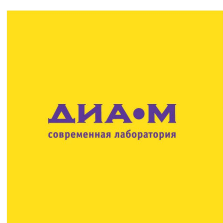


DNA Sequencing by Capillary Electrophoresis

Applied Biosystems Chemistry Guide | Third Edition



For Research Use Only. Not for use in diagnostic procedures.

Contents

Preface	v
How to use this guide	v
How to obtain support	vi
Safety and EMC compliance information.	vii
Safety conventions used in this document	viii
Chemical safety	ix
Chemical waste safety	ix
Biological hazard safety	ix
Chapter 1 Introduction to DNA sequencing	
DNA sequencing basics	2
Cycle sequencing	4
Capillary electrophoresis	8
Data analysis	10
Automated DNA sequencing workflow	13
Chapter 2 Applications overview	
DNA sequencing applications and approaches	16
<i>De novo</i> sequencing of genomes	16
Resequencing	21
Epigenetics	24
Microbial analysis	27

Chapter 3 DNA template preparation

Overview32
Preparing vector-based DNA templates33
Preparing genomic DNA34
Preparing PCR DNA templates.35
Primer design and quantitation.37
Purifying PCR products for sequencing40
DNA template quality40
DNA template quantity.42
Preparing templates for bisulfite sequencing.42

Chapter 4 Cycle sequencing

Overview46
Choosing a sequencing chemistry47
Reagent and equipment considerations52
DNA quantity54
Using DNA template controls.55
Using BigDye Direct Cycle Sequencing Kit57
Using BigDye Terminators and dGTP BigDye Terminators.60
Bisulfite sequencing.63

Chapter 5 Purification of extension products

Overview68
Purification with the BigDye XTerminator Purification Kit69
Purification by ethanol precipitation76
Purification with spin columns87
Sample preparation for electrophoresis89
Samples purified with other purification methods91

Chapter 6 Capillary electrophoresis

Applied Biosystems genetic analyzers	96
Instrument consumables	98
Calibrating the instrument	100

Chapter 7 Data analysis

Overview	104
Analysis software	105
Sequence Scanner Software	111
Sequencing Analysis Software	114
Variant Reporter Software	117
SeqScape Software	119
MicroSeq ID Analysis Software	122
MicrobeBridge Software	126
Sanger analysis modules on Thermo Fisher Cloud	128
Quality Check (QC) module	129
Variant Analysis (VA) module	130
Next-Generation Confirmation (NGC) module	131
Minor Variant Finder Software	133

Chapter 8 Troubleshooting

Troubleshooting overview	136
Troubleshooting workflow	136
Table of troubleshooting symptoms	141
Troubleshooting examples	143

Appendix A Product information

Peak color/base relationships	192
Control sequences	192

Bibliography	195
Literature cited in text	195
Websites	196
Additional references	197
Glossary	199
Index	203

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.

Audience

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

Assumptions

This guide assumes that your genetic analyzer has been installed by a Thermo Fisher Scientific 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 user documentation for Applied Biosystems products. 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 viii.

How to obtain support

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

At the Support page, you can:

- Search through frequently asked questions (FAQs)
- Submit a question directly to technical support
- Order user documents, material safety data sheets (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 Thermo Fisher Scientific technical support and sales facilities.

Safety and EMC compliance information

This section covers:

Safety conventions used in this document	viii
Chemical safety	ix
Chemical waste safety	ix
Biological hazard safety	ix

Safety conventions used in this document

Safety alert words

Four safety alert words appear in the user documentation at places in the document where you need to be aware of relevant hazards. Each alert word—**IMPORTANT**, **CAUTION**, **WARNING**, and **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.



DANGER

—indicates a potentially hazardous situation that, if not avoided, could result in death or serious injury.



WARNING

—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 **IMPORTANT!**, each safety alert word in the 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 down 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 material safety data sheet (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 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 warning

**CAUTION**

HAZARDOUS WASTE. Refer to MSDSs 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:

Safety and EMC compliance information

- U.S. Department of Health and Human Services guidelines published in *Biosafety in Microbiological and Biomedical Laboratories* (stock no. 017-040-00547-4)
<http://www.cdc.gov/biosafety/publications/bmb15/>
- Occupational Safety and Health Standards, Bloodborne Pathogens Standard (29 CFR 1910.1030) https://www.osha.gov/SLTC/bloodbornepathogens/bloodborne_quickref.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>.

Chapter 1

Introduction to DNA sequencing

This section covers:

DNA sequencing basics	2
Cycle sequencing	4
Capillary electrophoresis	8
Data analysis	10
Automated DNA sequencing workflow	13

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."

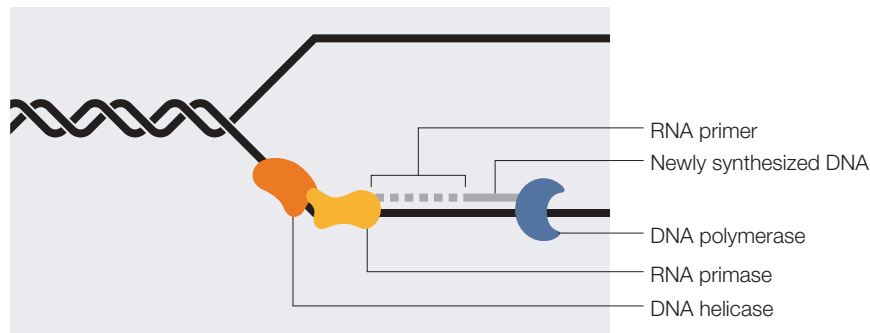


Figure 1. DNA replication fork.

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 [1] (Figure 2).

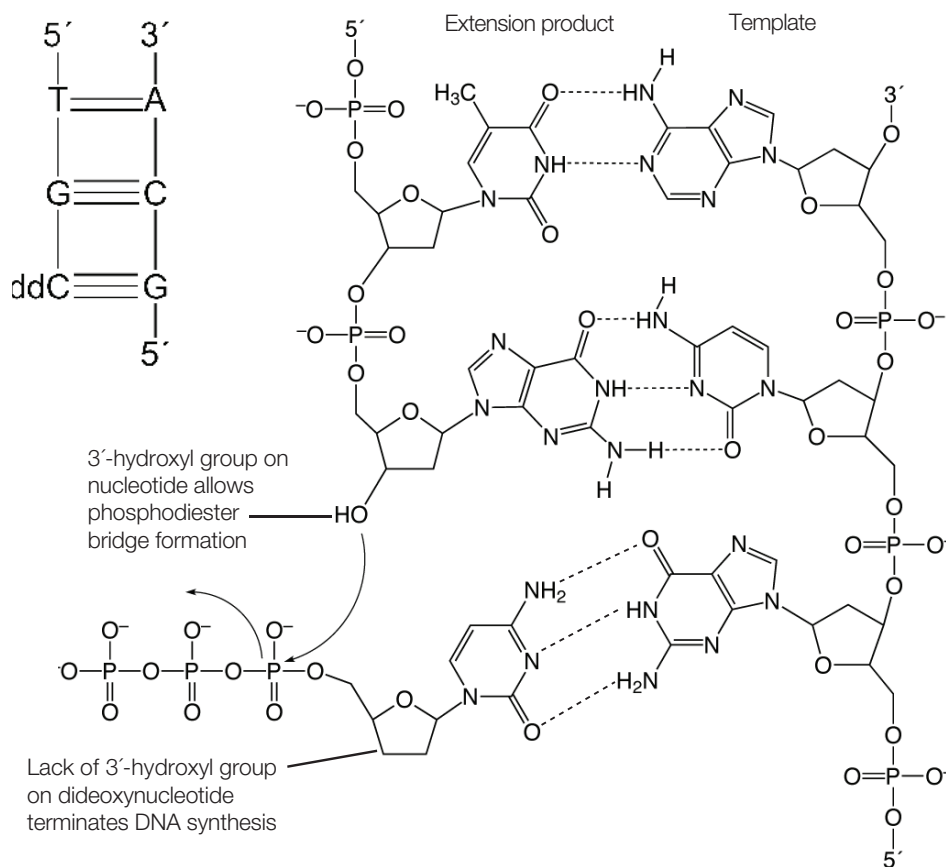


Figure 2. DNA strand synthesis and termination.

The DNA sequence is copied with high fidelity because at each base on the DNA template, DNA polymerase incorporates 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 2).

History of Sanger dideoxy sequencing

The principles of DNA replication were used by Sanger et al. [2] 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, deoxynucleotides (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 DNA polymerase has the optimal enzyme activity.

DNA polymerase adds 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, "DNA template preparation")
2. Cycle sequencing (Chapter 4, "Cycle sequencing")
3. Purification after cycle sequencing (Chapter 5, "Purification of extension products")
4. Capillary electrophoresis (Chapter 6, "Capillary electrophoresis")
5. Data analysis (Chapter 7, "Data analysis")

Cycle sequencing

Process overview

Like Sanger sequencing, fluorescence-based cycle sequencing requires a DNA template, a sequencing primer, a thermal stable DNA polymerase, deoxynucleoside triphosphates/deoxynucleotides (dNTPs), dideoxynucleoside triphosphates/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 3). The ratio of deoxynucleotides to dideoxynucleotides is optimized to produce a balanced population of long and short extension products.

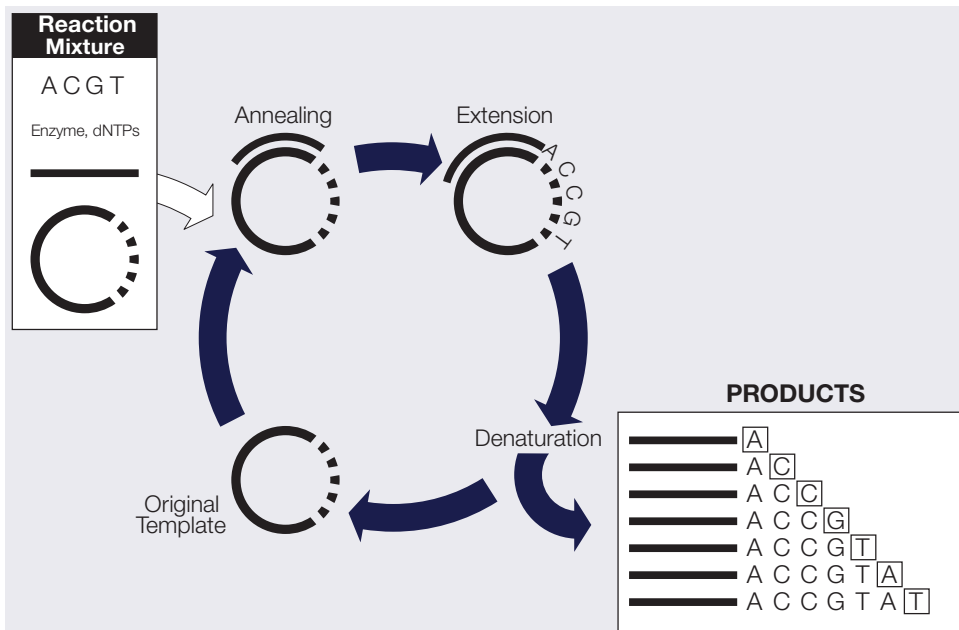


Figure 3. Example cycle sequencing reactions in a thermal cycler.

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 (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 4).

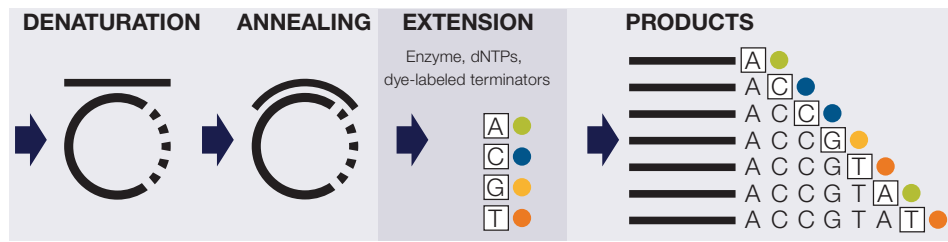


Figure 4. Diagram of dye terminator cycle sequencing.

The advantages of dye terminator chemistry compared to dye primer chemistry include:

- 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.
- Applied Biosystems BigDye™ Terminators v1.1 and v3.1 and dRhodamine Dye Terminators are formulated with dITP in place of dGTP to reduce peak compressions.
- Applied Biosystems ABI Prism™ 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 primers 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 5).

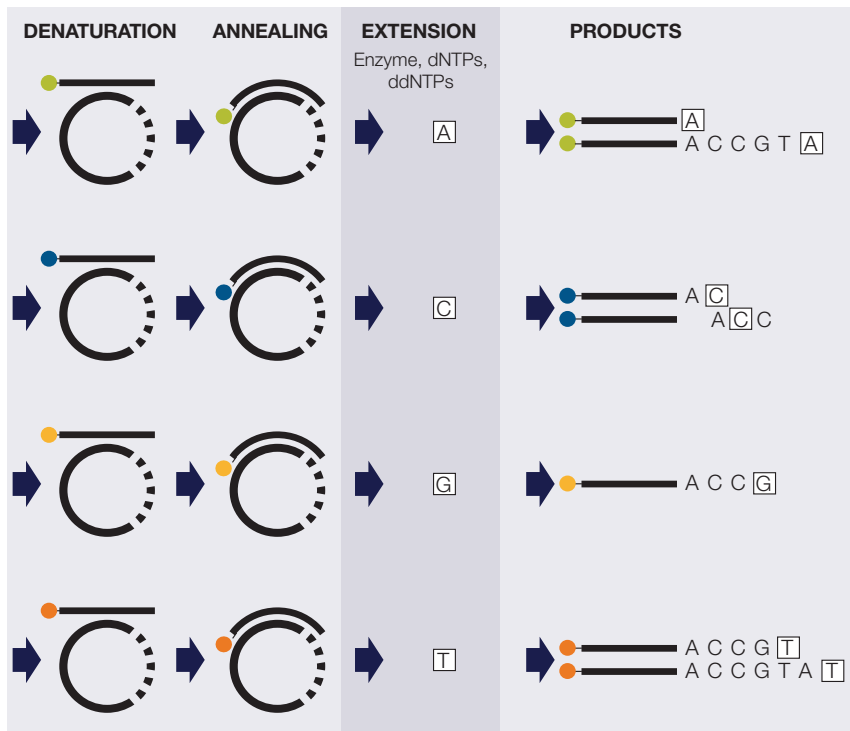


Figure 5. Diagram of dye primer cycle sequencing.

Advantages of dye primer chemistry compared to 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 dye terminator chemistries are:

- BigDye Terminator v1.1 and v3.1 Cycle Sequencing Kits
- dGTP BigDye Terminator v1.0 and v3.0 Cycle Sequencing Kits
- BigDye Direct Cycle Sequencing Kits

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 BigDye Direct 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 6 shows the normalized emission spectra and spectral overlap of the four dyes in the BigDye Terminator Cycle Sequencing Kit.

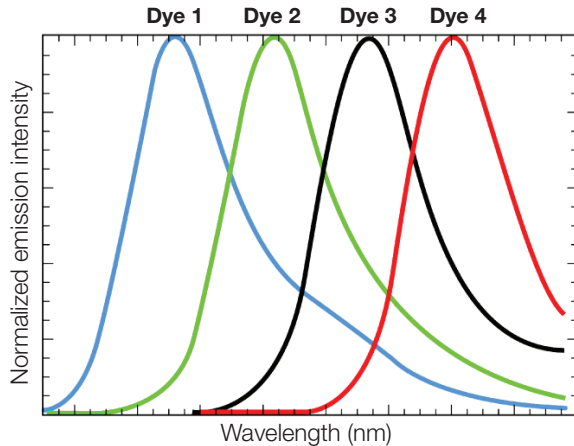


Figure 6. Emission spectra of the four BigDye dyes. Dye 1 = Big-d110, Dye 2 = R6G, Dye 3 = Big-dTAMRA, and Dye 4 = Big-dROX.

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 (.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 7).

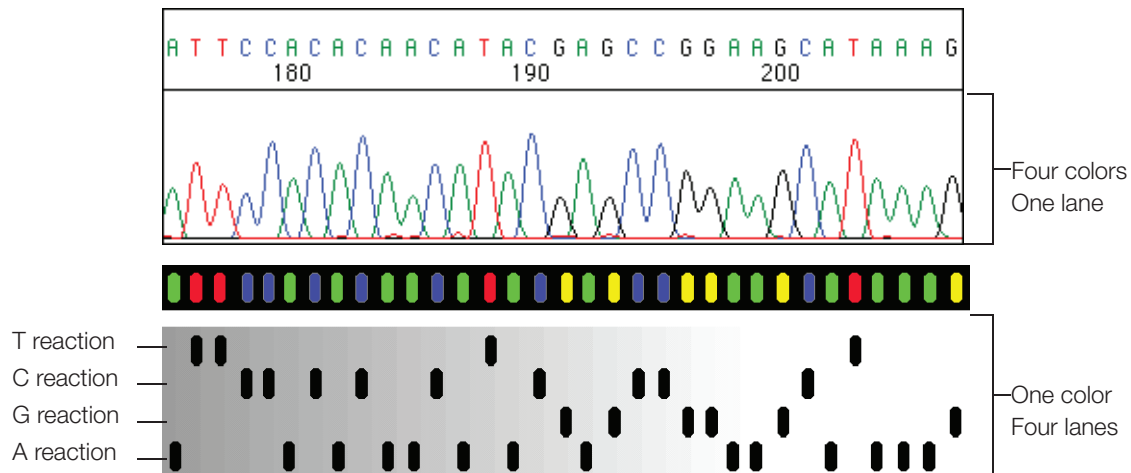


Figure 7. Fluorescent sequencing compared with radioactive sequencing.

Available instruments

Thermo Fisher Scientific offers the following automated Applied Biosystems genetic analyzers (Table 1).

Table 1. Applied Biosystems genetic analyzers.

Instrument	Number of capillaries	Capillary array length ^{†‡} (cm)	Polymer type	Sample capacity [§]
3730xl DNA Analyzer	96	36, 50	POP-7 POP-6 POP-CAP	96- and 384-well plates
3730 DNA Analyzer	48	36, 50	POP-7 POP-6 POP-CAP	96- and 384-well plates
3500xL Genetic Analyzer	24	36, 50	POP-7 POP-4 POP-6	96- and 384-well plates
3500 Genetic Analyzer	8	36, 50	POP-7 POP-4 POP-6	96- and 384-well plates
3130xl Genetic Analyzer	16	36, 50, 80	POP-7 POP-4 POP-6 POP-CAP	96- and 384-well plates
3130 Genetic Analyzer	4	36, 50, 80	POP-7 POP-4 POP-6 POP-CAP	96- and 384-well plates
3100 Genetic Analyzer*	16	36, 50, 80	POP-4 POP-6	96- and 384-well plates
3100-Avant Genetic Analyzer*	4	36, 50, 80	POP-4 POP-6	96- and 384-well plates
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.

*Systems have been discontinued and are no longer supported.

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 6 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 signals from dyes with emission spectra overlap.
- **Base calling**—The selected basecaller processes the fluorescence signals, then assigns a base to each peak (A, C, G, T, or N). If the Applied Biosystems KB™ Basecaller is used, it also provides per-base quality value predictions, optional mixed base calling, and automatic identification of failed samples.
- **Mobility shift correction**—The mobility file corrects electrophoretic mobility changes imposed by the presence of different fluorescent dye molecules associated with differently labeled reaction extension products. 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 are basecalled, you can simplify your workflow and use the KB Basecaller instead. The KB Basecaller can perform base calling and assign QVs. Then, you can generate PHD (phd.1) or SCF (.scf) files using the KB Basecaller to integrate with your downstream pipeline.

Analyzed sample data are displayed as an electropherogram, a sequence of peaks in four colors. Each color represents the base called for that peak (Figure 8).

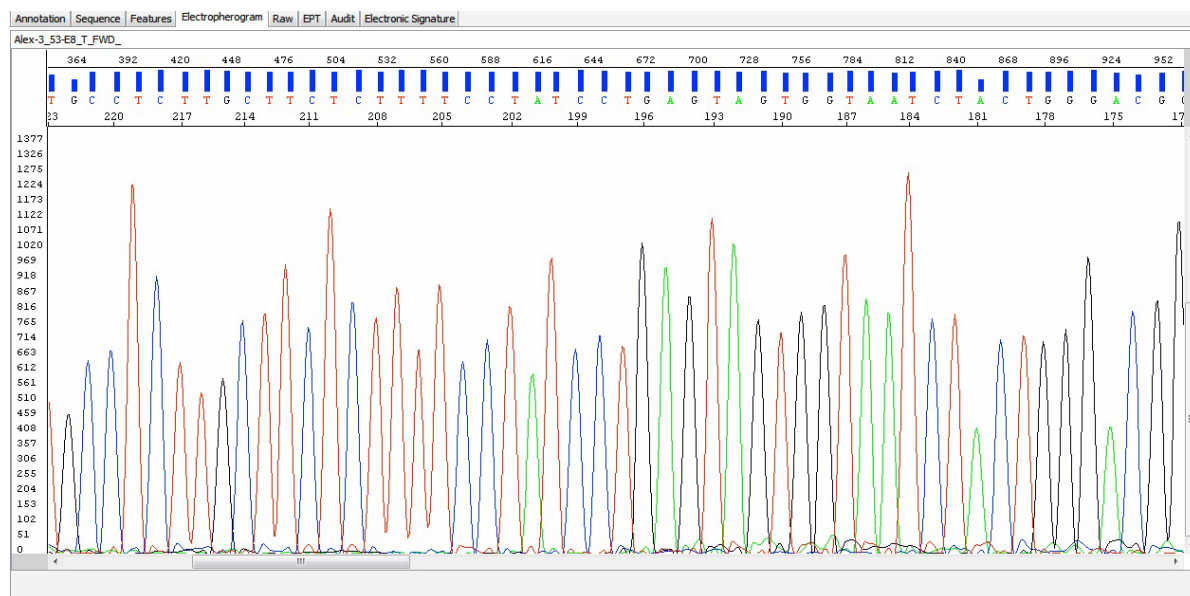


Figure 8. Example of electropherogram showing data analyzed with the KB Basecaller.

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, and making trace QC reports
Sequencing Analysis Software	Viewing or editing traces, evaluating trace quality, making trace QC reports, and reanalyzing traces
Variant Reporter Software	Mutation detection, SNP discovery, and validation
SeqScape Software	Mutation detection, SNP discovery and validation, sequence comparison, and typing
MicroSeq ID Analysis Software	With MicroSeq ID Kit for bacterial and fungal identification
New! MicrobeBridge Software	Bacterial identification using the CDC bacterial identification repository
New! Minor Variant Finder Software	High sensitivity detection of somatic variants to a level of 5% using Sanger sequencing
New! Sanger Analysis Modules on Thermo Fisher Cloud	FREE cloud-based secondary data analysis tools for Sanger sequencing. Available modules include Quality Check (QC), Variant Analysis (VA), and NGS Confirmation (NGC).

For more detailed information, see “Analysis software” on page 105.

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 can 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, BACs, or yeast artificial chromosomes (YACs), or prepare the 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
- BACs
- YACs
- Cosmids

Cycle sequencing (Chapter 4)



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

Cycle Sequencing Kits:

- BigDye Terminator v1.1 and v3.1 kits
- dGTP BigDye Terminator v1.0 and v3.0 kits
- BigDye Direct Cycle Sequencing Kits (streamlined workflow)



9700 thermal cycler

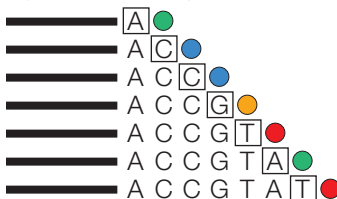


Veriti™ thermal cycler



ProFlex™ PCR system

Cycle sequencing output:
Dye terminator products



Purification of extension products (Chapter 5)



Purify extension products using one method:

- Applied Biosystems 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

Automated DNA sequencing workflow, continued

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.

Applied Biosystems instruments:



3730/3730xl analyzer



3500 analyzer

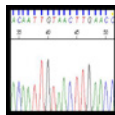


3130/3130xl analyzer



310 analyzer


Capillary electrophoresis output:



AB1 file

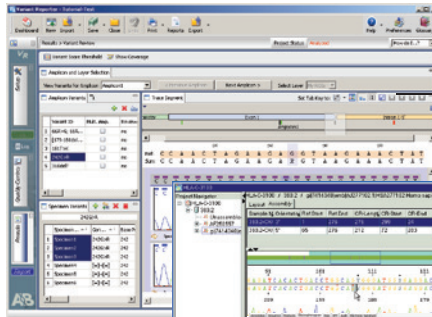
Data analysis
(Chapter 7)



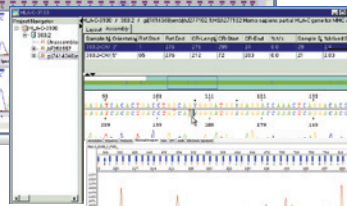
1. Apply analysis protocols:
 - Basecaller
 - Mobility file
2. Run analysis. 
3. Review the data.

Applied Biosystems software:

- Sequencing Analysis Software
- Variant Reporter™ Software
- SeqScape Software
- Sequence Scanner Software
- MicroSeq ID Analysis Software
- MicrobeBridge™ Software—New!
- Minor Variant Finder Software—New!
- Sanger Analysis Modules on Thermo Fisher Cloud—New!



Analyzed project in Variant Reporter Software



Analyzed project in SeqScape Software



Analyzed sample file in Sequencing Analysis Software



Analyzed sample file in Minor Variant Finder Software

Chapter 2

Applications overview

This section covers:

DNA sequencing applications and approaches	16
<i>De novo</i> sequencing of genomes	16
Resequencing	21
Epigenetics	24
Microbial analysis	27

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

To learn more about current applications using Sanger sequencing and to download the latest application notes, please visit [thermofisher.com/sangerapplications](https://www.thermofisher.com/sangerapplications).

Factors in selecting an approach

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

- **Length of the target** can range from determining the sequence of a single base to determining the sequence of a large genome.
- **Complexity of the sample** 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** can range from a single sample to thousands of samples.
- **Prior knowledge of the targeted sequencing region** can range 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 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 many different factors, including: 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 a 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 polymerase chain reaction (PCR) amplification than shorter genomic DNA fragments [3]. 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 page 34 for more information.

Template amplification using large insert vectors

Large-target DNA is cloned into bacterial artificial chromosomes (BACs) with insert sizes ranging from 100 to 300 kb. The BACs are then subcloned into smaller vectors that are more suitable for Sanger sequencing, with typical insert sizes of 1 to 10 kb. 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.

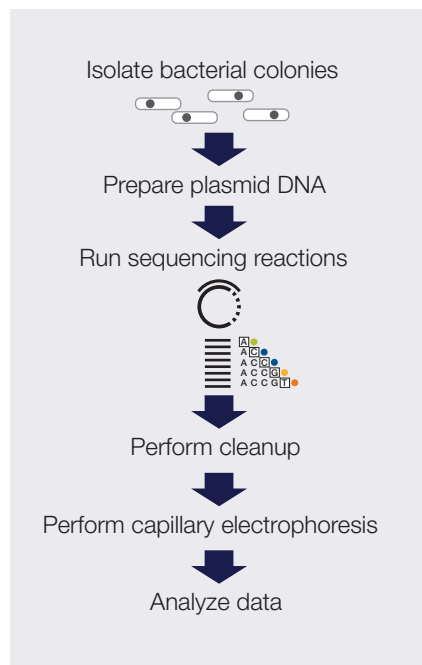


Figure 9. Proposed *de novo* sequencing workflow.

Clones with small inserts can be completely sequenced by 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 prime sites randomly 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 10).

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.

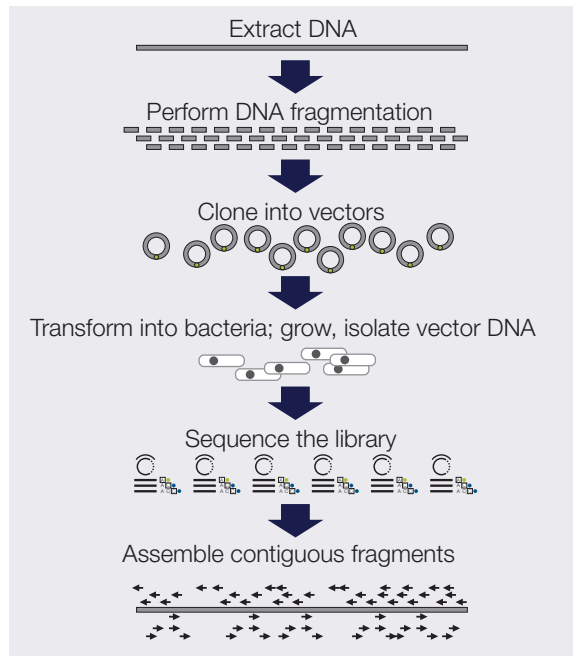


Figure 10. Shotgun sequencing.

To ensure full coverage of double-stranded template DNA, it may be necessary to sequence a region 7 to 10 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 11). These primers then serve as

sequencing start points to establish an additional >50 bp of sequence data. New primers are synthesized for the newly established sequence in the template DNA, and the process continues.

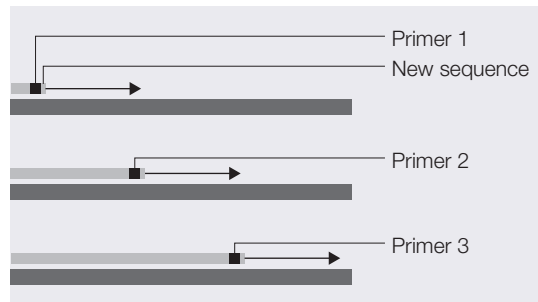


Figure 11. Primer walking.

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 12).

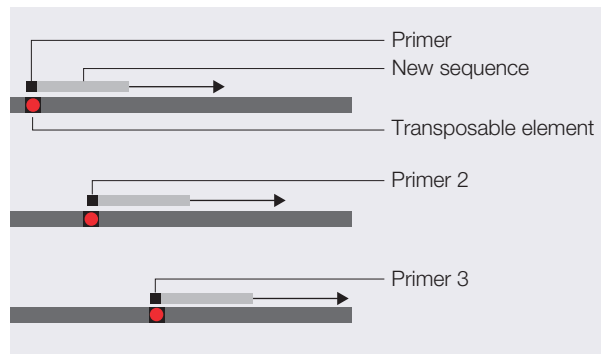


Figure 12. Transposon sequencing.

Nested deletions

Nested deletion strategies, with exonucleases or with restriction enzymes, help bring unknown DNA regions closer to the sequencing priming sites (Figure 13).

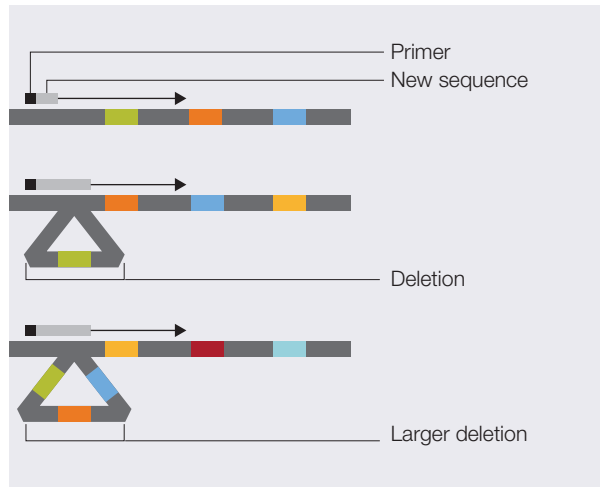


Figure 13. Nested-deletion sequencing.

PCR amplification of template

All of the above methods depend on template amplification by the 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 messenger RNA (mRNA) has been used to understand gene structure and to develop prediction rules for annotation of introns and exons 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. To this end, the mRNA is reverse transcribed into double-stranded cDNA. The cDNA is then cloned into a plasmid 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 amplification 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 bidirectionally 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 methylation analysis

PCR primer as the sequencing primer

Using the PCR primer as the sequencing primer (Figure 14) 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.

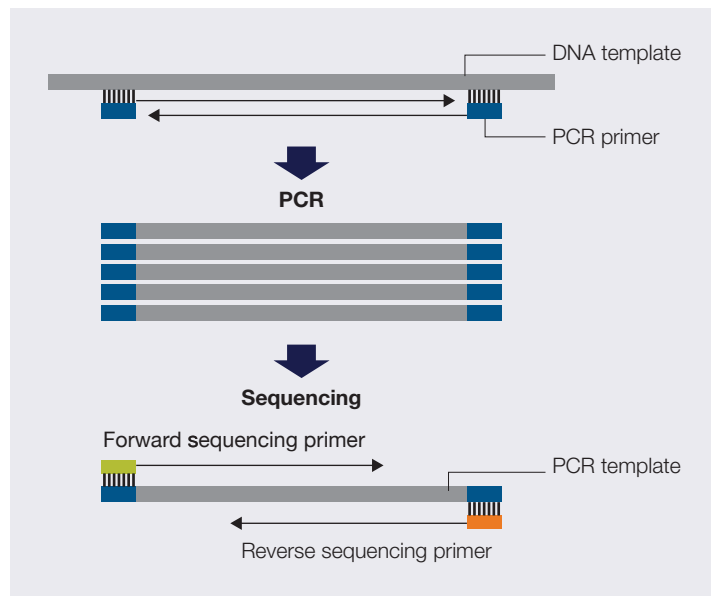


Figure 14. Sequencing with PCR primers.

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 setup (Figure 15). 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.

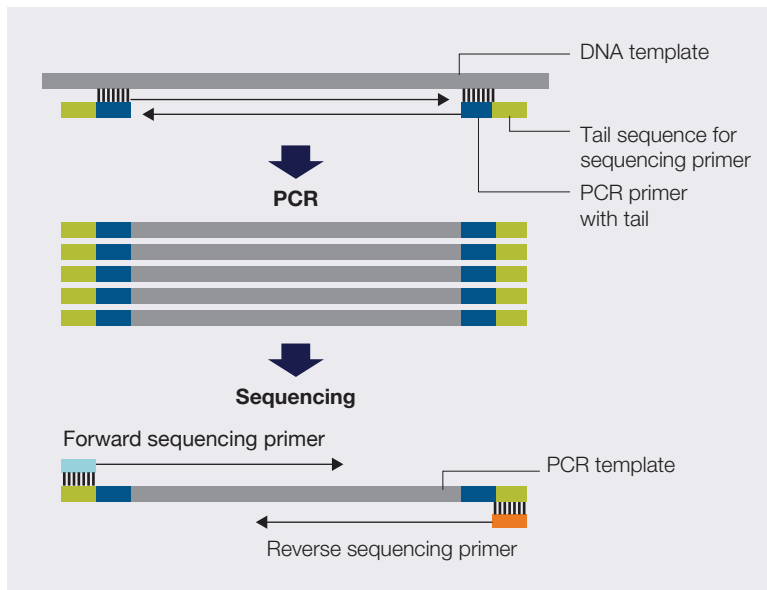


Figure 15. Sequencing with PCR primer and standard primer tail.

The advantage of M13-tagged primers for sequencing is the consistent use of only two primers (forward and reverse) which makes the process more robust and less failure prone and provides higher resolution, i.e., better readability of sequences of interest at the 5' end.

The DNA sequences for the most commonly used M13 sequencing primers are:

M13 forward: 5' TGTAACGACGGCCAGT 3'

M13 reverse: 5' CAGGAAACAGCTATGACC 3'

These sequence tags are added upstream (5') of the target-specific PCR primer part.

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.

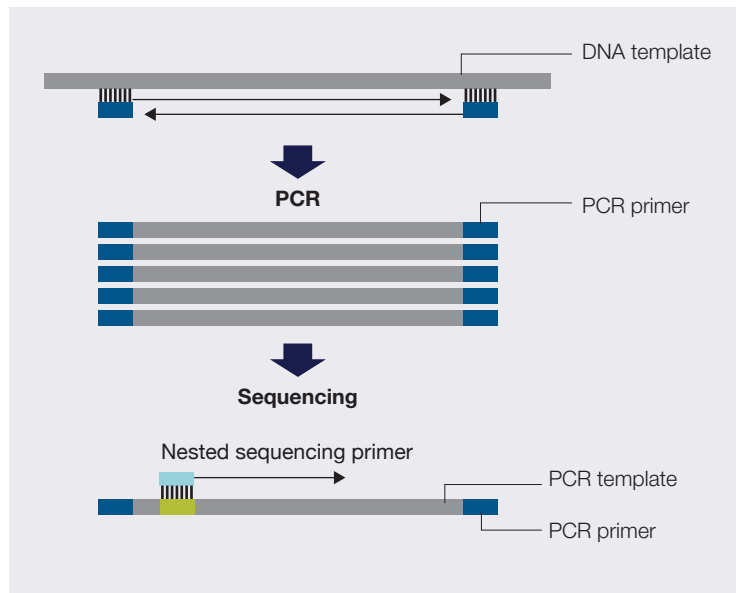


Figure 16. Sequencing with subtype-specific tail and PCR primer tail.

Nested sequencing primers are used in the primer walking method discussed in the *de novo* sequencing section (page 16), and also for genotyping applications such as those served by the Applied Biosystems 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 dideoxynucleotides (ddNTPs). Incorporation of a specific ddNTP (specific color) indicates the presence of that base on the template.

For more information, refer to the SNaPshot Multiplex Kit Protocol ([PN 4323357B](#)).

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 cytosine is often associated with a guanine residue as a CpG dinucleotide or CpG site (cytosine and guanine linked by only one phosphate). CpG islands are regions with a high frequency of CpG sites. Multiple CpG islands (that is, regions of >500 bp and higher than 55% GC content) 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 nonmethylated cytosine to uracil, while methylated cytosine is protected from bisulfite conversion (Figure 17). 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.

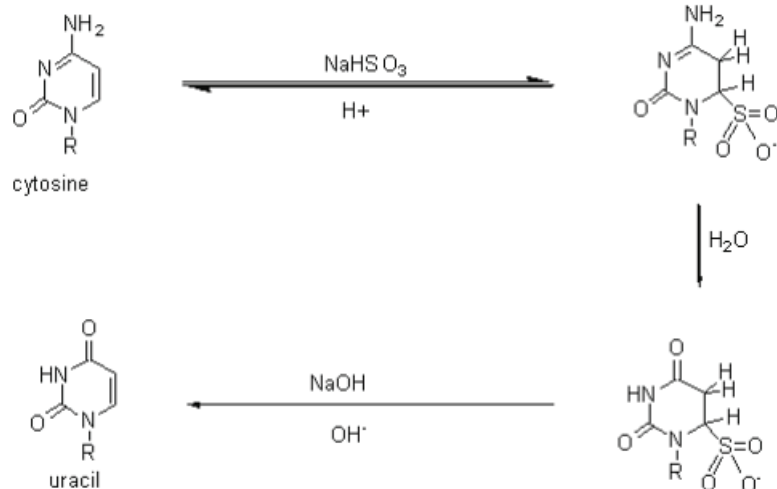


Figure 17. Conversion of methylated cytosine to cytosine and nonmethylated cytosine to uracil.

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 DNA sequences treated with sodium bisulfite with untreated genomic DNA sequences allows the precise identification of all methylated cytosines within a long stretch of DNA (Figures 18 and 19) [4].

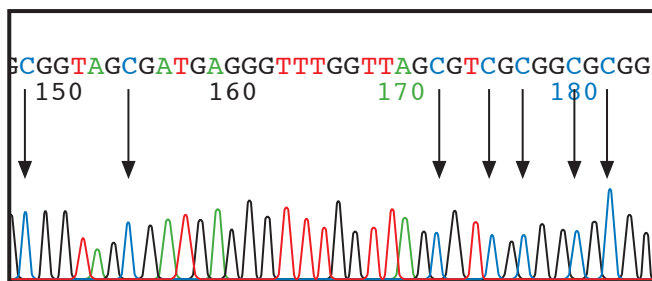


Figure 18. DNA sequence from untreated DNA. Arrows show locations of nonmethylated cytosine positioned before guanine. After bisulfite treatment, nonmethylated cytosine is converted to T.

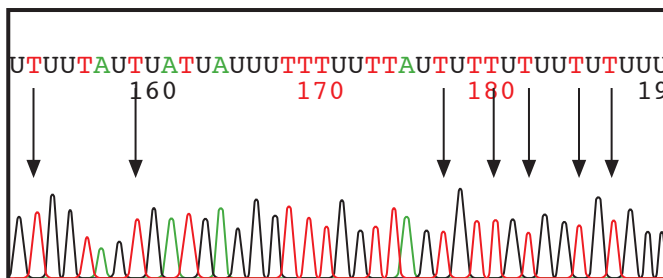


Figure 19. DNA sequence from bisulfite-treated DNA. Arrows show locations of nonmethylated cytosine converted to thymine after bisulfite sequencing.

Comparison of peaks from bisulfite sequencing and nonbisulfite sequencing is not quantitative. For a more quantitative analysis of methylation, fragment analysis is performed.

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 first cloned and then sequenced. The cloning method has been applied to genome-wide sequence analysis of methylation [5].

In the workflow shown in Figure 20, bisulfite sequencing without a cloning step (on the right) is compared with regular sequencing (on the left).

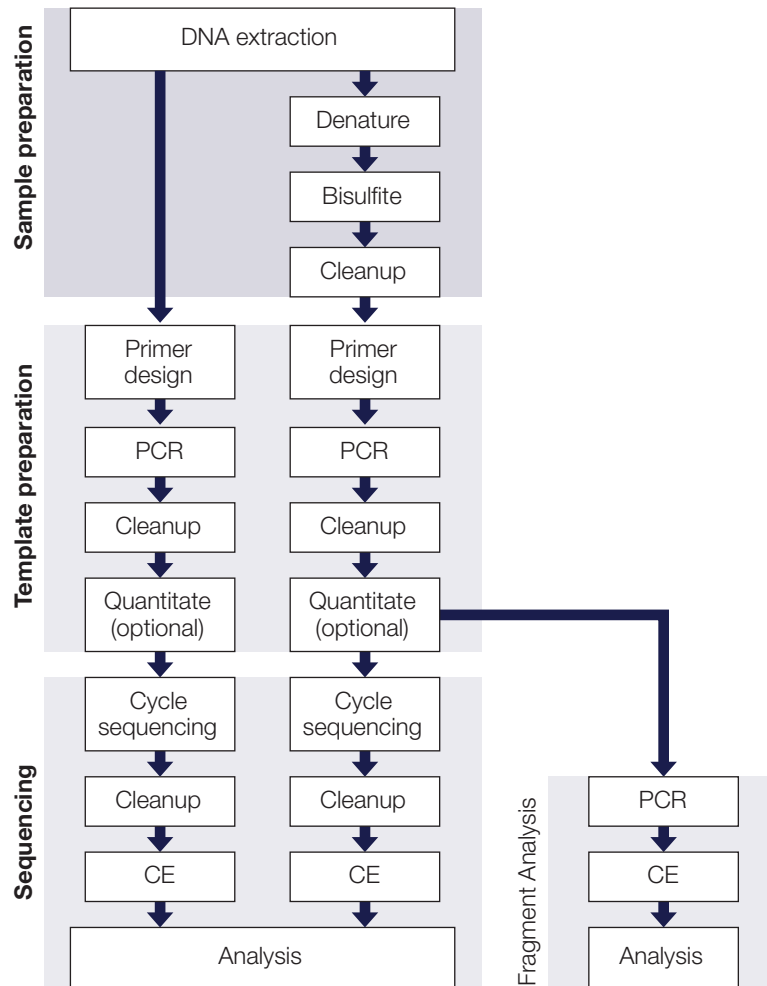


Figure 20. Steps for bisulfite sequencing compared with regular sequencing workflow.

For More Information

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

- *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 website)

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:

- Multilocus sequence typing (MLST) comparison of housekeeping genes to library standards
- Applied Biosystems MicroSeq ID Analysis Software comparison of ribosomal sequences to library standards
- Applied Biosystems MicrobeBridge software connects AB1 data files with the Centers for Disease Control and Prevention (CDC)'s MicrobeNet™ database for bacterial identification using 16S ribosomal RNA (rRNA) gene sequencing analysis

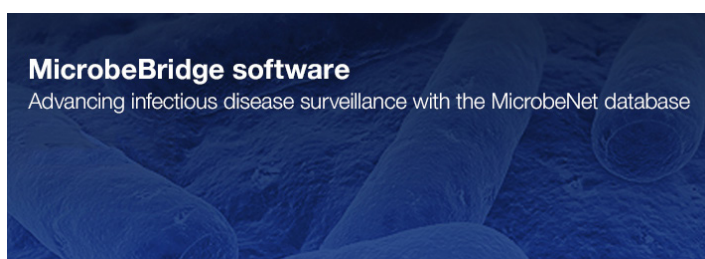


Figure 21. MicrobeBridge Software.

Multilocus sequence typing (MLST)

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.

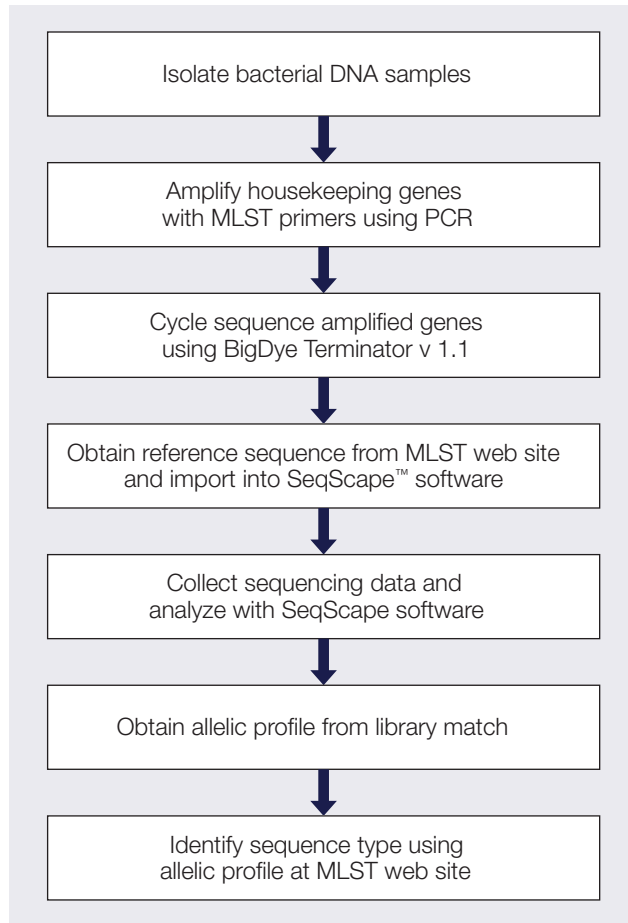


Figure 22. Multilocus sequence typing (MLST) workflow.

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 electrophoresis. 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 website at <http://www.mlst.net/>.

MicroSeq ID Analysis Software

Microbial identification based on rRNA gene sequencing is used to identify microbial species including bacteria, yeasts, molds, and fungi. Bacteria are identified by sequencing the universal 16S rRNA gene, which forms the basis for bacterial taxonomic classification in *Bergey's Manual* [6]. Fungi are typically 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.

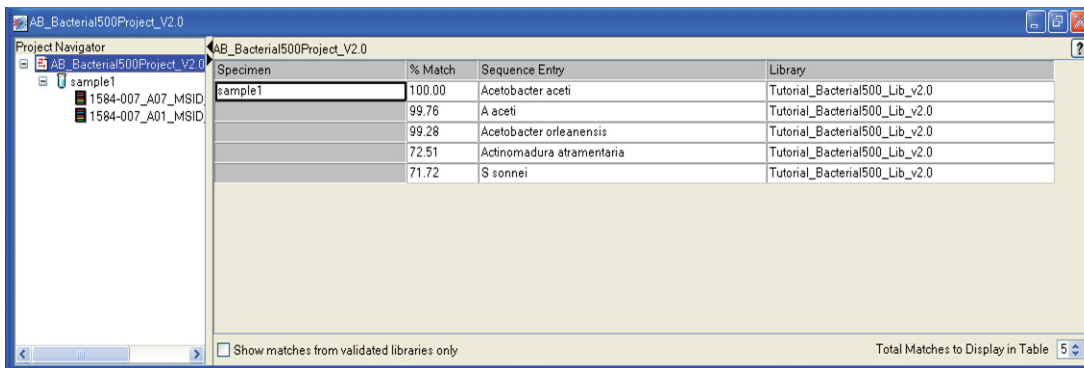


Figure 23. MicroSeq ID Analysis Software showing the top match of an unknown sequence to the library.

Libraries provided with the MicroSeq ID Analysis Software include entries for over 2,300 bacterial species, including gram-negative non-fermenters, *Bacillus*, coryneforms, *Mycobacteria*, and *Staphylococcus*. The library for fungal species includes over 1,100 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 sequences of a sample. The electropherograms of forward and reverse sequences are aligned to the consensus sequence generated by the software (at the top). The consensus sequence is compared to the validated library.

For more information

For more information, see the Applied Biosystems *MicroSEQ ID Microbial Identification Software Version 3.0 Getting Started Guide* ([PN 4465137](#)).

MicrobeBridge software

MicrobeBridge software is a desktop software solution that connects AB1 data files generated on Applied Biosystems Sanger sequencers with the Centers for CDC's MicrobeNet database for bacterial identification using 16S rRNA gene sequencing analysis.

MicrobeNet is a web-based tool built by the CDC for the identification of microbial pathogens through 16S rRNA gene sequence BLAST searches. It contains a highly curated database of genetic and phenotypic properties for the identification of bacterial and microbial species.

Current workflows require manual quality checks of sequence data assembly, manual examination of the assembled sequence, and a manual alignment search against GenBank to identify the species. MicrobeBridge software provides a streamlined workflow starting with importing AB1 data files generated on any Applied Biosystems capillary electrophoresis sequencing instrument. The software performs contig assembly, contig editing, primer trimming, and data quality check, and also copies the contig sequence and provides a one-click connection to MicrobeNet.

Features

- Overview of read coverage shows the range of forward and reverse sequences in a specimen.
- Contig review shows the forward and reverse sequences, identifies discrepancies in the assembled contig sequence, and allows editing of the contig sequence.
- Quality status displays color-coded trace files based on user-settable quality ranges and provides thumbnail trace views to examine raw data.
- One-click access to MicrobeNet provides one-click copy contig sequence function.
- Export contig file allows export of contig file in FASTA format.

Seamlessly connects Sanger results to the CDC

The software seamlessly connects Sanger sequencing data to the CDC MicrobeNet reference database, allowing researchers to identify microbes and other pathogens in one database. It automates the process of compiling raw sequence data for matching against known pathogenic sequences in MicrobeNet.

Chapter 3

DNA template preparation

This section covers:

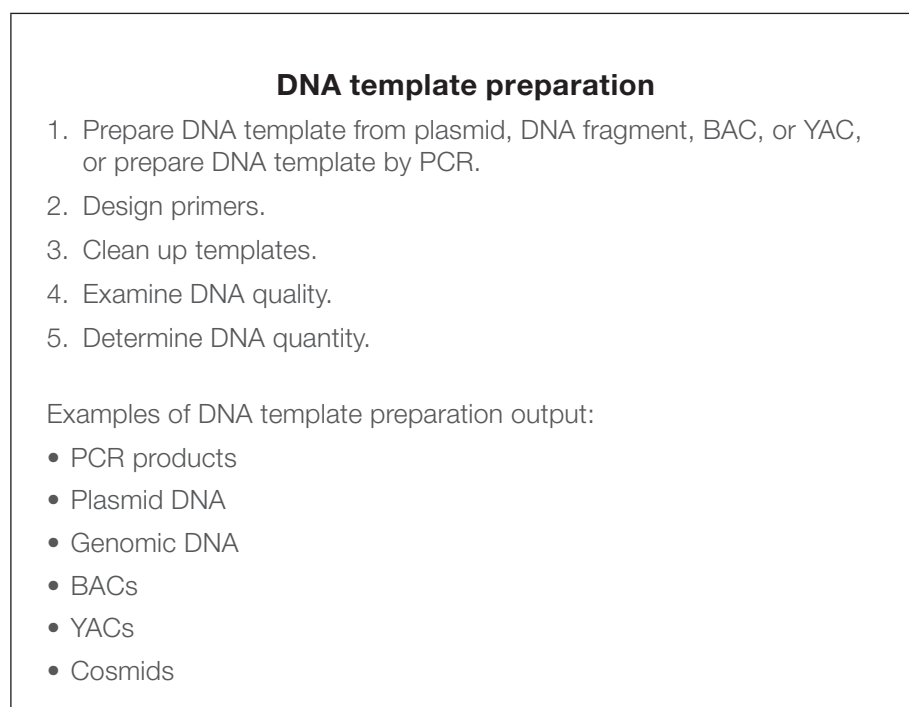
Overview	32
Preparing vector-based DNA templates	33
Preparing genomic DNA	34
Preparing PCR DNA templates	35
Primer design and quantitation	37
Purifying PCR products for sequencing	40
DNA template quality	40
DNA template quantity	42
Preparing templates for bisulfite sequencing	42

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 35)
- Guidelines for evaluating DNA quality (page 41)
- Procedure for cleaning up templates (page 41)
- Guidelines for quantitating the DNA (page 42)
- Procedures for performing bisulfite sequencing (page 42)

Workflow



Cycle sequencing (Chapter 4)



Purification of extension products (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.

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 [7].

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 include:

- 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, refer to [Molecular Cloning: A Laboratory Manual](#) [8].

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 include:

- Alkaline lysis, with extra phenol extraction and isopropanol precipitation if very clean DNA is desired [9]
- Cesium chloride (CsCl) banding

For other BAC DNA preparation protocols, refer to [Bacterial Artificial Chromosomes](#) [10].

- Applied Biosystems Application Note: *A Workflow for Obtaining High Quality Sequencing Data from Bacterial Artificial Chromosome (BAC) DNA* ([PN 107AP05-01](#))

Commercial products

Thermo Fisher Scientific features a variety of products for DNA extraction and purification. An overview can be found at www.thermofisher.com/us/en/home/life-science/dna-rna-purification-analysis.html.

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 and 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 tissues.

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 [11]. Only a few methods that reverse the effect of heparin have proven to be successful [12]. The manufacturers of the Invitrogen™ Dynabeads™ DNA Direct Universal Kit (DynaL Industrial, S.A.) report that because of the successful removal of heparin after the washing steps in these protocols, the genomic DNA obtained from heparin blood using their products is ready for PCR amplification.
- **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 [13]

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 Ambion™ RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE tissues ([PN AM1975](#)).

- **RNA as the starting material**—If you use RNA as your starting material via RT-PCR (for example, RNA viruses or 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, refer to [Molecular Cloning: A Laboratory Manual](#) [8].

For an overview of RNA preparation products from Thermo Fisher Scientific go to www.thermofisher.com/us/en/home/life-science/dna-rna-purification-analysis/rna-extraction.html.

Length of genomic DNA fragments

Genomic DNA fragments longer than 50 kb can be less efficient targets for PCR amplification than shorter genomic DNA fragments [3]. 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 Thermo Scientific™ 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 [8].

Commercial products

Commercial products for preparing RNA or cDNA

Information on a multitude of DNA and RNA purifications products offered by Thermo Fisher Scientific is available at

www.thermofisher.com/us/en/home/life-science/dna-rna-purification-analysis.html.

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 reagents and systems 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 39).

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 177).

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 192 in Appendix A for universal primer sequences). Universal-tailed PCR primers are useful in the following scenarios:

- In conjunction with dye terminator chemistries because universal sequencing primers have good annealing characteristics. However, longer PCR primers increase the reaction cost.
- In conjunction with commercially available dye-labeled 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 a 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 making decisions

For more information, see the Applied Biosystems application note *Advances in Fast PCR Contribute to a Fast Resequencing Workflow* (PN 104AP04-02).

Primer design and quantitation

When you perform dye terminator cycle sequencing reactions on a 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 greater than 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 of 30%–80%, preferably between 50%–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 greater than 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 website (thermofisher.com/sangersoftware).

Formulas

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

Feature	Formula
Melting temperature (T_m)	$T_m = (\text{number of A + T residues}) \times 2^\circ\text{C} + (\text{number of G + C residues}) \times 4^\circ\text{C}$
Primer concentration (derived from Beer's Law)	$C \text{ (pmol/}\mu\text{L or } \mu\text{M)} = (A_{260} \times 100) / (1.54n_A + 0.75n_C + 1.17n_G + 0.92n_T)$ 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): $\text{MW} = (N_A \times 335.2) + (N_C \times 311.2) + (N_G \times 351.2) + (N_T \times 326.2) + P$ where: N_x = number of residues of base x in the oligonucleotide $P = -101.0$ for dephosphorylated oligonucleotides, 40.0 for phosphorylated oligonucleotides



Predesigned primers through the Primer Designer Tool

The online Primer Designer Tool allows you to search for the right Sanger sequencing primer pair from a database of ~650,000 predefined primer pairs for resequencing the human exome and mitochondrial genome.

Benefits

- Find primers quickly with the user-friendly interface—search by genome position, gene symbol, SNP identifier, and other genome annotations.
- Get full primer coverage on the Sanger confirmation workflow for Ion Torrent™ research panels, including:
 - Ion AmpliSeq™ Exome Panel
 - Ion AmpliSeq Cancer Hotspot Panel v.2
- Compatible with all BigDye chemistries.
- Flexible primer configuration helps meet your research needs: primers can be ordered unmodified, M13-tailed, HPLC-purified, or desalted.
- View each primer pair and PCR amplicon on a gene map.
- All primers are checked by mass spectrometry and pass stringent bioinformatics metrics; lab bench validation tests show >95% success rate.
- Fully automated online ordering—including primer search and primer configuration.

Find out more at thermofisher.com/primerdesigner.

Custom primers

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

To order custom primers:

1. Go to the Thermo Fisher website at www.thermofisher.com/us/en/home/products-and-services/product-types/primers-oligos-nucleotides.html.
2. Follow the instructions for entering your primer names and sequences or for uploading them from a file.
3. Order primers.

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 cause 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:

- Optimize PCR through these parameters [14,15]:
 - Amount of starting DNA
 - Careful primer design
 - Primer concentration
 - Enzyme concentration
 - Magnesium ion (Mg^{2+}) concentration

- Nucleotide concentration
- Buffer composition
- Number of cycles
- pH
- Use manual hot-start method (if the enzyme does not have hot-start capability)
- Use 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.

Table 3. Commercial product for preparing PCR DNA templates.

Product	Source	Description
ExoSAP-IT™ PCR Product Cleanup	Affymetrix	PCR cleanup to remove excess primers and unincorporated nucleotides.
ExoSAP-IT Express PCR Product Cleanup	Affymetrix	A 5-minute protocol that delivers the same superior cleanup result as the original ExoSAP-IT reagent.

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 174)
- No or low signal (see page 147 and page 150)

- Early loss or termination of extension (see page 157)

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 include:

- Proteins
- RNA
- Chromosomal DNA
- Excess PCR primers, dNTPs, enzymes, 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 A260/A280 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)—The most efficient method for salt removal. See EMD Millipore's website (www.emdmillipore.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 40 for the name of the commercial product for preparing PCR DNA templates.
- **Phenol/chloroform extraction**—Refer to [Molecular Cloning: A Laboratory Manual](#) [8] 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 OD) of a sample at 260 nm in a spectrophotometer.

Measuring UV absorbance

One OD 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. A₂₆₀ values can be converted into µg/µL using Beer's Law:

Absorbance (260 nm) = sum of extinction coefficient contributions x cuvette path length x concentration

The following formulas are derived from Beer's Law [16]:

- Concentration of single-stranded DNA = A₂₆₀ x 33 µg/µL
- Concentration of double-stranded DNA = A₂₆₀ x 50 µg/µL

Note: Absorbance measurements of highly concentrated (OD > 1.0) or very dilute (OD < 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 33342 Fluorescent Stain or Invitrogen Quant-iT™ PicoGreen™ dsDNA reagent
- Fluorometric analysis using Invitrogen Quant-iT assays and the Qubit™ Fluorometer
- Measurement of UV-Vis absorbance using the Thermo Scientific NanoDrop 1000 Spectrophotometer, which does not require dilution for many sample types. If you have a real-time PCR instrument, you can use Applied Biosystems TaqMan™ RNase P Detection Reagents Kit (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 [17].

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 various commercial products to prepare high-quality template for bisulfite sequencing.

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

Performing bisulfite conversion

Applied Biosystems recommends the use of the Cells-to-CpG™ Bisulfite Conversion Kit ([catalog number 4445555](#)) for DNA sequence analysis of methylated DNA.

The bisulfite method is the most commonly used technique for identifying specific methylation patterns within a DNA sample. It consists of treating DNA with bisulfite, which converts unmethylated cytosines to uracil but does not change methylated cytosines. Bisulfite conversion has been utilized in DNA methylation research for the last 20 years with few improvements to the technology until now. With thorough optimization, the Cells-to-CpG Bisulfite Conversion Kit provides a quick, streamlined method for bisulfite conversion to reveal methylated cytosines in either loci-specific or genomewide analyses.

Sufficient materials are supplied in the Cells-to-CpG Bisulfite Conversion Kit (50) to perform bisulfite conversion of 50 samples.

Key product features:

- **Flexible DNA conversion**—A validated solution for our platforms to minimize the need for optimization.
- **Quantitative reliability**—Supports confidence in results with available controls to easily monitor conversion rate.
- **Streamlined workflow**—Gives you the ability to start with various sample input types including cell, tissue, blood, and FFPE samples.
- **Efficient application**—Enables reduced time and labor through direct conversion of cytosines in samples without the need for purifying genomic DNA.

Chapter 4

Cycle sequencing

This section covers:

Overview.	46
Choosing a sequencing chemistry	47
Reagent and equipment considerations.	52
DNA quantity	54
Using DNA template controls	55
Using BigDye Terminators and dGTP BigDye Terminators	60
Bisulfite sequencing	63

Overview

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

Workflow

DNA template preparation (Chapter 3)



Cycle sequencing

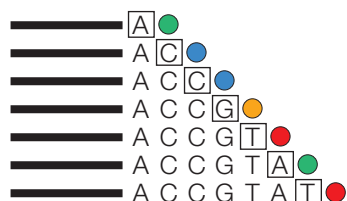
1. Select the sequencing chemistry.
2. Prepare cycle sequencing reactions.
3. Run sequencing reactions in a thermal cycler.

Thermo Fisher Scientific Cycle Sequencing Kits:

- BigDye Terminator v1.1 and v3.1 kits
- dGTP BigDye Terminator v1.0 and v3.0 kits
- BigDye Direct Cycle Sequencing Kit

Cycle sequencing output:

Dye terminator products



9700 thermal cycler



9800 thermal cycler



Veriti thermal cycler



Purification of extension products (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 4 and Table 5 to select the cycle sequencing kit that meets your needs:

Table 4. Thermo Fisher Scientific cycle sequencing kits.

Kit	Description	Page
BigDye Terminator v3.1 Cycle Sequencing Kits	<ul style="list-style-type: none"> • Compatible with automated genetic analyzers listed in Table 1 on page 10 • For comparative sequencing; <i>de novo</i> sequencing; resequencing; and sequencing PCR products, plasmids, cosmids, fosmids, and large templates, for example, bacterial artificial chromosome (BAC) clones • For long-range sequencing up to 1 kb (+) • Can sequence difficult templates and dinucleotide repeats • Formulated with dITP in place of dGTP to reduce peak compressions 	50
BigDye Terminator v1.1 Cycle Sequencing Kits	<ul style="list-style-type: none"> • Compatible with automated genetic analyzers listed in Table 1 on page 10 • For optimal base calling 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 	50
dGTP BigDye Terminator Cycle Sequencing Kits	<ul style="list-style-type: none"> • Compatible with automated genetic analyzers listed in 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 	50
BigDye Direct Cycle Sequencing Kits	<ul style="list-style-type: none"> • Compatible with automated genetic analyzers listed in Table 1 on page 10 • Combines template PCR and cycle sequencing steps without the need for additional PCR cleanup steps • For comparative sequencing; <i>de novo</i> sequencing; resequencing; and sequencing PCR products, plasmids, cosmids, fosmids, and large templates, for example, BAC clones 	51

*In sequences obtained using BigDye Terminators v1.1 run on rapid run modules 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 5 shows recommended chemistries, based on the sequencing application and/or characteristics of the DNA to be sequenced.

Ratings are:

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

Table 5. Chemistry recommendations for different sequencing applications and/or template characteristics.

Application	BigDye Terminator v3.1	BigDye Terminator v1.1	dGTP BigDye Terminator	BigDye Direct v3.1
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)	+	+	-	+
<i>De novo</i> 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				
Plasmid, BAC, cosmid, fosmid, lambda, large PCR product	++	++	-	++
Bacterial genomic DNA	++	++	-	++
Bisulfite-treated genomic DNA	+	++	-	+
PCR amplicon	++	++	+	++
PCR amplicon (heterozygous 10:90)	+	-	-	+
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.

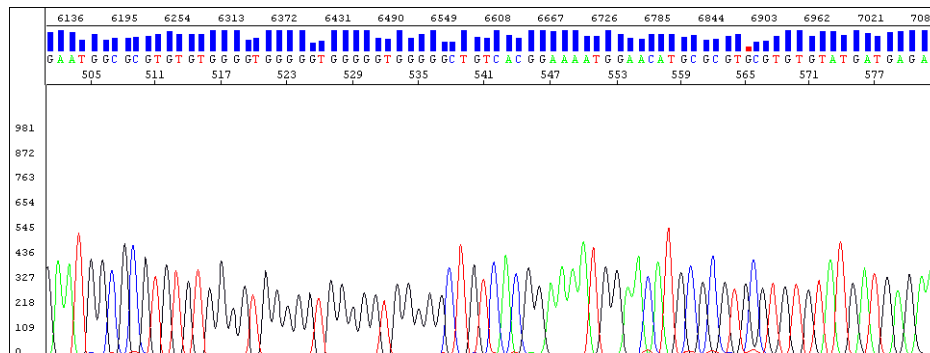


Figure 26. Sequence through a GT-rich region sequenced using dGTP BigDye Terminators.

IMPORTANT! Because of compressions, Thermo Fisher Scientific 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:

- *ABI Prism dGTP BigDye Terminator v3.0 Ready Reaction Cycle Sequencing Kit Protocol (PN 4390038)*.

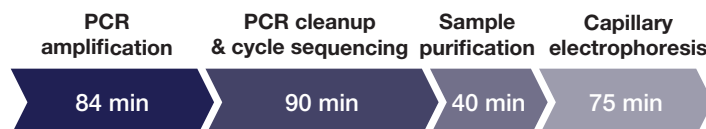
BigDye Direct Cycle Sequencing Kits

The BigDye Direct Cycle Sequencing Kit (PNs 4458688 through 4458690) was developed to simplify and streamline the Sanger sequencing workflow by combining post-PCR clean-up and cycle sequencing into a single step. The reproducibility and sequence quality of data generated by BigDye Direct Cycle Sequencing Kits is of the highest quality, generating long reads similar to that of BigDye Terminator 3.1, while retaining 5' read resolution close to the primer.

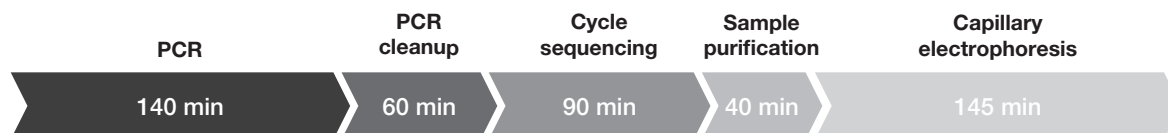
Leveraging M13 sequencing chemistry, Big Dye Direct can reduce the Sanger workflow by as much as 3 hours and several steps (Figure 27). Additionally, the BigDye Direct PCR and sequencing workflow requires use of only one plate, without having to transfer between steps. This helps reduce hands-on time and improve accuracy by reducing the possibility of pipetting errors.

BigDye Direct Cycle Sequencing Kit workflow, run with POP-7 polymer

Four steps in approximately 5 process hours.

**A traditional cycle sequencing workflow, run with POP-6 polymer**

Five steps in approximately 8 process hours.

**Sequencing with the BigDye Direct Cycle Sequencing Kit takes fewer steps and less hands-on time**

By combining PCR cleanup and cycle sequencing into a single step, the BigDye Direct workflow reduces the traditional Sanger sequencing workflow time by up to 40%. Time for each step is indicated in the diagram and includes hands-on time.

Figure 27. BigDye Direct Cycle Sequencing workflow versus traditional cycle sequencing.

Reagent and equipment considerations

Reagent handling and reaction storage

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

Store cycle sequencing reagents in uncolored tubes or vials at -15°C 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°C to -25°C .

Note: Do not store sequencing reactions at -70°C 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 (see pages 169 through 170).

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 3730/3730xl 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 and Veriti 96-Well Thermal Cycler

– 0.2 mL MicroAmp Reaction Tubes (PN N8010533)

or

– MicroAmp™ Optical 96-Well Reaction Plate (PN N8010560)

- Thermo Fisher Scientific Veriti 96-Well Fast Thermal Cycler

– 96-Well Fast Thermal Cycling Plate (PN 4346907)

Note: Fast plates require a 96-well fast (0.1 mL) plate base (PN 4367470) and a 96-well fast (0.1 mL) 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 Plate with Barcode (PN 4306737)

or

- MicroAmp Optical 384-Well Reaction Plate 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 Thermo Fisher Scientific Veriti 96-Well Thermal Cyclers. If you choose to use a thermal cycler not manufactured by Thermo Fisher Scientific, 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 Thermo Fisher Scientific 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/peak height and increases the chance for dye blobs because a greater proportion of unincorporated dye molecules are left behind. 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 6 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 6. 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	BigDye Direct
PCR product:				
100 to 200 bp	1 to 3 ng	0.5 to 3 ng	1 to 3 ng	1 to 3 ng
200 to 500 bp	3 to 10 ng	1 to 10 ng	3 to 10 ng	3 to 10 ng
500 to 1000 bp	5 to 20 ng	2 to 20 ng	5 to 20 ng	5 to 20 ng
1000 to 2000 bp	10 to 40 ng	5 to 40 ng	10 to 40 ng	10 to 40 ng
>2000 bp	40 to 100 ng	10 to 50 ng	40 to 100 ng	40 to 100 ng
Bisulfite converted genomic DNA-PCR product	3 to 10 ng	3 to 10 ng	Not recommended	3 to 10 ng
Single-stranded DNA	50 to 100 ng	10 to 50 ng	50 to 100 ng	50 to 100 ng
Double-stranded DNA	200 to 500 ng	50 to 300 ng	200 to 500 ng	200 to 500 ng
Cosmid, BAC	0.5 to 1.0 µg	200 to 1,000 ng	0.5 to 1.0 µg	0.5 to 1.0 µg
Bacterial genomic DNA	2 to 3 µg	1,000 to 3,000 ng	2 to 3 µg	2 to 3 µg

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.

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

Sequencing control reactions

Table 7. Sequencing control reactions.

Reagent	Quantity per reaction (μL)	
	96-well	384-well*
Ready Reaction Mix	8.0	4.0
pGEM-3Zf(+) 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.

Table 8. Sequencing control reactions for samples prepared with BigDye XTerminator Purification Kit.

Reagent	Quantity per reaction (μL)		
	96-well (20 μL reaction)	96-well (10 μL reaction)	384-well (5 μL reaction)*
Ready Reaction Mix	8.0	4.0	2.0
pGEM-3Zf(+) 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.

DNA template		Thermal cycling conditions		
Veriti 96-Well Thermal Cycler	Stage	Description	Temp. (°C)	Time
	1	Denaturation	96	1 min
	2	Amplification: 25 cycles	96 50 60	10 sec 5 sec 4 min
	3	Hold	4	Indefinite hold
Veriti Fast 96-Well Thermal Cycler	Stage	Description	Temp. (°C)	Time
	1	Denaturation	96	1 min
	2	Amplification: 25 cycles	96 50 60	10 sec 5 sec 75 sec
	3	Hold	4	Indefinite hold

Using BigDye Direct Cycle Sequencing Kit

The BigDye Direct Cycle Sequencing Kit (PNs 4458688 through 4458690) was developed to simplify and streamline the Sanger sequencing workflow by combining post-PCR cleanup and cycle sequencing into a single step (Figure 28).

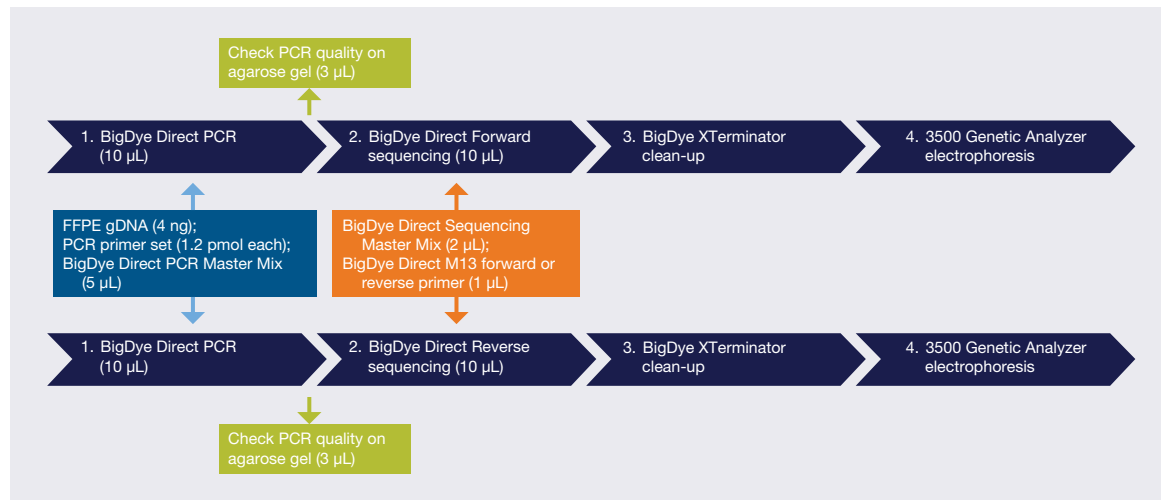


Figure 28. Workflow for BigDye Direct cycle sequencing.

PCR reaction components

Table 10. Reagents for BigDye Direct PCR.

Reagent	Quantity per 96-well or 384-well reaction (10 μ L reaction)*
Genomic DNA (4 ng/ μ L)	1.0 μ L
BigDye Direct PCR Master Mix	5.0 μ L
M13-tailed PCR Primer (0.8 μ M each primer)	1.5 μ L
Deionized water	2.5 μ L
Total volume	10 μL

*Performing 10 μ L reactions in 384-well reaction plates allows you to perform the post-reaction cleanup step in the same well.

Table 11. Thermal cycling conditions for BigDye Direct PCR.

System	Thermal cycling conditions		
Veriti Thermal Cycler	Description	Temp. ($^{\circ}$C)	Time
	Denature hold	95	10 min
	Cycle 35 times	96	3 sec
		62	15 sec
		68	30 min
	Hold	72	2 min
Hold	4	Until used	
PCR System 9700 Thermal Cycler	Description	Temp. ($^{\circ}$C)	Time
	Denature hold	96	5 min
	Cycle 35 times	94	30 sec
		62	45 sec
		68	45 min
	Hold	72	2 min
Hold	4	Until used	

According to the detailed instructions found in the *BigDye Direct Cycle Sequencing Kit Protocol* ([4458040C](#)), 1 μ L of the PCR product is run on a standard agarose gel. The amount of PCR product needed for the sequencing reaction is 20 ng.

Sequencing reaction components

Table 12. Reagents for BigDye Direct sequencing.

Reagent	Quantity per 96-well or 384-well reaction (10 μ L reaction)*
BigDye Direct Sequencing Master Mix	2.0 μ L
BigDye Direct M13 Fwd Primer or BigDye Direct M13 Rev Primer	1.0 μ L
Total volume	3.0 μL

*Performing 10 μ L reactions in 384-well reaction plates allows you to perform the post-reaction cleanup step in the same well.

Three microliters of the forward or the reverse sequencing reaction mix is added to the appropriate well of the products in the PCR amplification plate. Cycle sequencing is performed according to the chart below.

Table 13. Thermal cycling conditions for sequencing reaction.

System	Thermal cycling conditions		
Veriti Thermal Cycler	Description	Temp. ($^{\circ}$C)	Time
	Hold	37	15 min
	Hold	80	2 min
	Hold	96	1 min
	Cycle 25 times	96 50 60	10 sec 5 sec 75 sec
	Hold	4	Until used
PCR System 9700 Thermal Cycler	Description	Temp. ($^{\circ}$C)	Time
	Hold	37	15 min
	Hold	80	2 min
	Hold	96	1 min
	Cycle 25 times	96 50 60	10 sec 5 sec 4 min
	Hold	4	Until used

The reaction products are cleaned up using one of the methods outlined in the next chapter before sequencing.

Using BigDye Terminators and dGTP BigDye Terminators

Sequencing reaction components

Table 14. Reagents for sequencing reaction using Using BigDye Terminators and dGTP BigDye Terminators.

Reagent	Quantity per reaction		
	96-well (20 μ L reaction)	96-well or 384-well (10 μ L reaction)*	384-well (5 μ L reaction)
Ready Reaction Mix	8.0 μ L	4.0 μ L	2.0 μ L
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 μ L reactions in 384-well reaction plates allows you to perform post-reaction cleanup in the same well.

**See "Table 6. Recommended DNA template quantities for cycle sequencing." on page 55.

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 5x BigDye Terminator Sequencing Buffer to bring the final buffer concentration to 1x. The chemistry is optimized for a full-strength reaction. Different instruments have different sensitivities and different detection systems. Dilution of Big Dye reagents can impact signal intensity, read length, performance through difficult sequences, and robustness with different sample types.

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 in a 20 μ L reaction:

$$0.5 (8 \mu\text{L} - \text{Ready Reaction Mix Volume}) = \text{Volume of BigDye Terminator Sequencing Buffer to add}$$

Example for a 20 μ L sequencing reaction:

	1X rxn (= recommended full strength)	1/4 rxn
Ready Reaction Mix	8 μ L	2 μ L
5x sequencing buffer	0 μ L	3 μ L

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 15 work for a variety of templates and primers. These thermal cycling conditions were optimized using 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 15. Thermal cycling conditions for BigDye Terminators and dGTP BigDye Terminators.

BigDye Terminators v1.1 and v3.1 (Veriti 96-Well Thermal Cycler)					
DNA template	Thermal cycling conditions				
<ul style="list-style-type: none"> • Double-stranded DNA • Single-stranded DNA • PCR product 	Stage	Description	Temp. (°C)	Time	
		1	Denaturation	96	1 min
		2	Amplification: 25 cycles	96 50 60	10 sec 5 sec 4 min
		3	Hold	4	Indefinite hold
BAC DNA	Stage	Description	Temp. (°C)	Time	
	1	Denaturation	95	1 min	
	2	Amplification: 50 cycles*	95 50 to 55** 60	30 sec 10 sec 4 min	
	3	Hold	4	Indefinite hold	
Bacterial genomic DNA	Stage	Description	Temp. (°C)	Time	
	1	Denaturation	95	5 min	
	2	Amplification: 45 cycles	95 50 to 55** 60	30 sec 20 sec 4 min	
	3	Hold	4	Indefinite hold	
Bisulfite-treated template PCR amplicon	Stage	Description	Temp. (°C)	Time	
	1	Denaturation	96	10 min	
	2	Amplification: 25 cycles	96 50	10 sec 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 15. Thermal cycling conditions for BigDye Terminators and dGTP BigDye Terminators (continued).

dGTP BigDye Terminators v1.0 (Veriti 96-Well Thermal Cycler)				
DNA template		Thermal cycling conditions		
<ul style="list-style-type: none"> • Double-stranded DNA • Single-stranded DNA • PCR product 	Stage	Description	Temp. (°C)	Time
	1	Amplification: 25 cycles	96 50 60	10 sec 10 sec 4 min
	2	Hold	4	Indefinite hold
dGTP BigDye Terminators v3.0 (Veriti 96-Well Thermal Cycler)				
DNA template		Thermal cycling conditions		
<ul style="list-style-type: none"> • Double-stranded DNA • Single-stranded DNA • PCR product 	Stage	Description	Temp. (°C)	Time
	1	Amplification: 25 cycles	96 68	10 sec 2 min
	2	Hold	4	Indefinite hold
BigDye Terminators v1.1/v3.1 and dGTP BigDye Terminators v1.0/v3.0 (Veriti FAST 96-Well Thermal Cycler)				
DNA template		Thermal cycling conditions		
<ul style="list-style-type: none"> • Double-stranded DNA • Single-stranded DNA • PCR product 	Stage	Description	Temp. (°C)	Time
	1	Denaturation	96	1 min
	2	Amplification: 25 cycles	96 50 60	10 sec 5 sec 75 sec
	3	Hold	4	Indefinite hold
BigDye Terminators v3.1 (Veriti FAST 96-Well Thermal Cycler)				
DNA template		Thermal cycling conditions		
BAC DNA	Stage	Description	Temp. (°C)	Time
	1	Denaturation	96	2 min
	2	Amplification: 50 cycles	96 50 60	20 sec 10 sec 2 min
	3	Hold	4	Indefinite hold

Modifying Thermal Cycling Conditions

You may modify the thermal cycling conditions. Thermo Fisher Scientific makes the following suggestions for modifying these conditions. These suggestions have not been exhaustively tested at Thermo Fisher Scientific 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 $>60^\circ\text{C}$, try eliminating the annealing step.
- If you observe low signals and the T_m of a primer is $<50^\circ\text{C}$, increase the annealing time to 30 seconds or decrease the annealing temperature to 48°C .
- For sequencing large templates such as BACs and fosmids, increasing the number of cycles may help increase signal.

Further optimization strategies can be found in [Improved DNA sequencing quality and efficiency using an optimized fast cycle sequencing protocol](#) [18].

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 five 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 more difficult to amplify.

There are two ways of sequencing bisulfite converted DNA to assess the amount and extent of DNA methylation.

1) 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 four bases 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

Protocols for direct PCR sequencing of bisulfite-converted DNA can be found in the following references:

[Rapid quantification of DNA methylation by measuring relative peak heights in direct bisulfite-PCR sequencing traces](#) [19]

[DNA methylation detection: Bisulfite genomic sequencing analysis](#) [20]

[Optimizing methodologies for PCR-based DNA methylation analysis](#) [21]

Information regarding sequence data analysis can be found in the application note [Detection and Quantification of Sequence Variants from Sanger Sequencing Traces](#), which can be downloaded from the product literature section of the Thermo Fisher website.

DNA polymerase selection

After bisulfite conversion of gDNA, the double-stranded nucleic acid is transformed into single-stranded template, composed of five 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 complement 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 archaeobacteria 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 four bases in design.
- Use a PCR denaturant such as glycerol.
- Set the annealing temperature approximately 2°C 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).

Chapter 5

Purification of extension products

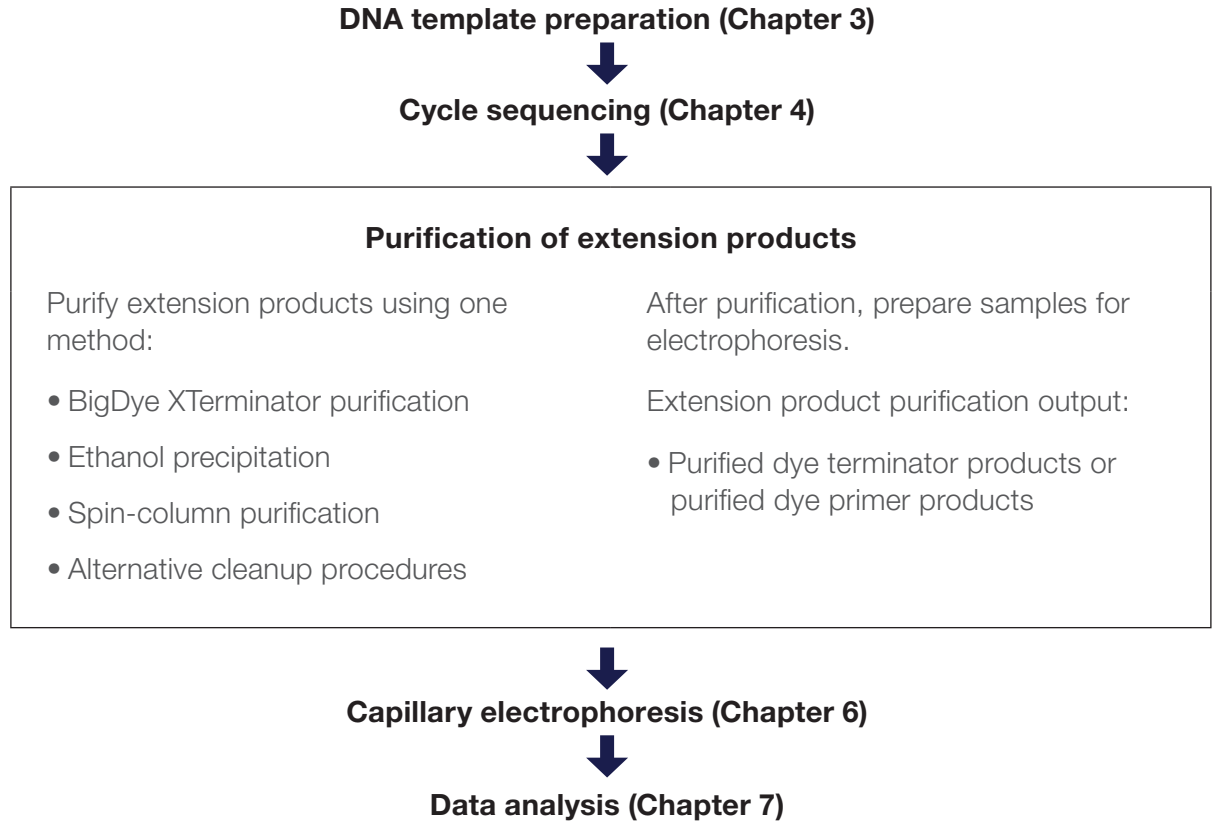
This section covers:

Overview	68
Purification with the BigDye XTerminator Purification Kit	69
Purification by ethanol precipitation	76
Purification with spin columns	87
Sample preparation for electrophoresis	89
Samples purified with other purification methods	91

Overview

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

Workflow



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 comigrate with sequencing reaction extension products obscure the desired signal and interfere with base calling. 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 v3.1
- dGTP BigDye Terminators v1.0 and v3.0
- BigDye Direct 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.

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 deoxynucleotides (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/v3.1 and BigDye Direct. 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 3500/3500xL, 3730/3730xl, 3130/3130xl, 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 6, “Recommended DNA template quantities for cycle sequencing.” on page 55.

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 72). This may be preferable when you have more samples for two reasons:
 1. Premix pipetting reduces extra pipetting.
 2. The premix can be vortexed frequently (which is important for consistent cleanup, and therefore consistent results) while adding it to the samples.
- **Sequential pipetting**—Add SAM™ Solution to the reaction plate first, followed by the XTerminator Solution (see Performing purification using sequential pipetting on page 74).

For more information see the *BigDye XTerminator Purification Kit Protocol* ([PN 4374408](#)).

Advantages of using the BigDye XTerminator Purification Kit

- Simplified workflow:
 - No need for spin column or ethanol precipitation
 - Fast
- 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)
- Efficient removal of dye “blobs”
- 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

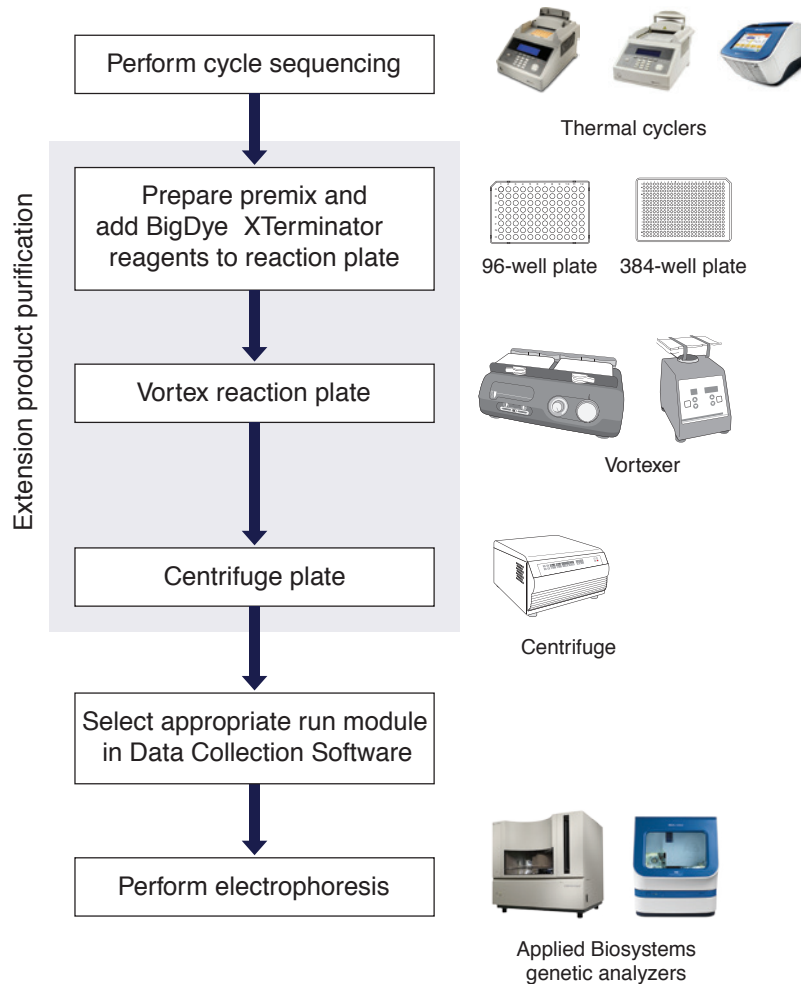


Figure 29. Sequencing workflow with extension product purification using the BigDye XTerminator Purification Kit.

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.
 - Vortex, or mix, the XTerminator more frequently (revortexing) while adding directly to the samples, especially when doing a lot of samples in a plate.
 - 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 material safety data sheet (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

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 centrifuge tube, 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 centrifuge tube, 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 the next section (Performing purification using the premix).

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.
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! 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 Film. Important: Verify that each well is sealed. Otherwise, spillover and contamination can occur.

IMPORTANT! If you use a 3730/3730xl 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.

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

Vortexer	Plate type	Speed
Digital Vortex-Genie 2	96-well 384-well	1800 rpm 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 1,000 x g for 2 minutes.
7. Proceed with Sample preparation for electrophoresis on page 89.

Performing purification using sequential pipetting



WARNING

CHEMICAL HAZARD. SAM Solution 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.
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. Vortex, or mix, the XTerminator Solution more frequently (revortexing) while adding directly to the samples, especially when doing a lot of samples in a plate.
 - e. Discard the pipette tip.
4. Seal the plate using:
 - A heat seal at 160°C for 1.5 seconds.

or

 - MicroAmp Clear Adhesive Film. Verify that each well is sealed.

IMPORTANT! If you use a 3730/3730xl 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.

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

Vortexer	Plate type	Speed
Digital Vortex-Genie 2	96-well 384-well	1800 rpm 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.

- In a swinging-bucket centrifuge, spin the plate at 1,000 x g for 2 minutes.
- Proceed with Sample preparation for electrophoresis on page 89.

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, NH₄OAc, KCl, or LiCl), temperature (–20°C to 0°C) and additives such as EDTA and Mg²⁺. 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 166).

- 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 77
 - Ethanol/EDTA/sodium acetate precipitation: page 80
- dGTP BigDye Terminators v1.0 and v3.0
 - Ethanol/sodium acetate precipitation: page 85
 - Ethanol precipitation: page 8587

Ethanol precipitation for BigDye Terminators v1.1/v3.1 and BigDye Direct Terminators v1.1/v3.1

Purification methods

- Ethanol/EDTA precipitation:
 - Precipitating in 96-well reaction plates (this page)
 - Precipitating in 384-well reaction plates (page 79)
- Ethanol/EDTA/sodium acetate precipitation:
 - Precipitating in 96-well reaction plates (page 81)
 - Precipitating in 384-well reaction plates (page 83)

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 μ L or 20 μ L reactions in 96-well reaction plates:

1. Remove the 96-well reaction plate from the thermal cycler and centrifuge the plate at 100 x 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%.

Sequencing reaction volume	Amount of 100% ethanol to add	Amount of 95% ethanol to add
10 μL	30 μL	36 μL
20 μL	60 μL	72 μL

4. Seal the plate securely with self-adhesive film, then invert the plate four times to mix the contents.

or

Mix the contents well by pipetting up and down in a multichannel pipette three to four times, then seal the plate securely with self-adhesive film.

5. Incubate 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 1,650 x g for 45 minutes
Any other centrifuge	Use a plate adapter and centrifuge the plate at the maximum speed at 4°C as follows: <ul style="list-style-type: none"> • 1,400 to 2,000 x g for 45 minutes or • 2,000 to 3,000 x g for 30 minutes

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 x 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 1,650 x g for 15 minutes.
9. Remove the self-adhesive film, invert the plate onto a paper towel, centrifuge up to 185 x 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 “Minimum sample volume” on page 92.

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 x 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 four times to mix the contents.

or

Mix the contents well by pipetting up and down in a multichannel pipette three to four times, then seal the plate securely with self-adhesive film.

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 1,650 x g for 45 minutes
Any other centrifuge	Use a plate adapter and centrifuge the plate at the maximum speed at 4°C as follows: <ul style="list-style-type: none"> • 1,400 to 2,000 x g for 45 minutes or • 2,000 to 3,000 x 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 x 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 1,600 to 2,000 x g for 15 minutes.

8. Remove the self-adhesive film, invert the plate onto a paper towel, centrifuge at 185 x 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 “Minimum sample volume” on page 90.

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 77).

**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. 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 x 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 μ L reactions—1 μ L
 - For 20 μ L reactions—2 μ L

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 μ L reactions—1 μ L
 - For 20 μ L reactions—2 μ L

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 μ L	25 μ L	30 μ L
20 μ L	50 μ L	60 μ L

5. Seal the plate securely with self-adhesive film, then mix by inverting the plate four times.
or

Mix well by pipetting up and down in a multichannel pipette three to four times, then seal the plate securely with self-adhesive film.

6. 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 1,650 x g for 45 minutes
Any other centrifuge	Use a plate adapter and centrifuge the plate at the maximum speed at 4°C as follows: <ul style="list-style-type: none"> • 1,400 to 2,000 x g for 45 minutes or <ul style="list-style-type: none"> • 2,000 to 3,000 x 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.

- Remove the self-adhesive film, invert the plate onto a paper towel, centrifuge at 185 x g for 1 minute, then remove the plate from the centrifuge.

Note: Start timing the spin when the rotor starts moving.

- Add the appropriate amount of 70% ethanol to each well.

Sequencing reaction volume	Amount of 70% ethanol to add
10 µL	35 µL
20 µL	70 µL

- Seal the plate with self-adhesive film, then with the centrifuge set to 4°C, spin the plate at 1,650 x g for 15 minutes.

- Remove the self-adhesive film, invert the plate onto a paper towel, centrifuge up to 185 x g for 1 minute, then remove the plate from the centrifuge.

Note: Start timing when the rotor starts moving.

- 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.

- (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.

- Proceed with “Minimum sample volume” on page 90.

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.

**WARNING**

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 μ L sequencing reactions in 384-well reaction plates:

1. Remove the 384-well reaction plate from the thermal cycler, centrifuge the plate at 100 x g for 1 minute, then remove the seal.
2. Add 1 μ L 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 μ L of 3 M sodium acetate to each well.

Note: Make sure that the sodium acetate reaches the bottom of the wells.

4. Add 25 μ L of 100% ethanol to each well.

Note: The final volume in the 384-well plate should not exceed 38 μ L.

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 μ L.

5. Seal the plate securely with self-adhesive film, then mix by inverting the plate four times.

or

Mix well by pipetting up and down in a multichannel pipette three to four times, then seal the plate securely with self-adhesive film.

6. Incubate at room temperature for 15 minutes and 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 1,650 x g for 45 minutes
Any other centrifuge	Use a plate adapter and centrifuge the plate at the maximum speed at 4°C as follows: <ul style="list-style-type: none"> • 1,400 to 2,000 x g for 45 minutes or <ul style="list-style-type: none"> • 2,000 to 3,000 x 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.

- Remove the self-adhesive film, invert the plate onto a paper towel, centrifuge at 185 x g for 1 minute, then remove the plate from the centrifuge.
- 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 1,650 x g for 15 minutes.
- Remove the self-adhesive film, invert the plate onto a paper towel, centrifuge at 185 x g for 1 minute, then remove the plate from the centrifuge.

Note: Start timing when the rotor starts moving.

- 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.

- (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.
- Proceed to “Minimum sample volume” on page 92.

Ethanol precipitation for dGTP BigDye Terminators v1.0 and v3.0

Purification methods

- Ethanol/sodium acetate precipitation (next section)
 - dGTP BigDye Terminators v3.0
 - dGTP BigDye Terminators v1.0
- Ethanol precipitation (page 87)
 - Precipitating in 96-well reaction plates
 - Precipitating in microcentrifuge tubes

Ethanol/sodium acetate precipitation

dGTP BigDye 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](#)).

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 μ L 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 μ L of deionized water
 - 64 μ L of non-denatured 95% ethanol

The final ethanol concentration should be 60% \pm 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 $\geq 1,400 \times g$ but $< 3,000 \times g$:

- 1,400 to 2,000 x g: 45 minutes
- 2,000 to 3,000 x g: 30 minutes

Note: A MicroAmp tube in a MicroAmp plate can withstand 3000 x 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 x g for 1 minute.
9. Add 150 μ L 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.
12. Place the inverted plate with the paper towel into the table-top centrifuge and spin at 50 x 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 90.

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 μ L 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 μ L of deionized water
 - 64 μ L of non-denatured 95% ethanol

The final ethanol concentration should be 60% \pm 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.

- 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.

- 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.

- Add 250 μ L of 70% ethanol to the tubes and vortex them briefly.
- Place the tubes in the microcentrifuge in the same orientation as in step 5 and spin at maximum speed for 10 minutes.
- Aspirate the supernatants carefully, as in step 6.
- Dry the samples in a vacuum centrifuge for 10 to 15 minutes or to dryness. Do not over-dry.
- Proceed with “Minimum sample volume” on page 92.

Purification with spin columns

Overview

- Prepare the spin column (page 88).
- Perform purification with the spin column (page 91).

Performing SDS/heat treatment

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).

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

Optimizing spin column purification

IMPORTANT! When using the BigDye terminators v3.1, hydrate the column for 2 hours (see step 3 in “Preparing the spin column,” 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.

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

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

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 87.
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. Remove any air bubbles by inverting or tapping the column and allowing the gel to settle.

4. 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.

5. Insert the column into the wash tube provided.
6. Centrifuge the column at 730 x g for 2 minutes to remove the interstitial fluid.
7. 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 x 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.

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 page 91

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°C 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

Due to 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	3730/3730xl	Heat seal	Place the plate directly in the instrument.
	3730/3730xl	MicroAmp Clear Adhesive Film	Remove the seal, replace it with a septa mat, then place the plate in the instrument.
	3130/3130xl 3500/3500xL	Either	Remove the seal, replace it with a septa mat, then place the plate in the instrument.
	310 Genetic Analyzer	Either	Transfer 10 μ L of supernatant to a clean plate, cover the plate with a septa mat, then place the plate in the instrument.
384-well	3730/3730xl	Heat seal	Place the plate directly in the instrument.
	3730/3730xl	MicroAmp Clear Adhesive Film	<ul style="list-style-type: none"> • 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.
	3500/3500xL, 3130/3130, 310 Genetic Analyzer	Either	Transfer 10 μ L of supernatant to a clean plate, cover with a septa mat, then place in the instrument.

2. Select the appropriate BigDye XTerminator run module for your instrument and plate type.

Note: Use standard run modules if you transferred the supernatant to a clean plate after centrifuging. On the 3130 and 3730 models, the BDX installer has to be installed initially on the instrument before using BDX to ensure that the autosampler is correctly working together with the BDX modules.

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 170).

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

Sample preparation procedure



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 Applied Biosystems 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 3730/3730xl instruments only.

Chapter 6

Capillary electrophoresis

This section covers:

Applied Biosystems genetic analyzers	96
Instrument consumables	98
Calibrating the instrument	100

Applied Biosystems genetic analyzers

Find the right instrument for your application and throughput needs.

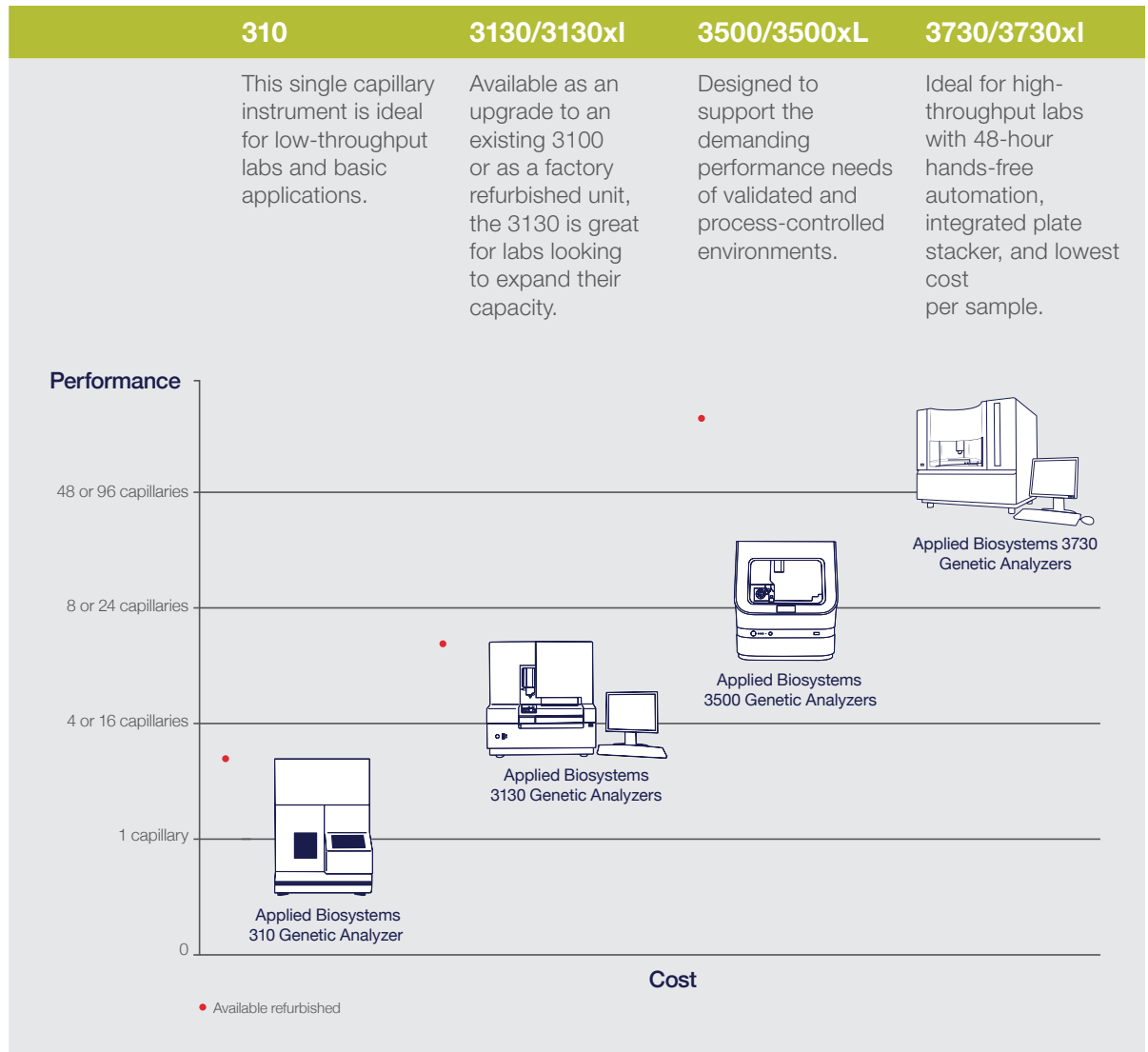


Figure 30. Comparison of performance, throughput, and cost of Applied Biosystems genetic analyzers.

Table 16. Applied Biosystems genetic analyzers.

	310	3130/3130xl	3500/3500xL	3730/3730xl
Number of capillaries	1	4 (3130), 16 (3130xl)	8 (3500), 24 (3500xL)	48 (3730), 96 (3730xl)
Number of dyes	5	5	6	5
Capillary array length (cm)	47, 61	22, 36, 50, 80	36, 50	36, 50
Consumable Tracking with RFID	No	No	Yes	No
Polymer type	POP CAP, POP-4, POP-6	POP CAP, POP-4, POP-6, POP-7	POP-4, POP-6, POP-7	POP CAP, POP-6, POP-7
Sample capacity	1–96 sample tubes	2 sample plates (96- or 384-well)	2 sample plates (96- or 384-well)	16 sample plates (96- or 384-well)
Hands-free automation	24 hours	24 hours	24 hours	48 hours
Integrated plate stacker	No	No	No	Yes
Data Collection Software version	310 Data Collection Software v3.1 or earlier	3130 Data Collection Software v4 or earlier	3500 Software Data Collection v3.1 or earlier	3730 Data Collection Software v4.0 or earlier

Electrophoresis workflow for all instruments

The user should refer to the appropriate instrument user manual for details concerning the setup and operation of their specific instrument. The part numbers for the user manual for each instrument are listed below:

Instrument	Catalog/Part Number
310	903565
3130/xl	4352715
3500/xL	100031809
3730/xl	4331468

1. Prepare the instrument:
 - a. Start the computer, the instrument, and the Data Collection Software.
 - b. Review status of consumables and maintenance notifications on dashboard. Replace with fresh material as needed.
 - c. Review/set user and general preferences. There are a number of preferences within the User Preference tab.

2. Perform spatial calibration, if necessary.
3. Perform spectral calibration, if necessary.
4. Set up the run using Data Collection Software:
 - a. Create or import a plate.
 - b. Assign plate contents.
 - c. Print the plate layout.
 - d. Prepare and load sample plates.
5. Load the prepared samples:
 - a. Load the prepared samples onto 8-tube strip tubes, or 96-well or 384-well plates.
 - b. Place the tubes or the plate assembly into the instrument.
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 pre-labeled sequencing reactions that require only resuspension before use. Standards available:

Table 17. Available BigDye sequencing standards.

Standards	Part number
3500/3500xL Sequencing Standards, BigDye Terminator v1.1	4404314
3500/3500xL Sequencing Standards, BigDye Terminator v3.1	4404312
BigDye Terminator v3.1 Sequencing Standard (310, 3130, and 3130xl)	4336935
BigDye Terminator v3.1 Sequencing Standard (3730/3730xl)	4336943
BigDye Terminator v1.1 Sequencing Standard (310, 3130, and 3130xl)	4336791
BigDye Terminator v1.1 Sequencing Standard (3730, 3730xl)	4336799

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. The 3500/3500xL instruments use POP polymer in a pouch that has a built-in RFID tag. It is the only polymer that can be used on this instrument.

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. For the 3500, please use the conditioning pouch.
- Discard capillaries that are exposed to dust or are dried out.
- Change the buffer and water and discard the waste according to the recommended timeline.

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 validated for 300 runs for 3730/3730xl instruments, 160 runs for 3500/3500xl instruments, 150 runs for 3130 and 3100-Avant instruments, and 100 runs for 3130xl, 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 page 188)
- High baseline
- Noisy data or peaks under peaks throughout the sequence (see page 174)
- Shoulders on peaks or irregular peaks (see pages 167, 169, and 170)

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 310 and 3730/3730xl genetic analyzer running buffers are supplied at 10X concentrations. Dilute to 1X with distilled water before use.

- **3500/3500xL instruments**—Applied Biosystems Anode Buffer Container (ABC) 3500/3500xL (PN 4393927) contains 1X running buffer to support all electrophoresis applications on the 3500 or 3500xL analyzer. Applied Biosystems Cathode Buffer Container (CBC) 3500/3500xL (PN 4408256) contains 1X running buffer to support all electrophoresis applications on the 3500 or 3500xL analyzer.
- **3730/3730xl instruments**—Applied Biosystems 3730/3730xl 10X Running Buffer with EDTA (PN 4335613).
- **310, 3130, and 3130xl instruments**—10X Running 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 charge-coupled device (CCD) camera to a position in the capillary array. Spatial calibration is required for all multicapillary 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 four-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 or 3500 instruments. Use sequencing standards for spectral calibration of 3730 and 3500 instruments.

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

Chapter 7

Data analysis

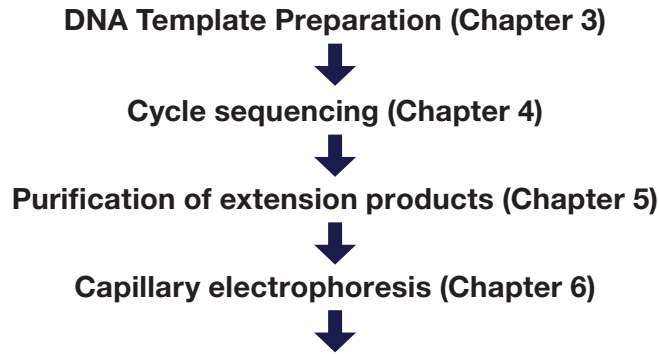
This section covers:

Overview	104
Analysis software	105
Sequence Scanner Software	111
Sequencing Analysis Software	114
Variant Reporter Software	117
SeqScape Software	119
MicroSeq ID Analysis Software	122
MicrobeBridge Software	126
Sanger analysis modules on Thermo Fisher Cloud	128
Quality Check (QC) module	129
Variant Analysis (VA) module	130
Next-Generation Confirmation (NGC) module	131
Minor Variant Finder Software	133

Overview

This chapter provides an overview of data analysis using Thermo Fisher Scientific 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

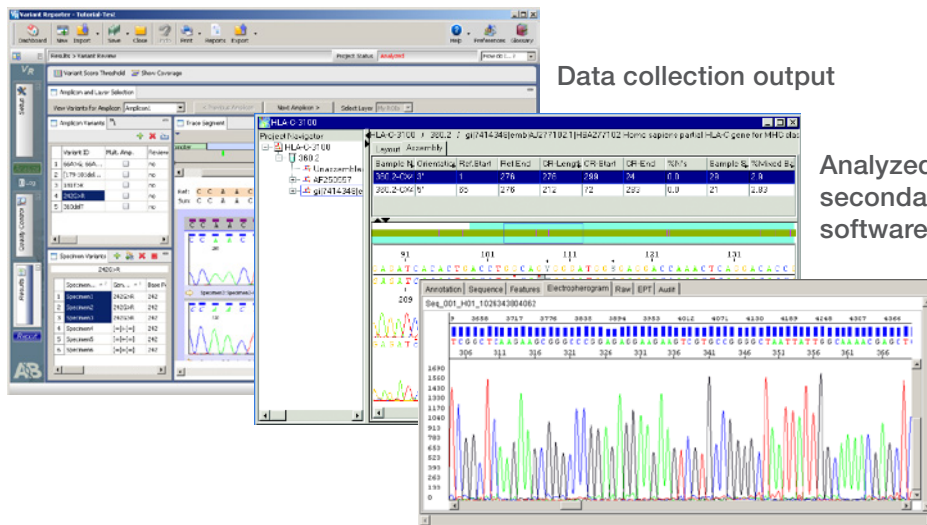


Data analysis

1. Choose software for your application.
2. Apply analysis protocols, including:
 - Basecaller
 - Mobility file
3. Run analysis.
4. Review the data.

Thermo Fisher Scientific software:

- Sequence Scanner Software
- Sequencing Analysis Software
- Variant Reporter™ Software
- “Minor Variant Finder Software” Software
- SeqScape Software
- MicroSeq ID Analysis Software
- MicrobeBridge Software
- Sanger Analysis Modules on Thermo Fisher Cloud



Data collection output

Analyzed project in secondary sequencing software

Analysis software

The following analysis software is available from Thermo Fisher Scientific at [thermofisher.com/sangersoftware](https://www.thermofisher.com/sangersoftware).

Table 18. Analysis software available from Thermo Fisher Scientific.

Product	Cloud or desktop	Suggested use
Sequence Scanner Software (free)	Desktop	Viewing or editing traces, evaluating trace quality, making trace QC reports
Sequencing Analysis Software	Desktop	Viewing or editing traces, evaluating trace quality, making trace QC reports, reanalyzing traces
Variant Reporter Software (free trial available)	Desktop	Detecting mutations, discovering and validating SNPs
SeqScape Software (free trial available)	Desktop	Detecting mutations, discovering and validating SNPs, comparing sequences, typing
MicroSeq ID Analysis Software	Desktop	For identification of bacterial and fungi strains (to be used in conjunction with the MicroSeq ID PCR and Sequencing kits)
Sanger Analysis Modules—QC module (free)	Cloud	Viewing or editing traces, evaluating trace quality, making trace QC reports
Sanger Analysis Modules—Next-Generation Confirmation (NGC) module (free)	Cloud	Variant identification of NGS sequencing data, QC data
Sanger Analysis Modules—Variant Analysis (VA) module (free)	Cloud	Detecting mutations, discovering and validating SNPs
MicrobeBridge Software v1.0	Desktop	Streamlined analysis for microbial 16S RNA sequencing with direct link to CDC MicrobeNet database
Minor Variant Finder Software	Desktop	Detecting and validating low-frequency (minor) single nucleotide variations (SNVs), NGS verification

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 109.

1. **What applications are performed in your lab with your genetic analyzer?** Use Table 19 below to select an analysis software package. If the table recommends more than one type of software, go to question 2.

Table 19. Analysis software recommendations, by application.

Sequencing application	Sequencing Analysis Software	Variant Reporter Software	SeqScape Software	Minor Variant Finder Software	QC module	VA module	NGC module
Sequence quality control	Yes	Yes	No	Yes	Yes	Yes	Yes
BAC end sequencing	Yes	No	No	No	No	No	No
Checking clone constructs	Not recommended	Yes	Yes	Yes	No	Yes	Yes
<i>De novo</i> sequencing	Yes	Yes	Yes	No	Yes	No	No
Multilocus sequence typing (MLST)	Not recommended	No	Yes	No	No	No	No
HLA typing	Not recommended	No	No	No	No	No	No
Methylation analysis using bisulfite DNA conversion	Not recommended	Yes	Yes	Yes	No	Yes	Yes
Mitochondrial DNA (mtDNA) sequencing	No	Yes	Yes	Yes	No	Yes	Yes
Resequencing—substitutions (SNPs), heterozygous mutations, insertions, and deletions	Not recommended	Yes	Yes	Yes	No	Yes	Yes
Virus research subtyping	Not recommended	No	Yes	No	No	No	No
NGS confirmation	No	No	No	Yes	No	No	Yes

2. **Are electronic signature, audit trail, and security compliance features required in your lab?**

- If yes, SeqScape Software and MicroSeq ID Software have electronic signature, audit trail, and security features.
- If no, continue to question 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 5,000 traces in only one project. Base calling, the most time-consuming part of the analysis, can be skipped if data are pre-basecalled by Sequencing Analysis Software.

- If the answer is no to both questions, continue to question 4.

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

- Examine Table 20 for more information.
- To download trial versions of our commercially available software and to learn more about our software portfolio, visit thermofisher.com/sangersoftware.
- To learn more, you can register for a web-based training course at learn.thermofisher.com/sequencing.
- Contact your Thermo Fisher Scientific sales representative or field application specialist.

Table 20. Features in Thermo Fisher Scientific analysis software.

Software	Features and functions
Sequencing Analysis Software	<p>With Sequencing Analysis Software you can:</p> <ul style="list-style-type: none"> • Perform base calling and trim low-quality bases at the 5' and 3' ends. With the KB Basecaller, you can also: <ul style="list-style-type: none"> — 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	<p>With Variant Reporter Software, you can:</p> <ul style="list-style-type: none"> • 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	<p>With SeqScape Software, you can:</p> <ul style="list-style-type: none"> • 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 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.

Table 20. Features in Thermo Fisher Scientific analysis software (continued).

Software	Features and functions
MicroSeq ID Analysis Software	<p>With MicroSeq ID Analysis software, you can:</p> <ul style="list-style-type: none"> • 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.
Sequence Scanner Software	<p>With Sequence Scanner Software, you can:</p> <ul style="list-style-type: none"> • 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 .fasta, .seq, .phd1, and .scf.
MicrobeBridge Software	<ul style="list-style-type: none"> • No setup required. • QC and assemble sequence data into 1,400 bp consensus contigs. • No external library inside software, provides one-click button to connect to CDC MicrobeNet database for bacterial identification. • Exports assembled data to .fasta format.
Sanger Analysis Modules: <ul style="list-style-type: none"> • Quality Check (QC) • Variant Analysis (VA) • Next-Generation Confirmation (NGC) 	<ul style="list-style-type: none"> • Cloud-based. • Has all QC functions provided by Sequence Scanner Software. • Reports variants from CE sequencing in genomic coordinates. • Exports variants in standard .vcf format. • Validates NGS variants by visualizing differences by Venn diagram.
Minor Variant Finder Software	<ul style="list-style-type: none"> • 5% somatic variant detection using Sanger sequencing. • Sequences 1–96 amplicons/targets at the lowest cost. • Confirms NGS findings in alignment view and Venn diagram.

Choosing analysis software by available features

This section describes the different analysis tasks that can be performed by Thermo Fisher Scientific analysis software for capillary electrophoresis (CE) sequencing instruments.

- **Base calling**—Translates the collected color-data images into the corresponding nucleotide basecalls.
- **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.

- **Variation 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**—Provides audit trail, access control, and e-signature features.

Table 21. Tasks performed by Thermo Fisher Scientific analysis software.

Feature	Software package				
	Sequence Scanner Software	Sequencing Analysis Software	Variation Reporter Software	SeqScape Software	MicroSeq ID Analysis Software
Base calling	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
Variation detection	No	No	Yes	Yes	No
Typing**	No	No	No	Yes	Yes
Auditing and electronic signature features	No	Yes	No	Yes	Yes

*For reanalysis

**With sequence library

Table 21. Tasks performed by Thermo Fisher Scientific analysis software (continued).

Feature	Software package				
	Microbe-Bridge Software	QC module	VA module	NGC module	Minor Variant Finder
Base calling	Yes	No	No	No	No
Autoanalysis	Yes	No	No	No	No
Sequence alignment with reference	No	No	Yes	Yes	Yes
Sequence alignment, no reference	Yes	No	No	No	Yes
Variant detection	No	No	Yes	Yes	Yes
Typing**	No	No	No	No	No
Auditing and electronic signature features	No	No	No	No	No

**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.

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 base calling 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 at thermofisher.com/sangsoftware.

Features of Sequence Scanner Software:

- Six instantaneous views of a trace
- Dynamic zoom to an exact base location
- Simultaneous display of raw and analyzed data
- Powerful thumbnail viewing and sorting
- Multiple export options for traces and reports
- Seven visually expressive summary reports
- Expert hotkey navigation

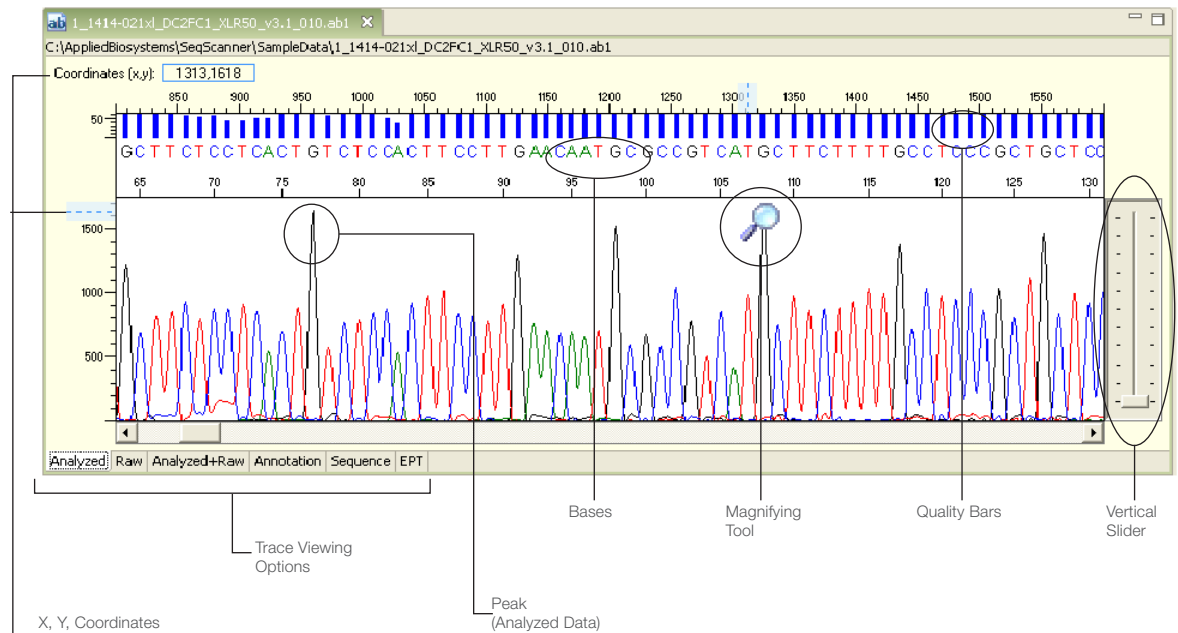


Figure 31. The sequence scanner tool allows user to explore traces using six different views.

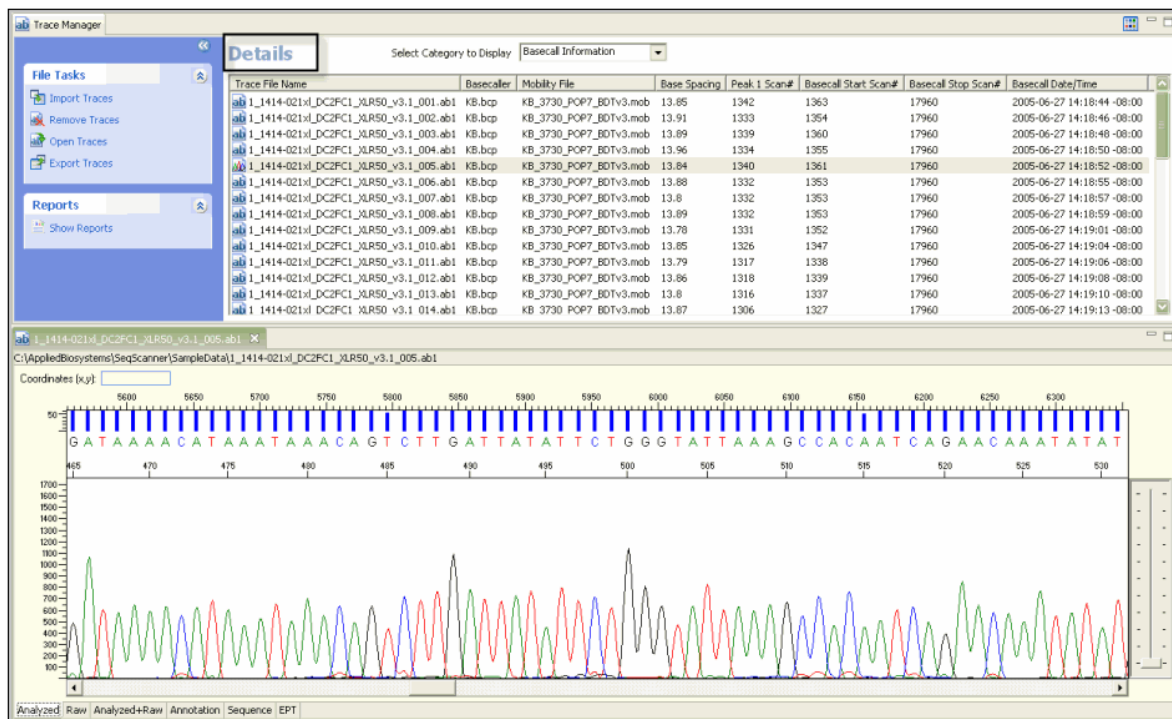


Figure 32. The trace manager allows you to handle and manage your traces.

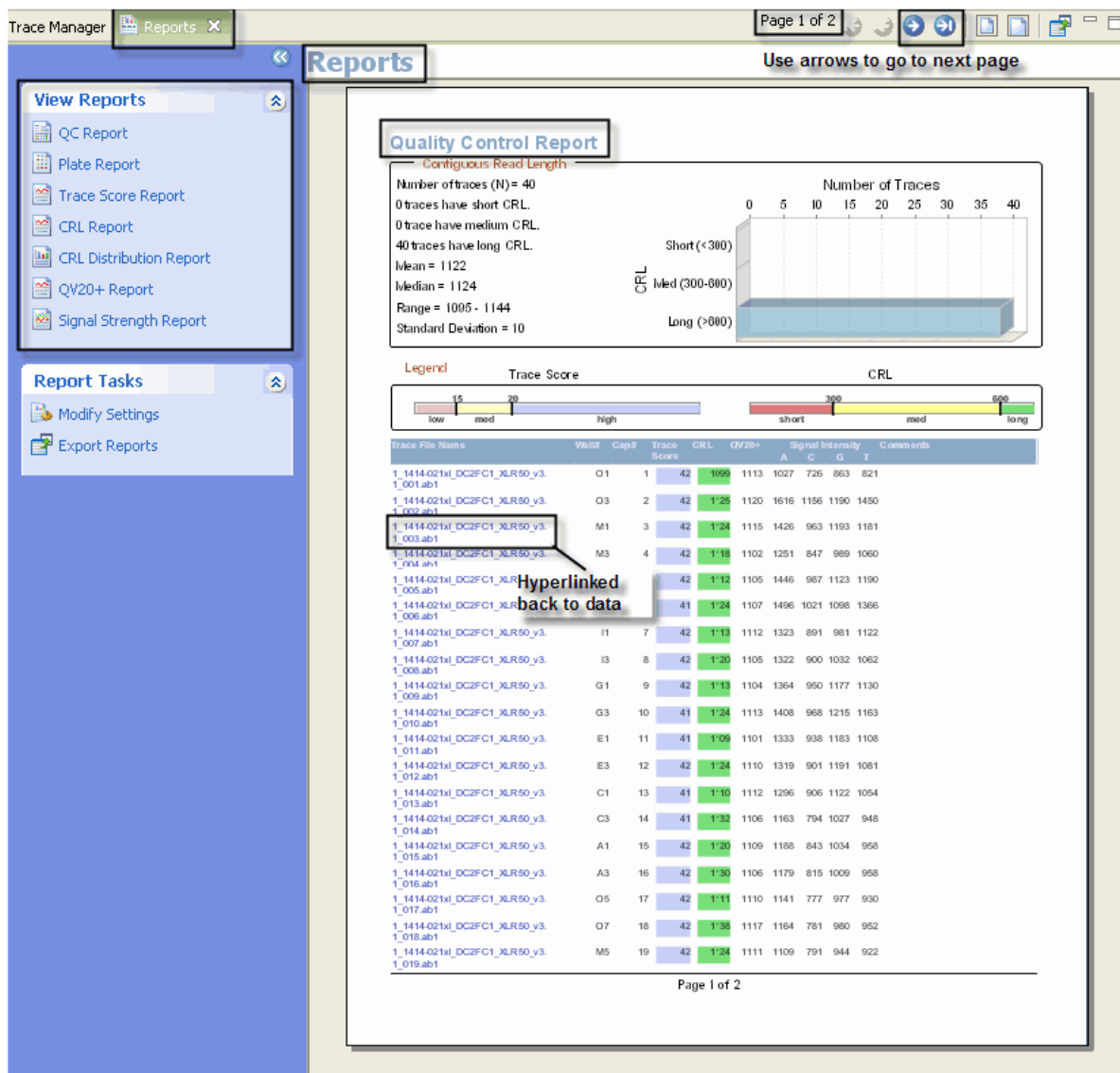


Figure 33. The sequence scanner also allows you to generate reports that suit your needs.

Sequencing Analysis Software

Overview and applications

Sequencing Analysis Software analyzes, displays, edits, saves, and prints sample files that are generated from Applied Biosystems DNA analyzers and genetic analyzers.

The Sequencing Analysis Software:

- Uses a basecaller algorithm that performs base calling for pure and mixed base calls
- Generates quality values to provide basecall accuracy information for pure and mixed base calls
- Generates analysis reports to help troubleshoot and provide easy assessment of data quality
- Can generate an audit trail of base changes

- Allows you to use electronic signatures

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 v5.4 Quick Reference Card* ([PN 4401738](#))
- *Sequencing Analysis Software v5.4 User Bulletin* ([PN 4413697](#))

To obtain the latest software updates and patches, go to thermofisher.com/sangersoftware.

Tips and hints

Performing analysis with Sequencing Analysis Software can reduce processing time in Variant Reporter Software (except for 3500, since the basecaller is already integrated into the data collection software).

Input sample files

Sequencing Analysis Software is compatible with sample files that are generated from:

- Applied Biosystems 3730/3730xl DNA Analyzers and 3130/3130xl Genetic Analyzers
- ABI Prism 310 Genetic Analyzers
- Applied Biosystems 3500/3500xL and 3500 Dx/3500xL Dx Genetic Analyzers

Output files for sequencing analysis

You can generate sample files as:

- Analyzed ABI files (.ab1)
- Text file of the sequence in ABI or FASTA files (.seq; all bases or only clear range bases)
- Phred (.phd.1) files
- Standard chromatogram format (.scf) files
- Analysis reports (TXT, HTML, PDF, or XML files)



Figure 34. The sample manager.

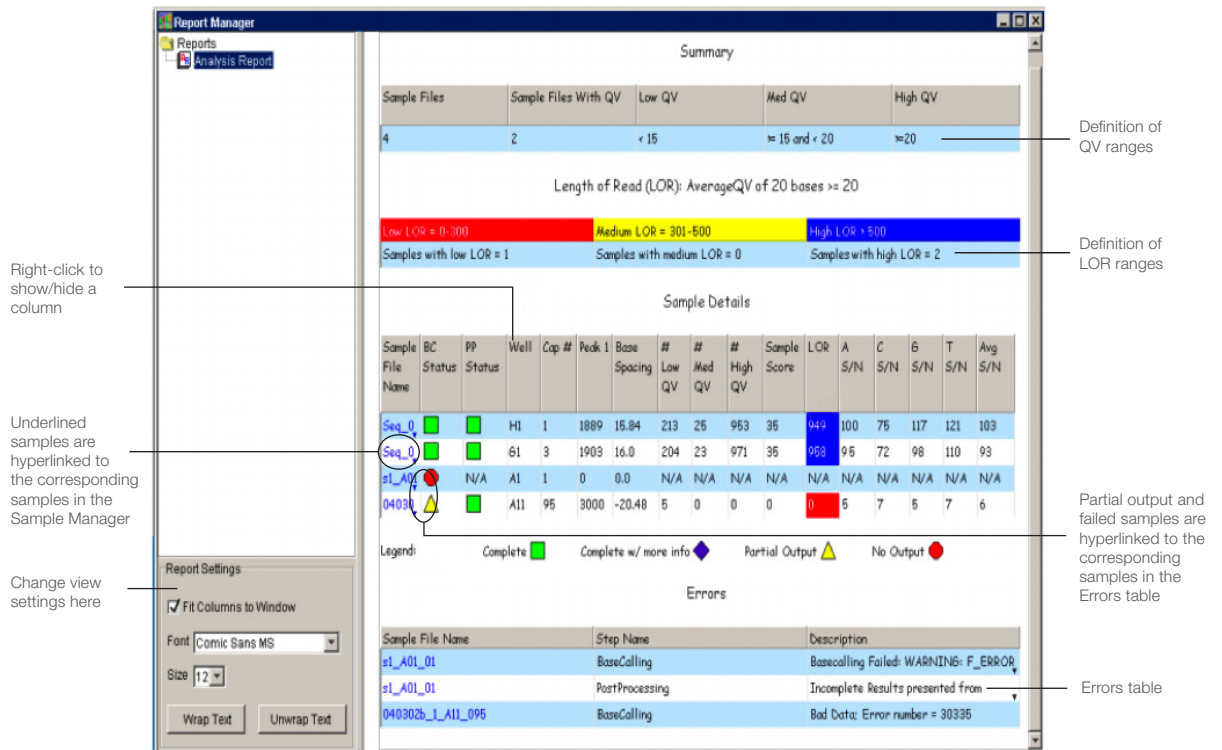


Figure 35. The analysis report.

Variant Reporter Software

Overview and applications

Variant Reporter Software is an intuitive resequencing software designed to accurately discover and validate mutations and single nucleotide polymorphisms (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 *Quick Reference Card: Variant Reporter Software v1.1—Analyzing a Project without a Reference* (PN 4401735) and *Quick Reference Card: Variant Reporter Software v1.1—Analyzing a Project with a Reference* (PN 4401734) for more information.

To obtain the latest software updates and patches, go to thermofisher.com/sangersoftware.

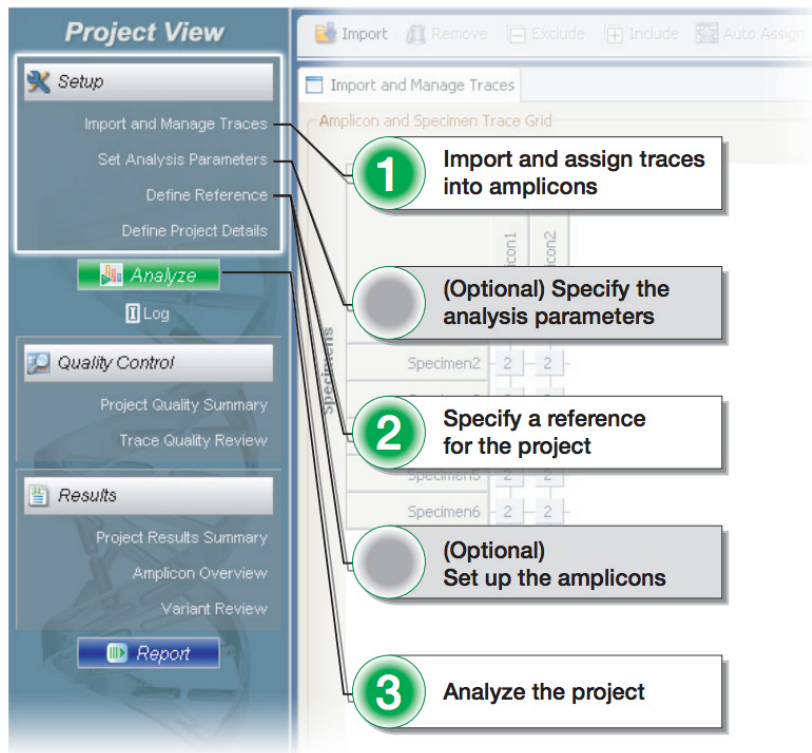


Figure 36. Preanalysis workflow with reference.

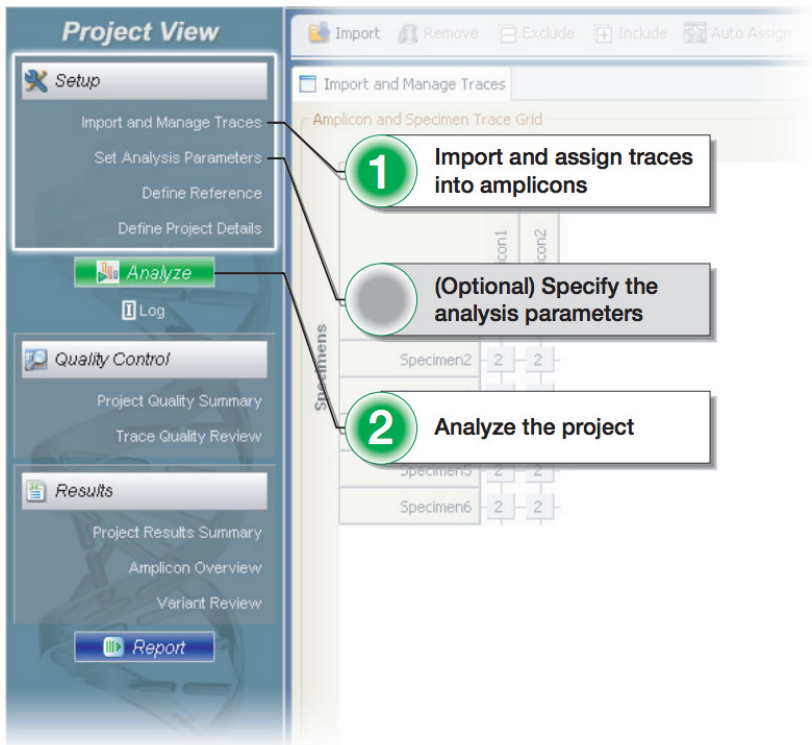


Figure 37. Preanalysis workflow without reference.

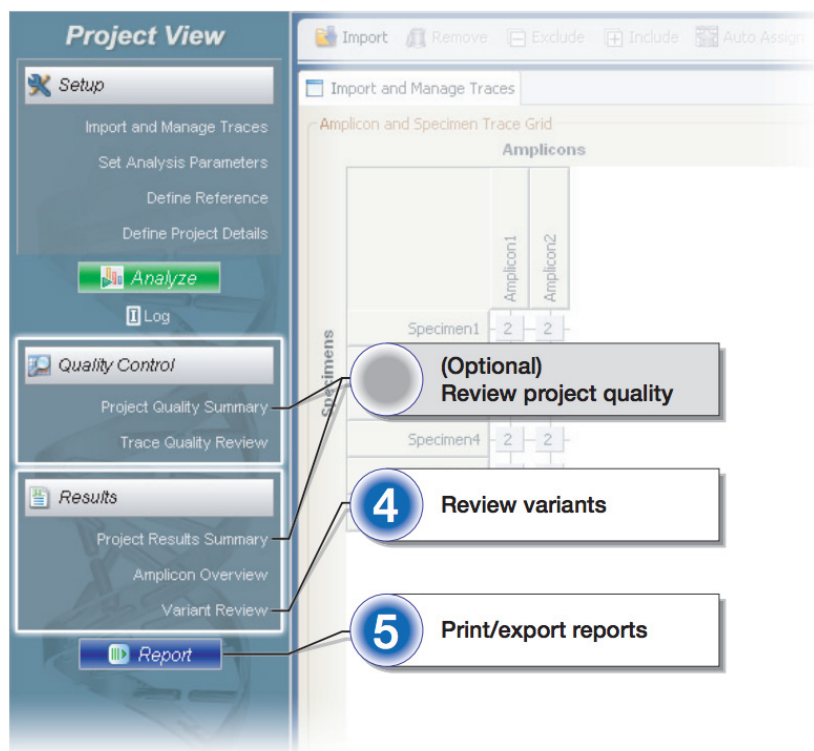


Figure 38. Post analysis workflow.

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 *Thermo Fisher Scientific SeqScape Software v3 User Guide* (PN 4474242) and online help for more information.

To obtain the latest software updates and patches, go to thermofisher.com/sangersoftware.

Tips and hints

Creating a project in SeqScape involves the following steps:

1. Import a reference sequence.
2. Define analysis and display settings.
3. Create a project template.
4. Add sample files to the project template.
5. Analyze the data.

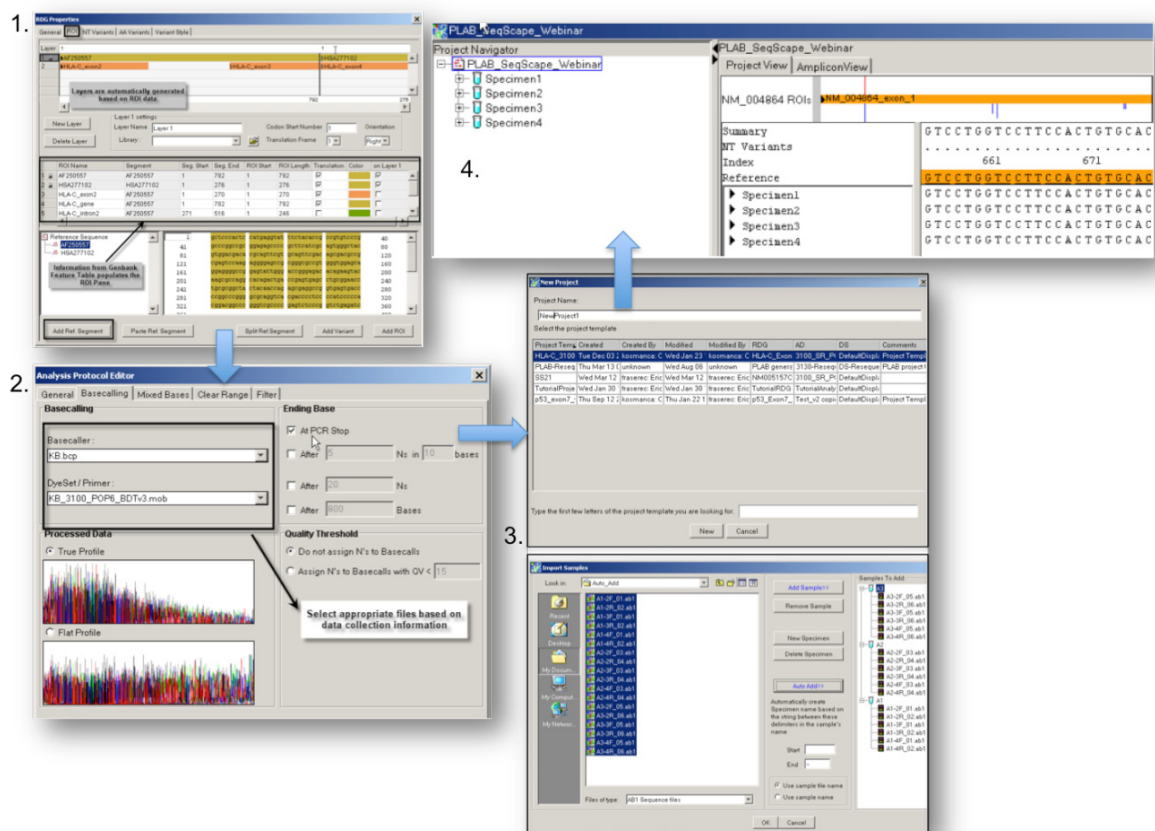


Figure 39. Steps for creating a project in SeqScope.

The results can be reviewed as following:

1. Review data per specimen and assign pass/fail status.
2. Review all data in the project view.
3. View variant results in mutations and AA variants reports.
4. View resequencing results in the genotyping report.

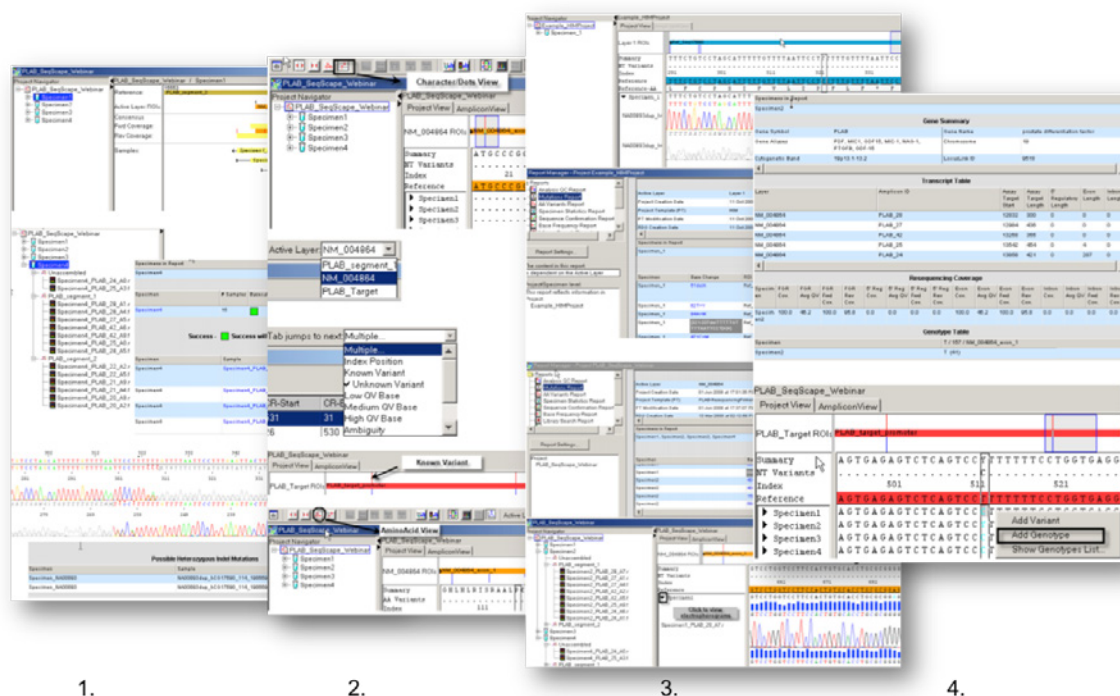


Figure 40. Options for reviewing results in SeqScope.

MicroSeq ID Analysis Software

Note: MicroSeq ID Analysis Software is specialized for bacterial and fungal identification and is not suitable for generalized sequence analysis. Contact your Thermo Fisher Scientific 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.

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.

With MicroSeq ID Microbial Identification Software Version 3.1, you can:

- Start and process a MicroSeq ID run directly with the Applied Biosystems 3500 Series Data Collection (DC) Software v3.1, without running Autoanalysis Manager software.

- Perform an analysis and automatically receive (Auto-ID) or manually enter a specimen identification.
- View phylogenetic relationships between specimens by:
 - Calculating the genetic distance for selected sequence pairs in an unrooted phylogenetic tree.
 - Zooming in/out on the horizontal branches in the phylogenetic tree diagram as needed.
- Search entries in MicroSeq ID Software's validated and custom libraries.
- Manage a series of sequence edits (multiple undo).
- Export multiple projects at one time.
- Copy an entire sample or consensus sequence using the MicroSeq ID software analysis toolbar and paste it into the National Library of Medicine's BLAST™ database search window.
- In the Raw view for sample data files, zoom in and out on the traces in the raw data profile.
- View, archive, and restore audit records for all users at the application level (administrators only).

MicroSeq ID Microbial Identification Software Version 3.1 supports 3500 Series (3500/3500xL) and 3130 Series (3130/3130xL) Genetic Analyzers.

Additionally, MicroSeq ID Software reads the AB1 files, and consequently supports data analysis, although not autoanalysis, from the following instruments:

- 310 Genetic Analyzer
- 3100 Series genetic analyzers
- 3730 Series genetic analyzers

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.

To obtain the latest software updates and patches, go to thermofisher.com/sangersoftware.

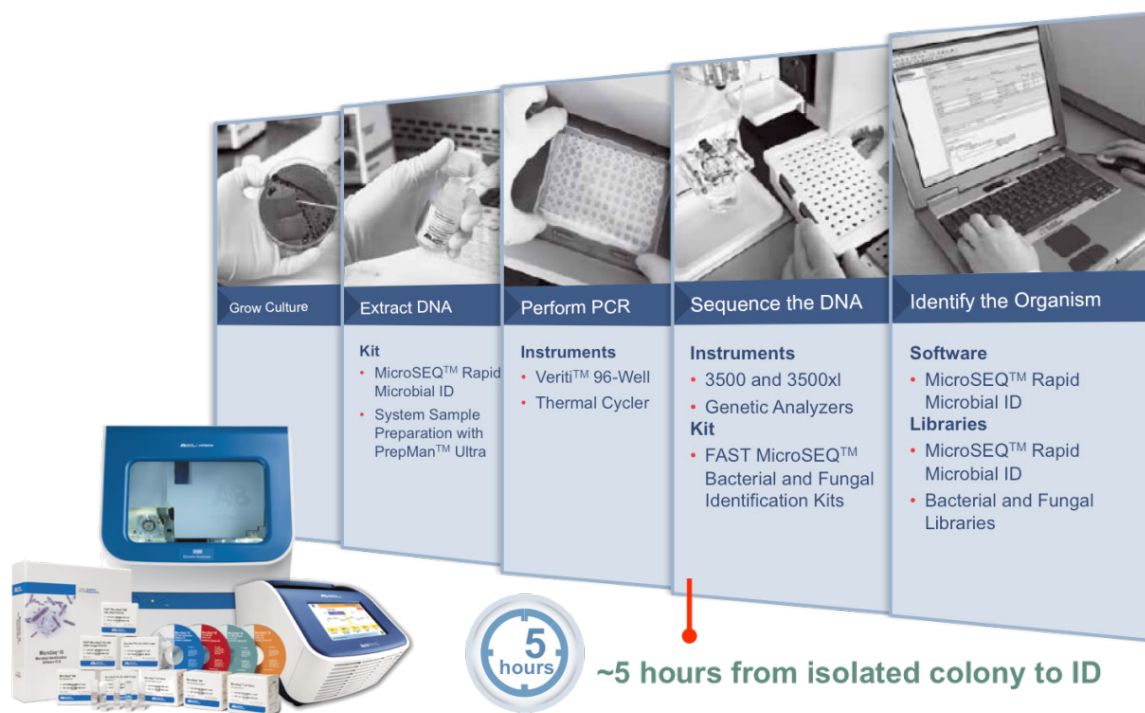


Figure 41. Overview of MicroSeq ID.

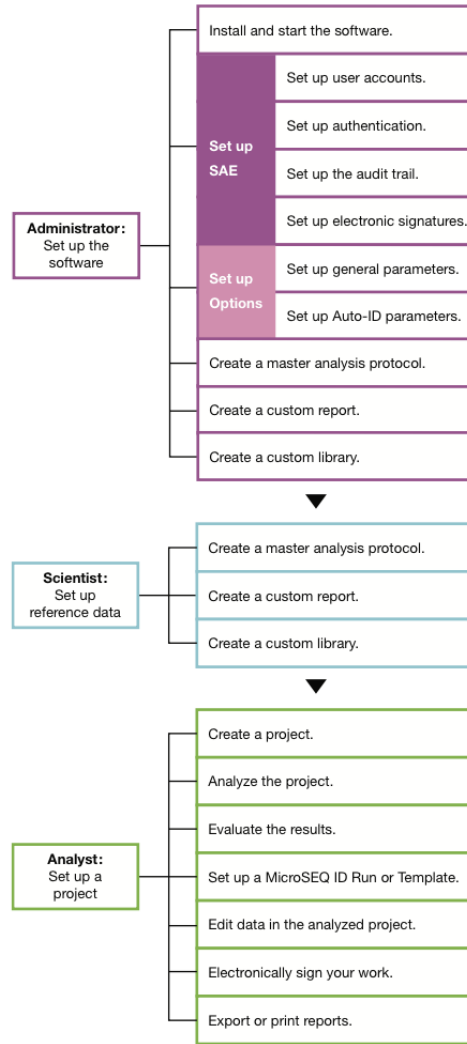


Figure 42. User workflow.

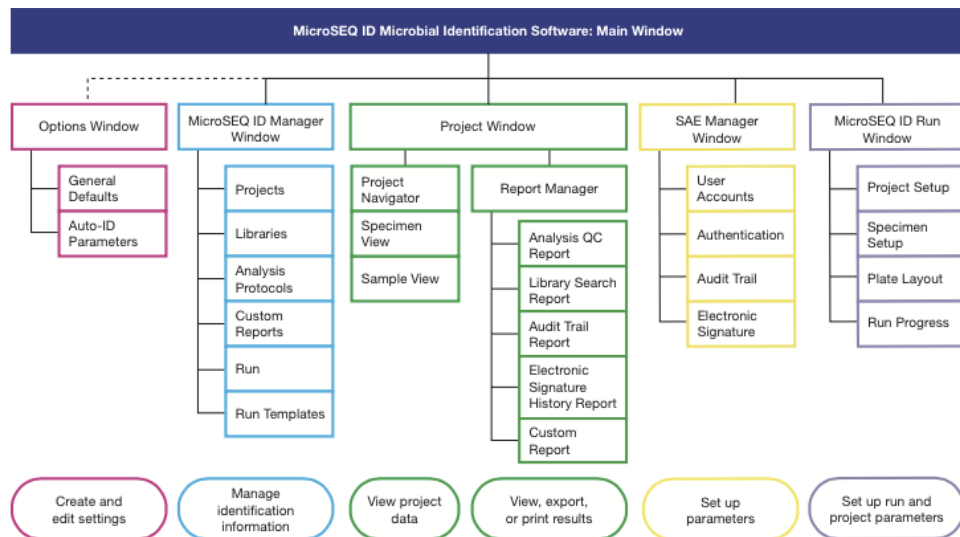


Figure 43. MicroSeq ID Software structure.

MicrobeBridge Software

Overview and applications

MicrobeBridge is a streamlined, desktop software solution which connects DNA sequences generated on Applied Biosystems Sanger Sequencers with the CDC's MicrobeNet database for bacterial identification using 16S rRNA gene sequencing analysis. There is no need for local database setup, so computer resources are easily developed.

- The MicrobeBridge Software processes data files (AB1) generated on an Applied Biosystems Sanger Sequencer.
- The software automatically assembles contig sequences when AB1 files are imported.
- The contig file can then be easily compared to the current 16S rRNA database using the CDC MicrobeNet tool (<https://microbenet.cdc.gov/>).

The MicrobeBridge Software provides the following review features:

- **Overview of read coverage**—Shows the range of forward and reverse sequences in a specimen.
- **Contig review**—Shows the forward and reverse sequences, identifies discrepancies in the assembled contig sequence, and allows editing of the contig sequence.
- **Quality status**—Displays color-coded trace files based on user-settable quality ranges and provides thumbnail trace views to examine raw data.
- **One-click access to MicrobeNet**—Provides one-click copy contig sequence function and one-click access to MicrobeNet.

More information on the technical details of MicrobeBridge can be found in *MicrobeBridge Software v1.0 Release Notes* (PN 100033483) on the www.thermofisher.com website.

Software workflow

1. Create and/or open a project.
2. Import samples and organize into specimens.
3. Review sequence and trace quality, and delete low-quality AB1 files from the project as needed.
4. Edit the contig sequence, change analysis settings, and reanalyze as needed.
5. Copy the contig sequence.
6. Click Open MicrobeNet (<https://microbenet.cdc.gov/>).
7. Log in, then paste the contig sequence into MicrobeNet.

For more information

See Chapter 8 for information on interpreting and troubleshooting results.

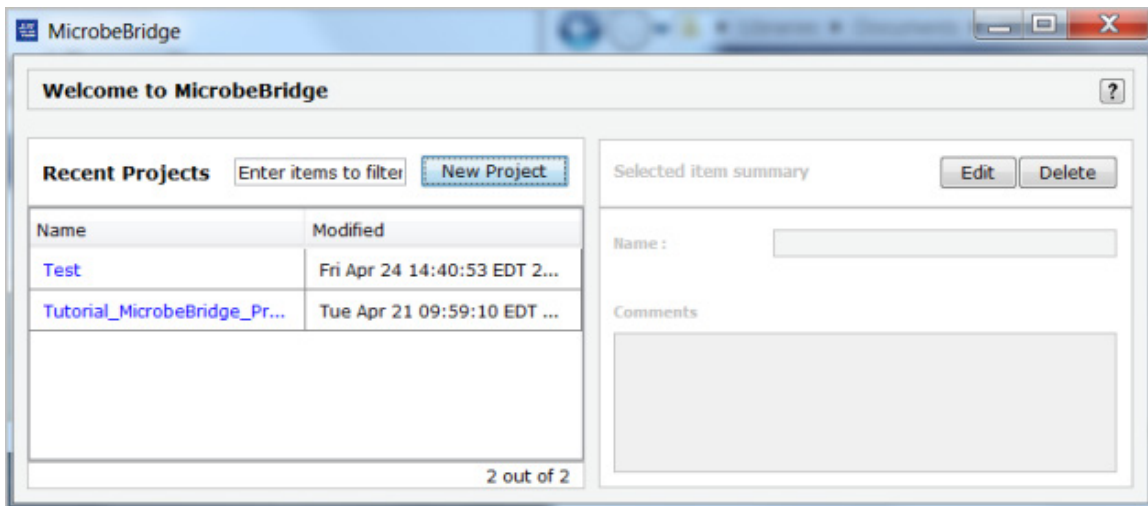


Figure 44. Create or open a project.

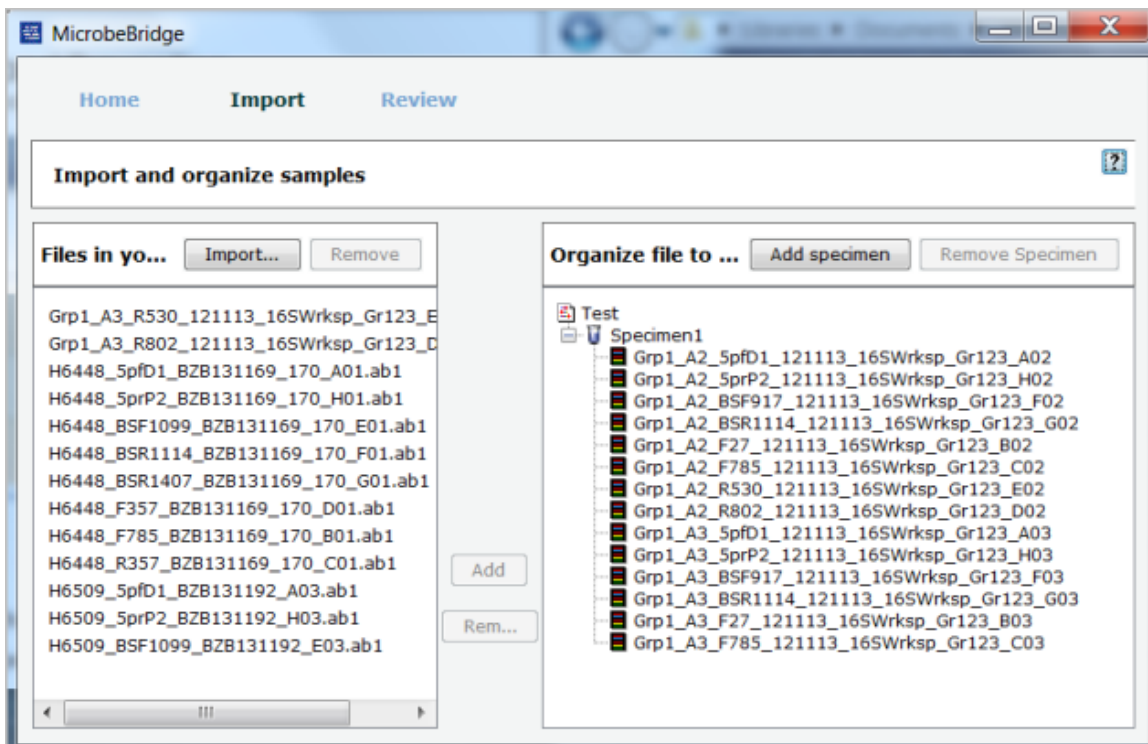


Figure 45. Import samples.

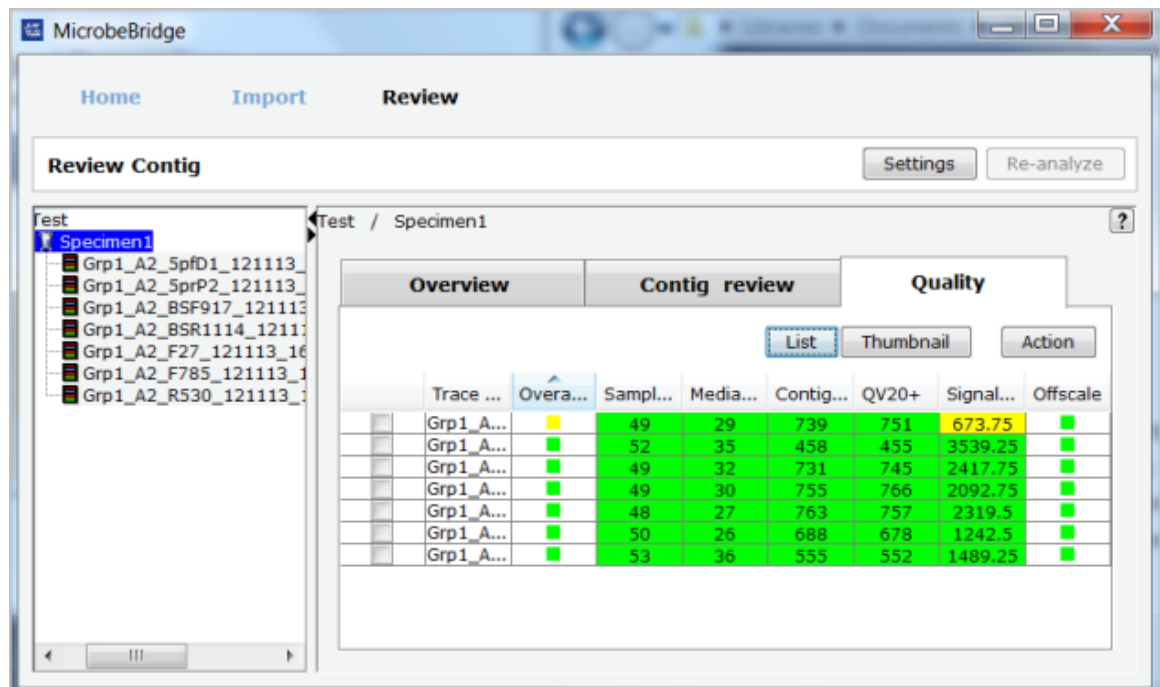


Figure 46. Contig review.

Sanger analysis modules on Thermo Fisher Cloud



Thermo Fisher Scientific has recently released a suite of cloud-based downstream analysis packages designed to enhance the functionality of Sanger sequencing data for variant detection using very large numbers of AB1 files. Users can now quickly assess the quality of their Sanger sequencing data using the Quality Check module. A wide range of sequence variants, insertions/deletions, and base changes are called with high confidence with the Variant Analysis module. Finally, variants called in their data can be seamlessly merged to NGS datasets in order to validate new variants using the Next-Generation Confirmation (NGC) module. Details on the step-by-step use of these tools can be found on the Thermo Fisher Scientific website at [thermofisher.com/sangermodules](https://www.thermofisher.com/sangermodules).

Quality Check (QC) module

Overview and application

The Thermo Fisher Scientific Quality Check (QC) module evaluates trace quality. The easy-to-interpret analysis summary gives a snapshot of Sanger sequence trace quality. The Trace Details page allows you to evaluate and edit traces as needed. The Flag Settings page allows you to adjust quality thresholds.

Software workflow

1. Select QC application.
2. Import samples into a new project or click existing samples from a current project.
3. Review sequence and trace quality, and delete low-quality AB1 files from the project as needed.
4. Edit bases or change quality flag settings.
5. Click Results to view QC results.
6. Generate PDF reports as needed.

For more information

See Chapter 8 for information on interpreting and troubleshooting results. The QC module can be accessed at apps.thermofisher.com.

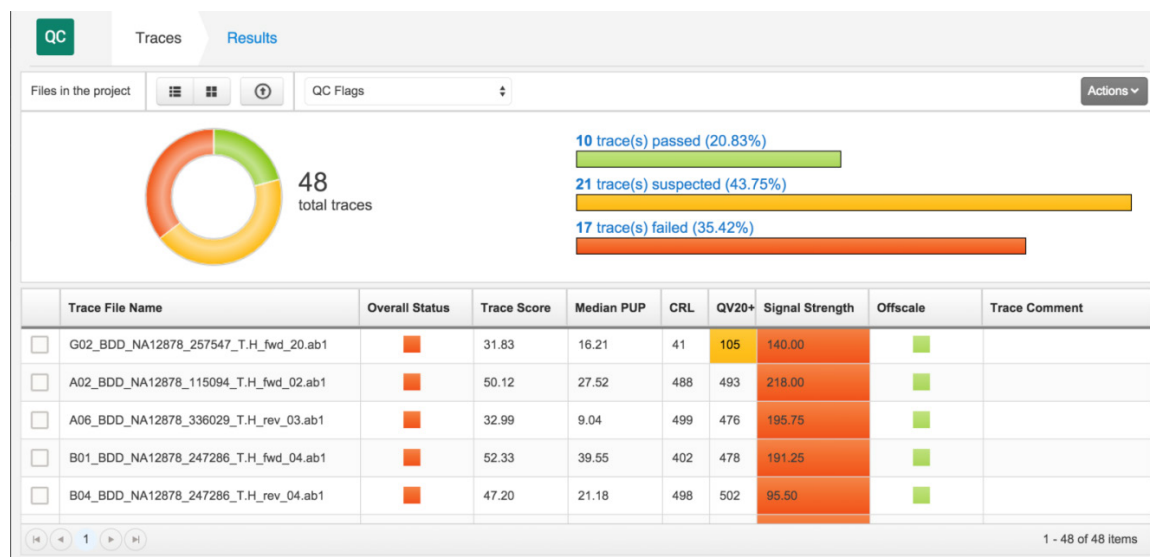


Figure 47. QC module filter view.

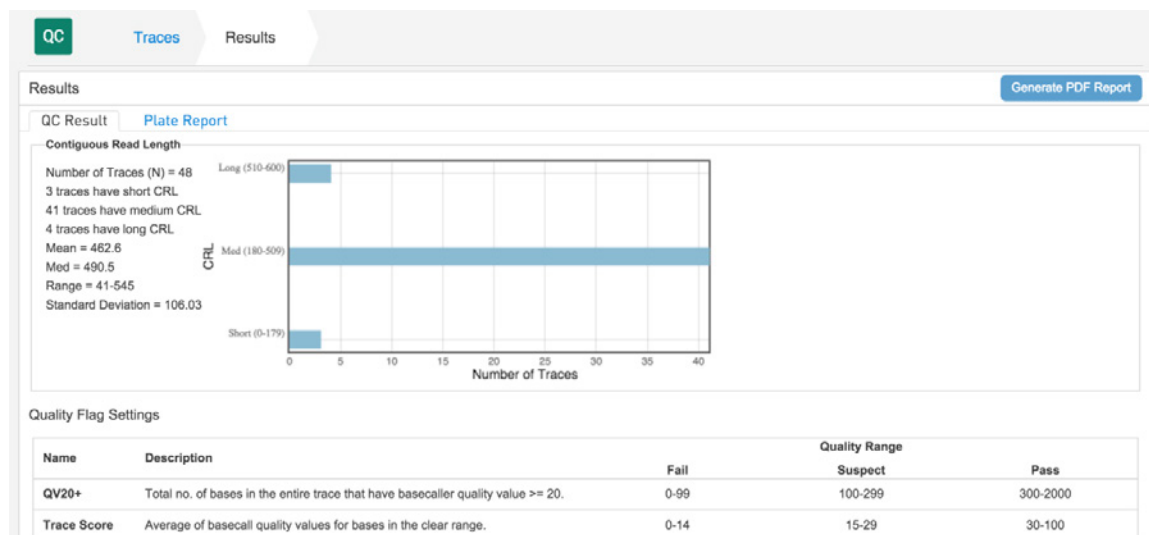


Figure 48. QC module results view.

Variant Analysis (VA) module

Overview and application

The Variant Analysis module provides fast analysis of Sanger sequencing data.

Up to 1,000 AB1 files can be analyzed and results displayed within 1 minute, and 10,000 AB1 files can be analyzed within 2 minutes—speeds that traditional desktop software cannot match. The VA module can automatically retrieve reference sequences from the genomic database, report variants with genomic coordinates, and report genomic annotations for SNPs. With highly overlapped forward/reverse strands, the VA module reports very high sensitivity for SNP calls. The VA module also reports and exports variant files in standard .vcf format. There is no software maintenance required from users.

Software workflow

1. Select VA application.
2. Import your Sanger AB1 files from any capillary electrophoresis instrument.
3. Create a Reference to map the variants.
4. Organize your data into Amplicons for grouping of reads.
5. Set parameters for Trimming, Filtering, and Alignment.
6. Review your settings and run the analyses.

For more information

See Chapter 8 for information on interpreting and troubleshooting results. The VA module can be accessed at apps.thermofisher.com.

An example of the output from the VA module is shown in the following figure.

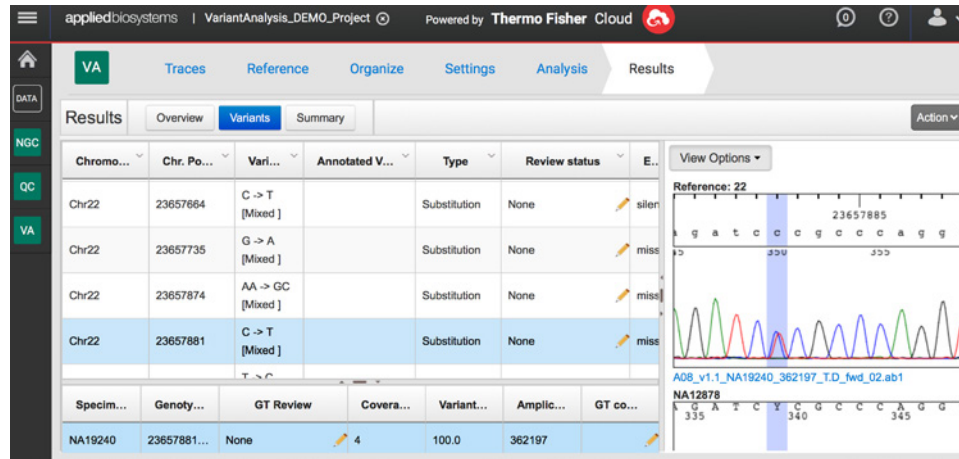


Figure 49. Example of output from the VA module.

Next-Generation Confirmation (NGC) module

Overview and application

The Next-Generation Confirmation (NGC) module lets users compare results from standard NGS variant files with results from Sanger sequencing instruments within the Thermo Fisher Cloud environment.

Critical decisions often require validation of NGS results using robust Sanger sequencing. The NGC module provides fast analysis of AB1 files and reports variants in genomic coordinates. The results are automatically annotated with known SNPs from the current genomic database.

Software workflow

1. Select NGC application.
2. Import your Sanger AB1 files from any capillary electrophoresis instrument.
3. Create a Reference to map the variants and upload your VCF file containing NGS variants.
4. Organize your data into Amplicons for grouping of reads.
5. Set parameters for Trimming, Filtering, and Alignment.
6. Review your settings and run the analyses.

For more information

See Chapter 8 for information on interpreting and troubleshooting results. The NGC module can be accessed at apps.thermofisher.com.

The NGC module is able to display both variants in summary view as shown in the following figure.

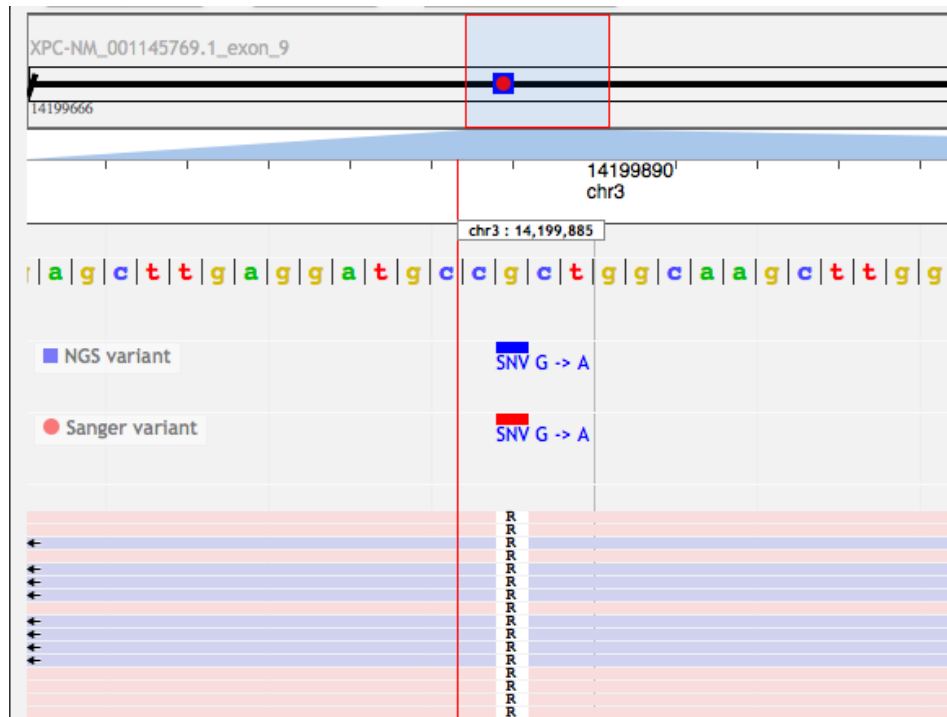


Figure 50. Summary view in the NGC module.

The results can also be displayed as a Venn diagram showing the intersect between the NGS and Sanger variants identified. This is shown in the following figure.

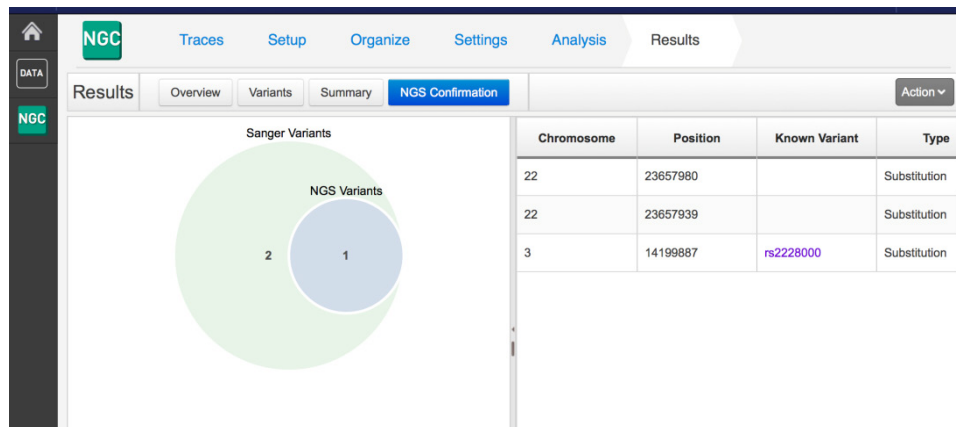
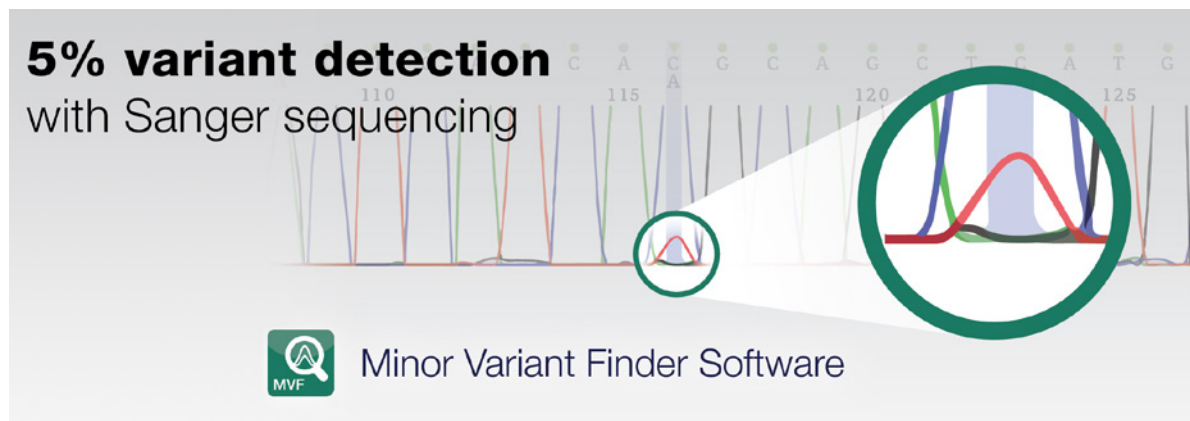


Figure 51. Venn diagram display in the NGC module.

Further decisions to accept or reject confirmation can be made after inspecting the electropherogram. The variant file can then be exported in standard .vcf format for any other downstream analysis.

Minor Variant Finder Software



Overview and applications

Thermo Fisher Scientific Minor Variant Finder Software is new software developed for the detection and reporting of minor mutations by Sanger sequencing. The ability to detect low-level somatic mutations is critical in research areas such as oncology, infectious disease, and inherited disease.

The improved sensitivity achieved through Minor Variant Finder Software makes Sanger sequencing the ideal choice for oncologists and pathologists to call low-frequency somatic variants (5% or below) where the number of relevant targets is often limited. Depending on the cancer type, the moderate number of relevant variants in oncogenes (for example, KRAS, NRAS, etc.) and tumor suppressor genes (for example, TP53) could be detected by the gold-standard Sanger sequencing with Minor Variant Finder, quickly and cost-effectively. This is also an important confirmatory method for NGS results.

Software workflow

1. Upload traces.
2. Create references.
3. Find minor variants.
4. Review data.
5. Export reports.

Learn more or download a free demo version at thermofisher.com/mvf.

Chapter 8

Troubleshooting

This section covers:

Troubleshooting overview	136
Troubleshooting workflow	136
Table of troubleshooting symptoms	141
Troubleshooting examples	143

Troubleshooting overview

This chapter provides information for troubleshooting automated DNA sequencing results from capillary electrophoresis (CE) 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 Thermo Fisher Scientific analysis software program, such as Sequence Scanner Software, Sequencing Analysis Software, or the Quality Check (QC) module on Thermo Fisher Cloud.

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, Thermo Fisher Scientific 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 55)—Results can help you determine whether failed reactions are caused by poor template quality or sequencing reaction failure.
- **Sequencing standards** (page 98)—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 "Sequencing Analysis Software" on page 114.

1. Review the electropherogram (page 137).
2. Review data analysis settings (page 137).
3. Review run and data analysis information (page 138).
4. Review experimental setup (page 140).
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?

- If you have not resolved your problem, identify the symptom in "Table of troubleshooting symptoms." on page 142. Then, determine the cause and perform the actions to resolve the problem.
- If the problem persists, contact Thermo Fisher Scientific Technical Support.

Reviewing the electropherogram

- 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.
- 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.

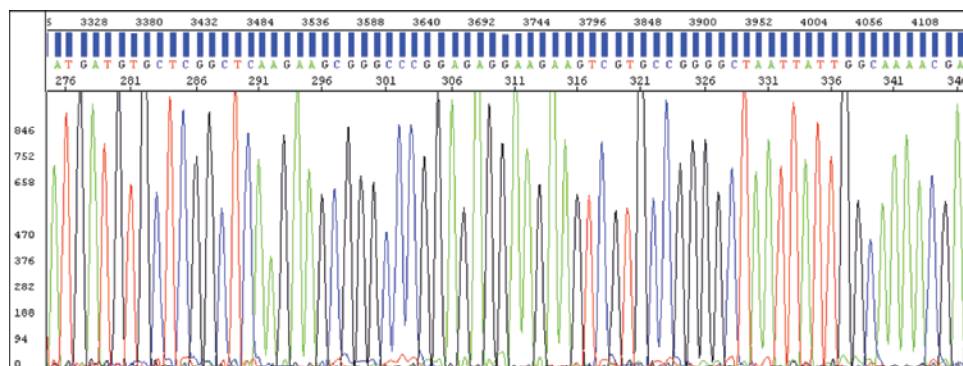


Figure 52. Example of electropherogram with high-quality data.

Reviewing data analysis settings

- 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

Row	Show	Sample File Name	Sample Name	BC	PP	P	BaseCaller	DyeSet/Primer	Matrix File	Spacing	Peak 1	Start	Stop
1	<input type="checkbox"/>	sample1	LRSv1.1	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	KB.bcp	KB_3730_POP7_BDTv1.mob	None	16.30	1872	1874	18760
2	<input type="checkbox"/>	sample2	B17_07_ID030_122_Gv...	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	Basecaller-3730POP7LR.bcp	DT3730POP7(BDv3).mob	None	15.06	2113	2113	18760
3	<input type="checkbox"/>	sample3	Control_144	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	Basecaller-3100RRv2.bcp	DT3100POP6(BDv3)v1.mob	None	11.13	2488	2488	14950

Bold, italic text indicates the file is not in the appropriate location

Matrix files are required for 310 instruments only

- 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)

The *Applied Biosystems DNA Sequencing Analysis Software User Guide* (PN 4346366) offers more information on troubleshooting these files.

3. In the analysis protocol and settings, verify the basecaller settings.

Reviewing run and data 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 163.
 - **Peak heights**—Are peaks well-resolved, with reasonable heights (Figure 53)? For examples of low or no signal, see pages 147 through 150; for examples of top-heavy data, see pages 160 through 162.
 - **Data start points**—Do any data start points deviate from others in the run? For examples of start-point deviation, see pages 151 and 152.
 - **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 157 through 159.
 - **Baseline**—Is there background noise for all the peaks? Zoom in horizontally and vertically to verify the baseline noise.

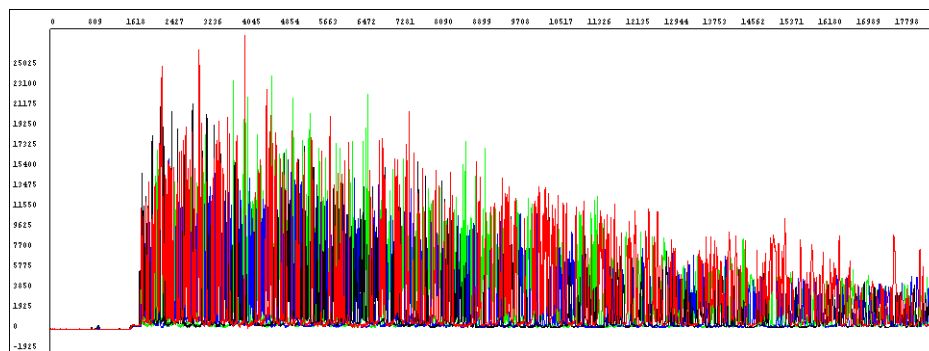


Figure 53. Example of high-quality raw data with tightly resolved peaks from the 3730 Genetic Analyzer.

3. Select the Annotation tab and review the data collection and data analysis settings and values for the sample file (Table 22).

Table 22. Annotation tab information.

Setting	Comments
Data collection settings	
Note: Incorrect data collection settings can result in base calling errors during data analysis.	
Instrument model	Make sure that the run parameters were appropriate for the instrument model (for more information, see page 97 for user manuals of each instrument.)
Length to detector	Capillary length. If the incorrect length was set, peaks can begin later than expected (for an example, see page 151). 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 151) or base calling may be affected.
Data analysis settings	
Basecaller name	To ensure that the correct basecaller name is selected for your run, please refer to the run parameter indicated in the instrument manual.
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 165) and/or base assignments may be incorrect.
Ave Signal Intensity	Low or high values can produce low-quality data (for examples, see pages 167, 169, 176, and 179). Generally acceptable values: <ul style="list-style-type: none"> • 3500 and 3730 series instruments: 500 to 10,000 rfus • 310 and 3130 series instruments: 100 to 1000 rfus Note: The values listed above are not specifications.
Signal:Noise	Average relative fluorescent units (rfus) divided by the noise level for each dye. High-quality data normally yields a signal-to-noise ratio >100, although accurate base calling can be achieved with values as low as 25.
Base Spacing Used	A negative number indicates abnormal peak spacing values. Base calling 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.

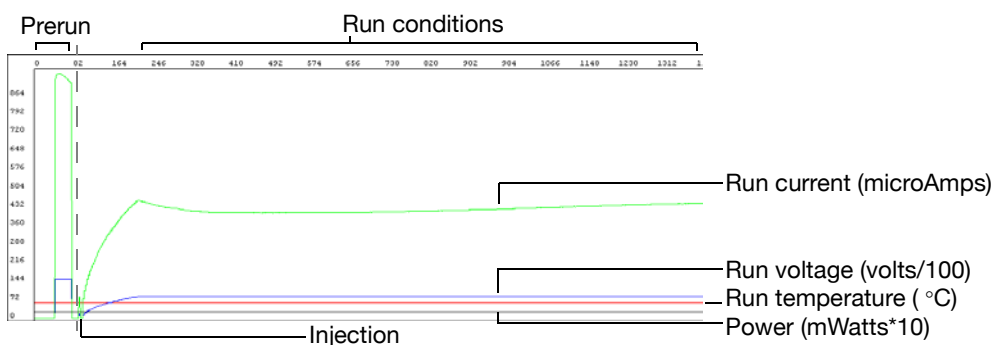


Figure 54. Example of EPT tab information for high-quality data.

Reviewing experimental setup

1. Confirm that you used the optimal quality and quantity of DNA using the table below.

Table 23. Reviewing DNA quality and quantity checklist.

Recommendation	Comments
Run an agarose gel to detect any contaminating DNA or RNA.	Purified DNA should run 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 μ g can be tolerated in the sequencing reaction, but it affects DNA quantitation greatly.
Measure the A260/A280 ratio of your samples.	For pure preparations of DNA (in TE), the A260/A280 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. 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 (A260).	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 A260 reading between 0.05 and 1.00.	A260 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 6, "Recommended DNA template quantities for cycle sequencing." on page 55. Calculate the template concentration using the formulas on page 42.	Too little template can result in no or low signal. Too much template can result in top-heavy data (pages 160 through 162).
Use the primer concentrations recommended in Chapter 4: 3.2 pmol in a 20 μ L reaction (dye terminator chemistry). Calculate the primer concentrations using the formula on page 38.	Too little primer can result in no or low signal (page 147 through page 150). Too much primer can lead to overamplification of the 5' end of the template, resulting in top-heavy data (pages 160 and 161).

2. Confirm that the primer design and quality are optimal using the table below.

Table 24. Reviewing primer design checklist.

Recommendation	Comments
Ensure that the primer has $T_m > 45^\circ\text{C}$.	If the T_m is too low, it may result in poor priming and low or no signal (pages 147 through 150).
Ensure that primers are at least 18 bases long.	Primers that are too short may have T_m s 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 174).
Choose primers that do not have runs of identical nucleotides, especially four or more Gs.	Runs of identical nucleotides in primers can cause n+1 or n-1 effects (page 182). 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 $T_m > 45^\circ\text{C}$.
Design primers to minimize the potential for secondary structure and/or hybridization (see page 37).	Primer-dimer formation from hybridization can result in mixed sequence at the beginning of the sequence (page 177). Secondary structure in the primer, particularly at the 3' end, can result in poor priming and low or no signal (pages 147 through 150).
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, and sequencing reaction issues.

Table 25. Table of troubleshooting symptoms.

Symptom	Example on page
Sample manager errors	
Spacing value is red in Sequence Analysis Software or Sequence Scanner Software	143
Incorrect base calling	
Mixed base not called correctly	144
Too many mixed bases called	145
Irregular signal	
No signal or low signal:	
• No signal	147
• Low peak intensity (too little signal)	148
• Low signal throughout	150
Signal starts later than expected:	
• Signal starts later than expected: No resolution loss	151
• Signal starts later than expected: With resolution loss	152
Irregular baseline:	
• Negative baseline: One color	154
• Negative baseline: All four bases	155
• Waterfall baseline	156
Sudden drop in signal:	
• Sudden drop in signal: Corresponds to base calling stop when sequencing short template	157
• Sudden drop in signal: Early sudden drop with sequence termination	158
• Sudden drop in signal: Sudden drop with continued base calling	159
Top-heavy data:	
• Top-heavy data: Gradual loss of signal	160
• Top-heavy data: Ski slope profile	161
• Top-heavy data: Preferential amplification of short sequence	161
• Top-heavy data: Split peaks with excessive signal	162
Abnormal peak shapes	
Spikes:	
• Four-color spikes	163
• One-color spikes	164
• Large spike at the end of the run	164
Improperly spaced peaks, especially peaks in the first 100 to 150 bases	165
Large peaks (blobs) in the first 120 bases	166
Irregular C peaks using BigDye Terminators v3.1	167
Irregular G peaks using BigDye Terminators v1.1 and v3.1	169

Table 25. Table of troubleshooting symptoms (continued).

Symptom	Example on page
Shoulders on all peaks	170
Peak compressions	171
"BigDye Direct GC compression"	171
"Peak compressions dGTP sequencing (GC-rich templates)"	172
Broad peaks for bisulfite-converted sequences	173
Double peaks:	
• Double peaks: Peaks under peaks throughout	174
• Double peaks with high average signal intensity values	176
• Double peaks at the beginning of the sequence	177
• Double peaks at the beginning of the sequence (bisulfite conversion)	178
• Double peaks: Specific peaks under specific bases	179
• Double peaks: Specific peaks under specific bases	180
• Double peaks: Peaks under peaks throughout (bisulfite conversion)	181
• "Double peaks: double sequence (n+1 or n-1) throughout"	182
• Double peaks after a homopolymer or repeated sequence	183
• Double peaks after a homopolymer or repeated sequence (bisulfite sequencing)	184
• Double peaks: Double sequence after clean sequence	185
Low resolution	
Resolution loss at the beginning of the run	186
Resolution loss in the middle of the run	187
Resolution loss: Gradual early loss	188
SeqScape Software symptoms	
High-quality sequence in unassembled category in SeqScape Software	190

Troubleshooting examples

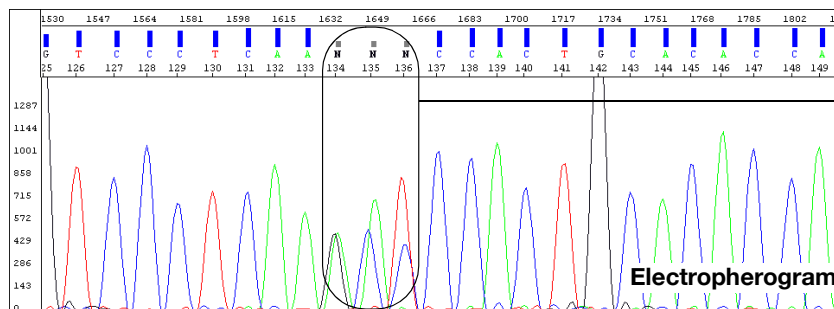
Spacing value is red in Sequence Analysis Software or Sequence Scanner Software

Row	Show	Sample File Name	Sample Name	BC	PP	P	BaseCaller	DyeSet/Primer	Matrix File	Spacing	Peak 1	Start	Stop
1	<input type="checkbox"/>	BDT7946_P21_m9	6463	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	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	
<p>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.</p>	<p>Verify that analysis settings are appropriate for the run setup.</p> <p>Manually set a spacing value and reanalyze the data. To estimate a spacing value:</p> <ol style="list-style-type: none"> 1. Refer to the raw data after 1,000 scan points. 2. Measure the distance between the crests of two adjacent peaks with the same color. <p>For more information, see the appropriate Sequencing Analysis Software user guide.</p>

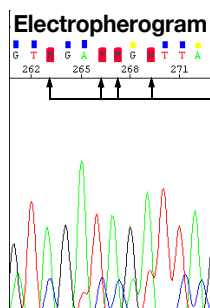
Mixed base not called correctly



Ns or low QVs for pure bases are assigned instead of mixed bases (analysis using the KB basecaller only)

Possible cause(s)	Recommended action
Data analysis issue	
<p>The quality threshold setting and the mixed bases settings are not correctly defined in the analysis protocol.</p>	<ol style="list-style-type: none"> 1. Review the quality threshold setting and the mixed bases settings in the analysis protocol that you used for the analysis. 2. Correct the settings if necessary, then reanalyze the data. <p>Note: Significant improvements in mixed base calling have been made with later versions of Sequencing Analysis Software and the KB Basecaller. Please check the Thermo Fisher Scientific website for the latest updates.</p>

Too many mixed bases called

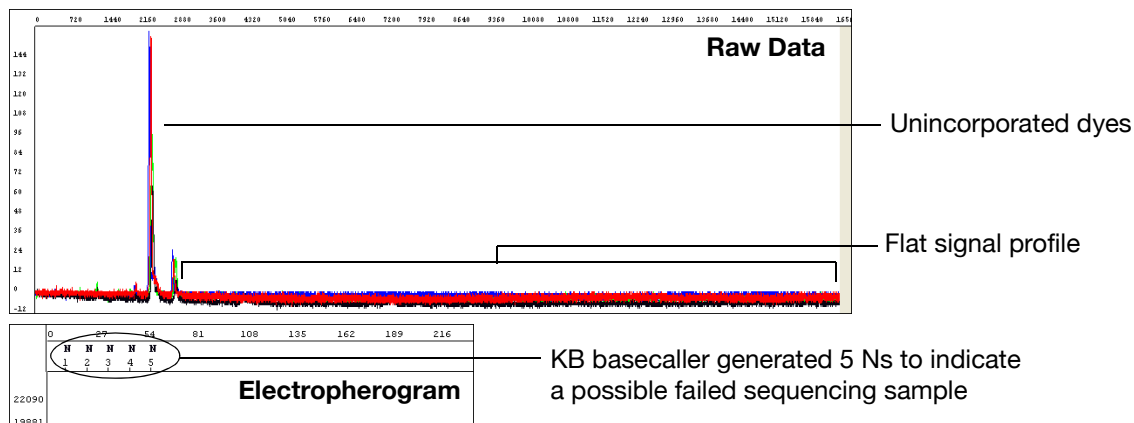


Too many mixed bases are called (analysis using the KB basecaller only)

Possible cause(s)	Recommended action
Biological cause	
Heterozygous insertion/deletion.	Verify on reverse strand.
Data analysis issue	
Second 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. 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 pull-up).	Perform the spectral calibration again.
Shifted spatial calibration.	Perform the spatial calibration again.
Poor charge-coupled device (CCD) alignment.	Contact Thermo Fisher Scientific 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 37).
Secondary amplification product in the PCR product used as a sequencing template or template contamination.	Use gel purification to isolate the desired product. For more information, see "Purifying PCR products for sequencing" on page 40. Design new PCR primers or optimize amplification parameters to obtain a single product. For more information, see "Preparing PCR DNA templates" on page 35.

Possible cause(s)	Recommended action
Sequencing reaction issues (in individual samples or multiple samples)	
PCR primers 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 40.
Mixed templates.	Review the DNA quality.
Sequencing primer contaminated with n-1 primer.	Resynthesize sequencing primer or purify by HPLC or PAGE.
Pull-up caused by overloading the capillaries with too much product.	<p>Review DNA quantity. Use standard run modules. Click the Annotation tab and examine the Ave Signal Intensity. Excessive signal:</p> <ul style="list-style-type: none"> •3500 and 3730 series instruments: >10,000 rfus •310 and 3130 series instruments: >1,000 rfus <p>Load less labeled sample by performing one of the following:</p> <ul style="list-style-type: none"> • 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 6, "Recommended DNA template quantities for cycle sequencing," on page 55).
<p>Stutter during either PCR amplification and/or cycle sequencing. Stutter is most common in any homopolymeric region greater than two 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.</p>	<p>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:</p> <ul style="list-style-type: none"> • 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 3X terminal dinucleotide or 2-base anchors.

No signal

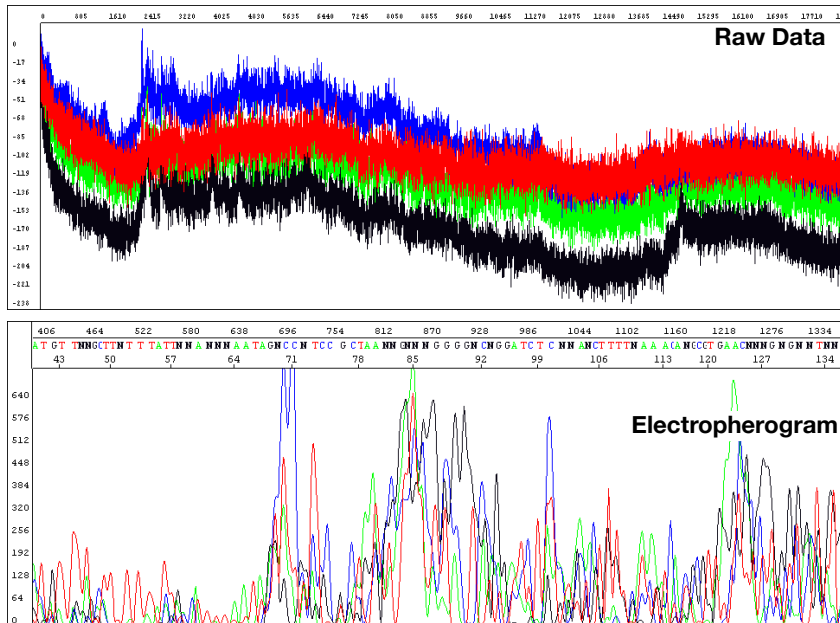


Possible cause(s)	Recommended action
PCR issues	
PCR failure.	Verify that a PCR product is present: perform agarose gel electrophoresis. If amplicon is present (i.e., clearly visible as a single band on agarose gel), perform sequencing troubleshooting (see below).
Sequencing reaction issues (likely with 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.	<p>Review the entire experiment carefully.</p> <ol style="list-style-type: none"> 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 55).
Insufficient template added to sequencing reactions, leading to too few sequencing products generated during PCR.	Check DNA quantitation and quality (page 42 and page 41).
Template contains sequencing inhibitors such as phenol (page 39).	Follow recommended procedures to prepare templates. Check DNA quality (page 41). 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 55).
Weak priming due to poor primer design.	Review primer design (page 37). Make new primers, then repeat the sequencing experiment.
Operator error: wrong sequencing primer used.	Use correct sequencing primer.

Low peak intensity (too little signal)

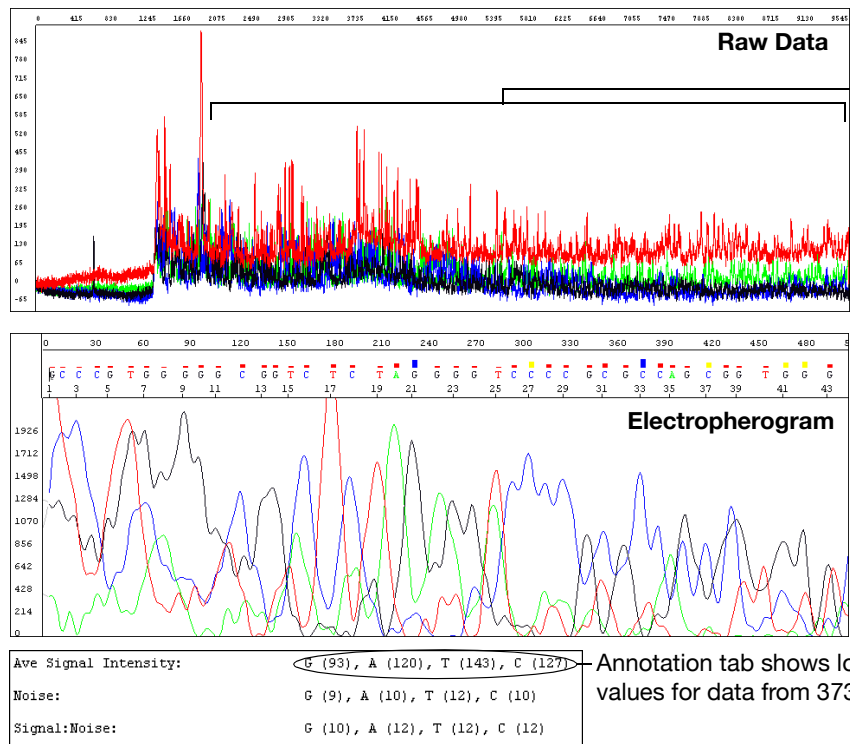
Low or no signal can be the result of many things, from thermal cycler malfunction (in the case of an entire plate failure) to insufficient sequencing template quantity or quality. This troubleshooting section will assume that there is some sequencing signal, but that raw signal is generally below 750 rfu. Data quality for general sequencing purposes may be adequate for base calling at signals as low as 200–750 rfu, but for the purpose of low-level variant detection, signal-to-noise patterns of samples with insufficient signal may be different enough to impact successful variant detection.



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.
Sample evaporated because water was used as the injection solution.	Use Hi-Di Formamide to resuspend your samples (see page 90). For future experiments, consider using the BigDye XTerminator Purification Kit to purify samples (see page 69). Use a heat sealer to seal the plates (3730/3730xl instruments only). Add more resuspension solution to the samples before loading them.
Sample volume too low.	Resuspend samples using sufficient volumes (at least 10 μ L) (see page 90).
Autosampler alignment is off and the tips did not enter the sample.	Follow the steps below depending on your instrument model. 3130 and 3730: 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 Updater Utility. Select Start > All Programs > Thermo Fisher Scientific > BDX Updater . (The utility is installed with the BigDye XTerminator run modules.) 310: Contact Thermo Fisher Scientific to arrange a service engineer visit. 3500: Use as is. The utility is not required since the feature above is built into the module.
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.	<ul style="list-style-type: none"> • Verify correct run module was used. • Verify correct volume in well. • Verify capillaries are not broken or blocked.

Low signal throughout



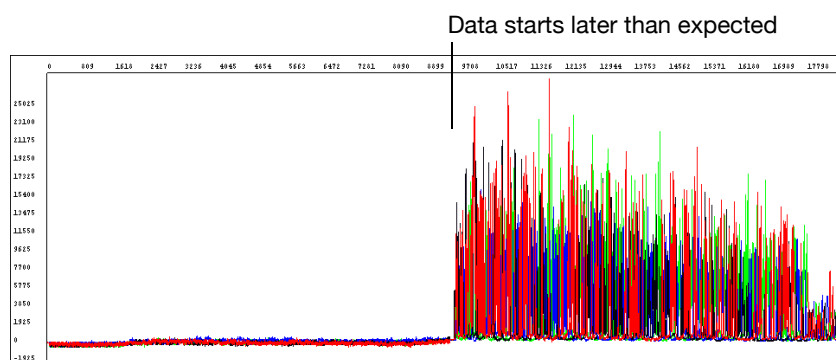
Low signal throughout the entire sequence

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 60 for recommended procedures. Thermo Fisher Scientific 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 140). Use the amounts recommended on page 55. Run a DNA template control to check sequencing reaction quality (page 56).
Poor template quality.	Follow recommended procedures to prepare templates. Check DNA quality (page 41). If necessary, clean up dirty templates. Run a DNA template control to check sequencing reaction quality (page 55).

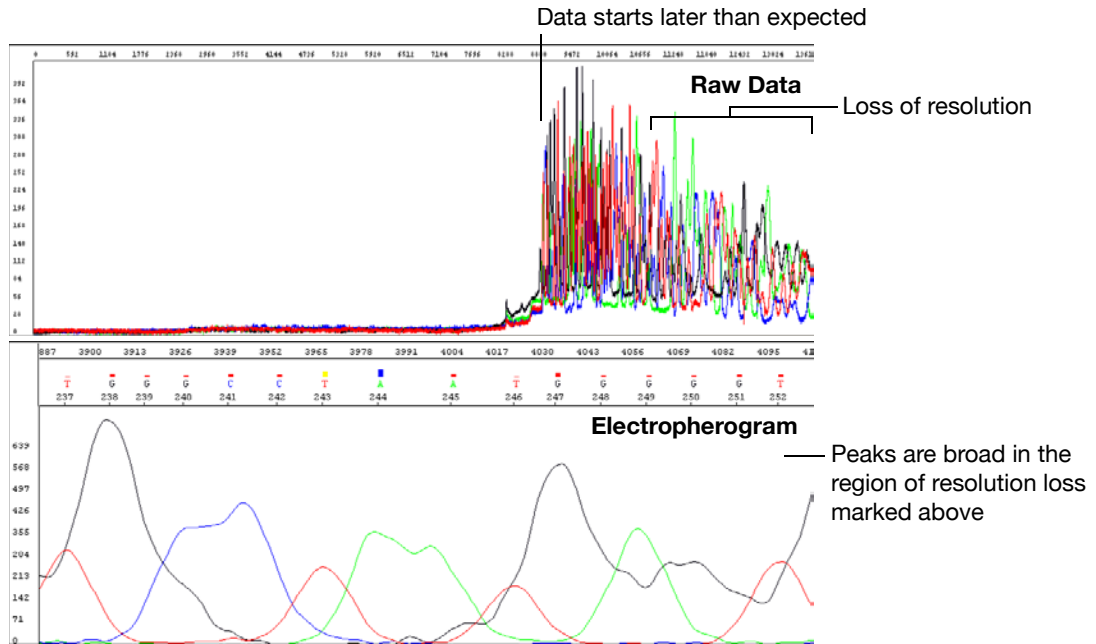
Possible cause(s)	Recommended action
Failure caused by difficult template sequence.	Use Table 5 on page 49 to select a chemistry kit for certain difficult templates.
Autosampler alignment is off and the tips did not enter the sample.	<ol style="list-style-type: none"> 1. Verify the correct run module was used. 2. If you are using samples purified with BigDye XTerminator Purification Kit and your autosampler was recently calibrated, run the BDX Updater Utility. Select Start > All Programs > AppliedBiosystems > BDX Updater. (The utility is installed with the BigDye XTerminator run modules.) 3. Contact Technical Support to arrange a service engineer visit.

Signal starts later than expected: No resolution loss



Possible cause(s)	Recommended action
Electrophoresis issues	
Incorrect capillary length (length to detector) or run module selected.	<ol style="list-style-type: none"> 1. Review run information in the Annotation tab using Sequencing Analysis Software (see page 139): <ul style="list-style-type: none"> — 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.	<ol style="list-style-type: none"> 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.

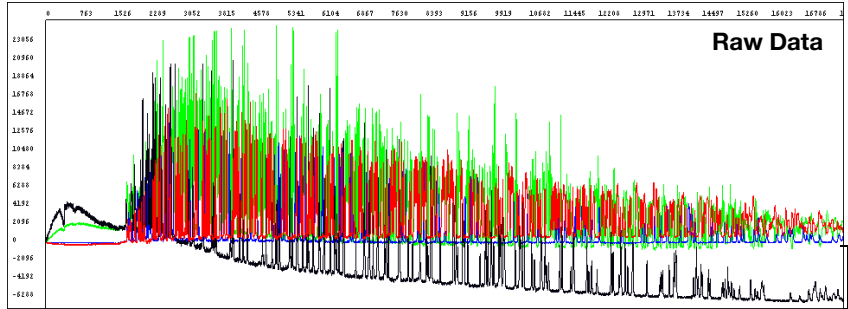
Signal starts later than expected: With resolution loss



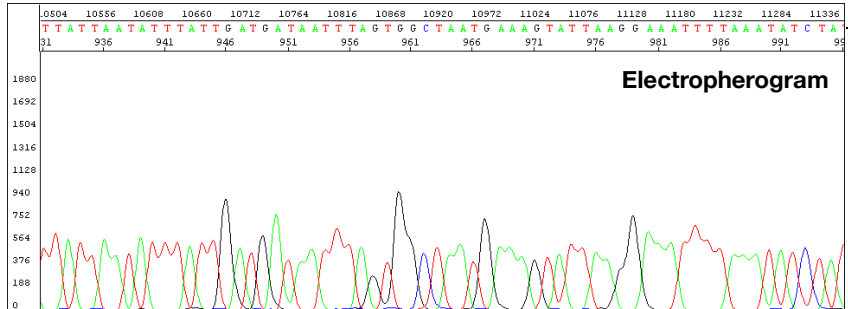
Possible cause(s)	Recommended action
Electrophoresis issues	
Capillaries overloaded with sequencing product, possibly unlabeled DNA or RNA.	<p>Click the Annotation tab and examine the Ave Signal Intensity. Excessive signal:</p> <ul style="list-style-type: none"> • 3500 and 3730 series instruments: >10,000 rfus • 310 and 3130 series instruments: >1,000 rfus <p>Reinject the samples using decreased injection time and/or lower voltage. Load less labeled sample by using less template in the sequencing reaction (see Table 6, "Recommended DNA template quantities for cycle sequencing," on page 55).</p>
Temperature in room and/or oven fluctuating.	Review the EPT tab using Sequencing Analysis Software (see page 139). If the oven temperature is fluctuating, the oven may be leaking because of a poor seal. Contact Thermo Fisher Scientific 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 Thermo Fisher Scientific to arrange a service engineer visit if necessary.
Temperature in the array heater fluctuating more than $\pm 0.5^{\circ}\text{C}$ (3730/3730xl and 3130/3130xl instruments and POP-7 only).	Using Data Collection Software, check the array heater temperature. If it fluctuates more than $\pm 0.5^{\circ}\text{C}$, contact Thermo Fisher Scientific 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 injected without removing the magnetic beads—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.	<ul style="list-style-type: none"> • Replace buffer in system with fresh 1X 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 rfu



Software corrects the baseline fluorescence during data analysis

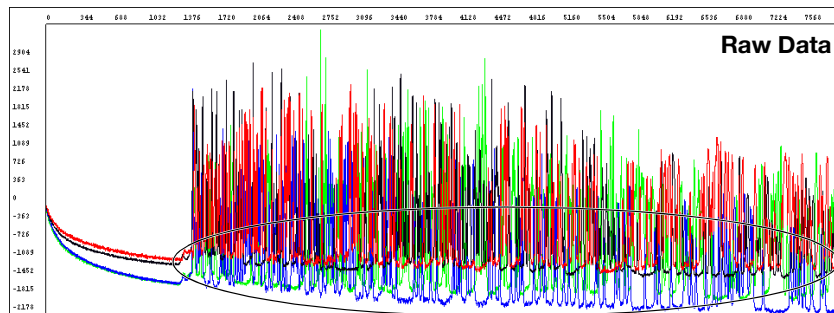
Possible cause(s)

You are using an early version of Sequencing Analysis Software. This error, found in versions earlier than v5.2, was corrected in Basecaller Updater v2.0.

Recommended action

Upgrade to Sequencing Analysis Software v5.2 or later. To obtain the latest software updates and patches, go to www.thermofisher.com/sangersoftware.

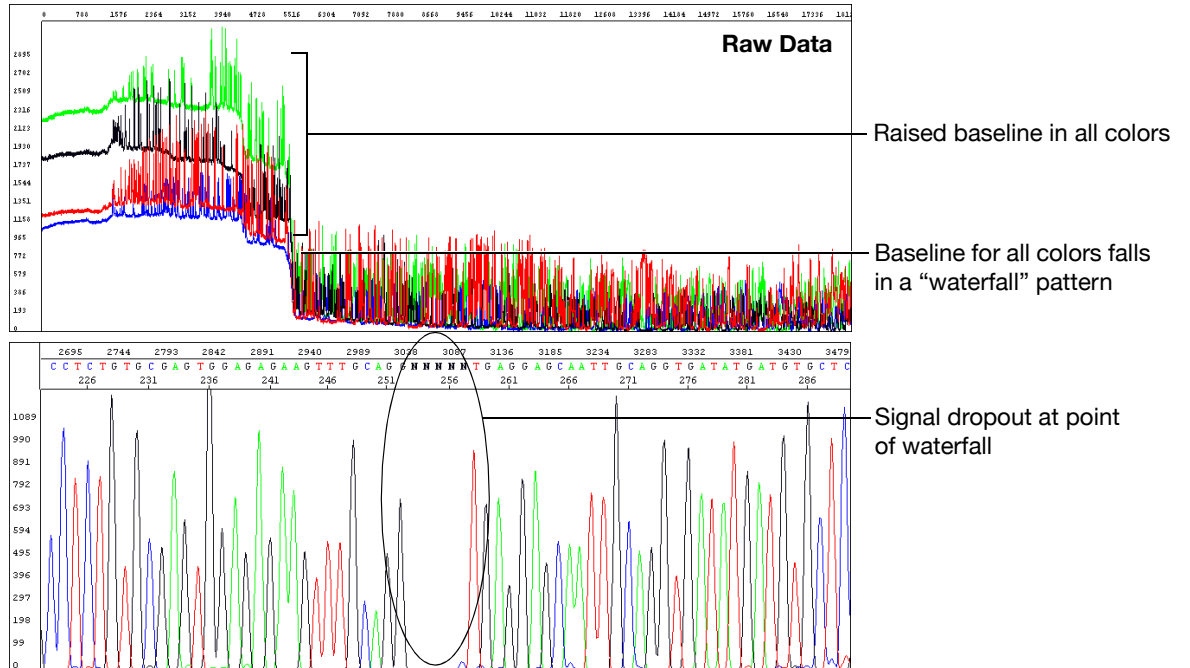
Negative baseline: All four bases



Baseline fluorescence for all four colors is below 0 rfus

Possible cause(s)	Recommended action
<p>Electrophoresis issue</p> <p>Excessive fluorescent contamination in the detection area that bleaches out over the duration of the run (3730/3730xl instruments only).</p>	<p>Use manual control to turn on the laser before starting the run to negate the effects of excessive fluorescent contaminant. Contact Thermo Fisher Scientific technical support or a field applications specialist.</p> <p>Perform a water wash on all components of the system using the wizard in Data Collection Software, then replace the capillary array.</p>

Waterfall baseline



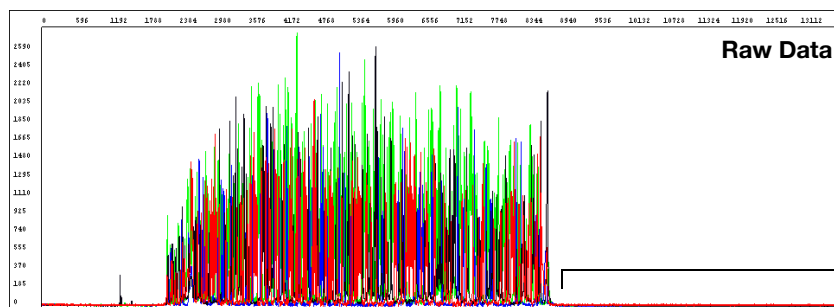
Possible cause(s)

Residue from cleansers used on glassware.
Note: Primarily observed in syringe-based instruments.

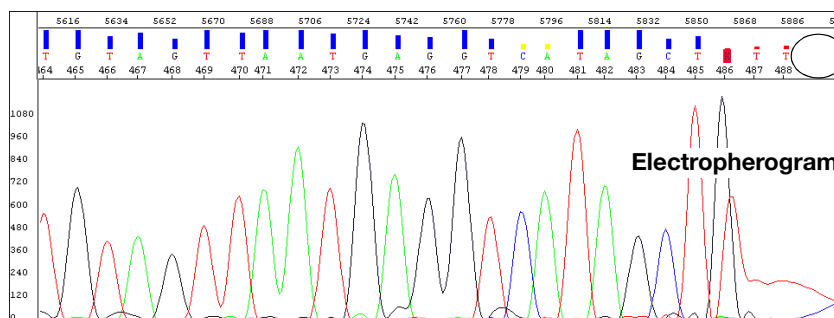
Recommended action

Rinse all components with deionized water.
 Check gasket on syringe(s), replace if necessary. Replace syringe(s).

Sudden drop in signal: Corresponds to base calling stop when sequencing short template



Sudden drop in signal



Basecalling stops when the signal drops

Possible cause(s)

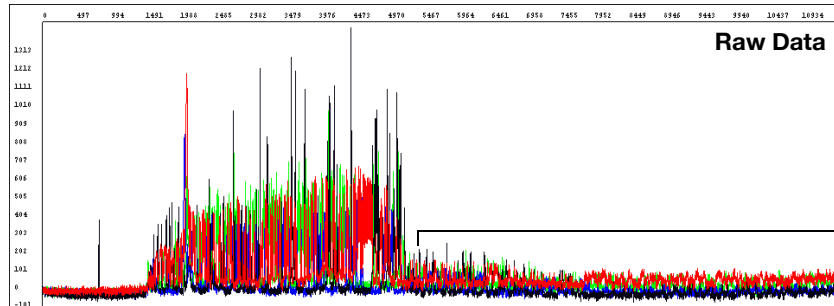
Recommended action

Data analysis issue

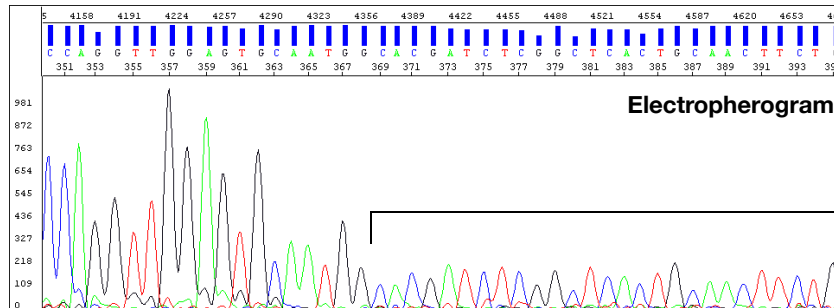
The drop in signal identifies a PCR stop point and the basecaller stops calling bases beyond this point. With the ABI basecaller, you observe Ns beyond the PCR stop. With the KB Basecaller, the analyzed trace is displayed until the last basecall.

Select the At PCR Stop check box in the analysis protocol using Sequencing Analysis Software.

Sudden drop in signal: Sudden drop with continued base calling



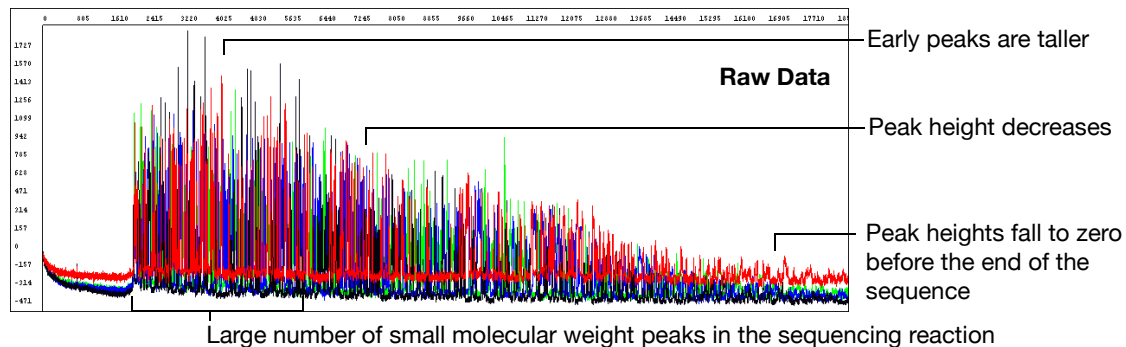
Sudden drop in signal



Basecalling continues beyond the drop in signal

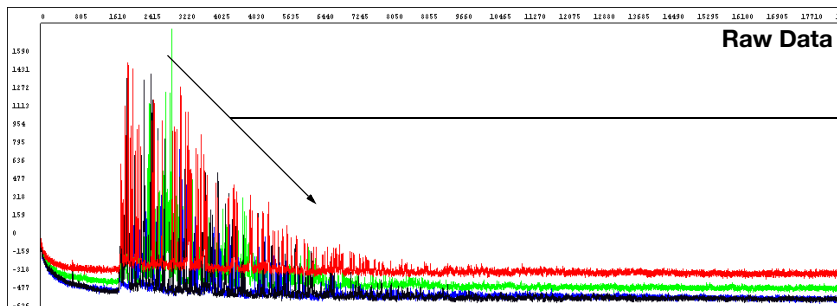
Possible cause(s)	Recommended action
Sequencing reaction issues	
DNA polymerase had difficulty processing through a particular sequence context.	Depending on sequence contexts, you can try sequencing some template with dGTP kits.
Not enough Ready Reaction Mix used in the sequencing reaction.	Follow recommended procedures to prepare sequencing reactions with Ready Reaction Mixes. See page 60 for recommended procedures. Thermo Fisher Scientific 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



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 to 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 41).
Not enough Ready Reaction Mix was used in the sequencing reaction.	Follow recommended procedures to prepare sequencing reactions with Ready Reaction Mixes. See page 60 for recommended procedures. Thermo Fisher Scientific 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 .

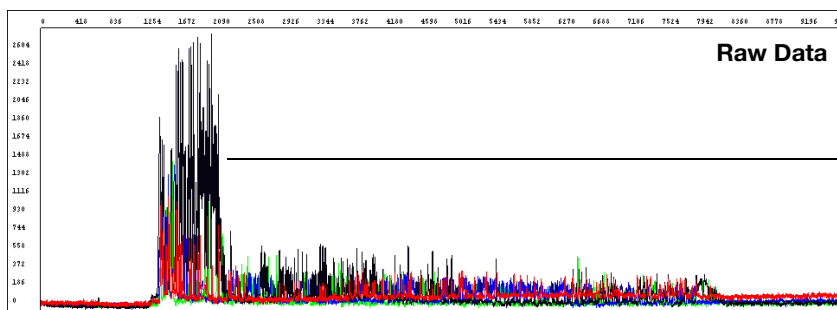
Top-heavy data: Ski slope profile



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 used in the sequencing reaction.	Review the DNA quantity (page 140).
Not enough or too much primer used in the sequencing reaction.	Review the DNA quantity (page 140).
Not enough Ready Reaction Mix used in the sequencing reaction.	Follow recommended procedures to prepare sequencing reactions with Ready Reaction Mixes. See page 60 for recommended procedures. Thermo Fisher Scientific 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.

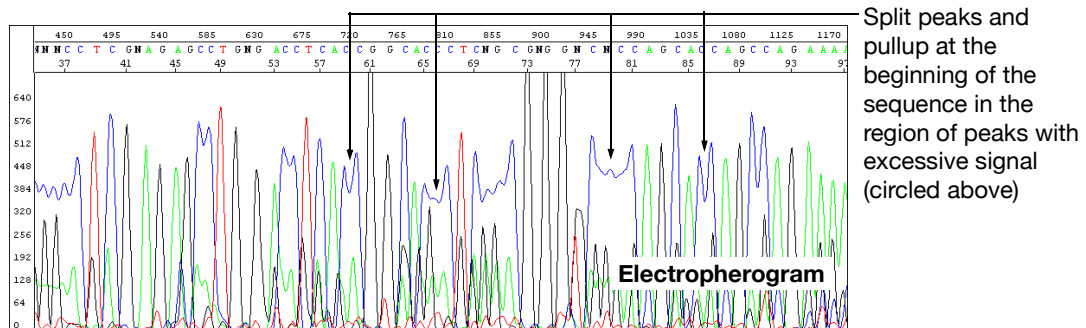
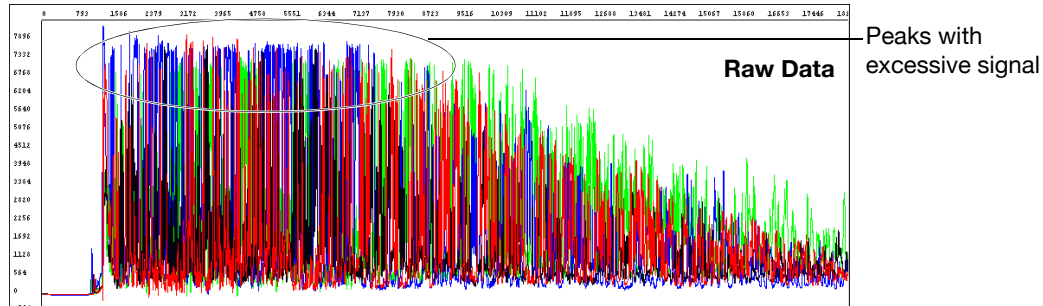
Top-heavy data: Preferential amplification of short sequence



Short 5' sequence, instead of the entire template, is amplified preferentially, (see also page 177)

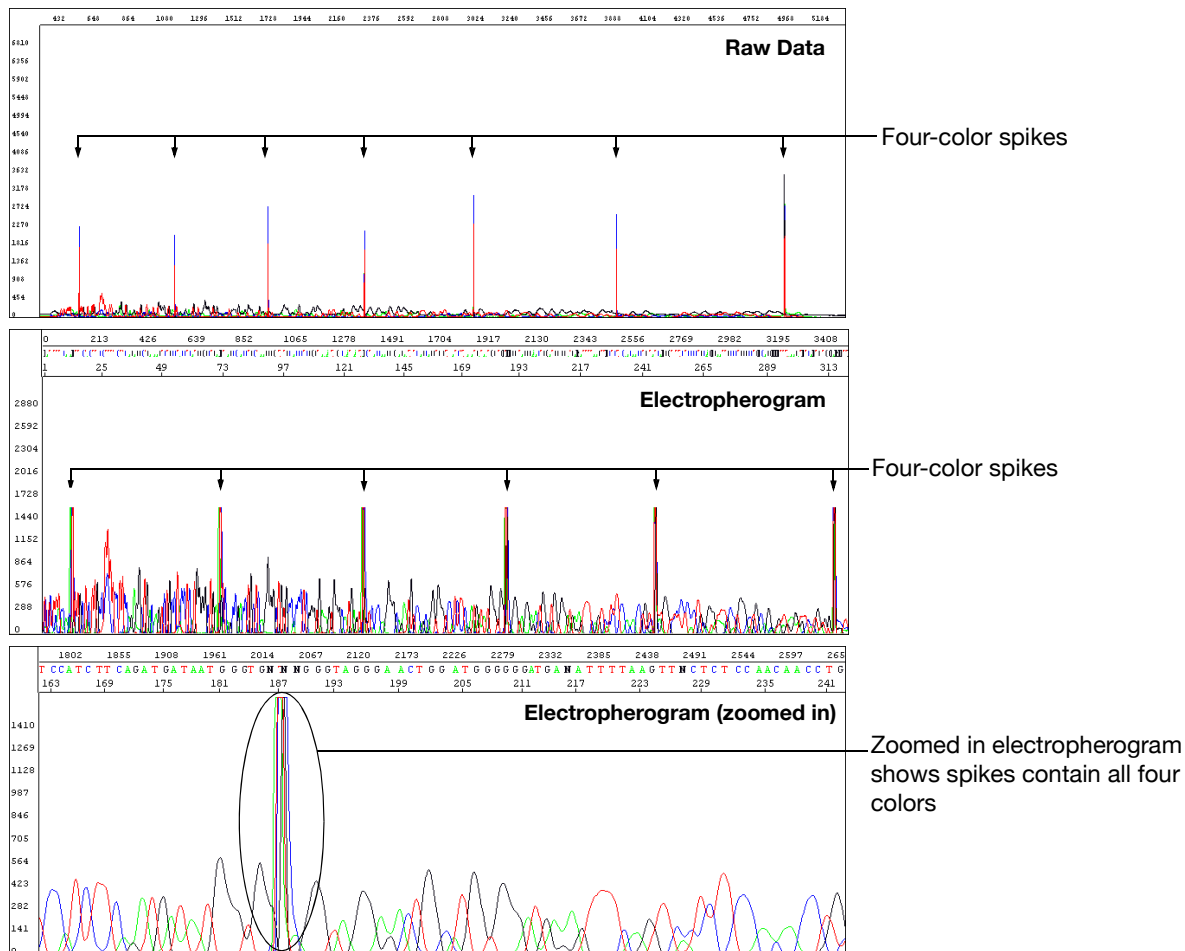
Possible cause(s)	Recommended action
Sequencing reaction issue	
Primer-dimer formation during the PCR reaction.	Redesign the PCR primers to eliminate the sequences that allow primer-dimer formation. Use a "hot start" PCR enzyme to inhibit primer-dimer formation.

Top-heavy data: Split peaks with excessive signal



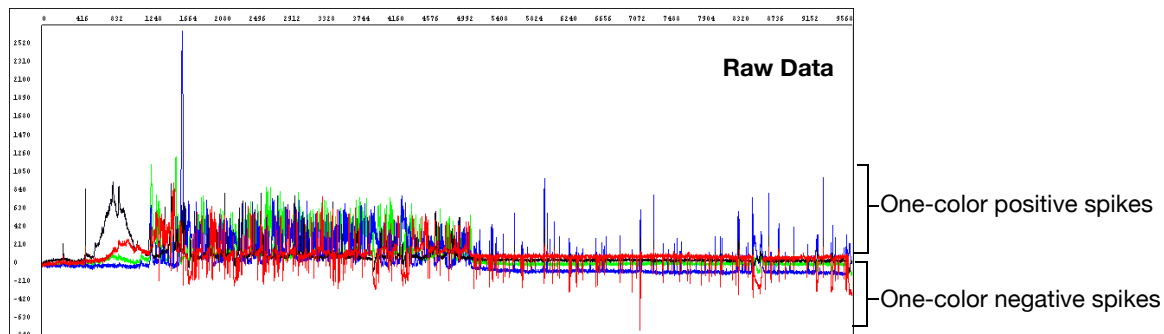
Possible cause(s)	Recommended action
Sequencing reaction issues	
Signal too high because too much template was used in the sequencing reaction and too much sequencing product was created.	<p>You do not have to repeat the reaction. Click the Annotation tab and check the Ave Signal Intensity. Excessive signal:</p> <ul style="list-style-type: none"> • 3500 and 3730 series instruments: >10,000 rfus • 310 and 3130 series instruments: >1,000 rfus <p>Especially if your samples were purified using the BigDye XTerminator Purification Kit, load less labeled sample by performing one of the following:</p> <ul style="list-style-type: none"> • 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 6, "Recommended DNA template quantities for cycle sequencing," on page 55) <p>For future reactions, reduce the amount of template in the sequencing reaction.</p>
Injection height incorrect due to incorrect run module.	Use correct run module, especially for samples purified with the BigDye XTerminator Purification Kit (refer to the instrument manuals on page 97 for the correct run modules).

Four-color spikes



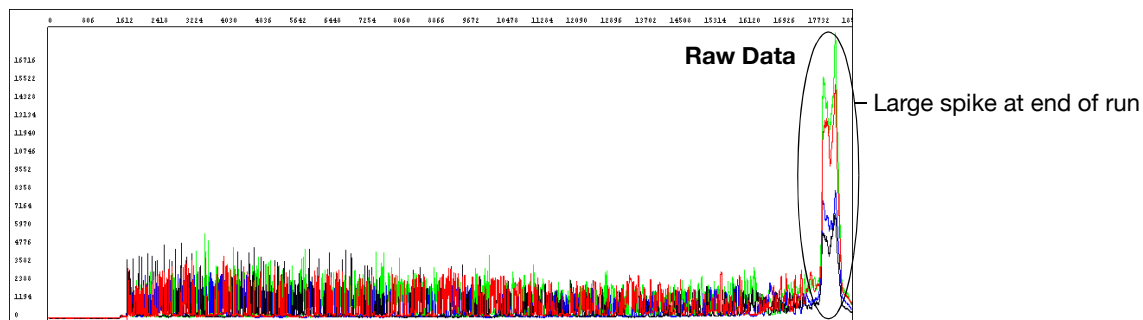
Possible cause(s)	Recommended action
Electrophoresis issues	
Dust, bubbles, or crystals in polymer passed through the path of the laser beam.	<ol style="list-style-type: none"> 1. Eliminate large amounts of dust in the environment. 2. Inspect the upper gel block for bubbles. If present, flush all bubbles 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.
Polymer is frozen.	Replace the polymer. Do not use frozen polymer or previously frozen polymer even after rewarming.

One-color spikes



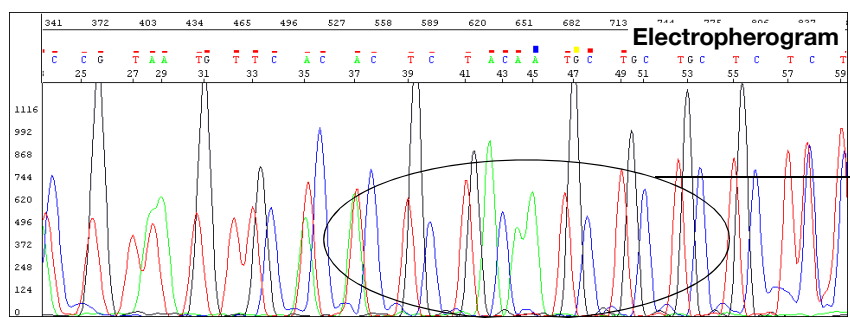
Possible cause(s)	Recommended action
Electrical noise or power fluctuations.	Verify the power source. Use an uninterruptible power supply.
Polymer temperature is too high.	<ul style="list-style-type: none"> Verify the shipping temperature of the polymer. Verify lab temperature is below 26°C.
Well volume is too low.	<ul style="list-style-type: none"> Verify volume is $\geq 10 \mu\text{L}$ for 96-well plates and $\geq 5 \mu\text{L}$ for 384-well plates. If using septa, verify septa are fresh to minimize evaporation.

Large spike at the end of the run



Possible cause(s)	Recommended action
<p>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.</p>	<p>None needed. Shorten the data collection time a few minutes to remove a persistent spike from your data.</p>

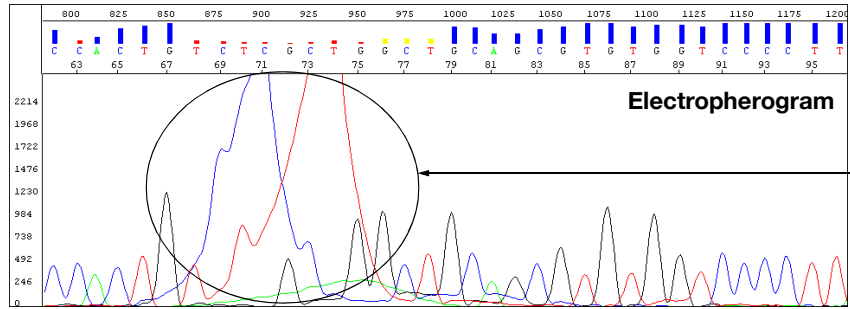
Improperly spaced peaks, especially peaks in the first 100 to 150 bases



Peaks are improperly spaced, especially peaks in the first 100 to 150 bases

Possible cause(s)	Recommended action
Data analysis issue	
<p>Wrong mobility file applied to the sequence data.</p>	<p>Reanalyze the data using the correct mobility file to observe proper spacing of all peaks (refer to the instrument manuals on page 97 for the correct run modules).</p>

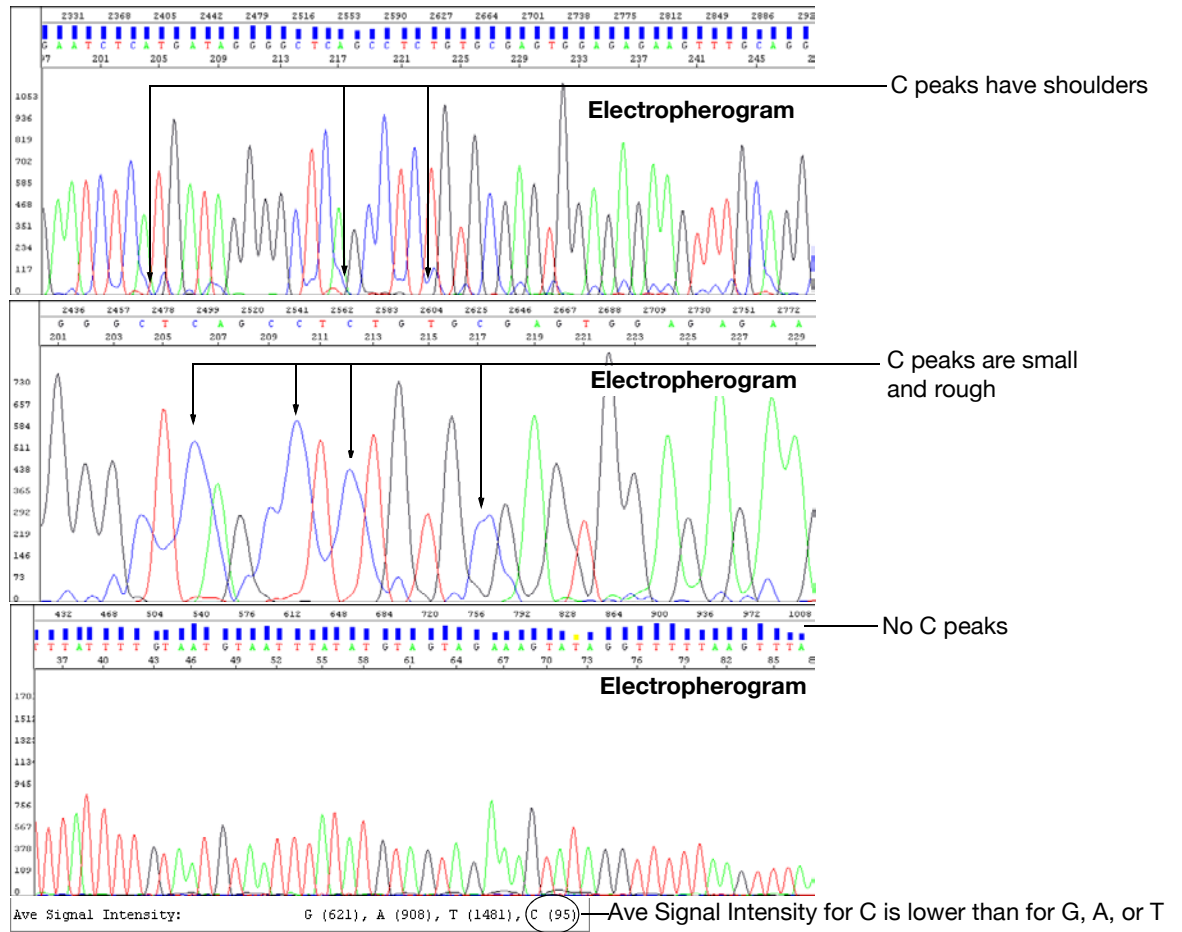
Large peaks (blobs) in the first 120 bases



Blobs in the first 120 bases
Precise location of the blobs
varies according to the dye
used and the specific
configuration

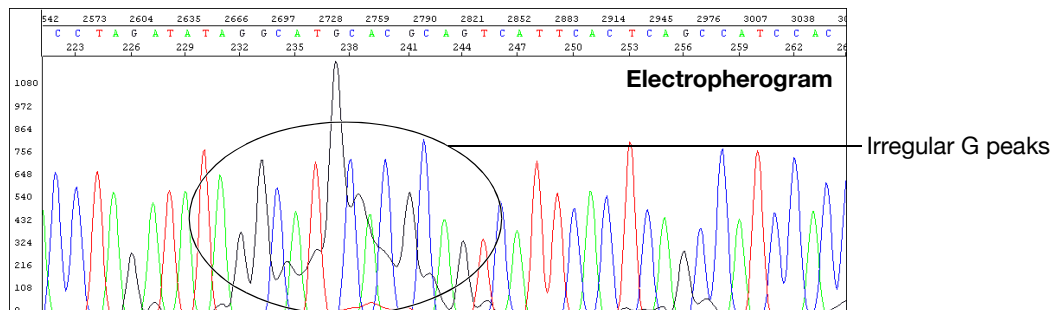
Possible cause(s)	Recommended action
Sequencing reaction issues	
Incomplete removal of dye-labeled terminators after the cycle sequencing reaction.	Review the methods described in Chapter 5, "Purification of extension products" If you are using a third-party product for purifying extension products, contact the manufacturer for troubleshooting help. For future experiments, consider using the BigDye XTerminator Purification Kit to purify samples (see page 69).
Poor incorporation of terminators, leaving excess unincorporated terminators.	Review the entire experiment carefully. <ul style="list-style-type: none"> • Check the quantitation (page 42). • 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 55). • Check expiration dates on all reagents and replace any that have expired.
If using BigDye XTerminator Purification Kit, insufficient mixing during vortexing step.	<ul style="list-style-type: none"> • Verify plate is firmly attached to vortexer. • Follow protocol for vortexing.
If using BigDye XTerminator Purification Kit, incorrect ratio of BigDye XTerminator reagents.	<ul style="list-style-type: none"> • 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



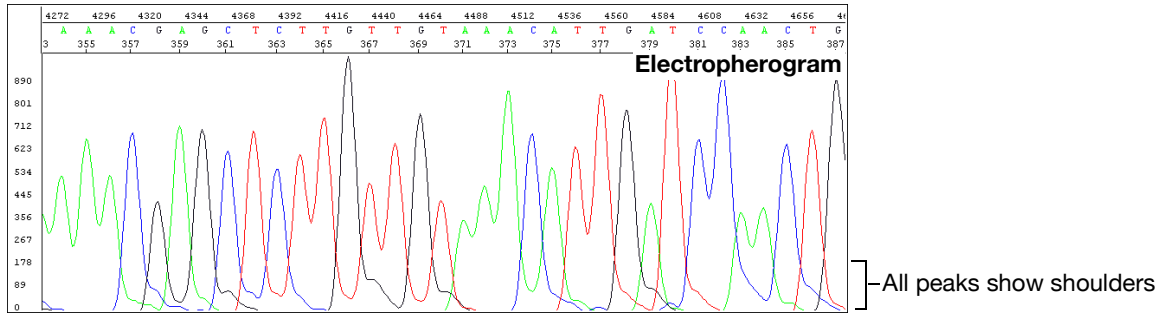
Possible cause(s)	Recommended action
Sequencing reaction issues	
The dye labels attached to the ddC terminators are degraded. Initial degradation results in shoulders on all C peaks. With further degradation, the C peaks appear very small or rough or disappear completely.	Protect the fluorescently labeled DNA from light, heat, acidic conditions, and oxygen(see "Storing sequencing reactions" on page 91). 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.
Electrophoresis issues	
The buffer heater is powered on (3730/3730xl instruments only).	Verify that the buffer heater is not powered on.
Severe arcing events can mask the C signal.	<ul style="list-style-type: none"> • 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 v3.1



Possible cause(s)	Recommended action
Electrophoresis issue	
The buffer heater is powered on (3730/3730xl 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 Terminator v1.1 and less frequently with BigDye Terminator v3.1.	Protect the fluorescently labeled DNA from light, heat, acidic conditions, and oxygen (see "Storing sequencing reactions" on page 91).
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.

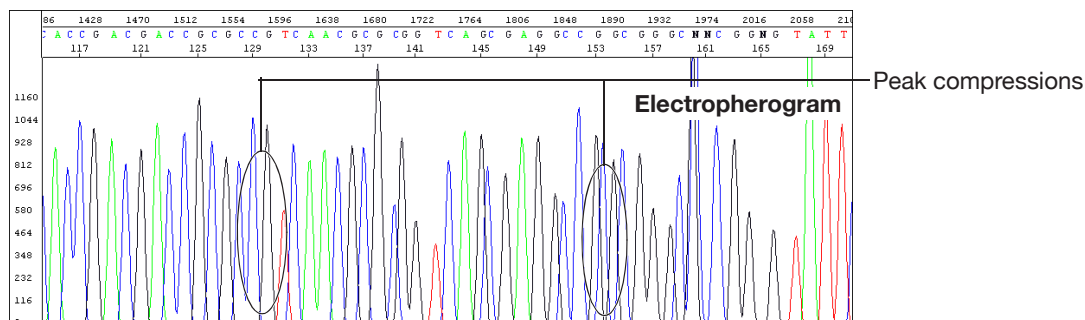
Shoulders on all peaks



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.
<p>Stutter during either PCR amplification and/or cycle sequencing</p> <p>Stutter is most common in any homopolymeric region greater than two bases. It can also be seen with simple repeated DNA sequences. The results are worse when the stutter occurs during PCR amplification.</p> <p>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.</p>	<p>If stutter occurs during PCR amplification, little can be done to correct the problem, except using anchored sequencing primers.</p> <p>If stutter occurs during cycle sequencing:</p> <ul style="list-style-type: none"> 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 3X 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

At times, incomplete denaturing of GC-rich regions of sequencing template or the use of too much sequencing template may lead to subtle G or C peak shoulders or unresolvable regions of GC bases. These can decrease the quality of sequencing as well as impact resolution and baseline noise.



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.

BigDye Direct GC compression

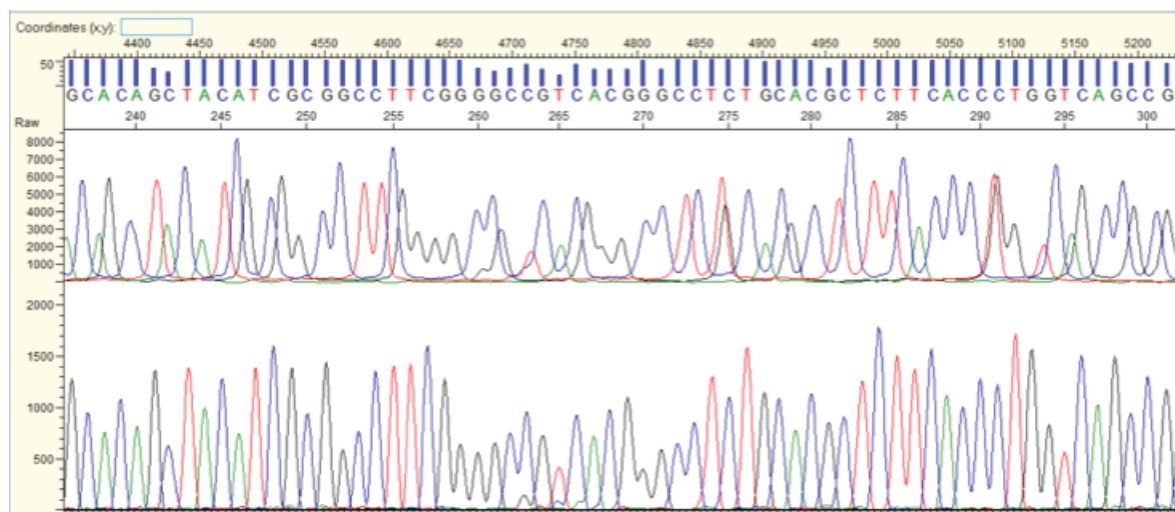


Figure 55. An example of a BigDye Direct sequencing sample with the raw data trace on top and the basecalled/analyzed data trace on the bottom where subtle GC peak shoulders are encountered and the resolution of a triplet of G peaks is less than desirable. In most cases, this is the result of too much sequencing template, a potential by-product of too much input DNA, and usually shows up near the 260–270 bp region of the electropherogram when using BigDye Direct.

Peak compressions dGTP sequencing (GC-rich templates)

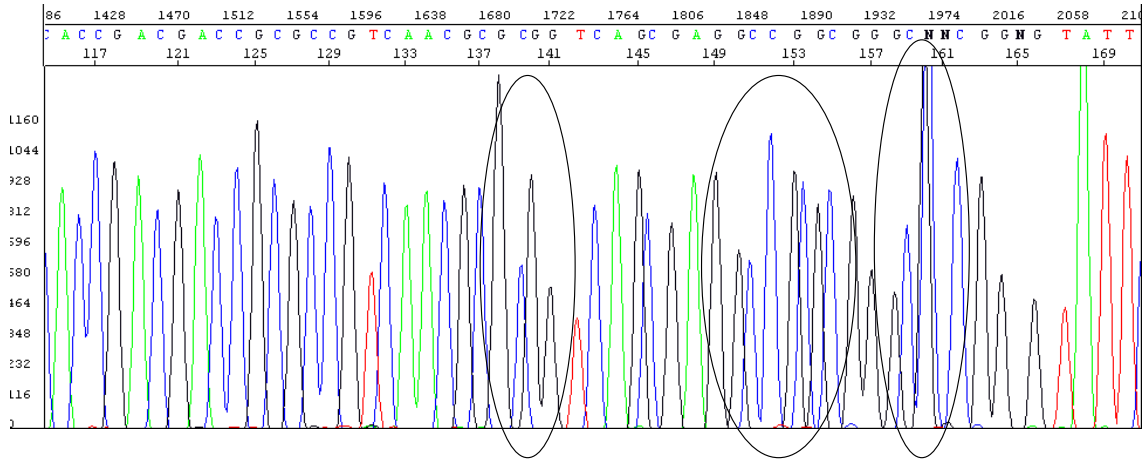


Figure 56. Represents compressions encountered using the dGTP sequencing chemistry kit, an alternative non-standard kit.

Possible cause(s)

GC-rich region, complicated by excessive sequencing template, occasionally encountered when using the BigDye Direct Kit and too much input gDNA during PCR.

Recommended action

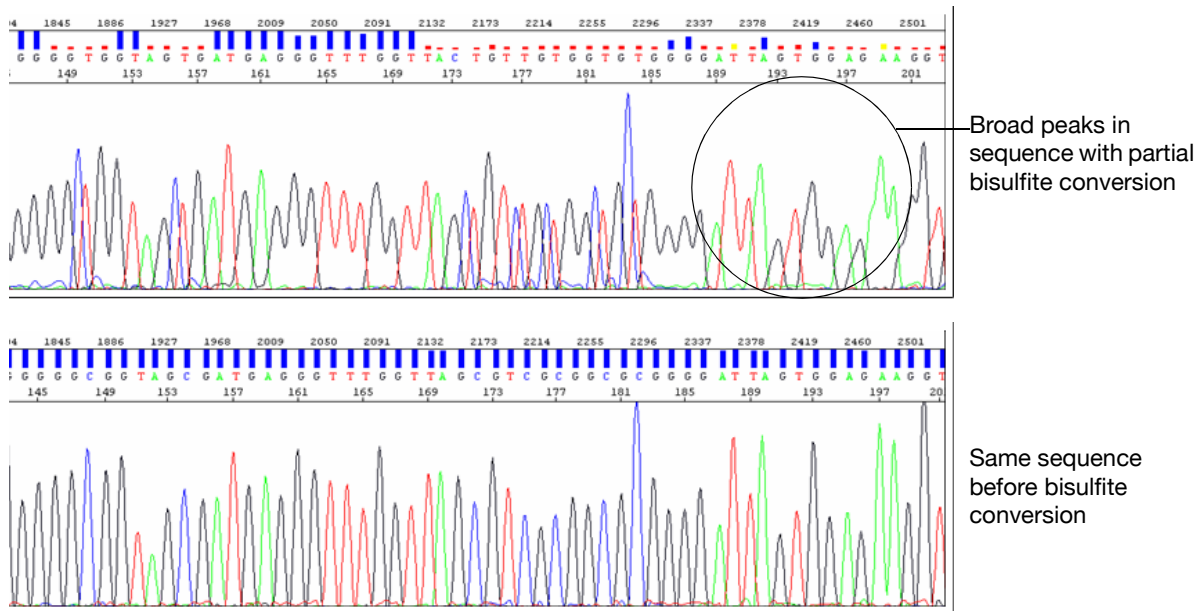
- If using BigDye Direct, reduce the amount of input DNA. Ensure not more than 20 ng is used.
- Because this is thought to be caused by excess sequencing template, in most cases these compressions are mitigated by a second injection of the BigDye Direct sample.

dGTP 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.

Broad peaks for bisulfite-converted sequences



Possible cause(s)

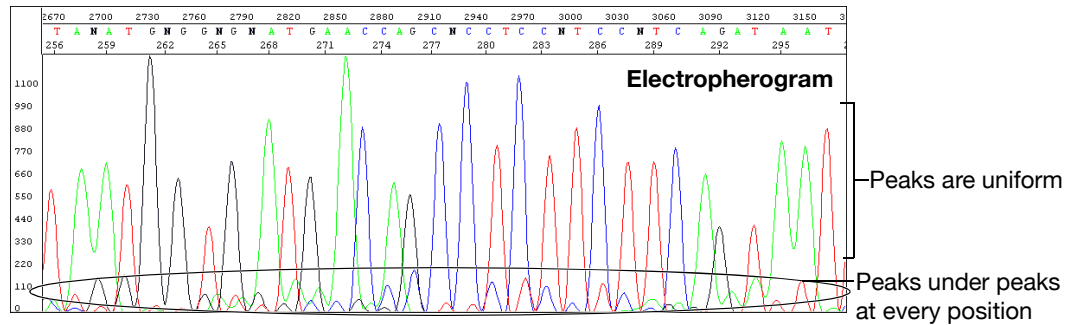
Recommended action

Sequencing reaction issue

Mobility of fragments uneven because the sample contains both Cs (from methylated samples) and no Cs (from unmethylated samples).

- Repeat bisulfite conversion.
- Ensure amplicon is 250 to 400 bp for cloning and 100 to 250 bp for direct sequencing.
- Include an extra incubation at the end of the thermal cycling run for non-templated A addition.

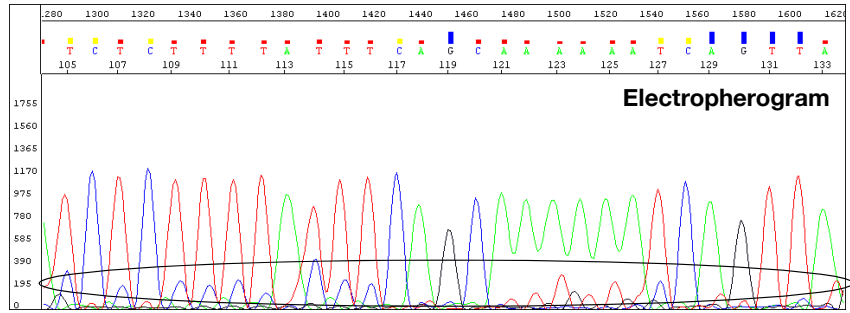
Double peaks: Peaks under peaks throughout



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 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 Thermo Fisher Scientific 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 37).
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 35.
PCR primers 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 35.
Mixed or contaminated templates or primers.	Review the DNA quality.
If peaks under peaks occur after a stretch of clean beginning sequence trace, this may indicate the presence of an insertion/deletion.	Verify by sequencing the opposite DNA strand.

Possible cause(s)	Recommended action
<p>Stutter during either PCR amplification and/or cycle sequencing.</p> <p>Stutter is most common in any homopolymeric region greater than two bases. It can also be seen with simple repeated DNA sequences. The results are worse when the stutter occurs during PCR amplification.</p> <p>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.</p>	<p>If stutter occurs during PCR amplification, little can be done to correct the problem, except using anchored sequencing primers.</p> <p>If stutter occurs during cycle sequencing:</p> <ul style="list-style-type: none"> • 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 3X terminal dinucleotide or 2-base anchors.
<p>Very strong or offscale data.</p>	<p>Reduce the signal:</p> <ul style="list-style-type: none"> • 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



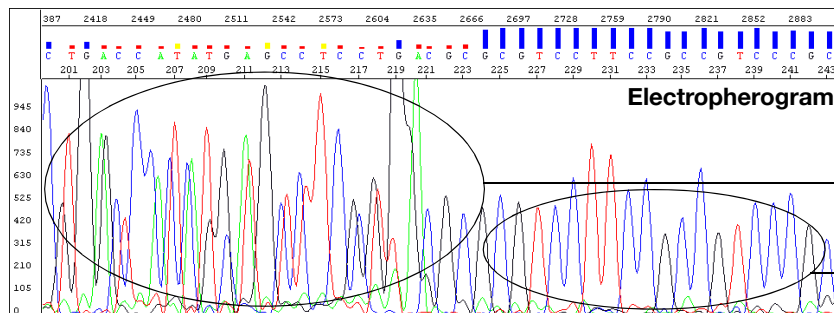
Secondary peaks under each peak

Number of Bases Detected:	565	Annotation
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)	

Ave Signal Intensity values are too high for data from a 3100 instrument

Possible cause(s)	Recommended action
Electrophoresis issues	
Modified run module with increased injection time was used.	Use an unmodified standard run module.
Sequencing reaction issues	
Signal is too high for data from the instrument. See page 139 for valid ranges.	Click the Annotation tab and examine the Ave Signal Intensity. Excessive signal: <ul style="list-style-type: none"> • 3500 and 3730 series instruments: >10,000 rfus • 310 and 3130 series instruments: >1,000 rfus Load less labeled sample by performing one of the following: <ul style="list-style-type: none"> • 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 6, "Recommended DNA template quantities for cycle sequencing," on page 55).

Double peaks at the beginning of the sequence



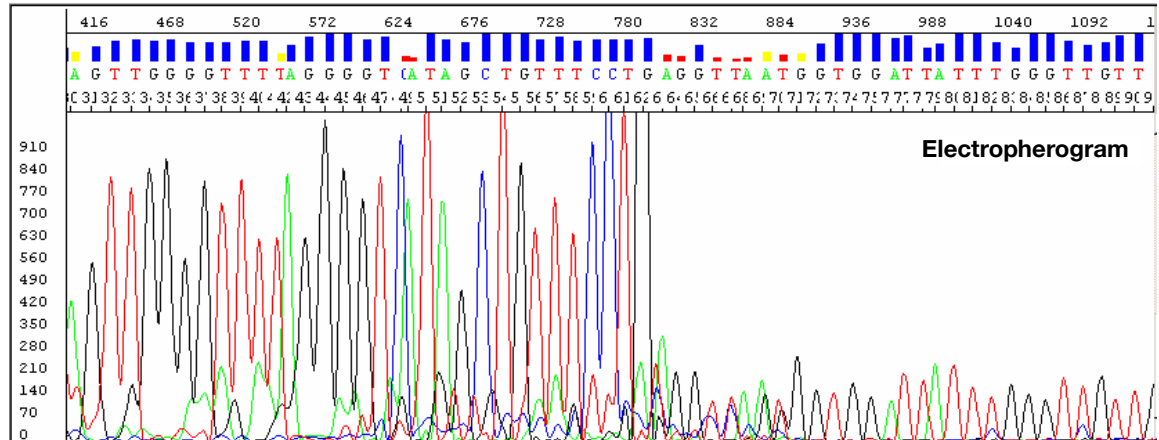
See also page 161 for the raw data view

Double peaks at the beginning of the sequence

Single peaks for the remaining sequence

Possible cause(s)	Recommended action
Sequencing reaction issues	
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: <ul style="list-style-type: none"> • Redesign the PCR primers to eliminate the sequences that allow primer-dimer formation. or <ul style="list-style-type: none"> • Use a "hot start" PCR enzyme to inhibit primer-dimer formation.
More than one PCR product present in the PCR reaction.	Re-examine the sequence for primer-site homology. Redesign as necessary.
More than one priming site (either upstream or downstream) on the sequencing template.	Re-examine the sequence for primer-site homology. Redesign as necessary.

Double peaks at the beginning of the sequence (bisulfite conversion)

**Possible cause(s)****Sequencing reaction issue**

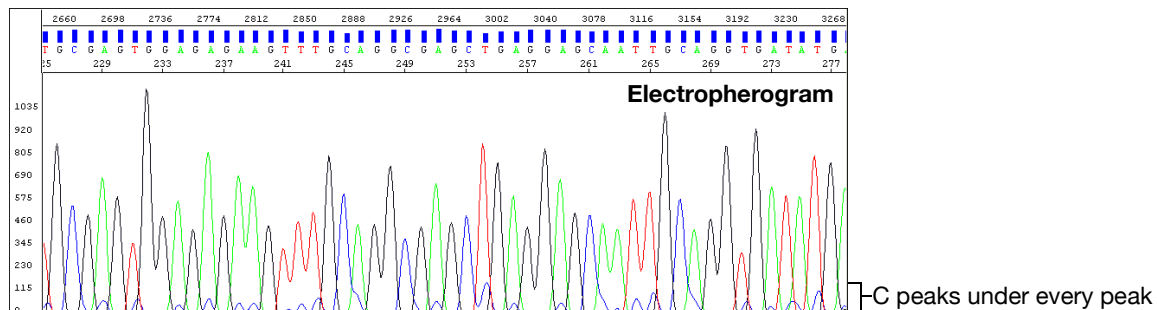
Observed when a PCR product is used as a sequencing template, caused by the formation of primer-dimers during the PCR reaction. The primer-dimers anneal and are filled in to create short, non-template PCR products.

Recommended action

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:

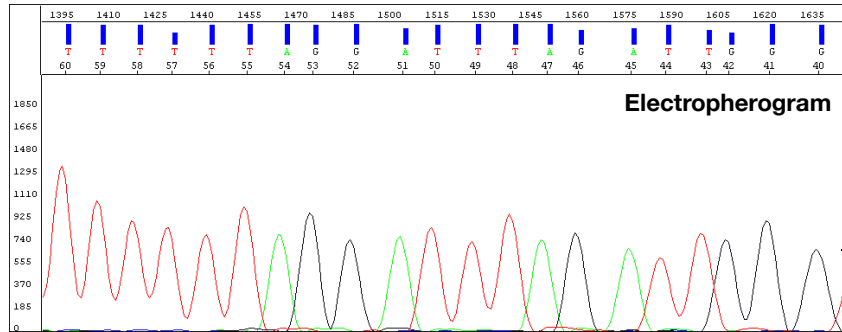
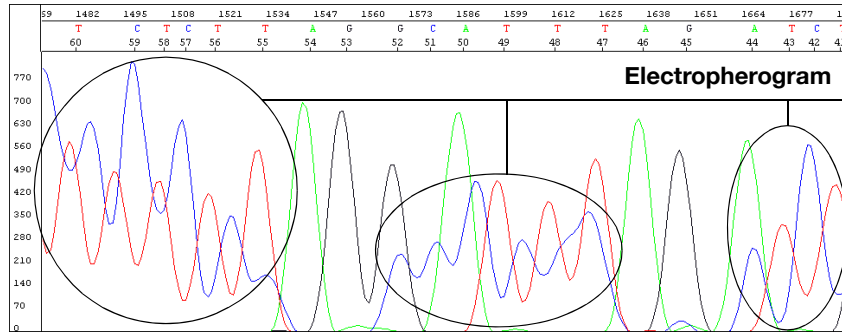
- Redesign the PCR primers to eliminate the sequences that allow primer-dimer formation.
- or
- Use a "hot start" PCR enzyme to inhibit primer-dimer formation.

Double peaks: Specific peaks under specific bases



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 issues	
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: <ul style="list-style-type: none"> • 3500 and 3730 series instruments: >10,000 rfus • 310 and 3130 series instruments: >1,000 rfus Load less labeled sample by performing one of the following: <ul style="list-style-type: none"> • 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 (Table 6, "Recommended DNA template quantities for cycle sequencing," on page 55).

Double peaks: Specific peaks under specific bases



Possible cause(s)

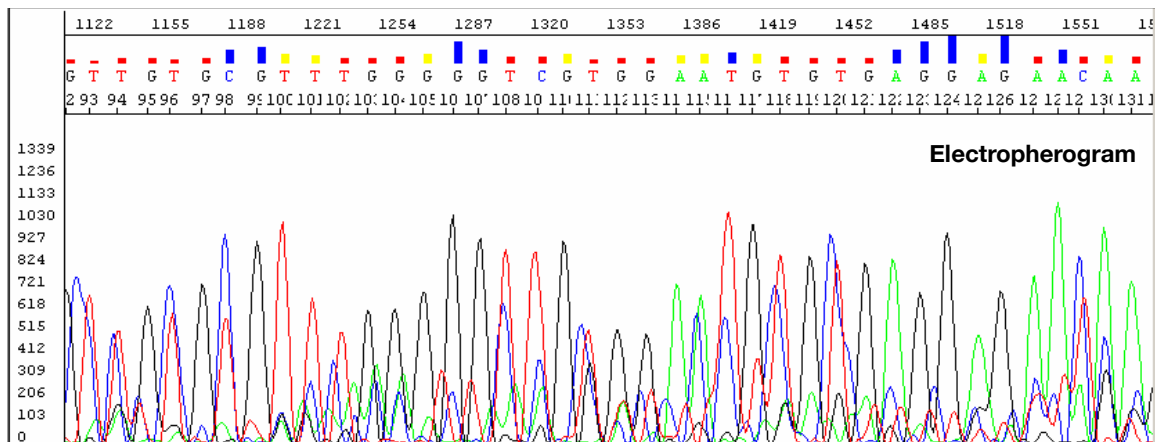
Recommended action

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

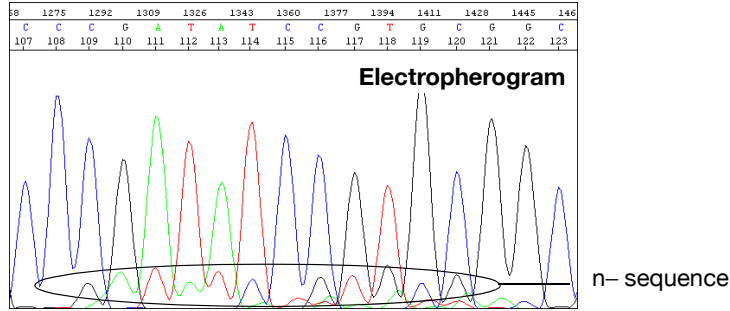
Incomplete bisulfite conversion, indicated by the presence of Cs (blue) not adjacent to Gs (black). A C at a non-CpG position serves as an internal control for complete bisulfite conversion.

Incomplete bisulfite conversion may be due to:

- Impure gDNA
- Too much gDNA
- Inadequate denaturation of gDNA prior to bisulfite conversion

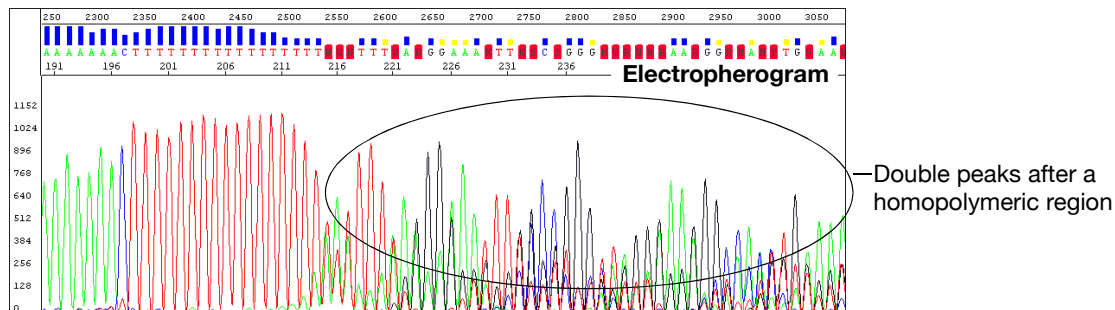
1. Check DNA quantitation and quality (page 42 and page 41).
2. Repeat the bisulfite conversion.
3. Repeat the sequencing.

Double peaks: Double sequence (n+1 or n-1) throughout



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.	Use the primers with a different template. If the problem persists, resynthesize the primers before repeating the experiment.
Sequencing primer contains a run of identical nucleotides, especially four or more Gs.	Design new sequencing primers, avoiding runs of identical nucleotides, especially four or more Gs.
Homopolymer at the beginning of the sequence.	See page 183.

Double peaks after a homopolymer or repeated sequence



Possible cause(s)

Recommended action

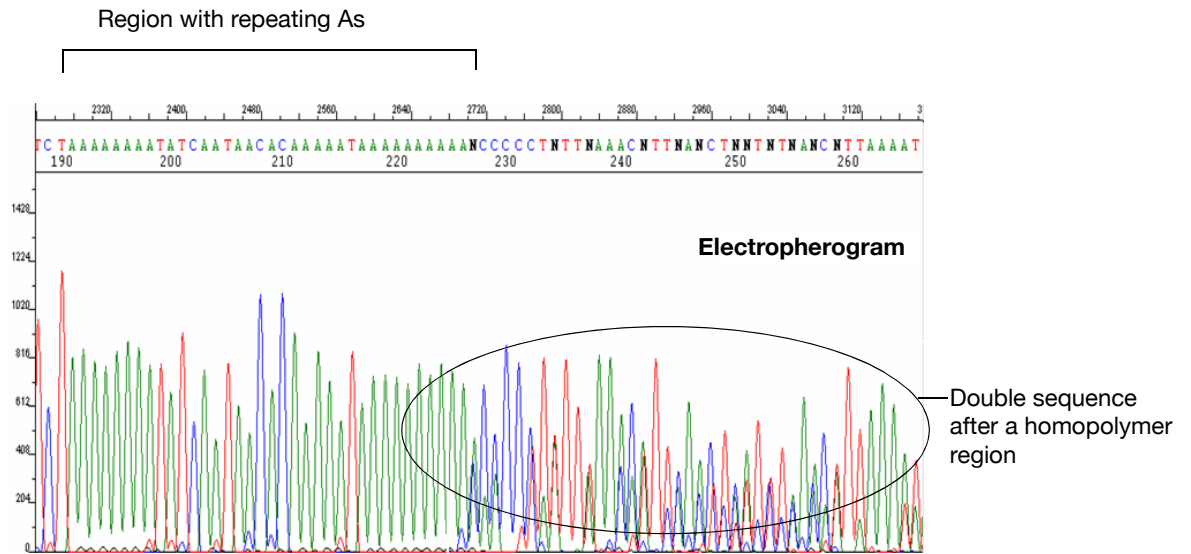
Sequencing reaction issue

Stutter during either PCR amplification and/or cycle sequencing.
 Stutter is most common in any homopolymeric region greater than two 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:

- 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 3X terminal dinucleotide or 2-base anchors.

Double peaks after a homopolymer or repeated sequence (bisulfite sequencing)



Possible cause(s)

Sequencing reaction issue

Stutter during either PCR amplification and/or cycle sequencing. Stutter is most common in any homopolymeric region greater than two 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.

Recommended action

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:

- 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 3X 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

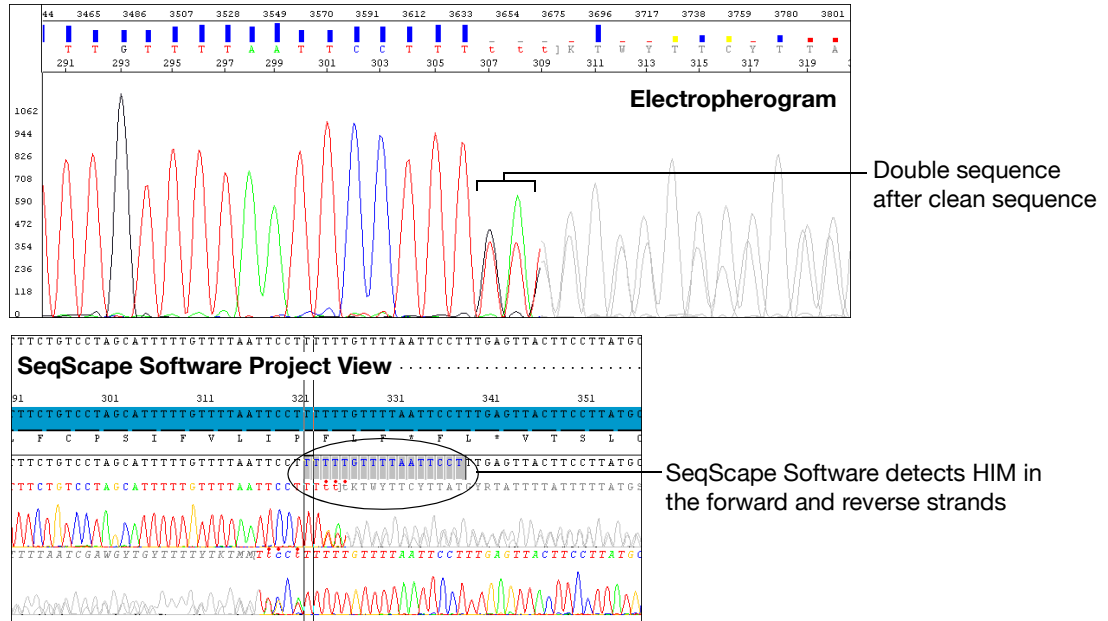
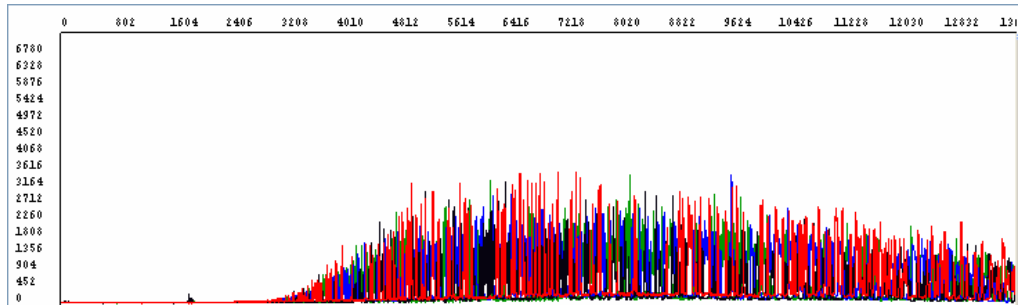


Figure 57. Sequence scanner view of a heterozygous insertion deletion (het indel). Het indel is a scenario in which one allele contains a specific insertion or deletion that the other allele lacks, putting the sequence content out of phase at the point of the particular insertion or deletion. This is a true biological event and can usually be confirmed through alignment of both forward and reverse trace files against a reference sequence to determine the insertion or deletion.

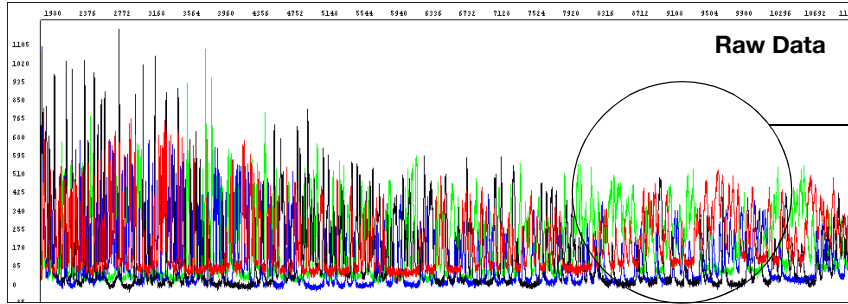
Possible cause(s)	Recommended action
Heterozygous indel mutation.	<ul style="list-style-type: none"> Assemble both forward and reverse sequence data to determine sequence of insertion or deletion. Redesign PCR target to exclude the region where the insertion or deletion occurs if additional low-level variants are potentially suspected to be present.

Resolution loss at the beginning of the run

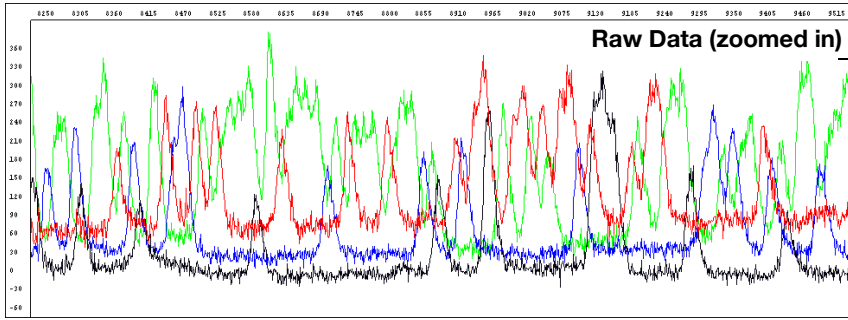


Possible cause(s)	Recommended action
Sequencing reaction issues	
XTerminator Solution or premix exposed to temperature over 25°C.	<ul style="list-style-type: none"> Use appropriate adapter for vortexer. Make sure plate does not heat up during vortexing step.
BigDye XTerminator Purification Kit reagents past their expiration date.	<ul style="list-style-type: none"> Verify expiration dates on reagents and discard if expired. Store XTerminator Solution at 4°C. Store SAM Solution at room temperature.

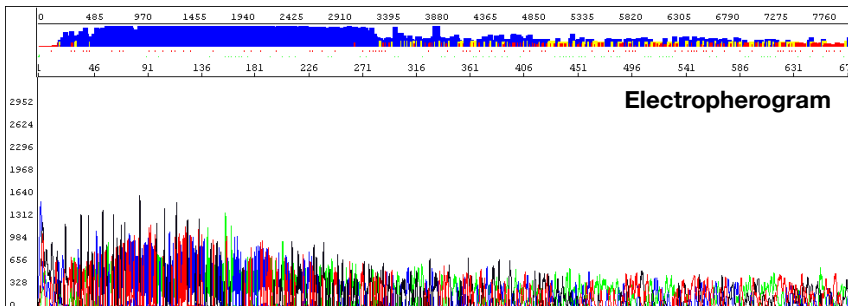
Resolution loss: Gradual early loss



Resolution loss marked by broad peaks



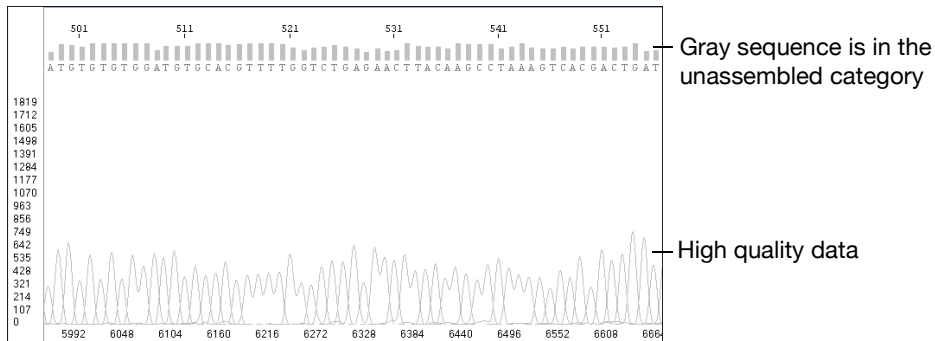
Zooming in on raw data (circled above) shows broad peaks



Possible cause(s)	Recommended action
Electrophoresis issues	
Capillary array degrading.	<ol style="list-style-type: none"> 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 90, then run the samples again.
Expired or old reagents: polymer, Hi-Di Formamide, buffer, or water.	Replace the reagent, then run your samples again.
Faulty electrophoresis source, resulting in unstable current.	Contact Thermo Fisher Scientific to arrange a service engineer visit.

Possible cause(s)	Recommended action
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.	<p>Click the Annotation tab and examine the Ave Signal Intensity. Excessive signal:</p> <ul style="list-style-type: none"> • 3500 and 3730 series instruments: >10,000 rfus • 310 and 3130 series instruments: >1,000 rfus <p>Load less labeled sample by performing one of the following:</p> <ul style="list-style-type: none"> • 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 6, "Recommended DNA template quantities for cycle sequencing," on page 55).
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-Thermo Fisher Scientific reagents.	<ol style="list-style-type: none"> 1. Perform a water wash on all components of the system using the wizard in Data Collection Software. 2. Replace reagents with Thermo Fisher Scientific products. <p>Note: the performance of non-Thermo Fisher Scientific reagents cannot be guaranteed.</p>

High-quality sequence in unassembled category in SeqScape Software



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.	Make sure that the sample is included in the right project.

Appendix A

Product information

This section covers:

Peak color/base relationships	192
Control sequences	192

Peak color/base relationships

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.

Peak color in raw data

Table 26. 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 compensates for the different color/base relationships when the correct mobility file is used. The software always displays analyzed data in the electropherogram as shown in Table 27.

Table 27. 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´TGTA AACGACGGCCAGT 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	CTGTTCGTGCC	AGCTGCATTA
	320		340		360
ATGAATCGGC	CAACGCGCGG	GGAGAGGCGG	TTTGCGTATT	GGGCGCTCTT	CCGCTTCCTC
	380		400		420
GCTCACTGAC	TCGCTGCGCT	CGGTCGTTTCG	GCTGCGGCCGA	GCGGTATCAG	CTCACTCAAA
	440		460		480
GGCGGTAATA	CGGTTATCCA	CAGAATCAGG	GGATAACGCA	GGAAAGAACA	TGTGAGCAAA
	500		520		540
AGGCCAGCAA	AAGGCCAGGA	ACCGTAAAAA	GGCCGCGTTG	CTGGCGTTTTT	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	CCCCCGTTC	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.

	2		4		60
AATTCCCTGC	AGGCGTGGCT	GCAGCCTGGT	TATGATTACT	GTTAATGTTG	CTACTACTGC
	8		10		120
TGACAATGCT	GCTGCTGCTT	CTCCTCACTG	TCTCCACTTC	CTTGAACAAT	GCGCCGTCAT
	14		16		180
GCTTCTTTTG	CCTCCCGCTG	CTCCAGAAAG	CTAGGCCGCA	GATCAGAACC	ACCACAGTCA
	20		22		240
ATATCACCAC	CTTCTCTTA	TAGATTCGGA	ATCTCATGAT	AGGGGCTCAG	CCTCTGTGCG
	26		28		300
AGTGGAGAGA	AGTTTGCAGG	CGAGCTGAGG	AGCAATTGCA	GGTGATATGA	TGTGCTCGGC
	32		34		360
TCAAGAAGCG	GGCCCGGAGA	GGAAGAAGTC	GTGCCGGGGC	TAATTATTGG	CAAAACGAGC
	38		40		420
TCTTGTTGTA	AACATTGATC	CAACTGGAAT	GTCACTAATG	GCGAATCAAT	ATTCCATAAG
	44		46		480
GCATGATGGT	TGCTCAGAGG	CAGGAGAAGA	GCAACGAATA	CGATCCTATA	AAAGATAAAA
	50		52		540
CATAAATAAA	CAGTCTTGAT	TATATTCTGG	GTATTAAAGC	CACAATCAGA	ACAAATATAT
	56		58		600
GCTTTGTATC	TTTTCTTGCC	TTCTTCATTA	CCAACTGCTT	CCGCGGCCAC	ATTAAGAGAA
	62		64		660
CTTGTGGTAA	GATAAGAAGA	TATTTTATTC	GTTCTGCTGA	CTTGCTGGAT	GTCGGGAAAT
	68		70		720
ATTCTGCATT	TGATAAGAGG	CGGTTAATTG	CAGATATAAT	TGGTAGTGAA	AAGGGTCGTT
	74		76		780
GCTATGGTCA	CCGTGAAGCG	AGTACAGCAG	CACAAGAATG	TGTGCCGTTT	TCAGTTAATA
	80		82		840
TTGTTTGAAT	ATGGTAACCT	GTTTTAGTCG	GTTTAAAGGT	AAGAAGATCT	AACCAAAAAC
	86		88		900
AACACTGCAG	TGACTGATTG	TAGTATTTAT	TTTTTTACTT	AATCTTAATT	TTGGTGTAAT
	92		94		960
CATCAACGGC	GCACTTCAAC	CAATACTCCA	ATGTTTTATC	CATCGACATG	ACGTTTCGAGA
	98		100		1020
TAGGGTTGAG	TGTTGTTCCA	GTTTGGAACA	AGAGTCCACT	ATTAAAGAAC	GTGGACTCCA
	104		106		1080
ACGTCAAAGG	GCGAAAACC	GTCTATCAGG	GCGATGGCCC	ACTACGTGAA	CCATCACCCA
	110		112		1140
AATCAAGTTT	TTTGGGGTCG	AGGTGCCGTA	AAGCACTAAA	TCGGAACCCCT	AAAGGGAGCC
	116		118		1200
CCCGATTTAG	AGCTTGACGG	GGAAAGCCGG	CGAACGTGGC	GAGAAAGGAA	GGGAAGAAAG

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QIAGEN: www.qiagen.com

Wellcome Trust Sanger Institute: 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

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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: base calling, 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 multicolor picture of a sequence showing peaks that represent the bases.
ept	A multicolor 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	A file format that can be generated during sample analysis. The file contains basecalls 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: $QV = -10 \log_{10}(Pe)$, where Pe stands for the estimated probability that the call is in error. Quality values are a measure of the certainty of the base calling 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 multicolor 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 base calling 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 file	A text file 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

Symbols

310 instrument. *See also* genetic analyzers
matrix files 137
polymer type 10, 97
sample capacity 10, 97
sequencing standards 98
user manual part number 97

3100-Avant instrument. *See also* genetic analyzers
polymer type 10
sample capacity 10

3100 instrument. *See also* genetic analyzers
polymer type 10
sample capacity 10

3130/3130xl instrument. *See also* genetic analyzers
polymer type 10, 97
sample capacity 10, 97
sequencing standards 98
user manual part number 97

3500/3500xL instrument. *See also* genetic analyzers
polymer type 10, 97
sample capacity 10, 97
sequencing standards 98
user manual part number 97

3730/3700xl instrument. *See also* genetic analyzers
polymer type 10, 97
sample capacity 10, 97
sequencing standards 98
user manual part number 97

.phd.1 file, format 200

.scf file, format 201
.seq file, format 201

A

ABI basecaller 199
absorbance
using to examine DNA quality 41
using to quantitate DNA 42
allele, defined 199
analysis. *See* data analysis
analysis protocol, definition 199
Annotation tab, reviewing 139
artifacts, reviewing data for 138
assembly, defined 199
Assembly view, defined 199
assumptions for using this guide v
AT-rich sequences, recommended kits for 49
autoanalysis, overview 12
average signal intensity
high values 139, 146, 152, 162, 176, 179, 189
low for C peaks 167
reviewing 139

B

bacterial artificial chromosomes (BACs)
cycle sequencing kits 48
preparing 33
recommended quantities for cycle sequencing 55
bacterial genomic DNA
recommended kits for 49
recommended quantity 55
thermal cycling conditions 61

- basecaller
 - file, location of 137
 - using 11
- base calling
 - defined 11
 - mixed bases not called correctly 144, 145
 - sources of interference 68
 - sudden stop 157
 - too many mixed bases called 145
- base complements 3
- baseline
 - negative, all colors 155
 - negative, one-color 154
 - reviewing 138
 - waterfall 156
- base spacing
 - definition 199
 - reviewing 139
- BDX Updater Utility 149, 151
- Beer's Law
 - in calculating DNA template quantity 42
 - in deriving primer concentration 38
- BigDye Direct Cycle Sequencing Kits
 - advantages 50
 - application recommendations 49
 - description 48
 - DNA template quantity recommended 55
 - fluorescent spectrum 8
 - workflow 52, 57
- BigDye primers
 - chemistry overview 6
 - color/base relationships in raw data 192
 - fluorescent spectra 8
- BigDye sequencing standards 98
- BigDye Terminators
 - advantages 50
 - application recommendations 49
 - chemistry overview 5
 - color/base relationships in raw data 192
 - description 48
 - DNA template quantity recommended 55
 - electropherogram example 50
 - ethanol purification of extension products 76
 - example electropherogram 50
 - irregular C peaks 167
 - irregular G peaks 169
 - M13 control primer sequences 192
 - pGEM-3Zf(+) control sequence 193
 - reactions using 60
 - sequencing standards 98
 - sequencing standard sequence 194
 - spin column purification of extension products 87
 - thermal cycling conditions 61
 - using 60
- BigDye XTerminator Purification Kit
 - about 69
 - advantages 70
 - BDX Updater Utility 149, 151
 - DNA template quantity recommended 55
 - premix pipetting protocol 73
 - premix preparation 72
 - sample preparation 91
 - sequencing control reactions 56
 - sequential pipetting protocol 74–76
 - symptom of incorrect run module 162
 - workflow 71
- biological hazard safety ix
- bisulfite conversion 24, 43
 - broad peaks 173
 - double peaks after a homopolymer 183
 - double peaks at beginning of sequence 178
 - peaks under peaks throughout 181
 - recommended DNA template quantities 55
 - sources of bias 64
- bisulfite sequencing 63–66
 - advantages of sequencing clones 63
 - broad peaks 173
 - double peaks after homopolymer 184
 - double peaks at beginning 178
 - peaks under peaks throughout 181
 - sources of bias 64
 - workflow 26

bisulfite-treated genomic DNA, recommended kits for 49

blobs in electropherograms 166

bold text, use in this guide v

buffer recommendations 99

C

calibrating genetic analyzers 100

capillaries

- handling 99
- possible signs of failure 99
- storage 99
- validation 99

capillary electrophoresis. *See also* electrophoresis

- array lengths supported 10
- BigDye XTerminator 69
- capillaries 99
- capillaries per instrument 10
- consumables 98
- overview 8
- sample preparation 92
- workflow 97

CAUTION, definition viii

chemical hazard warnings ix

chemical safety ix

chemical waste hazard warning ix

chemical waste safety ix

chromatogram. *See* electropherograms

clear range, definition 199

clones, advantages in bisulfite sequencing 63

color/base relationships 192

comparative sequencing, recommended kits for 49

complement, definition 199

compressions of peaks 171

consensus sequence, definition 199

controls

- DNA template controls 55
- M13 control primer sequence 192
- pGEM-3Zf(+) sequence 193
- sequencing standards 98
- sequencing standard sequence 194

troubleshooting using 136

conventions used in this guide

- bold text v
- Important vi
- italic text v
- menu commands v
- notes vi
- safety viii
- safety alert words vi
- user attention words vi

cosmid DNA

- recommended DNA template quantities 55
- recommended kits for 49

C peaks

- irregular 167
- shoulders on 168, 171

current, reviewing during runs 139

cycle sequencing. *See also* sequencing

- Applied Biosystems kits 7
- choosing chemistry 47
- DNA polymerase 7
- dye terminator chemistry 5
- kit descriptions 48
- kit recommendations 49
- PCR contaminants 39
- peak color/base relationships 192
- process overview 4–5
- reagent handling 52
- recommended DNA template quantities 55
- storing reactions 52
- ThermoFisher Scientific kits for 48
- Using BigDye dGTP Terminators 60
- Using BigDye Terminators 60
- using DNA template controls 55

D

DANGER, definition viii

data analysis

- autoanalysis 12
- manual analysis 12
- microbial 27

- MicroSeq ID Analysis Software 109
- options 12
- overview 10–11, 104
- PCR stop point 157
- reviewing information 138
- reviewing settings 137
- Sequencing Analysis Software 114
- settings on Annotation tab 139
- software recommendations by application 106
- ThermoFisher Scientific software for 105
- using MicrobeBridge Software 126
- using MicroSeq ID Analysis Software 122
- using Minor Variant Finder Software 133
- using Next Generation Confirmation (NGC) module 131
- using Quality Check (QC) module 129
- using SeqScape Software 119
- using Variant Analysis (VA) module 130
- using Variant Reporter Software 117
- when mobility file is incorrect 165
- with incorrect mixed bases settings 145
- with incorrect quality threshold setting 144
- with Sequence Scanner Software 111
- Data Collection Software
 - Annotation tab settings 139
 - compatibility with Applied Biosystems genetic analyzers 97
 - in electrophoresis workflow 97
 - in instrument calibration 100
- data start points, reviewing 138
- de novo* sequencing
 - methods 17
 - overview 16
 - recommended kits for 49
 - shotgun sequencing 18
- dGTP BigDye Terminators
 - advantages 50
 - application recommendations 49
 - chemistry overview 6
 - color/base relationships in raw data 192
 - DNA template quantity recommended 55
 - electropherogram example 51
 - example electropherogram 51
 - M13 control primer sequences 192
 - peak compressions 171
 - pGEM-3Zf(+) control primer sequence 193
 - purification with ethanol/sodium acetate precipitation 85
 - reactions using 60
 - thermal cycling conditions 61
 - using 60
- dideoxy sequencing 3
- DNA
 - assessing quality 41
 - calculating weight of oligonucleotide 38
 - cleaning up templates 41
 - extraction 42
 - genomic fragment length 35
 - methylation 24
 - preparing genomic 34
 - preparing plasmid DNA 33
 - preparing single-stranded 33
 - quantitating via absorbance 42
 - quantitation via NanoDrop 1000 Spectrophotometer 42
 - quantitation via fluorometric analysis 42
 - recommended quantities for sequencing 55
 - replication 2
 - resequencing 21
 - sequencing basics 2
 - shotgun sequencing 18
 - template quantities 54
 - template quantity 42
- DNA polymerase
 - in cycle sequencing 7
 - in DNA synthesis and replication 2
 - in Sanger dideoxy sequencing 3
 - selection 64
- DNA template
 - BAC DNA, preparing 33
 - cleaning up dirty templates 41
 - commercial products for preparing 35
 - contaminants in 41
 - controls for 55–57

- determining quality 41
 - difficult to sequence 158
 - genomic DNA, preparing 34
 - host strain variability 33
 - PCR DNA, preparing 35
 - plasmid DNA, preparing 33
 - quantitation of 42
 - recommended quantities for cycle sequencing 55
 - single-stranded DNA, preparing 33
 - vector-based DNA, preparing 33
 - DNA template controls 55. *See also* template controls
 - DNA template quality 40–41
 - effect of host cell variability 33
 - troubleshooting 140
 - DNA template quantity
 - determining 42
 - recommended for cycle sequencing 55
 - troubleshooting 140
 - double peaks 174–185
 - double-stranded DNA
 - quantitating 42
 - recommended quantities 55
 - dRhodamine terminators
 - formulation 6
 - peak colors 192
 - standard sequence 194
 - dye degradation
 - preventing 53, 92
 - symptoms 160, 168, 169
 - dye primers
 - chemistry 6
 - emission spectra of dyes 8
 - DyeSet/Primer file. *See* mobility files
 - dye terminators
 - chemistry 6
 - emission spectra of dyes 8
- E**
- editing bases
 - with Quality Check (QC) module 129
 - with Variant Reporter Software 108
 - electrokinetic injection
 - in capillary electrophoresis 8
 - salt interference 150
 - electropherograms
 - blobs in 166
 - definition 199
 - example 11, 137
 - example using BigDye Terminators 50
 - example using dGTP BigDye Terminators 51
 - peak color/base relationships 192
 - reviewing 137
 - electrophoresis
 - description 4
 - in DNA sequencing 4
 - sample preparation 89
 - to examine DNA quality 41
 - workflow 97
 - epigenetics. *See* methylation
 - EPT tab
 - in troubleshooting 139
 - reviewing 139
 - ethanol precipitation, for purifying extension products 76
 - ExoSAP-IT, for purifying PCR reactions 40
 - expressed sequence tags 20
 - extension product purification
 - using magnetic beads 69
 - using size-exclusion membranes 69
 - extension products
 - creation of 2
 - how labeled 5
 - purifying with BigDye XTerminator Purification Kit 69
 - purifying with ethanol precipitation 76
 - purifying with spin columns 87
- F**
- failure of capillaries, symptoms 99
 - FASTA format, definition 200
 - file formats
 - FASTA 200

- .phd.1 200
 - sample files 201
 - .scf 201
 - .seq 201
 - fluorometric analysis, for DNA quantitation 42
 - formulas
 - Beer's Law 42
 - DNA quantity using A260 42
 - melting temperature 38
 - oligonucleotide molecular weight 38
 - primer concentration 38
 - fosmid DNA, recommended kits for 49
- G**
- gap closure (custom primers), recommended kits for 49
 - GC-rich sequences, recommended kits for 49
 - genetic analyzers
 - calibrating 100
 - capillaries 99
 - comparison chart 10
 - running buffer 99
 - sequencing standards 98
 - user manual part numbers 97
 - workflow 97
 - gene walking (custom primers), recommended kits for 49
 - genomic DNA
 - bacterial, recommended kits for 49
 - bacterial, recommended quantities 55
 - commercial products for preparing 35
 - fragment length 35
 - length of fragments 35
 - preparing 34
 - G peaks, irregular 169
 - GT-rich regions, recommended kits for 49
- H**
- hazard warnings. *See* See safety warnings
 - heat seals 93, 168, 169
 - heparin
 - effect on amplification 34
 - in genomic DNA 34
 - heterozygous indel mutation (HIM) detection 186
 - heterozygous insertion deletion (het indel) detection 185
 - Hi-Di Formamide, using 92
 - homopolymeric regions
 - recommended kits for 49
 - troubleshooting 146, 170, 175, 183, 184
 - host strain variability, impact on template quality 33
- I**
- IMPORTANT, definition viii
 - indel, detection 185
 - instrument calibration 100
 - instrument run. *See* runs
 - italic text, use in this guide v
 - IUB/IUPAC, definition 200
- K**
- KB Basecaller 11
 - defined 200
 - using 11
- L**
- lambda DNA, recommended kits for 49
 - large peaks 166
 - layout view, definition 200
 - literature references 195–198
 - low signal 150
- M**
- M13 control primers. *See also* M13mp18 control
 - advantages of 23
 - for reducing primer-dimer formation 178
 - forward primer DNA sequence 23
 - in BigDye Direct Cycle Sequencing Kits 51
 - in BigDye Direct PCR 58
 - in BigDye Direct sequencing 59
 - in sequencing control reactions 56
 - Primer Designer Tool 38

- reverse primer DNA sequence 23
 - sequences 192
 - use in sequencing 22
 - M13mp18 control
 - in troubleshooting 136
 - using 55
 - magnetic beads, for extension product purification 69
 - manual analysis, overview 12
 - matrix file
 - correct location for 138
 - verifying correct one used 137
 - matrix standards 101
 - melting temperature, calculating 38
 - menu commands, conventions for describing v
 - methylation 24–26
 - MicrobeBridge Software
 - capabilities 109
 - overview 29, 126
 - suggested uses 12
 - tasks performed 30, 111
 - workflow 126
 - microbial analysis 27–30
 - workflow 28
 - MicroSeq ID Analysis Software
 - capabilities 109
 - features 109
 - libraries 29
 - overview 28, 122
 - reviewing results 123
 - suggested uses 12, 105
 - tasks performed 110
 - workflow 123
 - Minor Variant Finder Software
 - capabilities 109
 - suggested uses 12, 105
 - tasks performed 111
 - workflow 133
 - mixed bases
 - defined 200
 - not called correctly 144
 - too many called 145
 - MLST. See multilocus sequence typing
 - mobility files
 - and color/base relationships 192
 - correct location for 138
 - definition 200
 - symptoms of using incorrect 165
 - verifying correct one used 137
 - mobility shift correction 11
 - molecular weight, calculating for DNA oligonucleotide 38
 - mRNA sequencing 20
 - MSDSs
 - description ix
 - obtaining vi
 - multicomponent analysis, description 11
 - multilocus sequence typing (MLST) 27
 - mutation detection, with MicroSeq ID Analysis Software 28
- N**
- n+1 or n-1 throughout peaks 182
 - NanoDrop 1000 Spectrophotometer, for DNA quantitation 42
 - negative baselines 154
 - nested PCR 36
 - Next Generation Confirmation (NGC) module
 - capabilities 109
 - overview 131
 - suggested uses 12, 105
 - tasks performed 111
 - workflow 131
 - noise, definition 200
- O**
- oligonucleotide molecular weight, calculating 38
 - optimizing spin-column purification 87
 - overviews
 - Applied Biosystems automated DNA sequencing 4
 - capillary electrophoresis 8
 - data analysis 10, 104
 - dye primer cycle sequencing 4, 7

- dye terminator cycle sequencing 6
 - electrophoresis 4
 - Sanger dideoxy sequencing 3
- P**
- paraffin-embedded tissue
 - considerations 34
 - isolating DNA from 43
 - PCR
 - amplicons, recommended kits 49
 - amplification of template 20
 - bias 64
 - commercial products for cleaning up templates 40
 - ExoSAP-IT for purifying PCR product 40
 - fast vs. standard 36
 - nested 36
 - preparing PCR DNA templates 35
 - recommended quantities of product for cycle sequencing 55
 - semi-nested 36
 - stop point 157
 - strategies 35
 - PCR DNA template
 - commercial product for preparing 40
 - contaminants in 39
 - preparing 35
 - purifying for sequencing 40
 - quantity recommended 55
 - PCR stop point 157
 - peak heights, reviewing in raw data 138
 - peak spacing, troubleshooting 165
 - peak symptoms
 - blobs 166
 - broad peaks 173
 - compressions 171
 - double peaks 174–185
 - improperly spaced 165
 - irregular C peaks 167
 - irregular G peaks 169
 - large peaks 166
 - low intensity 148
 - missing C peaks 167
 - n+1 or n-1 throughout 182
 - peaks under peaks throughout 174
 - shoulders on peaks 170
 - spikes 163, 164
 - split peaks 162
 - sudden drop in signal 157–158
 - top-heavy data 160–161
 - unevenly spaced peaks 165
 - pGEM-3Zf(+) control
 - as a DNA template control 55
 - in BigDye XTerminator Purification Kit 56
 - in sequencing control reactions 56
 - sequence 193
 - use in troubleshooting 136
 - using 55–57
 - Phred software 11
 - plasmid DNA template
 - preparing 33
 - recommended kits for 49
 - poly(A) regions, sequencing 183, 184
 - poly(T) regions, sequencing 183, 184
 - POP polymers
 - about 98
 - problems caused by crystals 163
 - problems caused by freezing 163
 - problems caused by heating 163, 164
 - recommendations 98
 - recommended types for genetic analyzers 10
 - power during run, reviewing 139
 - Primer Designer Tool 38
 - primer-dimers
 - avoiding formation 161, 178
 - symptoms of 161
 - primers
 - avoiding primer-dimer formation 161, 178
 - calculating concentration 38
 - calculating concentration 38
 - design checklist 141
 - designing 37
 - ordering custom 39
 - predesigned with Primer Designer Tool 38

- primer walking 18
- software for designing 37
- universal-tailed PCR primers 36
- primer walking 18
- purifying extension products
 - ethanol precipitation 76
 - spin columns 87
 - using BigDye XTerminator Purification Kit 69
 - workflow 68

Q

- Quality Check (QC) module
 - capabilities 109
 - overview 129
 - suggested uses 12, 105
 - tasks performed 111
 - workflow 129
- quality value (QV)
 - defined 200
 - determination 11
- quantitation
 - DNA template 42
 - primers 37

R

- raw data
 - definition 200
 - example of high quality 138
 - peak color/base relationships in 192
 - reviewing 138
- Raw tab 138
- read length, reviewing 138
- Ready Reaction Mix
 - blended 170, 184, 189
 - effect of improper storage 148
 - insufficient 150, 158, 159, 160, 161
 - quality 147
- Reference Data Group (RDG), definition 200
- references, literature 195–198
- reptation peaks 165
- resequencing 21

- resolution loss, of peaks 152, 186–188
- resuspending purified sequencing products 90
 - Hi-Di Formamide 92
 - with BigDye XTerminator-purified products 90
- RNA, using as starting material 35
- Rolling circle amplified product, recommended kits for 49
- runs
 - reviewing information 138–141
 - reviewing temperature 139

S

- safety
 - biological hazard ix
 - chemical ix
 - conventions viii
 - warnings viii–x
- safety alert words, in this guide vi
- sample capacities, of Applied Biosystems genetic analyzers 10
- sample data, definition 200
- sample files, definition 201
- Sample Manager
 - definition 201
 - red spacing value in 143
 - reviewing data in 137
- samples
 - loading in electrophoresis 98
 - minimum volume 90
 - preparation 91
 - preparation for capillary electrophoresis 92
 - resuspending 90
- sample score, definition 201
- Sanger dideoxy sequencing 3
- SDS/heat treatment, of spin columns 87
- semi-nested PCR 36
- SeqScape Software
 - capabilities 108
 - detection of HIM using 185
 - High-quality sequence in unassembled category 190
 - overview 119

- suggested uses 12, 105
- tasks performed 110
- workflow 120
- sequences
 - M13 control primers 192
 - pGEM-3Zf(+) 193
- Sequence Scanner Software
 - capabilities 109
 - features 112
 - red spacing value 143
 - suggested uses 12, 105
 - tasks performed 110
 - workflow 112
- sequencing. *See also* cycle sequencing
 - basic principles 2
 - contaminants 41
 - control reactions 56
 - de novo* methods 17
 - mRNA sequencing 20
 - nested deletion strategies 19
 - PCR template amplification 20
 - random sequencing priming sites 19
 - recommended kits for 49
 - shotgun 18
 - storing sequencing reactions 91
- Sequencing Analysis Software
 - capabilities 108
 - overview 114
 - red spacing value 143
 - reviewing data analysis settings 137
 - reviewing run and data analysis 138
 - suggested uses 12, 105
 - tasks performed 110
 - workflow 115
- sequencing primers. *See also* primers
 - nested 23
 - PCR primers 22
- sequencing standards
 - definition 101
 - sequences 194
 - use in troubleshooting 98, 136
- shotgun sequencing
 - about 18
 - recommended kits for 49
- shoulders
 - on all peaks 170
 - on C peaks 168, 171
- Signal:Noise, reviewing 139
- signal symptoms
 - excessive signal 162
 - late start, no resolution loss 151
 - late start with resolution loss 152
 - low signal 148, 150
 - negative baseline 154
 - no signal 147
 - ski slope profile 161
 - spikes 163
 - sudden drop 157–159
 - top-heavy data 160–161
 - waterfall baseline 156
- single-stranded DNA
 - cycle sequencing kits recommended 49
 - preparing 33
 - quantitating 42
 - recommended quantities 55
- size-exclusion membranes, for extension product purification 69
- ski slope profile 161
- spacing value, red 144
- spatial calibration
 - about 100
 - in electrophoresis workflow 98
- specimen, definition 201
- spectral calibration
 - about 100
 - in electrophoresis workflow 98
 - poor or incorrect (spectral pull-up) 145
 - standard types 101
- spectral pull-up 145
- spectrophotometry, to examine DNA quality 41
- spikes
 - at end of run 164
 - four-color 163
 - one-color 164

- spin columns
 - for purifying extension products 87
 - optimizing purification 87
 - preparing 88
- spin plates, purification using 89
- split peaks 162
- standards
 - sequencing standards 98, 101
 - spectral calibration 101
- stutter (in data) 146, 170, 175, 183, 184
- symptoms, troubleshooting. *See* peak symptoms or signal symptoms

T

- technical support, obtaining vi
- template
 - amplification 20
 - BAC DNA, preparing 33
 - cleaning up 41
 - contaminants in 41
 - DNA template quantities, recommended 54
 - genomic DNA, preparing 34–35
 - PCR DNA, preparing 35
 - PCR DNA, purifying 40
 - plasmid DNA, preparing 33
 - quality 40
 - single-stranded DNA, preparing 33
 - template controls 55
 - vector-based DNA, preparing 33
- text conventions used in this guide v
- thermal cyclers 54
 - tubes or plates for 53
- thermal cycling conditions
 - BigDye Direct PCR 58
 - BigDye Direct sequencing 59
 - BigDye Terminators 61–62
 - dGTP BigDye Terminators 61–62
 - DNA template controls 57
 - modifying 63
- top-heavy data 160–162
- training, obtaining vi

- transposons 19
- troubleshooting. *See also* peak symptoms; *See also* signal symptoms
 - DNA quality and quantity, checklist 140
 - EPT tab 139
 - overview 136
 - primer design checklist 141
 - table of symptoms 141
 - workflow 136
- tube septa, using 93, 168, 169
- tubes or plates for thermal cyclers 53

U

- unevenly spaced peaks 165
- universal-tailed PCR primers 36
- user attention words, in this guide vi
- UV absorbance, for DNA quantitation 42

V

- Variant Analysis (VA) module
 - capabilities 109
 - overview 130
 - suggested uses 12, 105
 - tasks performed 111
 - workflow 130
- Variant Reporter Software
 - capabilities 108
 - overview 117
 - suggested uses 12, 105
 - tasks performed 110
 - workflow 117
- variants, definition 201
- vector-based DNA templates, preparing 33
- voltage, reviewing 139

W

- WARNING, definition viii
- warnings
 - biological hazard ix
 - chemical hazard ix
 - chemical waste hazard ix

hazardous waste ix
waterfall baseline 156
workflows
 automated DNA sequencing 13
 BigDye XTerminator Purification Kit 71
 bisulfite sequencing 26
 capillary electrophoresis 97
 extension product purification 68
 MicrobeBridge Software 126
 microbial analysis 28
 MicroSeq ID Analysis Software 123
 Minor Variant Finder Software 133
 multilocus sequence typing (MLST) 28
 Next-Generation Confirmation (NGC)
 module 131
 Quality Check (QC) module 129
 SeqScape Software 120
 Sequence Scanner Software 112
 Sequencing Analysis Software 115
 troubleshooting 136
 Variant Analysis (VA) module 130
 Variant Reporter Software 117

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