25 mL)	402824
Hi-Di Formamide (25 mL)	4311320
3100 Instrument Consumable: Reservoir Septa (20)	4315932
Lower Polymer Block, v1.0	638-3680

Product	Part Number
3100 POP-4™ Polymer (7 mL)	4316355
3100 POP-6™ Polymer (7 mL)	4316357
3100 22-cm capillary array	4319898
3100 36-cm capillary array	4315931
3100 50-cm capillary array	4315930
3100 80-cm capillary array	4319899
310 Capillary Consumables	
10 ✕ Genetic Analyzer Buffer With EDTA (25 mL)	402824
Hi-Di Formamide (25 mL)	4311320
310 capillaries, 47cm ✕ 50 µm i.d. (internally uncoated) (5 pkg.)	402839
310 capillaries, 61cm ✕ 50 µm i.d. (internally uncoated) (2 pkg.)	402840
48-tube sample tray kit	402867
96-tube sample tray kit	402868
Genetic analyzer 4.0-mL buffer vials (50)	401955
Genetic analyzer retainer clips (96-tube tray septa clips) (4 pkg.)	402866
Genetic analyzer 0.5-mL Sample tubes (500)	401957
Genetic analyzer septa for 0.5-mL sample tubes (500)	401956
Genetic analyzer septa strips (0.2-mL tube) (480)	4305547
POP-4™ Polymer for 310 genetic analyzers (5 mL)	402838
POP-6™ Polymer for 310 genetic analyzers (3 mL)	402837

**3730/3730*xl*
Instrument Parts
and Documents**

Product	Part Number
3730/3730*xl* Instrument Accessories	
384-Well Plate Autosampler Installation Kit	4336134
384-Well Plate Base (Heat Seal) (4)	4334877
384-Well Plate Base (Septa Seal) (4)	4334874
384-Well Plate Retainer (Septa Seal) (4)	4334868
96-Well and 384-Well Plate Retainer (Heat Seal) (4)	4334865
96-Well Plate Autosampler Installation Kit	4336131
96-Well Plate Base (Heat Seal) (4)	4334875
96-Well Plate Base (Septa Seal) (4)	4334873
96-Well Plate Retainer (Septa Seal) (4)	4334869
Anode Buffer Jar, 67-mL, v2.0	625-2045
Array Ferrule Knob	628-3730
Array Ferrule Sleeves, Peek (4)	628-0165
Buffer, Water, and Waste Labels (4 sheets)	625-1195
Buffer, Water, and Waste Retainer (3)	625-0500
Cathode Buffer Plate Base	625-0504
Drip Tray	625-1088
Lower Polymer Block, v2.0	625-2040
PDP/Lower Block Interconnect Line, Radel, v2.0 (2)	625-2043
Polymer Bottle Cap and Line, Radel, v2.0 (2)	625-2047
Reservoir Cap (3)	625-0503
Reservoir, Buffer Water Waste (3)	625-0501
Water and Waste Plate Base (4)	625-0502

3130/3130*xl* Instrument Parts and Documents

Product	Part Number
3130/3130*xl* Instrument Accessories	
Array comb holders	628-0164
Array port plug	628-3776
Buffer reservoir, 16 mL	4358351
Buffer/water/waste reservoir	628-0163
Ferrule knob	628-3730
Ferrule sleeves	628-0165
Polymer bottle	4362387
Polymer tubing assembly	628-3732
Septa strip, buffer reservoir	4315932
3130/3130*xl* Instrument Documents	
Applied Biosystems 3130/3130xl Genetic Analyzers Getting Started Guide	4352715
Applied Biosystems 3130/3130xl Genetic Analyzers Maintenance, Troubleshooting, and Reference Guide	4352716
Applied Biosystems 3130/3130xl Genetic Analyzers Quick Reference Card	4362825
Applied Biosystems 3730/3730xl DNA Analyzers and 3130/3130xl Genetic Analyzers AB Navigator Software Administrator Guide	4359472

3100-*Avant* Instrument Parts and Documents

Product	Part Number
3100-*Avant* Instrument Accessories	
Anode buffer reservoir jar	5402
96-well plate retainer (4)	4317241
96-well plate base (AB) (4)	4317237
384-well plate retainer (4)	4317240
384-well plate base (4)	4317236
3100-*Avant* Instrument Documents	
ABI PRISM® 3100-Avant Genetic Analyzer User Guide	4333549
ABI PRISM ® 3100 Genetic Analyzer and 3100-Avant Genetic Analyzer User Reference Guide	4335393

Product	Part Number
ABI PRISM® 3100/3100-Avant Data Collection v2.0 User Guide	4347102

3100 Instrument Parts and Documents

Item	Part Number
3100 Instrument Accessories	
250-µL glass syringe (array-fill syringe) (Contains syringe, o-rings, and ferrule)	4304470
5.0-mL glass syringe (polymer reserve syringe) (Contains syringe, 0-rings, and ferrule)	628-3731
96-well plate base (AB) (4)	4317237
96-well plate retainer (4)	4317241
96-well plate septa (20)	4315933
384-well plate base (4)	4317236
384-well plate retainer (4)	4317240
384-well plate septa (20)	4315934
Anode buffer reservoir jar	005402
Array calibration ruler	628-3214
Array ferrule knob	628-3730
Array ferrule sleeves, PEEK (4)	638-0165
Autosampler 384-well plate kit	4316472
Autosampler 96-well plate kit	4316471
Reservoirs (for buffer, water, and waste)	628-0163
Polymer block tubing assembly	628-3732
Syringe ferrule	005401
Syringe O-rings	221102
Drip tray, lower polymer block	628-3088
Drip tray, autosampler	628-3059
Array calibration ruler	628-3732
Array comb holders	628-3403
Array ferrule sleeves	628-0165
Array ferrule knob	628-3730

Item	Part Number
3100 Instrument Spare Parts	
Drip tray, autosampler	628-3059
Lower polymer block drip tray	628-3088
Upper polymer block drip tray	628-3720
3100 Computer Accessories	
ABI PRISM® 3100 Genetic Analyzer Operator Training CD	4322559
3100 Instrument Documents	
ABI PRISM® 3100 Genetic Analyzer Quick Start Guide For Sequencing	4315833
ABI PRISM® 3100 Genetic Analyzer and 3100-Avant Genetic Analyzer User Reference Guide	4335393
ABI PRISM® 3100 Genetic Analyzer User Guide	4334785
ABI PRISM® 3100/3100-Avant Data Collection v2.0 User Guide	4347102

310 Instrument Parts and Documents

Item	Part Number
310 Instrument Accessories	
0.5-mL sample tray	5572
1.0-mL glass syringe	4304471
2.5-µL glass syringe	604042
96-Well tray adaptor	4305051
Anode buffer reservoir jar	5402
Basic bundle for Applied Biosystems 310 Genetic Analyzer	4327421
Capillary fitting	5404
Filter, air 10 × 20 × 7/8	201356
FTG, 1/4-28 TFE Syringe Plug 310	110319
FTG, Capillary Plug	7023
G5v2 Module CD	4339037
Gel Block Waste Vial	603796
Genetic Analyzer Capillary Cutters (2)	401958

Item	Part Number
O-Ring, 5/64ID × 13/64OD × 1/16BUNA	221100
O-Ring, 5/8 × 1/2 × 1/16 Silicone	221085
O-Ring, 7/8ID × 0.7 BUNA-N	221101
Syringe Ferrule, Bushing Seal	5401
Syringe Fitting Assembly	604071
Thermal Tape	310021
Waste Fitting Assembly	604076
310 Instrument Documents	
ABI PRISM® 310 Genetic Analyzer User Guide	4317588

Software Products

Primer Design

Product	Part Number
Methyl Primer Express® Software‡	4376041

‡ Can be downloaded for free from the Applied Biosystems web site.

Sequence Scanner Software

Sequence Scanner software can be downloaded for free from the Applied Biosystems web site at **www.appliedbiosystems.com/sequencescanner**.

Sequencing Analysis Software

Product	Part Number
Sequencing Analysis Software v5.3.1 (single-user)	4360967

Variant Reporter™ Software

Product	Part Number
Variant Reporter™ Software v1.0, 30-Day Demo	4385270
Variant Reporter™ Software v1.0, Initial License	4385261

SeqScape® Software

Product	Part Number
SeqScape® Software version 2.5, 45-Day Demo (5 users)	4327099
SeqScape® Software version 2.5, Initial License	4327091

MicroSeq® ID Analysis Software

Product	Part Number
MicroSeq® ID 16S rDNA 500 Library v2.0	**4371294**
MicroSeq® ID 16S rDNA Full Gene Library v2.0	4371295
MicroSeq® ID Fungal Gene Library v2.0	4371296
MicroSeq® ID Microbial Identification Software v2.0 Demo	4371297
MicroSeq® ID Microbial Identification Software v2.0 bundle (MSID v2.0 analysis software with bacterial and fungal libraries)	4371298
MicroSeq® ID Microbial Identification Software v2.0 Upgrade Bundle from MicroSeq® Mac Software	4371300
MicroSeq® ID Microbial Identification Software v2.0	4371431

Documents

Product	Part Number
Applied Biosystems MicroSeq® ID Analysis Software Version 2.0 Getting Started Guide, Rev B	4364623
Applied Biosystems Variant Reporter™ Software Version 1.0 Getting Started Guide	4376590
Applied Biosystems DNA Sequencing Analysis Software v5.1 User Guide	4346366
Applied Biosystems SeqScape® Software v2.5 User Guide	4359442
Applied Biosystems Sequencing Analysis Software Version 5.2 Quick Reference Card	4359446
KB™ Basecaller Frequently Asked Questions: KB Basecaller Software v1.4	4362968
Sequencing Analysis Software v5.2 User Bulletin	4358355

Miscellaneous Documents

The following documents are cited in the text.

Document	Part or Publication Number
A Workflow for Obtaining High Quality Sequencing Data from Bacterial Artificial Chromosome (BAC) DNA	107AP05-01
Advances in Fast PCR Contribute to a Fast Resequencing Workflow	104AP04-01
BigDye® Terminator v3.1 Cycle Sequencing Kit Protocol	4337035
BigDye® XTerminator™ Purification Kit Protocol	4374408
dGTP BigDye® Terminator Ready Reaction Kit Protocol	4307169
dGTP BigDye® Terminator v3.0 Ready Reaction Cycle Sequencing Kit Protocol	4390038
How to Order Primers and Probes (Publication 132GU01-01)	113920
Introducing New 96-Well Fast Plate Adapters for Applied Biosystems Capillary Electrophoresis Systems User Bulletin	4370890
Precipitation Methods to Remove Residual Dye Terminators from Sequencing Reactions User Bulletin	4304655

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Centre National de Séquençage (CNS, or Génoscope): **www.cns.fr**

QIAGEN: **www.qiagen.com**

Sanger Centre: **www.sanger.ac.uk**

The Association of Biomolecular Resource Facilities DNA Sequencing Research Group: **www.abrf.org**

University of Oklahoma Advanced Center for Genome Technology (ACGT): **www.genome.ou.edu**

University of Washington Genome Center: **www.genome.washington.edu/uwgc**

Washington University School of Medicine Genome Sequencing Center (GSC): **genome.wustl.edu/gsc**

International Union of Biochemistry/International Union of Pure and Applied Biochemistry (IUB/IUPAC): **www.chem.qmw.ac.uk/iubmb/misc/naseq.html#300**.

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Glossary

ABI basecaller An algorithm used to determine the bases of a sequence. This algorithm is available in Sequencing Analysis Software v5.x, SeqScape Software v2.x, and MicroSeq ID Analysis Software. Development on this algorithm ended in 2003.

allele One member of a pair or series of alternative forms of a genetic locus.

analysis protocol The settings that govern sample analysis: basecalling, mixed base identification, clear range and trimming, sequence file formats, and filtering.

assembly The aligned and overlapping sample data that result from the sequencing of one PCR product or clone.

Assembly view In SeqScape software, a view of the specimen consensus sequence, the aligned sample sequences, electropherograms, and quality values.

base spacing The number of data points from one peak to the next. A negative spacing value or a spacing value shown in red indicates a problem with your samples, and/or the analysis parameters.

basecaller An algorithm that determines the bases of a sequence during analysis. The two types of basecallers are KB and ABI.

chromatogram See electropherogram.

clear range The region of sequence that remains after excluding the low-quality or error-prone sequence at both the 5′ and 3′ ends. If the KB basecaller was used for analysis, the clear range is calculated from the quality values (QVs). If an ABI basecaller was used, the range is calculated from the Ns in the data and/or trim by the number of bases at the start and end of the data.

complement The opposite strand of double-stranded DNA. For example, if you sequenced the 3′ to 5′ strand, then the 5′ to 3′ strand is the complement.

consensus sequence The output of the assembly from a biologically related group of samples.

data point A sampling of fluorescence. Each data point is associated with a scan number.

DyeSet/Primer file See mobility file.

electropherogram A multi-color picture of a sequence showing peaks that represent the bases.

ept	A multi-color graph displaying the values for the voltage, power, current, and temperature for the entire run.
FASTA format	A standard text-based file format for storing one or more sequences.
heterozygote	A position at which the electropherogram displays more than one base.
HIM	Heterozygous insertion/deletion (indel) mutation.
IUB/IUPAC	International Union of Biochemistry/International Union of Pure and Applied Biochemistry.
KB basecaller	An algorithm used to determine the bases of a sequence. The algorithm can calculate mixed or pure bases and determines sample quality values. This algorithm is available in Sequencing Analysis Software v5.x, SeqScape Software v2.x, MicroSeq ID Analysis Software and Variant Reporter Software. Development on this algorithm is ongoing.
layout view	View of the layout of the sample assembly with arrows indicating the placement and orientation of samples.
length	The number of characters a sequence contains, including gap characters. For example, GAATTC has a length of 6 and GAA-TTC has a length of 7.
length of read	The usable range of high-quality or high-accuracy bases, as determined by quality values. This information is displayed in the Analysis report.
mixed bases	One-base positions that contain 2, 3, or 4 bases. These bases are assigned the appropriate IUB code.
mobility file	Files that compensate for the mobility differences between the dyes and primers and correct the color-code changes due to the chemistry used to label the DNA. Mobility files are sometimes referred to as DyeSet/Primer files.
noise	Average background fluorescent intensity for each dye.
*.phd.1 file	File format that can be generated during sample analysis. The file contains base calls and quality values.
quality values	An estimate (or prediction) of the likelihood that a given basecall is in error. Typically, the quality value is scaled following the convention established by the phred program: $QV = -10 \log10(Pe)$, where *Pe* stands for the estimated probability that the call is in error. Quality values are a measure of the certainty of the basecalling and consensus-calling algorithms. Higher values correspond to lower chance of algorithm error. Sample quality values refer to the per-base quality values for a sample, and consensus quality values are per-consensus quality values.
raw data	A multi-color graph displaying the fluorescence intensity (signal) collected for each of the four fluorescent dyes.

Reference Data Group (RDG)	The data that contain the reference and associated data.
sample data	The output of a single lane or capillary on a sequencing instrument. Sample data is entered into Sequencing Analysis, SeqScape, and other sequencing analysis software.
sample files	A file containing raw DNA sequence data (as read by the electrophoresis instrument), and the basecalls, peak locations, and electropherogram created by the Sequencing Analysis software. For Applied Biosystems genetic analysis instruments, raw sample files are created and, optionally, analyzed by the Data Collection Software. Raw or previously analyzed sample files are analyzed by Sequencing Analysis software.
Sample Manager	A window that displays sample file name, name, and specimen; last used basecaller and mobility (DyeSet/Primer) files; calculated basecalling results (spacing, peak 1, start and stop); and assembly status. The sample name, basecaller, and/or mobility file can be changed here.
sample score	The average of the per-base quality values for the bases in the clear-range sequence for the sample.
scan number	On an Applied Biosystems genetic analysis instrument, one sampling taken during each scan and stored as a data point.
*.scf file	A file format that can be generated during sample analysis. The file contains base calls, an electropherogram, and quality values but no raw data. **Note:** When standard chromatogram file format is created, the .scf extension is not appended to the file name. However, the file format is correct.
*.seq files	Text files, created by the Sequencing Analysis software, containing only the characters of the sequence. The *.seq files can be saved in ABI or FASTA format for use with other software.
sequence	A linear series of nucleotide base characters that represent a linear DNA sequence, or a linear series of amino acid characters that represent a protein sequence, displayed in rows from left to right.
sequencing reactions	The reactions performed to incorporate fluorescent dye labels into DNA extension products.
signal	A number that indicates the intensity of the fluorescence from one of the dyes used to identify bases during a data run. Signal strength numbers are shown in the Annotation view of the sample file.
signal:noise	The average of the signal intensity of the 'A', 'C', 'G', or 'T' base divided by the average noise for that base.
spacing	See base spacing.
specimen	The container that holds all the sample data as assembled contigs from a biological source or PCR product.

specimen view   In SeqScape software, a view of the consensus sequence and all sample files that were used to create that consensus sequence.

variants   Bases where the consensus sequence differs from the reference sequence that is provided.

Index

preventing 61, 122
symptom of 231, 233, 234
dye primers
emission spectra of dyes 8
features of chemistry 7
dye set, correct set for cycle sequencing kits 133
dye terminators
emission spectra of dyes 8
features of chemistry 6
DyeSet/Primer files. See mobility files.

E

editing bases
Sequence Scanner Software 164
Variant Reporter™ Software 177
electrokinetic injection, modifying 141
electropherogram
defined 285
example 11, 195
example using BigDye® primers 60
example using BigDye® terminators 57
example using dGTP BigDye® terminators 58
example using dRhodamine terminators 59
peak color/base relationships in 262
reviewing 195
electrophoresis
description 4
modifying conditions for 142
optimizing conditions for 142
preparing samples for 122
procedural overview 129
run temperature and voltage 143
run time 143
epigenetics *See* methlyation.
EPT tab, reviewing 198
ethanol precipitation, to purify extension products 96
examples
electropherogram 195
EPT tab 198
raw data 196
troubleshooting symptoms 201
ExoSAP-IT, using for purifying PCR reactions 43
expressed sequence tags 20
extension product purification
alternative procedures 119
BigDye XTerminator Purification Kit 88
ethanol precipitation 96
spin column and spin plate purification 116
extension products
about creation of 2
how labeled 6

F

failure, of capillaries 130

FASTA format, defined 286
file format
.phd.1 286
.scf 287
.seq 287
FASTA 286
formamide, resuspending samples in 122
formulas
Beer's Law 45
DNA quantity using A_{260} 45
melting temperature 39
oligonucleotide molecular weight 39
primer concentration 39
fosmid DNA
recommended sequencing kits for 56

G

G peaks, irregular 233
gap closure (custom primers), recommended sequencing kits for 56
GC-rich sequences, recommended sequencing kits for 56
gene walking (custom primers), recommended sequencing kits for 56
general settings, for analysis 143
genomic DNA
commercial products for preparing 36
preparing 34
genomic DNA, bacterial
quantity recommended 63
recommended sequencing kits for 56
GT-rich regions, recommended sequencing kits for 56

H

hazard symbols. *See* safety symbols, on instruments
heat seal, using 122
heparin, in genomic DNA 35
heterozygotes, verifying 180, 188
heterozygous indel mutation (HIM) detection 248
Hi-Di™ Formamide, using 122
homopolymeric regions
recommended sequencing kits for 56
troubleshooting sequencing of 206, 234, 238, 246, 247

I

IMPORTANT, description x
indel, detection of 248
injection
modifying 141
overview 9
instrument

peak symptoms
  blobs   230
  compressed peaks   235
  double peaks   239, 240, 242, 243, 244, 246, 248
  irregular C peaks   231
  irregular G peaks   233
  large peaks   230
  missing C peaks   231
  n+1 or n-1 sequence throughout   244
  peaks under peaks throughout   237
  resolution loss   250, 251
  shoulders   231, 234
  spikes   226, 227
  split peaks   225
  unevenly spaced peaks   229
pGEM-3Zf(+) control
  sequence   263
  troubleshooting using   194
  using   64
Phred, creating Phred (.phd.1) file   144
plasmid DNA template
  commercial products for preparing   34
  preparing   33
  recommended sequencing kits for   57
plate linking, in electrophoresis workflow   129
plate record
  creation of in electrophoresis workflow   129
  list of settings   136
plates, part numbers for   269
poly(A) regions, sequencing   247
poly(T) regions, sequencing   247
polymer
  problems caused by crystals   226
  problems caused by heating   226, 227
  recommendations   130
  supported types   10
power during run, reviewing   198
primer walking   18
primers
  designing   38, 61
  ordering custom   39
  primer-dimer formation   224
  quantitation of   38, 61
  reviewing design of   200
  software for designing   278
protocol part numbers   269
purifying extension products
  ethanol precipitation   96
  overview   86
  using BigDye XTerminator Purification Kit   88
  using magnetic bead adsorption/elution   119
  using size exclusion products   119
  using spin columns or spin plates   116

Q

Q Value   134
quality values (QVs)
  chart   172
  defined   286
  overview   11
quantitation
  of DNA template   45
  of primers   38, 61

R

raw data
  defined   286
  example of high quality   196
  in calibration results   134
  peak color/base relationships in   262
  reviewing   196
reaction plates, part numbers   269
read length
  for 310 instrument run modules   140
  for 3100 instrument run modules   139
  for 3100-*Avant* instrument run modules   139
  for 3130/3130*xl* instrument run modules   138
  for 3730/3730*xl* instrument run modules   138
read length, reviewing   196
Ready Reaction Mix
  blended   234, 252
  improper storage   208
  insufficient quantity   207, 211, 220, 221, 222, 223
Reference Data Group (RDG)
  creating   184
  definition   287
references, literature   281 to 282
reptation peak   228
resequencing   20
resolution
  improving   142
  loss of   214, 250, 251
results group, creation of in electrophoresis
        workflow   129
resuspending purified sequencing products   122
RNA, using as starting material   35
rolling circle amplified product
  recommended sequencing kits for   57
run module
  modifying   141
  selecting   137
run. See instrument run.

S

safety
  biological hazards   xi
  chemical   xi