Applied Biosystems 3730/3730*xl*

DNA Analyzers

Sequencing Chemistry Guide

Applied Biosystems | HITACHI

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

NOTICE TO PURCHASER:

PLEASE REFER TO THE APPLIED BIOSYSTEMS 3730/3730*xl* DNA ANALYZERS USER GUIDE AND THE REAGENT PACKAGE INSERTS FOR LIMITED LABEL LICENSE OR DISCLAIMER INFORMATION.

TRADEMARKS:

Part Number 4331467Rev. B
11/2002

Contents

Preface

Safety Information

Chapter 1 About Applied Biosystems 3730/3730xl DNA Analyzers

Chapter 2 Preparing the Samples for Cycle Sequencing

Chapter 3 Purifying the Extension Products

Appendix A Purchasing or Preparing Formamide

Appendix B Troubleshooting

Index

Preface

About This Guide

This guide describes the chemistry protocols for the:

- Applied Biosystems 3730 DNA Analyzer (48-capillary array)
- Applied Biosystems 3730*xl* DNA Analyzer (48- and 96-capillary arrays)

This guide is organized into task-based topics, each with a step-by-step description of how to perform the task.

Note: This guide is specific to the 3730/3730*xl* DNA Analyzers. For general chemistry information about kits, dyes, enzymes, protocols, and troubleshooting, please refer to the documents listed below under "Related Documentation."

Terms

Unless otherwise specified, the term *DNA Analyzer* will be used throughout this guide to refer to both the 3730 and 3730*xl* instruments.

Audience

This guide is designed for those persons preparing DNA samples for loading onto the DNA Analyzer.

Conventions

This guide uses the following conventions:

- **Bold** text indicates user action. For example:
  Type **100**, then press **Enter** for each of the remaining fields.
- *Italic* text indicates new or important words and is also for emphasis. For example:
  Before analyzing, always prepare a *new* matrix.
- A right arrow bracket (>) separates successive menu commands. For example:
  Select **File** > **Open**.

Related Documentation

If you need more information on...	See the...	Part Number
operating the DNA Analyzer	*Applied Biosystems 3730/3730xl DNA Analyzers User Reference Manual*	4331466
	Applied Biosystems 3730/3730xl DNA Analyzers User Guide	4331468
analyzing DNA sequencing data	*ABI PRISM® DNA Sequencing Analysis Software v.5.0 Guide*	4331940
ABI PRISM® BigDye® Terminator v3.1 chemistry	*ABI PRISM® BigDye® Terminator v3.1 Ready Reaction Cycle Sequencing Kit Protocol*	4337035
	User Bulletin: Using an SDS/Heat Treatment with Spin Columns or 96-Well Spin Plates to Remove Unincorporated Dye Terminators	4330951
ABI PRISM® BigDye® Terminator v3.0 chemistry	*ABI PRISM® BigDye® Terminator v3.0 Ready Reaction Cycle Sequencing Kit Protocol*	4390037
	User Bulletin: Precipitation Method to Remove Unincorporated Dye Terminators from ABI PRISM® *BigDye® Terminator v3.0 Cycle Sequencing Reactions*	4333020
	User Bulletin: Using an SDS/Heat Treatment with Spin Columns or 96-Well Spin Plates to Remove Unincorporated Dye Terminators	4330951
	User Bulletin: Aliquoting Bulk Quantities of ABI PRISM® Cycle Sequencing Ready Reaction Mixes	4310588
	User Bulletin: Sequencing Large DNA Templates	4304656
ABI PRISM® BigDye® Terminator v1.1 chemistry	*ABI PRISM® BigDye® Terminator v1.1 Ready Reaction Cycle Sequencing Kit Protocol*	4337036

Obtaining Technical Support

For services and support, access the Applied Biosystems Web site:

http://www.appliedbiosystems.com

At the Applied Biosystems Web site, you can:

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

In addition, the Applied Biosystems Web site provides a list of telephone and fax numbers that can be used to contact Technical Support.

Safety Information

Safety Conventions Used in This Document

Safety Alert Words

Four safety alert words appear in Applied Biosystems user documentation. Each word implies a particular level of observation or action, as described below:

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

Indicates a potentially hazardous situation that, if not avoided, can result in minor or moderate injury. It can also alert against unsafe practices, damage to an instrument, or loss of data.

Indicates a potentially hazardous situation that, if not avoided, can result in serious injury or death.

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

Chemical Safety

Chemical Hazard Warning

CHEMICAL HAZARD. Some of the chemicals used with Applied Biosystems instruments and protocols are potentially hazardous and can cause injury, illness, or death.

Chemical Safety Guidelines

To minimize the hazards of chemicals:

- Read and understand the material safety data sheets (MSDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (*e.g.,* safety glasses, gloves, or protective clothing). For additional safety guidelines, consult the MSDS.
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (*e.g.,* fume hood). For additional safety guidelines, consult the MSDS.
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended on the MSDS.
- Comply with all local, state/provincial, or national laws and regulations related to chemical storage, handling, and disposal.

About MSDSs

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

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

Obtaining MSDSs

You can obtain from Applied Biosystems the MSDS for any chemical supplied by Applied Biosystems. This service is free and available 24 hours a day.

To obtain MSDSs:

1. Go to **https://docs.appliedbiosystems.com/msdssearch.html**
2. In the Search field, type in the chemical name, part number, or other information that appears in the MSDS of interest. Select the language of your choice, then click **Search**.
3. Find the document of interest, right-click the document title, then select any of the following:
   - **Open** – To view the document
   - **Print Target** – To print the document
   - **Save Target As** – To download a PDF version of the document to a destination that you choose
4. To have a copy of a document sent by fax or e-mail, select **Fax** or **Email** to the left of the document title in the Search Results page, then click **RETRIEVE DOCUMENTS** at the end of the document list.
5. After you enter the required information, click **View/Deliver Selected Documents Now**.

Chemical Waste Safety

Chemical Waste Hazard

WARNING **CHEMICAL WASTE HAZARD.** Some wastes produced by the operation of the instrument or system are potentially hazardous and can cause injury, illness, or death.

Chemical Waste Guidelines

To minimize the hazards of chemical waste:

- Read and understand the MSDSs for the chemicals in a waste container before you store, handle, or dispose of chemical waste.
- Provide primary and secondary waste containers
- Minimize contact with and inhalation of chemical waste. When handling chemicals, wear appropriate personal protective equipment such as safety glasses, gloves, and protective clothing.
- Handle chemical wastes in a fume hood.
- After you empty a chemical waste container, seal it with the cap provided.

- Dispose of the contents of a waste container in accordance with good laboratory practices and local, state/provincial, and/or national environmental and health regulations.

Site Preparation and Safety Guide

A site preparation and safety guide is a separate document sent to all customers who have purchased an Applied Biosystems instrument. Refer to the guide written for your instrument for information on site preparation, instrument safety, chemical safety, and waste profiles.

Waste profiles help you plan for the handling and disposal of waste generated by operation of the instrument. Read the waste profiles and all applicable MSDSs for your instrument before handling or disposing of chemical waste.

Waste Disposal

If potentially hazardous waste is generated when you operate the instrument, you must:

- Characterize (by analysis if necessary) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure the health and safety of all personnel in your laboratory.
- Ensure that the instrument waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.

Note: Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.

About Applied Biosystems 3730/3730*xl* DNA Analyzers

1

In This Chapter

This chapter includes the following topics:

Instrument Overview

Description

The Applied Biosystems 3730/3730*xl* DNA Analyzers are automated, high-throughput, capillary electrophoresis systems used for analyzing fluorescently labeled DNA fragments.

Note: This guide provides detailed information on the DNA Analyzer chemistry. For detailed information on the DNA Analyzer hardware, operation, and maintenance, please refer to the *Applied Biosystems 3730/3730xl DNA Analyzers User Guide* (PN 4331468).

Chemistries Supported

The following sequencing chemistries are currently supported for use with the DNA Analyzer.

BigDye Terminator v3.1 Chemistry

BigDye Terminator v3.1 Chemistry	No. of Reactions	Part Number
BigDye Terminator v3.1 Cycle Sequencing Kit	100	4337455
	1000	4337456
	5000	4337457
	25000	4337958
BigDye® Terminator v3.1 Cycle Sequencing Kit Protocol	—	4337035

BigDye Terminator v3.0 Chemistry

BigDye Terminator v3.0 Chemistry*	No. of Reactions	Part Number
ABI PRISM® BigDye® Terminator v3.0 Ready Reaction Cycle Sequencing Kit with AmpliTaq® DNA Polymerase, FS	100	4390242
	1000	4390244
	5000	4390246
	25,000	4390253
ABI PRISM® BigDye® Terminator v3.0 Ready Reaction Cycle Sequencing Kit Protocol	—	4390037

*The ABI PRISM® dGTP BigDye® Terminator Ready Reaction kit v3.0 is not supported on the 3730/3730XL DNA Analyzer.

BigDye Terminator v1.1 Chemistry

BigDye Terminator v1.1 Chemistry*	No. of Reactions	Part Number
BigDye Terminator v1.1 Cycle Sequencing Kit	100	4337450
	1000	4337451
	5000	4337452
	25,000	4337453
BigDye® Terminator v1.1 Cycle Sequencing Kit Protocol	—	4337036

*Protocols for the BigDye Terminator v1.1 chemistry are included in the kits.

POP-7 Polymer Required

The Applied Biosystems 3730/3730xl DNA Analyzers require POP-7™ Polymer (PN 4335615) and BigDye sequencing buffer (10X) with EDTA (500 mL - PN 4335613 and 4 L - PN 4318976). Use of other polymers and buffers will result in signal uniformity problems and loss of resolution.

Run Cycle Overview

Diagram of Run Cycle Steps

Below is a diagram of the DNA Analyzer run cycle. Steps 1 to 6 are described in more detail under "How It Works" below.

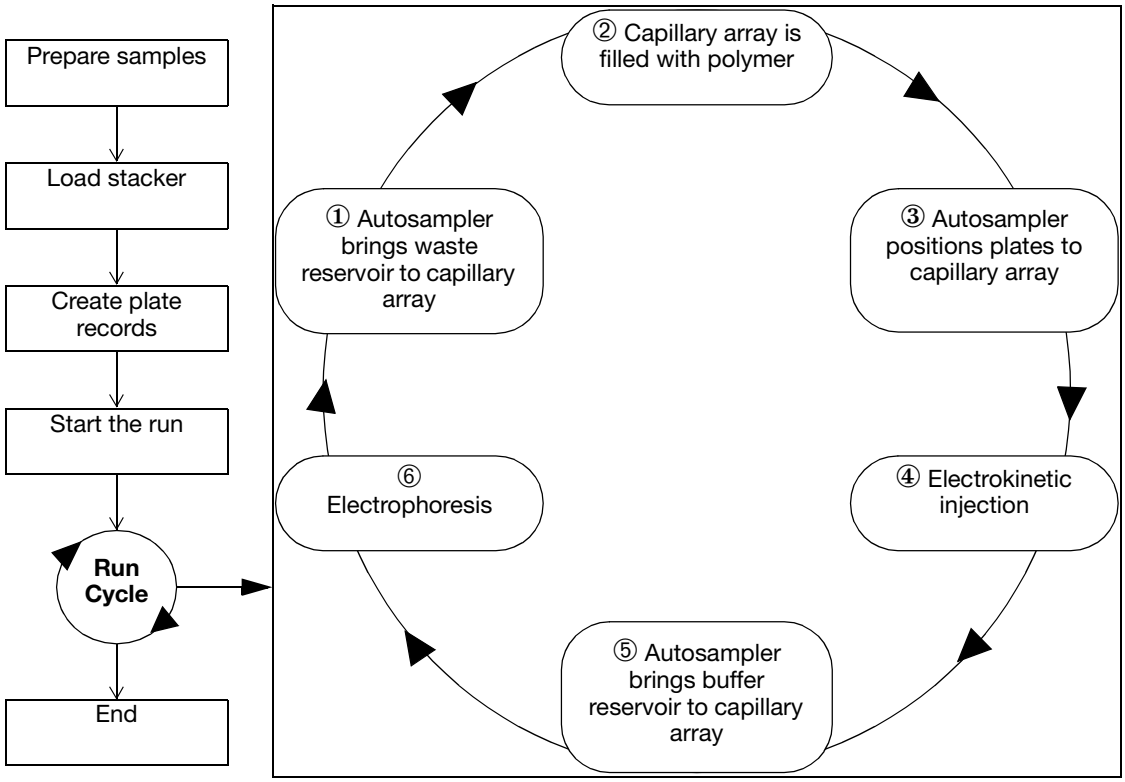


Figure 1-1 DNA analyzer run cycle

How It Works

How the DNA 3730/3730xl Analyzer Run Cycle Works (see Figure 1-1)

1.	The autosampler brings the waste reservoir to the capillary array.
2.	The capillary array is filled with POP-7™ polymer, a medium that separates the DNA fragments. Refer to the *Applied Biosystems 3730/3730xl DNA Analyzers User Guide* (PN 4331468) for information about how to fill the capillary array. • The 3730 DNA Analyzer is compatible with the 48-capillary array only. **Note:** A service upgrade is needed to run the 96-capillary array. • The 3730xl DNA Analyzer is compatible with both the 48- and 96-capillary arrays. **Note:** Always use adequate ventilation such as that provided by a fume hood. **WARNING** **CHEMICAL HAZARD**. **POP-7 polymer** causes eye, skin, and respiratory tract irritation. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.
3.	The autosampler removes the reaction plates from the instrument's stacker and positions the sample wells to the capillary array: • 48-capillary array for the 3730 DNA Analyzer, or • 48- and 96-capillary arrays for the 3730*xl* DNA Analyzer
4.	The fluorescently labeled DNA is loaded into the capillary array by a short period of electrophoresis called *electrokinetic injection*. The capillary array is rinsed with water to remove any sample adhering to the sides.
5.	The autosampler brings the buffer reservoir to the capillary array for electrophoresis: • 48-capillary array for the 3730 DNA Analyzer, or • 48- and 96-capillary arrays for the 3730*xl* DNA Analyzer
6.	The labeled DNA sequence fragments are separated by size as they travel through the polymer-filled capillary array (electrophoresis). As they reach the detection window, the laser beam excites the dye molecules and causes them to fluoresce (*electrophoresis*). The fluorescence emissions from 48 or 96 samples are collected simultaneously and spectrally separated by a spectrograph. The fluorescence emissions are focused as columns of light onto the CCD camera.
7.	The 3730/3730xl Data Collection software reads and interprets the fluorescence data, then displays the data as an electropherogram. For more information on data analysis, refer to the *ABI PRISM® DNA Sequencing Analysis Software v.5.0 Guide* (PN 4331940).

Preparing the Samples for Cycle Sequencing

2

In This Chapter

This chapter includes the following topics:

Template Type and Template Quality

Template Types

The following templates may be used with the BigDye® terminator chemistry:

- PCR product
- Single-stranded DNA (*e.g.,* M13)
- Double-stranded DNA
- Large DNA (*e.g.,* BACs, PACs, YACs, cosmids, and fosmids)
- Bacterial genomic DNA

Control DNA Template Recommended

It is strongly recommended that you include a control DNA template as one of the templates in a set of sequencing reactions. The results from the control can help determine whether failed reactions are the result of poor template quality or sequencing reaction failure.

Applied Biosystems recommends M13mp18 as a single-stranded control and pGEM®-3Zf(+) as a double-stranded control.

- All Applied Biosystems DNA sequencing kits provide pGEM control DNA.
- All dye terminator cycle sequencing kits include a –21 M13 forward primer for use in performing all control reactions.

Ensuring Template Quality

The quality of DNA in a reaction can affect the performance of the DNA Analyzer. When preparing DNA templates, it is critical to avoid the following:

- Residual salts
- Proteins
- Residual detergents
- Residual RNA

The presence of residual salts, proteins, RNA, and detergents can interfere with capillary electrophoresis and electrokinetic injection. Your current template purification methods may have to be modified to remove residual salts, proteins, and detergents.

Effect of Residual Salts

Capillary electrophoresis is especially susceptible to salt in samples, either from template preparation, from cycle sequencing reactions, or from precipitation methods using salts. The negative ions in salts can be preferentially injected into the capillary array during electrokinetic injection, leading to lower signal. In addition, the negative ions compete and interfere with the injection of larger DNA extension fragments, leading to shortened read lengths. The capillary array view in Figure 2-1 shows the effects of increasing concentrations of salt, both the Sodium Acetate and EDTA, during electrokinetic injection.

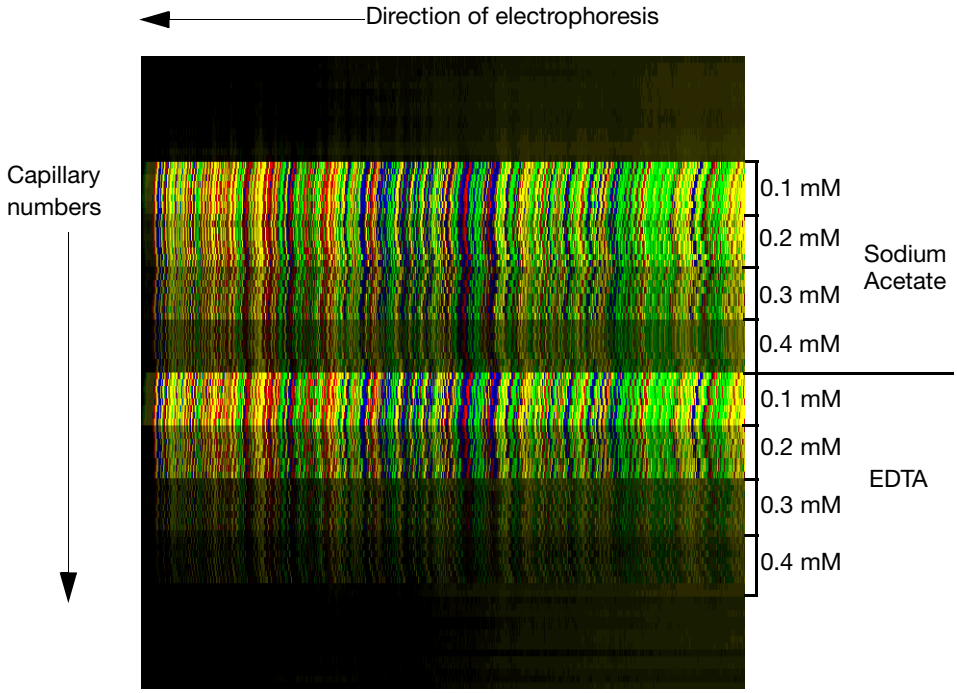


Figure 2-1 The effect of salt concentration during electrokinetic injection

Recommended Protocols for Removing Excess Salts

Applied Biosystems recommends implementing an efficient method to remove excess salts. For protocols, please refer to the documents listed in the table below.

Recommended Protocols for Removing Excess Salts

Chemistry	Document	Part Number
BigDye Terminator v3.1 chemistry	*BigDye® Terminator v3.1 Cycle Sequencing Kit Protocol*	4337035
	User Bulletin: *Using an SDS/Heat Treatment with Spin Columns or 96-Well Spin Plates to Remove Unincorporated Dye Terminators*	4330951
BigDye Terminator v3.0 chemistry	*ABI PRISM® BigDye® Terminator v3.0 Ready Reaction Cycle Sequencing Kit Protocol*	4390037
	User Bulletin: *Precipitation Method to Remove Unincorporated Dye Terminators from ABI PRISM® BigDye® Terminator v3.0 Cycle Sequencing Reactions*	4333020
	User Bulletin: *Using an SDS/Heat Treatment with Spin Columns or 96-Well Spin Plates to Remove Unincorporated Dye Terminators*	4330951

Recommended Protocols for Removing Excess Salts *(continued)*

BigDye Terminator v1.1 chemistry	*ABI PRISM*® BigDye® *Terminator v1.1 Ready Reaction Cycle Sequencing Kit Protocol*	4337036
BigDye Terminator v3.1, v3.0 and v1.1 chemistries	User Bulletin: *Sequencing Large DNA Templates*	4304656

Effect of Proteins

Many DNA preparation methods for sequencing require the recovery of DNA from lysed bacterial cultures. Unless DNA is carefully purified, protein can remain in the DNA samples. Protein can be injected and adhere to the walls of the capillary array, adversely affecting data resolution and capillary array lifetime.

Effect of Residual Detergents

Some methods of template preparation, such as the Thermomax method for M13 preparation, use detergents such as Triton X-100 to lyse the protein coat of phage particles. Other detergents, such as sodium dodecyl sulfate (SDS), are used in plasmid purification protocols to lyse bacterial cells. Small, negatively charged detergents may be preferentially injected over DNA during electrokinetic injection. If present at high levels, detergents such as Triton X-100 and SDS will adversely affect the life of the capillary array and the quality of the sequencing data.

Effect of Residual RNA

Residual RNA that is present in DNA template preparations competes with the DNA for injection into the capillary array. Residual RNA has the same effect as excess salt, that is, decreased signal and shortened read lengths.

Template Quantity

Recommended Template Quantity

The table below shows the recommended quantity of template to use in a cycle sequencing reaction.

Template	Recommended Quantity
PCR product:	
100–200 bp	1–3 ng
200–500 bp	3–10 ng
500–1000 bp	5–20 ng
1000–2000 bp	10–40 ng
>2000 bp	40–100 ng
Single-stranded DNA	50–100 ng*
Double-stranded DNA	200–500 ng*
Large DNA† (*e.g.,* BACs, PACs, YACs, cosmids, and fosmids)	0.5–1.0 µg
Bacterial genomic DNA	2–3 µg

*Because the DNA Analyzer is a highly sensitive instrument, you may not need this much template.

†For more information on large DNA templates, refer to *User Bulletin: Sequencing Large DNA Templates* (PN 4304656).

Effect of Too Little Template

Too little template or primer in cycle sequencing reactions reduces the signal strength and therefore the peak height of reaction products. In the worst case, the signal-to-noise level decreases so that bases cannot be called.

Effect of Excess Template

Excess template can affect data quality when:

- present in sample loaded onto the DNA Analyzer
- used in excess in the cycle sequencing reaction

Excess template inhibits the injection of labeled extension fragments, thus affecting signals generated from this instrument. Excess template can behave similarly to proteins and accumulate in the capillary array, which adversely affects data resolution and capillary array lifetime.

Excess template used in the cycle sequencing reaction results in generation of short extension fragments. During electrokinetic injection, short fragments are injected more efficiently resulting in “top heavy” peak characteristics and shortened reads (see Figure 2-2 on page 2-6). This phenomena becomes more pronounced with increased dilution of BigDye Terminator.

In Figure 2-2, the raw data from a BigDye Terminator reaction contains excess template, resulting in a “top heavy” peak profile.

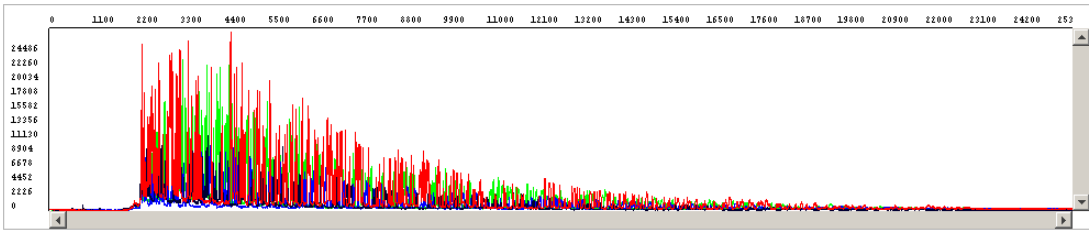


Figure 2-2 Raw data from a BigDye terminator reaction containing excess DNA template

In Figure 2-3 below, analyzed data from a BigDye Terminator reaction contains excess template (this is from the same sample shown in Figure 2-2 on page 2-6). The peaks are clearly off-scale and have overall shortened read lengths. The presence of excess template in the reaction and the preferential electrokinetic injection of small DNA fragments cause this effect.

Figure 2-3 Analyzed data from a BigDyeTerminator reaction

Reagent and Equipment Considerations

Reagent Handling and Reaction Storage

Properly stored reagents are likely to perform the best. The following methods are recommended for guaranteeing reagent quality.

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

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

- Avoid excess (more than 10) freeze-thaw cycles. Aliquot reagents in smaller amounts if necessary. For more information, refer to *User Bulletin: Aliquoting Bulk Quantities of ABI PRISM® Cycle Sequencing Ready Reaction Mixes* (PN 4310588).
- Shield reagents and sequencing reactions from light. Fluorescent dyes are susceptible to photo-bleaching.
- If you would like to store sequencing reactions for future use, purify and dry them. Store the reactions at –15 to –25 °C.

Recommended Thermal Cyclers

The cycle sequencing procedures provided in this chemistry guide (page 2-16) have been optimized for the following thermal cyclers:

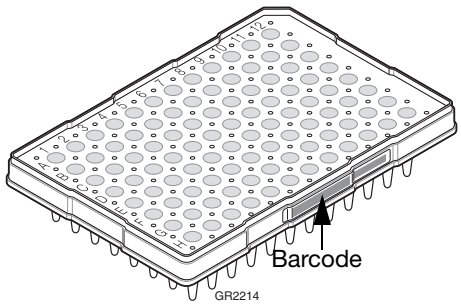
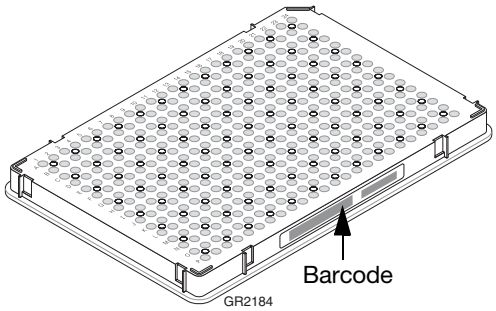
- GeneAmp® PCR System 9700 (in 9600 emulation mode)
- GeneAmp® PCR System 9700 Dual 384-Well Sample Block Module
- GeneAmp® PCR System 9600

Compatible Reaction Plates

Samples should either be prepared in a reaction plate or transferred to a reaction plate before being placed in the DNA Analyzer's stacker.

Applied Biosystems supplies two types of reaction plates that are compatible with the DNA Analyzer, as shown on page 2-8. Both reaction plates include barcodes, which are read by the DNA Analyzer to automatically assign the plate record associated with the plates.

IMPORTANT! Presently, these are the reaction plates recommended for use on the DNA Analyzer. Other plate types may be of different dimensions and thus affect instrument performance. Plates of varying tube depths may damage the capillary array, or the piercer and autosampler.

Reaction Plate		Part Number
ABI PRISM™ 96-Well Optical Reaction Plate with Barcode		4306737
ABI PRISM™ 384-Well Clear Optical Reaction Plate with Barcode		4309849

Choosing a Reaction Plate Format

The reaction plate format you choose depends on the thermal cycler that you are using. The table below lists the reaction plates and covers required for the recommended Applied Biosystems thermal cyclers.

IMPORTANT! If you are using heat-seal film (PN 4337570) to cover samples prior to electrokinetic injection, do not use the Optical Adhesive Covers (PN 4311971). The residual glue will interfere with the heat-sealing process.

GeneAmp® PCR System 9600		**Part Number**
Reaction Plate	ABI PRISM™ 96-Well Optical Reaction Plate with Barcode (code 128)	4306737
Reaction Plate Covers	ABI PRISM™ Optical Adhesive Cover Starter Kit	4313663
	ABI PRISM™ Optical Adhesive Covers	4311971
	MicroAmp® Clear Adhesive Film	4306311
	MicroAmp® 96-Well Full Plate Covers	N801-0550
GeneAmp® PCR System 9700		**Part Number**
Reaction Plate	ABI PRISM™ 96-Well Optical Reaction Plate with Barcode (code 128)	4306737
Reaction Plate Covers	ABI PRISM™ Optical Adhesive Cover Starter Kit	4313663
	ABI PRISM™ Optical Adhesive Covers	4311971
	MicroAmp® Clear Adhesive Film	4306311
	MicroAmp® 96-Well Full Plate Covers	N801-0550
GeneAmp® PCR System 9700 Dual 384-Well Sample Block Module		**Part Number**
Reaction Plate	ABI PRISM™ 384-Well Clear Optical Reaction Plate with Barcode (code 128)	4309849
Reaction Plate Covers	ABI PRISM™ Optical Adhesive Cover Starter Kit	4313663
	ABI PRISM™ Optical Adhesive Covers	4311971
	MicroAmp® Clear Adhesive Film	4306311

Preparing the Samples for Cycle Sequencing

Choosing a Reaction Volume

Use the table below to choose the reaction volume best suited to your template type.

Reaction Plate Type	Total Volume	Ready Reaction Mix	Refer to...
96-well	20 µL Full	8 µL 1X	Preparing 96-Well Full-Volume Reactions (1X) on page 2-12
96-well	20 µL Full	4 µL 0.5X	Preparing 96-Well Full-Volume Reactions (0.5X) on page 2-13
96-well	10 µL Half	4 µL 0.5X	Preparing 96-Well Half-Volume Reactions (0.5X) on page 2-14
384-well	10 µL Half	4 µL 0.5X	Preparing 384-Well Half-Volume Reactions (0.5X) on page 2-15

About Sequencing Buffers

The 5X Sequencing Buffer for the 3.0 chemistry cannot be used with the 3.1 and 1.1 chemistries. And, the BigDye® Terminator Sequencing Buffer v1.1/3.1 (5X) for the 3.1 and 1.1 chemistries cannot be used with the 3.0 chemistry. Please read the label carefully to determine that you are using the correct buffer.

Using BigDye Terminator Sequencing Buffers

The BigDye Terminator Sequencing Buffer is supplied at a 5X concentration. If you use it for sequencing reactions, be sure the final reaction volume is at a concentration of 1X. For example, for a half reaction in 20 µL final volume, use 4 µL of ready reaction premix and 2 µL of BigDye sequencing buffer as shown below.

Reagent	Buffer Concentration	Volume
Ready Reaction Premix	2.5X	4 µL
BigDye Sequencing Buffer*	5X	2 µL
Primer	—	3.2 pmol
Template	—	See "Template Quantity" on page 2-5
Water	—	to 20 µL
Final Volume	**1X**	**20 µL**

*Select either the 5X Sequencing Buffer for 3.0 chemistry or the BigDye® Terminator Sequencing Buffer v1.1/3.1 (5X) for 3.1 and 1.1 chemistries.

Note: The use of this buffer without optimization may result in deterioration of sequence quality. Applied Biosystems does not support diluted reactions or guarantee the performance of BigDye® chemistry when it is diluted.

Preparing 96-Well Full-Volume Reactions (1X)

To prepare full-volume reactions using 1X Ready Reaction Mix in 96-well reaction plates:

1. For each reaction, add the reagents listed below to a separate well.

Reagent	Quantity
Terminator Ready Reaction Mix	8.0 µL
Template	See "Recommended Template Quantity" on page 2-5.
Primer	3.2 pmol
Deionized water	*q.s.*
Total Volume	20 µL

2. Mix well.
3. Cover the reaction plate(s) with an appropriate cover (see the table on page 2-9).
4. Spin briefly.
5. Proceed to "Performing Cycle Sequencing for BigDye Terminator v1.1, v3.0, and v3.1 Chemistries" on page 2-16.

Preparing 96-Well Full-Volume Reactions (0.5X)

To prepare full-volume reactions using 0.5X Ready Reaction Mix in 96-well reaction plates:

1. For each reaction, add the reagents listed below to a separate well.

Reagent	Quantity
Terminator Ready Reaction Mix	4.0 µL
Template	See “Recommended Template Quantity” on page 2-5.
5X Sequencing Buffer (PN 4305605)	2 µL
Primer	3.2 pmol
Deionized water	*q.s.*
Total Volume	20 µL

2. Mix well.
3. Cover the reaction plate(s) with an appropriate cover (see the table on page 2-9).
4. Spin briefly.
5. Proceed to “Performing Cycle Sequencing for BigDye Terminator v1.1, v3.0, and v3.1 Chemistries” on page 2-16.

Preparing 96-Well Half-Volume Reactions (0.5X)

To prepare half-volume reactions using 0.5X Ready Reaction Mix in 96-well reaction plates:

1. For each reaction, add the reagents listed below to a separate well.

Reagent	Quantity
Terminator Ready Reaction Mix	4.0 µL
Template	See "Recommended Template Quantity" on page 2-5.
Primer	3.2 pmol
Deionized water	*q.s.*
Total Volume	10 µL

2. Mix well.
3. Cover the reaction plate(s) with an appropriate cover (see the table on page 2-9).
4. Spin briefly.
5. Proceed to "Performing Cycle Sequencing for BigDye Terminator v1.1, v3.0, and v3.1 Chemistries" on page 2-16.

Preparing 384-Well Half-Volume Reactions (0.5X)

To prepare half-volume reactions using 0.5X Ready Reaction Mix in 384-well reaction plates:

1. For each reaction, add the reagents listed below to a separate well.

Reagent	Quantity
Terminator Ready Reaction Mix	4.0 µL
Template	See “Recommended Template Quantity” on page 2-5.
Primer	3.2 pmol
Deionized water	*q.s.*
Total Volume	10 µL

2. Mix well.
3. Cover the reaction plate(s) with an appropriate cover (see the table on page 2-9).
4. Spin briefly.
5. Proceed to “Performing Cycle Sequencing for BigDye Terminator v1.1, v3.0, and v3.1 Chemistries” on page 2-16.

   Note: Use on a GeneAmp PCR System 9700 Dual 384-Well Sample Block Module.

Performing Cycle Sequencing for BigDye Terminator v1.1, v3.0, and v3.1 Chemistries

Applicable Kits

The cycle sequencing procedures provided in this section are applicable to the following ABI PRISM® BigDye® Terminator chemistry kits:

- BigDye® Terminator v3.1 Cycle Sequencing Kit
- ABI PRISM® BigDye® Terminator v3.0 Ready Reaction Cycle Sequencing Kit with AmpliTaq® DNA Polymerase, FS
- BigDye® Terminator v1.1 Cycle Sequencing Kit

Recommended Thermal Cyclers

The Applied Biosystems thermal cyclers listed below can be used to cycle sequence half- and full-volume reactions. The cycle sequencing procedures that follow have been optimized for these thermal cyclers.

- GeneAmp PCR System 9700 (in 9600 emulation mode)
- GeneAmp PCR System 9700 Dual 384-Well Sample Block Module
- GeneAmp PCR System 9600

IMPORTANT! If you use a thermal cycler not manufactured by Applied Biosystems, you may need to optimize thermal cycling conditions. Ramping time is very important. If the thermal ramping time is too fast (>1 °C/sec), poor (noisy) data may result.

Modifying Cycle Sequencing Parameters

The cycle sequencing procedures provided below work for a variety of templates. However, the following modifications may be made:

- For short PCR products, a reduced number of cycles can be used (*e.g.*, 20 cycles for a 300-bp or smaller fragment).
- If the T_m of a primer is >60 °C, the annealing step can be eliminated.
- If the T_m of a primer is <50 °C, increase the annealing time to 30 seconds or decrease the annealing temperature to 48 °C.
- For templates with high GC content (>70%), heat the tubes at 98 °C for 5 minutes before cycling to help denature the template.
- For sequencing large DNA templates such as BAC DNA, cosmid DNA, and genomic DNA.

Cycle Sequencing Large DNA Templates

For sequencing large DNA templates:

1.	Place the tubes in a thermal cycler and set the volume to 20 µL.
2.	Heat the tubes at 95 °C for 5 minutes.
3.	Repeat the following for 50 cycles:* • Rapid thermal ramp† to 95 °C • 95 °C for 30 sec • Rapid thermal ramp to 50-55 °C (depending on template) • 50–55 °C for 10 sec • Rapid thermal ramp to 60 °C • 60 °C for 4 min
4.	Rapid thermal ramp to 4 °C and hold until ready to purify.
5.	Spin down the contents of the tubes in a microcentrifuge.
6.	Proceed to Chapter 4, “Preparing for Electrophoresis.”

*Some laboratories have found that increasing the number of cycles gives better results.
†Rapid thermal ramp is 1 °C/sec.

Cycle Sequencing Half- and Full-Volume Reactions

To perform cycle sequencing for half-volume reactions:

1.	Place the reaction plate in a thermal cycler and set the appropriate volume.
2.	For 3.1 and 1.1 chemistries only, perform an initial denaturation step: • Rapid thermal ramp to 96 °C • 96 °C for 1 min
3.	Repeat the following for 25 cycles: • Rapid thermal ramp* to 96 °C • 96 °C for 10 sec • Rapid thermal ramp to 50 °C • 50 °C for 5 sec • Rapid thermal ramp to 60 °C • 60 °C for 4 min
4.	Rapid thermal ramp to 4 °C and hold until ready to purify.
5.	Spin down the contents of the wells in a microcentrifuge.
6.	Proceed to one of the following sections, as appropriate: • "Purifying the Extension Products: BigDye Terminator v3.1 Chemistry" on page 3-3 • "Purifying the Extension Products: BigDye Terminator v3.0 Chemistry" on page 3-19 • "Purifying the Extension Products: BigDye Terminator v1.1 Chemistry" on page 3-41

*Rapid thermal ramp is 1 °C/sec.

Purifying the Extension Products 3

In This Chapter

This chapter includes the following topics:

Section 3.1 Purifying the Extension Products: BigDye Terminator v3.1 Chemistry

About Purification for BigDye Terminator v3.1 Chemistry

Unincorporated dye terminators must be completely removed before the samples can be analyzed by electrophoresis. Excess dye terminators in sequencing reactions obscure data in the early part of the sequence and can interfere with basecalling.

Applicable Kit

The purification procedures provided in this section are applicable to the BigDye® Terminator v3.1 Cycle Sequencing Kit.

Purification Methods

For BigDye Terminator v3.1 chemistry, three methods for preparing extension products for electrophoresis are presented to offer a choice of reagents and processes, as shown in the table below. We recommend performing controlled reactions with each method first in order to determine the one that works best for you.

Purification Method	See Page
Ethanol/EDTA Precipitation Method for BigDye Terminator v3.1 Chemistry	3-4
Ethanol/EDTA/Sodium Acetate Precipitation Method for BigDye Terminator v3.1 Chemistry	3-10
Spin Plate Purification for BigDye Terminator v3.1 Chemistry	3-16

For More Information

If you would like more information purifying extension products for the BigDye Terminator v3.1 chemistry, refer to the *BigDye® Terminator v3.1 Cycle Sequencing Kit Protocol* (PN 4337035) or *User Bulletin: Using an SDS/Heat Treatment with Spin Columns or 96-Well Spin Plates to Remove Unincorporated Dye Terminators* (PN 4330951).

Ethanol/EDTA Precipitation Method for BigDye Terminator v3.1 Chemistry

For most applications, the ethanol/EDTA precipitation method can give clean data. This method produces consistent signal, while minimizing unincorporated dye terminators.

There are two procedures provided for the ethanol/EDTA precipitation method, as listed below.

Procedure	See Page
Precipitating in 96-Well Reaction Plates	3-5
Precipitating in 384-Well Reaction Plates	3-8

100% Ethanol

IMPORTANT! If you use absolute (100%) ethanol in the ethanol/EDTA precipitation procedures, you must remember that when exposed to air, 100% ethanol absorbs moisture from the air and becomes more dilute over time, resulting in slight variations in concentration.

95% Ethanol

IMPORTANT! If you use 95% ethanol in the ethanol/EDTA precipitation procedures, purchase non-denatured ethanol at this concentration.

Required Equipment

You need the following equipment and reagents for these procedures:

- Variable speed centrifuge with a plate adaptor, capable of reaching at least 1400 $\times g$.
- MicroAmp strip caps or adhesive-backed aluminum foil tape (3M Scotch Tape 431 or 439)

Note: Use of other tapes may result in leakage or contamination of the sample. To contact 3M in the USA, call (800) 364-3577 for a local 3M representative.

Precipitating in 96-Well Reaction Plates

Note: A final 70% ethanol wash step is required to remove residual unincorporated dyes. If salts and unincorporated dyes are not removed from the sequencing reaction, they will compete with the extension fragments during electrokinetic injection and result in weak signals.

To precipitate half- (10 µL) or full-volume (20 µL) reactions in 96-well reaction plates:

1.	Remove the 96-well reaction plate from the thermal cycler.
2.	Remove the cover from the reaction plate.
3.	Prepare the ethanol/EDTA solution. • For half-volume reactions, add the following to each 10 µL reaction in this order: – 2.5 µL of 125 mM EDTA – 30.0 µL of 100% ethanol or – 35.0 µL of non-denatured 95% ethanol • For full-volume reactions, add the following to each 20 µL reaction in this order: – 5.0 µL of 125 mM EDTA – 60.0 µL of 100% ethanol or – 70.0 µL of non-denatured 95% ethanol **WARNING** **CHEMICAL HAZARD**. **Ethanol** is a flammable liquid and vapor. Exposure may cause eye, skin, and upper respiratory tract irritation. Prolonged or repeated contact may dry the skin. Exposure may cause central nervous system depression and liver damage. Keep away from heat, sparks, and flame. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. **WARNING** **CHEMICAL HAZARD. EDTA.** Exposure causes eye irritation. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.
4.	Seal the wells with a piece of 3M Scotch Tape 431 or 439 adhesive-backed aluminum foil tape. Press the foil onto the wells to prevent any leakage. **IMPORTANT!** If you are using heat-seal film (PN 4337570) to cover samples prior to electrokinetic injection, do not use the Optical Adhesive Covers (PN 4311971). The residual glue will interfere with the heat-sealing process.
5.	Invert the reaction plate four times or vortex for 15 sec to mix.
6.	Leave the reaction plate at room temperature for at least 15 min to precipitate the extension products.

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

7.	Place the reaction plate in a centrifuge with a plate adaptor and spin at the maximum speed, which must be ≥1400 × *g* but <3000 × *g*: • 1400 to 2000 × *g*: 45 min • 2000 to 3000 × *g*: 30 min **Note:** The reaction plate can withstand 3000 × *g* for 30 min. **IMPORTANT!** Proceed to the next step immediately. If this is not possible, then spin the reaction plate for an additional 2 min immediately before performing the next step.
8.	Discard the supernatant as follows: a. Without disturbing the precipitates, remove the adhesive tape. b. Invert the reaction plate onto a paper towel folded to the size of the plate. c. Place the inverted reaction plate and paper towel into the centrifuge and spin up to 185 × *g*. Then remove from the centrifuge. **IMPORTANT!** The supernatants must be removed completely, as unincorporated dye terminators are dissolved in them. The more residual supernatant left in the wells, the more unincorporated dye terminators remain in the samples.
9.	Perform a 70% wash. • For half-volume reactions (10 µL), add 30 µL of 70% ethanol to each pellet. • For full-volume reactions (20 µL), add 60 µL of 70% ethanol to each pellet. **WARNING** **CHEMICAL HAZARD**. **Ethanol** is a flammable liquid and vapor. Exposure may cause eye, skin, and upper respiratory tract irritation. Prolonged or repeated contact may dry the skin. Exposure may cause central nervous system depression and liver damage. Keep away from heat, sparks, and flame. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.
10.	Seal the wells as in step 4, then invert the reaction plate a few times or vortex for 15 sec to mix. **IMPORTANT!** If you are using heat-seal film (PN 4337570) to cover samples prior to electrokinetic injection, do not use the Optical Adhesive Covers (PN 4311971). The residual glue will interfere with the heat-sealing process.
11.	Place the reaction plate in the centrifuge and spin for 15 min. at 1650 × *g*. **IMPORTANT!** Proceed to the next step immediately. If this is not possible, then spin the reaction plate for 2 min immediately before performing the next step.

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

12.	Repeat step 8. Except in step c, place the inverted reaction plate and paper towel into the centrifuge and spin up to 185 × *g* for 1 min. Then remove from the centrifuge. **Note:** Start timing when the rotor begins to move.
13.	Remove the reaction plate from the centrifuge and discard the paper towel. **IMPORTANT!** Make sure the wells are dry. Use a Speed-Vac for 15 min. to dry the plate. **IMPORTANT!** Make sure the samples are protected from light while they are drying.
14.	Proceed to Chapter 4, "Preparing for Electrophoresis," if you are loading the samples immediately on the 3730/3730xl DNA Analyzer. Otherwise, seal the wells as in step 4 for storage and keep in the dark at −15 °C to −25 °C.

Precipitating in 384-Well Reaction Plates

IMPORTANT! When you use the procedure below with 95% ethanol, the final precipitation volume is greater than 40.0 µL. The maximum volume that each well in a 384-well plate holds depends on the type of plate. If the type of 384-well plate you use will overflow with the 95% ethanol procedure, Applied Biosystems recommends that you use the procedure for 100% ethanol or use a plate type that has a larger volume capacity.

To precipitate half-volume (10 µL) reactions in 384-well plates:

1.	Remove the 384-well reaction plate from the thermal cycler.
2.	Remove the seal from the reaction plate.
3.	Add 2.5 µL of 125 mM EDTA to each half-volume reaction (10 µL) and mix. **CAUTION** **CHEMICAL HAZARD. EDTA** may cause eye, skin, and respiratory tract irritation. Please read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.
4.	Prepare the ethanol/EDTA solution by adding to each half-volume reaction: • 25 µL of 100% ethanol or • 30 µL of non-denatured 95% ethanol **WARNING** **CHEMICAL HAZARD**. **Ethanol** is a flammable liquid and vapor. Exposure may cause eye, skin, and upper respiratory tract irritation. Prolonged or repeated contact may dry the skin. Exposure may cause central nervous system depression and liver damage. Keep away from heat, sparks, and flame. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.
5.	Seal the wells with a piece of 3M Scotch Tape 431 or 439 adhesive-backed aluminum foil tape. Press the foil onto the wells to prevent any leakage.
6.	Invert the reaction plate four times or vortex for 15 sec to mix.
7.	Leave the reaction plate at room temperature for at least 15 min to precipitate the extension products.
8.	Place the reaction plate in a centrifuge with a plate adaptor and spin at the maximum speed, which must be ≥1400 × *g* but <3000 × *g*: • 1400 to 2000 × *g*: 45 min • 2000 to 3000 × *g*: 30 min **Note:** The reaction plate can withstand 3000 × *g* for 30 min. **IMPORTANT!** Proceed to the next step immediately. If this is not possible, then spin the reaction plate for an additional 2 min immediately before performing the next step.

To precipitate half-volume (10 µL) reactions in 384-well plates: *(continued)*

9.	Discard the supernatant as follows: a. Without disturbing the precipitates, remove the adhesive tape. b. Invert the reaction plate onto a paper towel folded to the size of the plate. c. Place the inverted reaction plate and paper towel into the centrifuge and spin up to 185 × *g*. **IMPORTANT!** The supernatants must be removed completely, as unincorporated dye terminators are dissolved in them. The more residual supernatant left in the wells, the more unincorporated dye terminators will remain in the samples.
10.	Remove the reaction plate from the centrifuge and discard the paper towel. **Note:** Pellets may or may not be visible.
11.	To avoid residual terminator peaks, before drying: a. Rinse the pellets with 30 µL of 70% ethanol. b. Seal the wells as in step 5, then invert the reaction plate a few times or vortex for 15 sec to mix. c. Place the reaction plate in the centrifuge and spin for 15 min at the same speed you used in step 8 above. **Note:** The reaction plate can withstand 3000 × *g* for 30 min. d. Repeat step 9.
12.	Dry the samples by: • Placing in a Speed-Vac for 15 min or • Air drying at room temperature for 1 h **IMPORTANT!** Make sure the samples are protected from light while they are drying.
13.	Proceed to Chapter 4, “Preparing for Electrophoresis,” if you are loading the samples immediately on the 3730/3730xl DNA Analyzer. Otherwise, seal the wells as in step 4 for storage and keep in the dark at –15 °C to –25 °C.

Ethanol/EDTA/Sodium Acetate Precipitation Method for BigDye Terminator v3.1 Chemistry

For most applications, the ethanol/EDTA/sodium acetate precipitation method can give clean data. This method produces consistent signal, while minimizing unincorporated dye terminators.

Ethanol/EDTA/sodium acetate precipitation is recommended when good signal from base 1 of a sequence product is required. 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.

There are two procedures provided for the ethanol/EDTA/sodium acetate precipitation method, as listed below.

Procedure	See Page
Precipitating in 96-Well Reaction Plates	3-11
Precipitating in 384-Well Reaction Plates	3-14

100% Ethanol

IMPORTANT! If you use absolute (100%) ethanol in the ethanol/EDTA precipitation procedures, you must remember that when exposed to air, 100% ethanol absorbs moisture from the air and becomes more dilute over time, resulting in slight variations in concentration.

95% Ethanol

IMPORTANT! If you use 95% ethanol in the ethanol/EDTA precipitation procedures, purchase non-denatured ethanol at this concentration.

Required Equipment

You will need the following equipment and reagents for these procedures:

- Variable speed centrifuge with a plate adaptor, capable of reaching at least 1400 × *g*.
- MicroAmp strip caps or adhesive-backed aluminum foil tape (3M Scotch Tape 431 or 439)

Note: Use of other tapes may result in leakage or contamination of the sample. To contact 3M in the USA, call (800) 364-3577 for a local 3M representative.

Precipitating in 96-Well Reaction Plates

Note: A final 70% ethanol wash step is required to remove residual unincorporated dyes. If salts and unincorporated dyes are not removed from the sequencing reaction, they will compete with the extension fragments during electrokinetic injection and result in weak signals.

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

1.	Remove the 96-well reaction plate from the thermal cycler.
2.	Remove the cover from the reaction plate.
3.	Prepare the ethanol/EDTA/sodium acetate solution. • For half-volume reactions, add the following to each 10 µL reaction in this order: – 1.0 µL of 125 mM EDTA – 1.0 µL of 3 M sodium acetate, pH 4.6 – 25.0 µL of 100% ethanol or – 29.0 µL of non-denatured 95% ethanol • For full-volume reactions, add the following to each 20 µL reaction in this order: – 2.0 µL of 125 mM EDTA – 2.0 µL of 3 M sodium acetate, pH 4.6 – 50.0 µL of 100% ethanol or – 58.0 µL of non-denatured 95% ethanol **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 buffer, pH 4.6,** causes 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 may cause eye, skin, and upper respiratory tract irritation. Prolonged or repeated contact may dry the skin. Exposure may cause central nervous system depression and liver damage. Keep away from heat, sparks, and flame. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

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

4.	Seal the wells with a piece of 3M Scotch Tape 431 or 439 adhesive-backed aluminum foil tape. Press the foil onto the wells to prevent any leakage. **IMPORTANT!** If you are using heat-seal film (PN 4337570) to cover samples prior to electrokinetic injection, do not use the Optical Adhesive Covers (PN 4311971). The residual glue will interfere with the heat-sealing process.
5.	Invert the reaction plate four times or vortex for 15 sec to mix.
6.	Leave the reaction plate at room temperature for at least 15 min to precipitate the extension products.
7.	Place the reaction plate in a centrifuge with a plate adaptor and spin at the maximum speed, which must be ≥1400 × *g* but <3000 × *g*: • 1400 to 2000 × *g*: 45 min • 2000 to 3000 × *g*: 30 min **Note:** The reaction plate can withstand 3000 × *g* for 30 min. **IMPORTANT!** Proceed to the next step immediately. If this is not possible, then spin the reaction plate for an additional 2 min immediately before performing the next step.
8.	Discard the supernatant as follows: a. Without disturbing the precipitates, remove the adhesive tape. b. Invert the reaction plate onto a paper towel folded to the size of the plate. c. Place the inverted reaction plate and paper towel into the centrifuge and spin up to 185 × *g*. Then remove from the centrifuge. **IMPORTANT!** The supernatants must be removed completely, as unincorporated dye terminators are dissolved in them. The more residual supernatant left in the wells, the more unincorporated dye terminators will remain in the samples.
9.	Perform a 70% wash. • For half-volume reactions (10 µL), add 35 µL of 70% ethanol to each pellet. • For full-volume reactions (20 µL), add 70 µL of 70% ethanol to each pellet. **WARNING** **CHEMICAL HAZARD**. **Ethanol** is a flammable liquid and vapor. Exposure may cause eye, skin, and upper respiratory tract irritation. Prolonged or repeated contact may dry the skin. Exposure may cause central nervous system depression and liver damage. Keep away from heat, sparks, and flame. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

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

10.	Seal the wells as in step 4, then invert the reaction plate a few times or vortex for 15 sec to mix. **IMPORTANT!** If you are using heat-seal film (PN 4337570) to cover samples prior to electrokinetic injection, do not use the Optical Adhesive Covers (PN 4311971). The residual glue will interfere with the heat-sealing process.
11.	Place the reaction plate in the centrifuge and spin for 15 min. at 1650 × *g*.
12.	Repeat step 8.
13.	Remove the reaction plate from the centrifuge and discard the paper towel. **Note:** Pellets may or may not be visible. Vacuum drying of the samples is not necessary.
14.	Proceed to Chapter 4, "Preparing for Electrophoresis," if you are loading the samples immediately on the 3730/3730xl DNA Analyzer. Otherwise, seal the wells as in step 4 for storage and keep in the dark at – 15 °C to –25 °C.

Precipitating in 384-Well Reaction Plates

IMPORTANT! When you use the procedure below with 95% ethanol, the final precipitation volume is greater than 40.0 µL. The maximum volume that each well in a 384-well plate holds depends on the type of plate. If the type of 384-well plate you use will overflow with the 95% ethanol procedure, Applied Biosystems recommends that you use the procedure for 100% ethanol or use a plate type that has a larger volume capacity.

To precipitate half-volume (10 µL) reactions in 384-well plates:

1.	Remove the 384-well reaction plate from the thermal cycler.
2.	Remove the seal from the reaction plate.
3.	Prepare the ethanol/EDTA/sodium acetate solution by adding to each half-volume reaction the following in this order: • 1 µL of 125 mM EDTA • 1 µL of 3 M sodium acetate, pH 4.6 • 25 µL of 100% ethanol or • 29 µL of non-denatured 95% ethanol **WARNING** **CHEMICAL HAZARD. EDTA** may cause eye, skin, and respiratory tract irritation. Please read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. **WARNING** **CHEMICAL HAZARD. 3 M Sodium Acetate buffer, pH 4.6,** causes 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 may cause eye, skin, and upper respiratory tract irritation. Prolonged or repeated contact may dry the skin. Exposure may cause central nervous system depression and liver damage. Keep away from heat, sparks, and flame. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.
4.	Seal the wells with a piece of 3M Scotch Tape 431 or 439 adhesive-backed aluminum foil tape. Press the foil onto the wells to prevent any leakage.
5.	Invert the reaction plate a few times or vortex for 15 sec to mix.
6.	Leave the reaction plate at room temperature for at least 15 min to precipitate the extension products.

To precipitate half-volume (10 µL) reactions in 384-well plates: *(continued)*

7.	Place the reaction plate in a centrifuge with a plate adaptor and spin at the maximum speed, which must be ≥1400 × *g* but <3000 × *g*: • 1400 to 2000 × *g*: 45 min • 2000 to 3000 × *g*: 30 min **Note:** The reaction plate can withstand 3000 × *g* for 30 min. **IMPORTANT!** Proceed to the next step immediately. If this is not possible, then spin the reaction plate for an additional 2 min immediately before performing the next step.
8.	Discard the supernatant as follows: a. Without disturbing the precipitates, remove the adhesive tape. b. Invert the reaction plate onto a paper towel folded to the size of the plate. c. Place the inverted reaction plate and paper towel into the centrifuge and spin up to 185 × *g*. **IMPORTANT!** The supernatants must be removed completely, as unincorporated dye terminators are dissolved in them. The more residual supernatant left in the wells, the more unincorporated dye terminators will remain in the samples.
9.	Remove the reaction plate from the centrifuge and discard the paper towel. **Note:** Pellets may or may not be visible.
10.	To avoid residual terminator peaks, before drying: a. Rinse the pellets with 35 µL of 70% ethanol. b. Seal the wells as in step 4, then invert the reaction plate a few times or vortex for 15 sec to mix. c. Place the reaction plate in the centrifuge. Spin at 1650 × *g* for 15 min. **Note:** The reaction plate can withstand 3000 × *g* for 30 min. d. Repeat step 8.
11.	Dry the samples by: • Placing in a Speed-Vac for 15 min or • Air drying at room temperature for 1 h **IMPORTANT!** Make sure the samples are protected from light while they are drying.
12.	Proceed to Chapter 4, “Preparing for Electrophoresis,” if you are loading the samples immediately on the 3730/3730xl DNA Analyzer. Otherwise, seal the wells as in step 4 for storage and keep in the dark at −15 °C to −25 °C.

Spin Plate Purification for BigDye Terminator v3.1 Chemistry

About Spin Plate Purification

The efficiency of removing all excess dye terminators depends on the spin plate purification kit. There are several commercially available purification kits. If not used properly, these commercially available kits can lead to dye blobs appearing in the sequencing data. To remove excess dye terminators efficiently when mixing spin plates, Applied Biosystems recommends the following protocol.

Preparing Extension Products with SDS/Heat Treatment

Use this procedure to prepare extension products for 96-well spin plate purification.

To prepare extension products:

1.	Prepare 2.2% SDS (sodium dodecyl sulfate) in deionized water. This SDS solution is stable at room temperature. **WARNING CHEMICAL HAZARD. Sodium dodecyl sulfate (SDS)** may cause an allergic respiratory reaction. It is harmful if inhaled, swallowed, or absorbed through the skin. Exposure causes eye, skin, and respiratory tract irritation. Please read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.
2.	Add an appropriate amount of the 2.2% SDS solution to your sample to bring the final concentration of SDS to 0.2%. For example: Add 2 µL of 2.2% SDS to each 20-µL completed cycle sequencing reaction.
3.	Seal the tubes with caps and mix thoroughly.
4.	Heat the tubes to 98 °C for 5 minutes, then allow the tubes to cool to ambient temperature before proceeding to the next step. A convenient way to perform this heating/cooling cycle is to place the tubes in a thermal cycler and set the thermal cycler as follows: 98 °C for 5 minutes 25 °C for 10 minutes
5.	Spin down the contents briefly.
6.	Continue with the manufacturer's recommended protocol for spin plate purification.

Recommended Spin Plates

For large-scale procedures, you can use the commercially available spin plates listed below, or an equivalent one of your choice.

- 96-Well spin plates, Gel Filtration Kit (Edge Biosystems, PN 94880)

IMPORTANT! When using the Edge Biosystems gel filtration kit, centrifuge at 850 × *g* for 2 min.

Section 3.2 Purifying the Extension Products: BigDye Terminator v3.0 Chemistry

About Purification for BigDye Terminator v3.0 Chemistry

Unincorporated dye terminators must be completely removed before the samples can be analyzed by electrophoresis. Excess dye terminators in sequencing reactions obscure data in the early part of the sequence and can interfere with basecalling.

Applicable Kit

The purification procedures provided in this section are applicable to the BigDye® Terminator v3.0 Cycle Sequencing Kit.

Purification Methods

For BigDye Terminator v3.0 chemistry, four methods for preparing extension products for electrophoresis are presented to offer a choice of reagents and processes, as shown in the table below. We recommend performing controlled reactions with each method first in order to determine the one that works best for you.

Purification Method	See Page
Ethanol/Sodium Acetate Precipitation Method for BigDye Terminator v3.0 Chemistry	3-20
Ethanol/EDTA Precipitation Method for BigDye Terminator v3.0 Chemistry	3-27
Ethanol/EDTA/Sodium Acetate Precipitation Method for BigDye Terminator v3.0 Chemistry	3-33
Spin Plate Purification for BigDye Terminator v3.0 Chemistry	3-39

For More Information

If you would like more information purifying extension products for the BigDye Terminator v3.0 chemistry, refer to *User Bulletin: Precipitation Method to Remove Unincorporated Dye Terminators from ABI PRISM® BigDye® Terminator v3.0 Cycle Sequencing Reactions* (PN 4333020) or *User Bulletin: Using an SDS/Heat Treatment with Spin Columns or 96-Well Spin Plates to Remove Unincorporated Dye Terminators* (PN 4330951).

Ethanol/Sodium Acetate Precipitation Method for BigDye Terminator v3.0 Chemistry

For most applications, the ethanol/sodium acetate precipitation method can give clean data. This method produces consistent signal, while minimizing unincorporated dye terminators.

While the ethanol/sodium acetate precipitation method produces a clean sequence (with a low amount of residual dye-terminator carryover), it may also remove more of the small molecular weight fragments.

There are two procedures provided for the ethanol/sodium acetate precipitation method, as listed below.

100% Ethanol

IMPORTANT! If you use absolute (100%) ethanol in the ethanol/EDTA precipitation procedures, you must remember that when exposed to air, 100% ethanol absorbs moisture from the air and becomes more dilute over time, resulting in slight variations in concentration.

95% Ethanol

IMPORTANT! If you use 95% ethanol in the ethanol/EDTA precipitation procedures, purchase non-denatured ethanol at this concentration.

Required Equipment

You will need the following equipment and reagents for these procedures:

- Variable speed centrifuge with a plate adaptor, capable of reaching at least $1400 \times g$.
- MicroAmp strip caps or adhesive-backed aluminum foil tape (3M Scotch Tape 431 or 439)

Note: Use of other tapes may result in leakage or contamination of the sample. To contact 3M in the USA, call (800) 364-3577 for a local 3M representative.

Precipitating in 96-Well Reaction Plates

Note: A final 70% ethanol wash step is required to remove residual unincorporated dyes. If salts and unincorporated dyes are not removed from the sequencing reaction, they will compete with the extension fragments during electrokinetic injection and result in weak signals.

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

1.	Remove the 96-well reaction plate from the thermal cycler.
2.	Remove the cover from the reaction plate.
3.	Prepare the ethanol/sodium acetate solution. • To prepare the ethanol/sodium acetate solution for half-volume reactions (10 µL), combine the following for each reaction: – 1.5 µL of 3 M sodium acetate, pH 4.6 – 31.25 µL of non-denatured 95% ethanol – 7.25 µL of deionized water The final volume should be 40 µL for each sample. • To prepare the ethanol/sodium acetate solution for full-volume reactions (20 µL), combine the following for each reaction: – 3.0 µL of 3 M sodium acetate, pH 4.6 – 62.5 µL of non-denatured 95% ethanol – 14.5 µL of deionized water The final volume should be 80 µL for each sample. **WARNING** **CHEMICAL HAZARD. 3 M Sodium Acetate buffer, pH 4.6,** causes 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 may cause eye, skin, and upper respiratory tract irritation. Prolonged or repeated contact may dry the skin. Exposure may cause central nervous system depression and liver damage. Keep away from heat, sparks, and flame. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.
4.	Add the ethanol/sodium acetate solution to each reaction. • For half-volume reactions (10 µL), add the following to each reaction: 40 µL of ethanol/sodium acetate solution (created in step 3 above). • For full-volume reactions (20 µL), add the following to each reaction: 80 µL of ethanol/sodium acetate solution (created in step 3 above).

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

5.	Seal the wells with a piece of 3M Scotch Tape 431 or 439 adhesive-backed aluminum foil tape. Press the foil onto the wells to prevent any leakage. **IMPORTANT!** If you are using heat-seal film (PN 4337570) to cover samples prior to electrokinetic injection, do not use the Optical Adhesive Covers (PN 4311971). The residual glue will interfere with the heat-sealing process.
6.	Invert the reaction plate a few times or vortex for 15 sec to mix.
7.	Leave the reaction plate at room temperature for at least 15 min to precipitate the extension products. **Note:** Precipitation times <15 min will result in the loss of very short extension products. Precipitation times >24 h will increase the precipitation of unincorporated dye terminators.
8.	Place the reaction plate in a centrifuge with a plate adaptor and spin at the maximum speed, which must be ≥1400 × *g* but <3000 × *g*: • 1400 to 2000 × *g*: 45 min • 2000 to 3000 × *g*: 30 min **Note:** The reaction plate can withstand 3000 × *g* for 30 min. **IMPORTANT!** Proceed to the next step immediately. If this is not possible, then spin the reaction plate for an additional 2 min immediately before performing the next step.
9.	Discard the supernatant as follows: a. Without disturbing the precipitates, remove the adhesive tape. b. Invert the reaction plate onto a paper towel folded to the size of the plate. c. Place the inverted reaction plate and paper towel into the centrifuge and spin at 50 × *g* for 1 min. **Note:** The reaction plate can withstand 3000 × *g* for 30 min. **IMPORTANT!** The supernatants must be removed completely, as unincorporated dye terminators are dissolved in them. The more residual supernatant left in the wells, the more unincorporated dye terminators will remain in the samples.

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

10.	Perform a 70% wash. • For half-volume reactions (10 µL), add 75 µL of 70% ethanol to each pellet. • For full-volume reactions (20 µL), add 150 µL of 70% ethanol to each pellet. **WARNING** **CHEMICAL HAZARD**. **Ethanol** is a flammable liquid and vapor. Exposure may cause eye, skin, and upper respiratory tract irritation. Prolonged or repeated contact may dry the skin. Exposure may cause central nervous system depression and liver damage. Keep away from heat, sparks, and flame. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.
11.	Seal the wells as in step 5, then invert the reaction plate a few times or vortex for 15 sec to mix. **IMPORTANT!** If you are using heat-seal film (PN 4337570) to cover samples prior to electrokinetic injection, do not use the Optical Adhesive Covers (PN 4311971). The residual glue will interfere with the heat-sealing process.
12.	Place the reaction plate in the centrifuge and spin for 10 min. at the same speed you used in step 8 above. **Note:** The reaction plate can withstand 3000 × g for 30 min.
13.	Repeat step 9.
14.	Remove the reaction plate from the centrifuge and discard the paper towel. **Note:** Pellets may or may not be visible. Vacuum drying of the samples is not necessary.
15.	Proceed to Chapter 4, “Preparing for Electrophoresis,” if you are loading the samples immediately on the 3730/3730xl DNA Analyzer. Otherwise, seal the wells as in step 4 for storage and keep in the dark at −15 °C to −25 °C.

Precipitating in 384-Well Reaction Plates

This is the recommended protocol for precipitating samples in 384-well reaction plates. This protocol uses 10 µL of reaction per well, which ensures you will not exceed the volume capacity of the 384-well reaction plates.

Note: A final 70% ethanol wash is optional.

To precipitate half-volume (10 µL) reactions in 384-well plates:

1.	Remove the 384-well reaction plate from the thermal cycler.
2.	Remove the seal from the reaction plate.
3.	Add 1 µL of 250 mM EDTA to each half-volume reaction (10 µL) and mix. **CAUTION** **CHEMICAL HAZARD. EDTA** may cause eye, skin, and respiratory tract irritation. Please read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.
4.	Prepare the ethanol/sodium acetate solution by combining the following for each reaction: • 1 µL of 3 M sodium acetate, pH 4.6 • 23 µL of non-denatured 95% ethanol • 1 µL of deionized water The final ethanol concentration should be 62%. **CAUTION** **CHEMICAL HAZARD. 3 M Sodium Acetate buffer, pH 4.6,** causes 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 may cause eye, skin, and upper respiratory tract irritation. Prolonged or repeated contact may dry the skin. Exposure may cause central nervous system depression and liver damage. Keep away from heat, sparks, and flame. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.
5.	Add 25 µL of ethanol/sodium acetate solution (created in step 4 above) to each 11-µL reaction/EDTA mixture. The final reaction volume should be 36 µL for each sample.
6.	Seal the wells with a piece of 3M Scotch Tape 431 or 439 adhesive-backed aluminum foil tape. Press the foil onto the wells to prevent any leakage.
7.	Invert the reaction plate a few times or vortex for 15 sec to mix.

To precipitate half-volume (10 µL) reactions in 384-well plates: *(continued)*

8.	Leave the reaction plate at room temperature for at least 15 min to precipitate the extension products. **Note:** Precipitation times <15 min will result in the loss of very short extension products. Precipitation times >24 h will increase the precipitation of unincorporated dye terminators.
9.	Place the reaction plate in a centrifuge with a plate adaptor and spin at the maximum speed, which must be ≥1400 × g but <3000 × g: • 1400 to 2000 × g: 45 min • 2000 to 3000 × g: 30 min **Note:** The reaction plate can withstand 3000 × g for 30 min. **IMPORTANT!** Proceed to the next step immediately. If this is not possible, then spin the reaction plate for an additional 2 min immediately before performing the next step.
10.	Discard the supernatant as follows: a. Without disturbing the precipitates, remove the adhesive tape. b. Invert the reaction plate onto a paper towel folded to the size of the plate. c. Place the inverted reaction plate and paper towel into the centrifuge and spin at 20 × g for 1 min. **IMPORTANT!** The supernatants must be removed completely, as unincorporated dye terminators are dissolved in them. The more residual supernatant left in the wells, the more unincorporated dye terminators will remain in the samples.
11.	Remove the reaction plate from the centrifuge and discard the paper towel. **Note:** Pellets may or may not be visible.
12.	*Optional*. To avoid residual terminator peaks, before drying: a. Rinse the pellets with 35 µL of 70% ethanol. b. Seal the wells as in step 6, then invert the reaction plate a few times or vortex for 15 sec to mix. c. Place the reaction plate in the centrifuge and spin for 10 min. at the same speed you used in step 9 above. **Note:** The reaction plate can withstand 3000 × g for 30 min. d. Repeat step 10.

To precipitate half-volume (10 µL) reactions in 384-well plates: *(continued)*

13.	Dry the samples by: • Placing in a Speed-Vac for 15 min, or • Air drying at room temperature for 1 h **IMPORTANT!** Make sure the samples are protected from light while they are drying.
14.	Proceed to Chapter 4, "Preparing for Electrophoresis," if you are loading the samples immediately on the 3730/3730xl DNA Analyzer. Otherwise, seal the wells as in step 4 for storage and keep in the dark at −15 °C to −25 °C.

Ethanol/EDTA Precipitation Method for BigDye Terminator v3.0 Chemistry

For most applications, the ethanol/EDTA precipitation method can give clean data. This method produces consistent signal, while minimizing unincorporated dye terminators.

There are two procedures provided for the ethanol/EDTA precipitation method, as listed below.

Procedure	See Page
Precipitating in 96-Well Reaction Plates	3-28
Precipitating in 384-Well Reaction Plates	3-31

100% Ethanol

IMPORTANT! If you use absolute (100%) ethanol in the ethanol/EDTA precipitation procedures, you must remember that when exposed to air, 100% ethanol absorbs moisture from the air and becomes more dilute over time, resulting in slight variations in concentration.

95% Ethanol

IMPORTANT! If you use 95% ethanol in the ethanol/EDTA precipitation procedures, purchase non-denatured ethanol at this concentration.

Required Equipment

You need the following equipment and reagents for these procedures:

- Variable speed centrifuge with a plate adaptor, capable of reaching at least 1400 $\times g$.
- MicroAmp strip caps or adhesive-backed aluminum foil tape (3M Scotch Tape 431 or 439)

Note: Use of other tapes may result in leakage or contamination of the sample. To contact 3M in the USA, call (800) 364-3577 for a local 3M representative.

Precipitating in 96-Well Reaction Plates

Note: A final 70% ethanol wash step is required to remove residual unincorporated dyes. If salts and unincorporated dyes are not removed from the sequencing reaction, they will compete with the extension fragments during electrokinetic injection and result in weak signals.

To precipitate half- (10 µL) or full-volume (20 µL) reactions in 96-well reaction plates:

1.	Remove the 96-well reaction plate from the thermal cycler.
2.	Remove the cover from the reaction plate.
3.	Prepare the ethanol/EDTA solution. • For half-volume reactions, add the following to each 10 µL reaction in this order: – 2.5 µL of 125 mM EDTA – 30.0 µL of 100% ethanol or – 35.0 µL of non-denatured 95% ethanol • For full-volume reactions, add the following to each 20 µL reaction in this order: – 5.0 µL of 125 mM EDTA – 60.0 µL of 100% ethanol or – 70.0 µL of non-denatured 95% ethanol **WARNING** **CHEMICAL HAZARD**. **Ethanol** is a flammable liquid and vapor. Exposure may cause eye, skin, and upper respiratory tract irritation. Prolonged or repeated contact may dry the skin. Exposure may cause central nervous system depression and liver damage. Keep away from heat, sparks, and flame. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. **WARNING** **CHEMICAL HAZARD. EDTA**. Exposure causes eye irritation. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.
4.	Seal the wells with a piece of 3M Scotch Tape 431 or 439 adhesive-backed aluminum foil tape. Press the foil onto the wells to prevent any leakage. **IMPORTANT!** If you are using heat-seal film (PN 4337570) to cover samples prior to electrokinetic injection, do not use the Optical Adhesive Covers (PN 4311971). The residual glue will interfere with the heat-sealing process.
5.	Invert the reaction plate a four times or vortex for 15 sec to mix.
6.	Leave the reaction plate at room temperature for at least 15 min to precipitate the extension products.

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

7.	Place the reaction plate in a centrifuge with a plate adaptor and spin at the maximum speed, which must be ≥1400 × *g* but <3000 × *g*: • 1400 to 2000 × *g*: 45 min • 2000 to 3000 × *g*: 30 min **Note:** The reaction plate can withstand 3000 × *g* for 30 min. **IMPORTANT!** Proceed to the next step immediately. If this is not possible, then spin the reaction plate for an additional 2 min immediately before performing the next step.
8.	Discard the supernatant as follows: a. Without disturbing the precipitates, remove the adhesive tape. b. Invert the reaction plate onto a paper towel folded to the size of the plate. c. Place the inverted reaction plate and paper towel into the centrifuge and spin up to 185 × *g*. Then remove from the centrifuge. **IMPORTANT!** The supernatants must be removed completely, as unincorporated dye terminators are dissolved in them. The more residual supernatant left in the wells, the more unincorporated dye terminators remain in the samples.
9.	Perform a 70% wash. • For half-volume reactions (10 µL), add 30 µL of 70% ethanol to each pellet. • For full-volume reactions (20 µL), add 60 µL of 70% ethanol to each pellet. **WARNING** **CHEMICAL HAZARD**. **Ethanol** is a flammable liquid and vapor. Exposure may cause eye, skin, and upper respiratory tract irritation. Prolonged or repeated contact may dry the skin. Exposure may cause central nervous system depression and liver damage. Keep away from heat, sparks, and flame. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.
10.	Seal the wells as in step 4, then invert the reaction plate a few times or vortex for 15 sec to mix. **IMPORTANT!** If you are using heat-seal film (PN 4337570) to cover samples prior to electrokinetic injection, do not use the Optical Adhesive Covers (PN 4311971). The residual glue will interfere with the heat-sealing process.
11.	Place the reaction plate in the centrifuge and spin for 15 min. at 1650 × *g*. **IMPORTANT!** Proceed to the next step immediately. If this is not possible, then spin the reaction plate for an additional 2 min immediately before performing the next step.

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

12.	Repeat step 8. Except in step c, place the inverted reaction plate and paper towel into the centrifuge and spin up to 185 × *g* for 1 min. Then remove from the centrifuge. **Note:** Start timing when the rotor begins to move.
13.	Remove the reaction plate from the centrifuge and discard the paper towel. **IMPORTANT!** Make sure the wells are dry. Use a Speed-Vac for 15 min to dry the plate. **IMPORTANT!** Make sure the samples are protected from light while they are drying.
14.	Proceed to Chapter 4, "Preparing for Electrophoresis," if you are loading the samples immediately on the 3730/3730xl DNA Analyzer. Otherwise, seal the wells as in step 4 for storage and keep in the dark at −15 °C to −25 °C.

Precipitating in 384-Well Reaction Plates

IMPORTANT! When you use the procedure below with 95% ethanol, the final precipitation volume is greater than 40.0 µL. The maximum volume that each well in a 384-well plate holds depends on the type of plate. If the type of 384-well plate you use will overflow with the 95% ethanol procedure, Applied Biosystems recommends that you use the procedure for 100% ethanol or use a plate type that has a larger volume capacity.

To precipitate half-volume (10 µL) reactions in 384-well plates:

1.	Remove the 384-well reaction plate from the thermal cycler.
2.	Remove the seal from the reaction plate.
3.	Add 2.5 µL of 125 mM EDTA to each half-volume reaction (10 µL) and mix. **CAUTION** **CHEMICAL HAZARD. EDTA** may cause eye, skin, and respiratory tract irritation. Please read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.
4.	Prepare the ethanol/sodium acetate solution by adding to each half-volume reaction: • 25 µL of 100% ethanol or • 30 µL of non-denatured 95% ethanol **WARNING** **CHEMICAL HAZARD**. **Ethanol** is a flammable liquid and vapor. Exposure may cause eye, skin, and upper respiratory tract irritation. Prolonged or repeated contact may dry the skin. Exposure may cause central nervous system depression and liver damage. Keep away from heat, sparks, and flame. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.
5.	Seal the wells with a piece of 3M Scotch Tape 431 or 439 adhesive-backed aluminum foil tape. Press the foil onto the wells to prevent any leakage.
6.	Invert the reaction plate four times or vortex for 15 sec to mix.
7.	Leave the reaction plate at room temperature for at least 15 min to precipitate the extension products.
8.	Place the reaction plate in a centrifuge with a plate adaptor and spin at the maximum speed, which must be ≥1400 × *g* but <3000 × *g*: • 1400 to 2000 × *g*: 45 min • 2000 to 3000 × *g*: 30 min **Note:** The reaction plate can withstand 3000 × *g* for 30 min. **IMPORTANT!** Proceed to the next step immediately. If this is not possible, then spin the reaction plate for an additional 2 min immediately before performing the next step.

To precipitate half-volume (10 µL) reactions in 384-well plates: *(continued)*

9.	Discard the supernatant as follows: a. Without disturbing the precipitates, remove the adhesive tape. b. Invert the reaction plate onto a paper towel folded to the size of the plate. c. Place the inverted reaction plate and paper towel into the centrifuge and spin up to 185 × *g*. **IMPORTANT!** The supernatants must be removed completely, as unincorporated dye terminators are dissolved in them. The more residual supernatant left in the wells, the more unincorporated dye terminators will remain in the samples.
10.	Remove the reaction plate from the centrifuge and discard the paper towel. **Note:** Pellets may or may not be visible.
11.	To avoid residual terminator peaks, before drying: a. Rinse the pellets with 30 µL of 70% ethanol. b. Seal the wells as in step 5, then invert the reaction plate a few times or vortex for 15 sec to mix. c. Place the reaction plate in the centrifuge and spin for 15 min at the same speed you used in step 8 above. **Note:** The reaction plate can withstand 3000 × *g* for 30 min. d. Repeat step 9.
12.	Dry the samples by: • Placing in a Speed-Vac for 15 min or • Air drying at room temperature for 1 h **IMPORTANT!** Make sure the samples are protected from light while they are drying.
13.	Proceed to Chapter 4, "Preparing for Electrophoresis," if you are loading the samples immediately on the 3730/3730xl DNA Analyzer. Otherwise, seal the wells as in step 4 for storage and keep in the dark at −15 °C to −25 °C.

Ethanol/EDTA/Sodium Acetate Precipitation Method for BigDye Terminator v3.0 Chemistry

For most applications, the ethanol/EDTA/sodium acetate precipitation method can give clean data. This method produces consistent signal, while minimizing unincorporated dye terminators.

Ethanol/EDTA/sodium acetate precipitation is recommended when good signal from base 1 is required. 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.

There are two procedures provided for the ethanol/EDTA/sodium acetate precipitation method, as listed below.

Procedure	See Page
Precipitating in 96-Well Reaction Plates	3-34
Precipitating in 384-Well Reaction Plates	3-37

100% Ethanol

IMPORTANT! If you use absolute (100%) ethanol in the ethanol/EDTA precipitation procedures, you must remember that when exposed to air, 100% ethanol absorbs moisture from the air and becomes more dilute over time, resulting in slight variations in concentration.

95% Ethanol

IMPORTANT! If you use 95% ethanol in the ethanol/EDTA precipitation procedures, purchase non-denatured ethanol at this concentration.

Required Equipment

You will need the following equipment and reagents for these procedures:

- Variable speed centrifuge with a plate adaptor, capable of reaching at least $1400 \times g$.
- MicroAmp strip caps or adhesive-backed aluminum foil tape (3M Scotch Tape 431 or 439)

Note: Use of other tapes may result in leakage or contamination of the sample. To contact 3M in the USA, call (800) 364-3577 for a local 3M representative.

Precipitating in 96-Well Reaction Plates

Note: A final 70% ethanol wash step is required to remove residual unincorporated dyes. If salts and unincorporated dyes are not removed from the sequencing reaction, they will compete with the extension fragments during electrokinetic injection and result in weak signals.

To precipitate half- (10 µL) or full-volume (20 µL) reactions in 96-well reaction plates:

1.	Remove the 96-well reaction plate from the thermal cycler.
2.	Remove the cover from the reaction plate.
3.	Prepare the ethanol/EDTA/sodium acetate solution. • For half-volume reactions, add the following to each 10 µL reaction in this order: – 1.0 µL of 125 mM EDTA – 1.0 µL of 3 M sodium acetate, pH 4.6 – 25.0 µL of 100% ethanol or – 29.0 µL of non-denatured 95% ethanol • For full-volume reactions, add the following for each 20 µL reaction in this order: – 2.0 µL of 125 mM EDTA – 2.0 µL of 3 M sodium acetate, pH 4.6 – 50.0 µL of 100% ethanol or – 58.0 µL of non-denatured 95% ethanol **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 buffer, pH 4.6,** causes 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 may cause eye, skin, and upper respiratory tract irritation. Prolonged or repeated contact may dry the skin. Exposure may cause central nervous system depression and liver damage. Keep away from heat, sparks, and flame. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

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

4.	Seal the wells with a piece of 3M Scotch Tape 431 or 439 adhesive-backed aluminum foil tape. Press the foil onto the wells to prevent any leakage. **IMPORTANT!** If you are using heat-seal film (PN 4337570) to cover samples prior to electrokinetic injection, do not use the Optical Adhesive Covers (PN 4311971). The residual glue will interfere with the heat-sealing process.
5.	Invert the reaction plate four times or vortex for 15 sec to mix.
6.	Leave the reaction plate at room temperature for at least 15 min to precipitate the extension products.
7.	Place the reaction plate in a centrifuge with a plate adaptor and spin at the maximum speed, which must be ≥1400 × *g* but <3000 × *g*: • 1400 to 2000 × *g*: 45 min • 2000 to 3000 × *g*: 30 min **Note:** The reaction plate can withstand 3000 × *g* for 30 min. **IMPORTANT!** Proceed to the next step immediately. If this is not possible, then spin the reaction plate for 2 min immediately before performing the next step.
8.	Discard the supernatant as follows: a. Without disturbing the precipitates, remove the adhesive tape. b. Invert the reaction plate onto a paper towel folded to the size of the plate. c. Place the inverted reaction plate and paper towel into the centrifuge and spin up to 185 × *g*. Then remove from the centrifuge. **IMPORTANT!** The supernatants must be removed completely, as unincorporated dye terminators are dissolved in them. The more residual supernatant left in the wells, the more unincorporated dye terminators will remain in the samples.
9.	Perform a 70% wash. • For half-volume reactions (10 µL), add 35 µL of 70% ethanol to each pellet. • For full-volume reactions (20 µL), add 70 µL of 70% ethanol to each pellet. **WARNING** **CHEMICAL HAZARD**. **Ethanol** is a flammable liquid and vapor. Exposure may cause eye, skin, and upper respiratory tract irritation. Prolonged or repeated contact may dry the skin. Exposure may cause central nervous system depression and liver damage. Keep away from heat, sparks, and flame. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

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

10.	Seal the wells as in step 4, then invert the reaction plate a few times or vortex for 15 sec to mix. **IMPORTANT!** If you are using heat-seal film (PN 4337570) to cover samples prior to electrokinetic injection, do not use the Optical Adhesive Covers (PN 4311971). The residual glue will interfere with the heat-sealing process.
11.	Place the reaction plate in the centrifuge and spin for 15 min at 1650 × *g*.
12.	Repeat step 8.
13.	Remove the reaction plate from the centrifuge and discard the paper towel. **Note:** Pellets may or may not be visible. Vacuum drying of the samples is not necessary.
14.	Proceed to Chapter 4, "Preparing for Electrophoresis," if you are loading the samples immediately on the 3730/3730xl DNA Analyzer. Otherwise, seal the wells as in step 4 for storage and keep in the dark at –15 °C to –25 °C.

Precipitating in 384-Well Reaction Plates

IMPORTANT! When you use the procedure below with 95% ethanol, the final precipitation volume is greater than 40.0 µL. The maximum volume that each well in a 384-well plate holds depends on the type of plate. If the type of 384-well plate you use will overflow with the 95% ethanol procedure, Applied Biosystems recommends that you use the procedure for 100% ethanol or use a plate type that has a larger volume capacity.

To precipitate half-volume (10 µL) reactions in 384-well plates:

1.	Remove the 384-well reaction plate from the thermal cycler.
2.	Remove the seal from the reaction plate.
3.	Prepare the ethanol/EDTA/sodium acetate solution by adding to each half-volume reaction the following in this order: • 1 µL of 125 mM EDTA • 1 µL of 3 M sodium acetate, pH 4.6 • 25 µL of 100% ethanol or • 29 µL of non-denatured 95% ethanol **WARNING** **CHEMICAL HAZARD. EDTA** may cause eye, skin, and respiratory tract irritation. Please read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. **WARNING** **CHEMICAL HAZARD**. **3 M Sodium Acetate buffer, pH 4.6,** causes 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 may cause eye, skin, and upper respiratory tract irritation. Prolonged or repeated contact may dry the skin. Exposure may cause central nervous system depression and liver damage. Keep away from heat, sparks, and flame. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.
4.	Seal the wells with a piece of 3M Scotch Tape 431 or 439 adhesive-backed aluminum foil tape. Press the foil onto the wells to prevent any leakage.
5.	Invert the reaction plate a few times or vortex for 15 sec to mix.
6.	Leave the reaction plate at room temperature for at least 15 min to precipitate the extension products.

To precipitate half-volume (10 µL) reactions in 384-well plates: *(continued)*

7.	Place the reaction plate in a centrifuge with a plate adaptor and spin at the maximum speed, which must be ≥1400 × *g* but <3000 × *g*: • 1400 to 2000 × *g*: 45 min • 2000 to 3000 × *g*: 30 min **Note:** The reaction plate can withstand 3000 × *g* for 30 min. **IMPORTANT!** Proceed to the next step immediately. If this is not possible, then spin the reaction plate for 2 min immediately before performing the next step.
8.	Discard the supernatant as follows: a. Without disturbing the precipitates, remove the adhesive tape. b. Invert the reaction plate onto a paper towel folded to the size of the plate. c. Place the inverted reaction plate and paper towel into the centrifuge and spin up to 185 × *g*. **IMPORTANT!** The supernatants must be removed completely, as unincorporated dye terminators are dissolved in them. The more residual supernatant left in the wells, the more unincorporated dye terminators will remain in the samples.
9.	Remove the reaction plate from the centrifuge and discard the paper towel. **Note:** Pellets may or may not be visible.
10.	To avoid residual terminator peaks, before drying: a. Rinse the pellets with 35 µL of 70% ethanol. b. Seal the wells as in step 4, then invert the reaction plate a few times or vortex for 15 sec to mix. c. Place the reaction plate in the centrifuge. Spin at 1650 × *g* for 15 min. **Note:** The reaction plate can withstand 3000 × *g* for 30 min. d. Repeat step 8.
11.	Dry the samples by: • Placing in a Speed-Vac for 15 min or • Air drying at room temperature for 1 h **IMPORTANT!** Make sure the samples are protected from light while they are drying.
12.	Proceed to Chapter 4, “Preparing for Electrophoresis,” if you are loading the samples immediately on the 3730/3730xl DNA Analyzer. Otherwise, seal the wells as in step 4 for storage and keep in the dark at −15 °C to −25 °C.

Spin Plate Purification for BigDye Terminator v3.0 Chemistry

About Spin Plate Purification

The efficiency of removing all excess dye terminators depends on the spin plate purification kit. There are several commercially available purification kits. If not used properly, these commercially available kits can lead to dye blobs appearing in the sequencing data. To remove excess dye terminators efficiently when mixing spin plates, Applied Biosystems recommends the following protocol.

Preparing Extension Products with SDS/Heat Treatment

Use this procedure to prepare extension products for 96-well spin plate purification.

To prepare extension products:

1.	Prepare 2.2% SDS (sodium dodecyl sulfate) in deionized water. This SDS solution is stable at room temperature. **WARNING CHEMICAL HAZARD. Sodium dodecyl sulfate (SDS)** may cause an allergic respiratory reaction. It is harmful if inhaled, swallowed, or absorbed through the skin. Exposure causes eye, skin, and respiratory tract irritation. Please read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.
2.	Add an appropriate amount of the 2.2% SDS solution to your sample to bring the final concentration of SDS to 0.2%. For example: Add 2 µL of 2.2% SDS to each 20-µL completed cycle sequencing reaction.
3.	Seal the tubes with caps and mix thoroughly.
4.	Heat the tubes to 98 °C for 5 minutes, then allow the tubes to cool to ambient temperature before proceeding to the next step. A convenient way to perform this heating/cooling cycle is to place the tubes in a thermal cycler and set the thermal cycler as follows: 98 °C for 5 minutes 25 °C for 10 minutes
5.	Spin down the contents briefly.
6.	Continue with the manufacturer's recommended protocol for spin plate purification.

Recommended Spin Plates

For large-scale procedures, you can use the commercially available spin plates listed below, or an equivalent one of your choice.

- 96-Well spin plates, Gel Filtration Kit (Edge Biosystems, PN 94880)

IMPORTANT! When using the Edge Biosystems gel filtration kit, centrifuge at $850 \times g$ for 2 min.

Section 3.3 Purifying the Extension Products: BigDye Terminator v1.1 Chemistry

About Purification for BigDye Terminator v1.1 Chemistry

Unincorporated dye terminators must be completely removed before the samples can be analyzed by electrophoresis. Excess dye terminators in sequencing reactions obscure data in the early part of the sequence and can interfere with basecalling.

The methods recommended in this section have produced clean sequencing data.

Applicable Kit

The purification procedures provided in this section are applicable to the BigDye® Terminator v1.1 Cycle Sequencing Kit .

Purification Methods

For BigDye Terminator v1.1 chemistry, three methods for preparing extension products for electrophoresis are presented to offer a choice of reagents and processes, as shown in the table below. We recommend performing controlled reactions with each method to determine the one that works best for you.

Purification Method	See Page
Ethanol/EDTA Precipitation Method for BigDye Terminator v1.1 Chemistry	3-42
Ethanol/EDTA/Sodium Acetate Precipitation Method for BigDye Terminator v1.1 Chemistry	3-48
Spin Plate Purification for BigDye Terminator v1.1 Chemistry	3-54

Choosing Spin Plate or Precipitation Methods

Use the method that works best for your particular application.

- The spin plate method removes more terminators, but is more costly and may take additional time to perform.
- Precipitation methods are cheaper and faster. However, they may remove less of the unincorporated dye-labeled terminators, which can obscure data at the beginning of the sequence.

For More Information

Note: If you would like more information purifying extension products for the BigDye Terminator v1.1 chemistry, refer to the *BigDye® Terminator v1.1 Cycle Sequencing Kit Protocol* (PN 4337036) or *User Bulletin: Using an SDS/Heat Treatment with Spin Columns or 96-Well Spin Plates to Remove Unincorporated Dye Terminators* (PN 4330951).

Ethanol/EDTA Precipitation Method for BigDye Terminator v1.1 Chemistry

For most applications, the ethanol/EDTA precipitation method can give clean data. This method produces consistent signal, while minimizing unincorporated dye terminators.

While the ethanol/EDTA precipitation method produces clean signal (*i.e.,* a low amount of residual dye-terminators), it may cause loss of small molecular weight fragments.

There are two procedures provided for the ethanol/EDTA precipitation method, as listed below.

Procedure	See Page
Precipitating in 96-Well Reaction Plates	3-43
Precipitating in 384-Well Reaction Plates	3-46

100% Ethanol

IMPORTANT! If you use absolute (100%) ethanol in the ethanol/EDTA precipitation procedures, you must remember that when exposed to air, 100% ethanol absorbs moisture from the air and becomes more dilute over time, resulting in slight variations in concentration.

95% Ethanol

IMPORTANT! If you use 95% ethanol in the ethanol/EDTA precipitation procedures, purchase non-denatured ethanol at this concentration.

Required Equipment

You will need the following equipment and reagents for these procedures:

- Variable speed centrifuge with a plate adaptor, capable of reaching at least $1400 \times g$.
- MicroAmp strip caps or adhesive-backed aluminum foil tape (3M Scotch Tape 431 or 439)

Note: Use of other tapes may result in leakage or contamination of the sample. To contact 3M in the USA, call (800) 364-3577 for a local 3M representative.

Precipitating in 96-Well Reaction Plates

Note: A final 70% ethanol wash step is required to remove residual unincorporated dyes. If salts and unincorporated dyes are not removed from the sequencing reaction, they will compete with the extension fragments during electrokinetic injection and result in weak signals.

To precipitate half- (10-µL) or full-volume (20-µL) reactions in 96-well reaction plates:

1.	Remove the 96-well reaction plate from the thermal cycler.
2.	Remove the cover from the reaction plate.
3.	Prepare the ethanol/EDTA solution. • For half-volume reactions, add the following to each 10 µL reaction in this order: – 2.5 µL of 125 mM EDTA – 30.0 µL of 100% ethanol or – 35.0 µL of non-denatured 95% ethanol • For full-volume reactions, add the following to each 20-µL reaction in this order: – 5.0 µL of 125 mM EDTA – 60.0 µL of 100% ethanol or – 70.0 µL of non-denatured 95% ethanol **WARNING** **CHEMICAL HAZARD**. **Ethanol** is a flammable liquid and vapor. Exposure may cause eye, skin, and upper respiratory tract irritation. Prolonged or repeated contact may dry the skin. Exposure may cause central nervous system depression and liver damage. Keep away from heat, sparks, and flame. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. **WARNING** **CHEMICAL HAZARD. EDTA**. Exposure causes eye irritation. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.
4.	Seal the wells with a piece of 3M Scotch Tape 431 or 439 adhesive-backed aluminum foil tape. Press the foil onto the wells to prevent any leakage. **IMPORTANT!** If you are using heat-seal film (PN 4337570) to cover samples prior to electrokinetic injection, do not use the Optical Adhesive Covers (PN 4311971). The residual glue will interfere with the heat-sealing process.
5.	Invert the reaction plate a four times or vortex for 15 sec to mix.
6.	Leave the reaction plate at room temperature for at least 15 min to precipitate the extension products.

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

7.	Place the reaction plate in a centrifuge with a plate adaptor and spin at the maximum speed, which must be ≥1400 × g but <3000 × g: • 1400 to 2000 × g: 45 min • 2000 to 3000 × g: 30 min **Note:** The reaction plate can withstand 3000 × g for 30 min. **IMPORTANT!** Proceed to the next step immediately. If this is not possible, then spin the reaction plate for an additional 2 min immediately before performing the next step.
8.	Discard the supernatant as follows: a. Without disturbing the precipitates, remove the adhesive tape. b. Invert the reaction plate onto a paper towel folded to the size of the plate. c. Place the inverted reaction plate and paper towel into the centrifuge and spin up to 185 × g. Then remove from the centrifuge. **IMPORTANT!** The supernatants must be removed completely, as unincorporated dye terminators are dissolved in them. The more residual supernatant left in the wells, the more unincorporated dye terminators will remain in the samples.
9.	Perform a 70% wash. • For half-volume reactions (10 µL), add 30 µL of 70% ethanol to each pellet. • For full-volume reactions (20 µL), add 60 µL of 70% ethanol to each pellet. **WARNING** **CHEMICAL HAZARD**. **Ethanol** is a flammable liquid and vapor. Exposure may cause eye, skin, and upper respiratory tract irritation. Prolonged or repeated contact may dry the skin. Exposure may cause central nervous system depression and liver damage. Keep away from heat, sparks, and flame. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.
10.	Seal the wells as in step 4, then invert the reaction plate a few times or vortex for 15 sec to mix. **IMPORTANT!** If you are using heat-seal film (PN 4337570) to cover samples prior to electrokinetic injection, do not use the Optical Adhesive Covers (PN 4311971). The residual glue will interfere with the heat-sealing process.
11.	Place the reaction plate in the centrifuge and spin for 15 min. at 1650 × g. **IMPORTANT!** Proceed to the next step immediately. If this is not possible, then spin the reaction plate for an additional 2 min immediately before performing the next step.

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

12.	Repeat step 8. Except in step c, place the inverted reaction plate and paper towel into the centrifuge and spin up to 185 × *g* for 1 min. Then remove from the centrifuge. **Note:** Start timing when the rotor begins to move.
13.	Remove the reaction plate from the centrifuge and discard the paper towel. **Note:** Pellets may or may not be visible. Vacuum drying of the samples is not necessary.
14.	Proceed to Chapter 4, "Preparing for Electrophoresis," if you are loading the samples immediately on the 3730/3730xl DNA Analyzer. Otherwise, seal the wells as in step 4 for storage and keep in the dark at −15 °C to −25 °C.

Precipitating in 384-Well Reaction Plates

IMPORTANT! When you use the procedure below with 95% ethanol, the final precipitation volume is greater than 40.0 µL. The maximum volume that each well in a 384-well plate holds depends on the type of plate. If the type of 384-well plate you use will overflow with the 95% ethanol procedure, Applied Biosystems recommends that you use the procedure for 100% ethanol or use a plate type that has a larger volume capacity.

To precipitate half-volume (10 µL) reactions in 384-well plates:

1.	Remove the 384-well reaction plate from the thermal cycler.
2.	Remove the seal from the reaction plate.
3.	Add 2.5 mL of 125 mM EDTA to each half-volume reaction (10 mL) and mix. **CAUTION** **CHEMICAL HAZARD. EDTA** may cause eye, skin, and respiratory tract irritation. Please read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.
4.	Prepare the ethanol/sodium acetate solution by adding to each half-volume reaction: • 25 µL of 100% ethanol or • 30 µL of non-denatured 95% ethanol **WARNING** **CHEMICAL HAZARD**. **Ethanol** is a flammable liquid and vapor. Exposure may cause eye, skin, and upper respiratory tract irritation. Prolonged or repeated contact may dry the skin. Exposure may cause central nervous system depression and liver damage. Keep away from heat, sparks, and flame. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.
5.	Seal the wells with a piece of 3M Scotch Tape 431 or 439 adhesive-backed aluminum foil tape. Press the foil onto the wells to prevent any leakage.
6.	Invert the reaction plate four times or vortex for 15 sec to mix.
7.	Leave the reaction plate at room temperature for at least 15 min to precipitate the extension products.

To precipitate half-volume (10 µL) reactions in 384-well plates: *(continued)*

8.	Place the reaction plate in a centrifuge with a plate adaptor and spin at the maximum speed, which must be ≥1400 × *g* but <3000 × *g*: • 1400 to 2000 × *g*: 45 min • 2000 to 3000 × *g*: 30 min **Note:** The reaction plate can withstand 3000 × *g* for 30 min. **IMPORTANT!** Proceed to the next step immediately. If this is not possible, then spin the reaction plate for an additional 2 min immediately before performing the next step.
9.	Discard the supernatant as follows: a. Without disturbing the precipitates, remove the adhesive tape. b. Invert the reaction plate onto a paper towel folded to the size of the plate. c. Place the inverted reaction plate and paper towel into the centrifuge and spin up to 185 × g. **IMPORTANT!** The supernatants must be removed completely, as unincorporated dye terminators are dissolved in them. The more residual supernatant left in the wells, the more unincorporated dye terminators will remain in the samples.
10.	Remove the reaction plate from the centrifuge and discard the paper towel. **Note:** Pellets may or may not be visible.
11.	To avoid residual terminator peaks, before drying: a. Rinse the pellets with 30 µL of 70% ethanol. b. Seal the wells as in step 5, then invert the reaction plate a few times or vortex for 15 sec to mix. c. Place the reaction plate in the centrifuge and spin for 15 min at the same speed you used in step 8 above. **Note:** The reaction plate can withstand 3000 × g for 30 min. d. Repeat step 9.
12.	Dry the samples by: • Placing in a Speed-Vac for 15 min or • Air drying at room temperature for 1 h **IMPORTANT!** Make sure the samples are protected from light while they are drying.
13.	Proceed to Chapter 4, "Preparing for Electrophoresis," if you are loading the samples immediately on the 3730/3730xl DNA Analyzer. Otherwise, seal the wells as in step 4 for storage and keep in the dark at −15 °C to −25 °C.

Ethanol/EDTA/Sodium Acetate Precipitation Method for BigDye Terminator v1.1 Chemistry

For most applications, the ethanol/EDTA/sodium acetate precipitation method can give clean data. This method produces consistent signal, while minimizing unincorporated dye terminators.

Ethanol/EDTA/sodium acetate precipitation is recommended when good signal from base 1 is required. 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.

There are two procedures provided for the ethanol/EDTA/sodium acetate precipitation method, as listed below.

100% Ethanol

IMPORTANT! If you use absolute (100%) ethanol in the ethanol/EDTA precipitation procedures, you must remember that when exposed to air, 100% ethanol absorbs moisture from the air and becomes more dilute over time, resulting in slight variations in concentration.

95% Ethanol

IMPORTANT! If you use 95% ethanol in the ethanol/EDTA precipitation procedures, purchase non-denatured ethanol at this concentration.

Required Equipment

You will need the following equipment and reagents for these procedures:

- Variable speed centrifuge with a plate adaptor, capable of reaching at least $1400 \times g$.
- MicroAmp strip caps or adhesive-backed aluminum foil tape (3M Scotch Tape 431 or 439)

Note: Use of other tapes may result in leakage or contamination of the sample. To contact 3M in the USA, call (800) 364-3577 for a local 3M representative.

Precipitating in 96-Well Reaction Plates

Note: A final 70% ethanol wash step is required to remove residual unincorporated dyes. If salts and unincorporated dyes are not removed from the sequencing reaction, they will compete with the extension fragments during electrokinetic injection and result in weak signals.

To precipitate half- (10 µL) or full-volume (20 µL) reactions in 96-well reaction plates:

1.	Remove the 96-well reaction plate from the thermal cycler.
2.	Remove the cover from the reaction plate.
3.	Prepare the ethanol/EDTA/sodium acetate solution. • For half-volume reactions, add the following to each 10 µL reaction in this order: – 1.0 µL of 125 mM EDTA – 1.0 µL of 3 M sodium acetate, pH 4.6 – 25.0 µL of 100% ethanol or – 29.0 µL of non-denatured 95% ethanol • For full-volume reactions, add the following to each 20 µL reaction in this order: – 2.0 µL of 125 mM EDTA – 2.0 µL of 3 M sodium acetate, pH 4.6 – 50.0 µL of 100% ethanol or – 58.0 µL of non-denatured 95% ethanol **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 buffer, pH 4.6,** causes 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 may cause eye, skin, and upper respiratory tract irritation. Prolonged or repeated contact may dry the skin. Exposure may cause central nervous system depression and liver damage. Keep away from heat, sparks, and flame. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

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

4.	Seal the wells with a piece of 3M Scotch Tape 431 or 439 adhesive-backed aluminum foil tape. Press the foil onto the wells to prevent any leakage. **IMPORTANT!** If you are using heat-seal film (PN 4337570) to cover samples prior to electrokinetic injection, do not use the Optical Adhesive Covers (PN 4311971). The residual glue will interfere with the heat-sealing process.
5.	Invert the reaction plate four times or vortex for 15 sec to mix.
6.	Leave the reaction plate at room temperature for at least 15 min to precipitate the extension products.
7.	Place the reaction plate in a centrifuge with a plate adaptor and spin at the maximum speed, which must be ≥1400 × *g* but <3000 × *g*: • 1400 to 2000 × *g*: 45 min • 2000 to 3000 × *g*: 30 min **Note:** The reaction plate can withstand 3000 × *g* for 30 min. **IMPORTANT!** Proceed to the next step immediately. If this is not possible, then spin the reaction plate for an additional 2 min immediately before performing the next step.
8.	Discard the supernatant as follows: a. Without disturbing the precipitates, remove the adhesive tape. b. Invert the reaction plate onto a paper towel folded to the size of the plate. c. Place the inverted reaction plate and paper towel into the centrifuge and spin up to 185 × *g*. Then remove from the centrifuge. **Note:** The reaction plate can withstand 3000 × *g* for 30 min. **IMPORTANT!** The supernatants must be removed completely, as unincorporated dye terminators are dissolved in them. The more residual supernatant left in the wells, the more unincorporated dye terminators will remain in the samples.

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

9.	Perform a 70% wash. • For half-volume reactions (10 µL), add 35 µL of 70% ethanol to each pellet. • For full-volume reactions (20 µL), add 70 µL of 70% ethanol to each pellet. **WARNING** **CHEMICAL HAZARD**. **Ethanol** is a flammable liquid and vapor. Exposure may cause eye, skin, and upper respiratory tract irritation. Prolonged or repeated contact may dry the skin. Exposure may cause central nervous system depression and liver damage. Keep away from heat, sparks, and flame. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.
10.	Seal the wells as in step 4, then invert the reaction plate a few times or vortex for 15 sec to mix. **IMPORTANT!** If you are using heat-seal film (PN 4337570) to cover samples prior to electrokinetic injection, do not use the Optical Adhesive Covers (PN 4311971). The residual glue will interfere with the heat-sealing process.
11.	Place the reaction plate in the centrifuge and spin for 15 min at 1650 × *g*.
12.	Repeat step 8.
13.	Remove the reaction plate from the centrifuge and discard the paper towel. **Note:** Pellets may or may not be visible. Vacuum drying of the samples is not necessary.
14.	Proceed to Chapter 4, “Preparing for Electrophoresis,” if you are loading the samples immediately on the 3730/3730xl DNA Analyzer. Otherwise, seal the wells as in step 4 for storage and keep in the dark at −15 °C to −25 °C.

Precipitating in 384-Well Reaction Plates

IMPORTANT! When you use the procedure below with 95% ethanol, the final precipitation volume is greater than 40.0 µL. The maximum volume that each well in a 384-well plate holds depends on the type of plate. If the type of 384-well plate you use will overflow with the 95% ethanol procedure, Applied Biosystems recommends that you use the procedure for 100% ethanol or use a plate type that has a larger volume capacity.

To precipitate half-volume (10 µL) reactions in 384-well plates:

1.	Remove the 384-well reaction plate from the thermal cycler.
2.	Remove the seal from the reaction plate.
3.	Prepare the ethanol/sodium acetate solution by combining the following for each reaction: • 1 µL of 125 mM EDTA • 1 µL of 3 M sodium acetate, pH 4.6 • 25 µL of 100% ethanol or • 29 µL of non-denatured 95% ethanol **WARNING** **CHEMICAL HAZARD. EDTA** may cause eye, skin, and respiratory tract irritation. Please read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. **WARNING** **CHEMICAL HAZARD. 3 M Sodium Acetate buffer, pH 4.6,** causes 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 may cause eye, skin, and upper respiratory tract irritation. Prolonged or repeated contact may dry the skin. Exposure may cause central nervous system depression and liver damage. Keep away from heat, sparks, and flame. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.
4.	Seal the wells with a piece of 3M Scotch Tape 431 or 439 adhesive-backed aluminum foil tape. Press the foil onto the wells to prevent any leakage.
5.	Invert the reaction plate a few times or vortex for 15 sec to mix.
6.	Leave the reaction plate at room temperature for at least 15 min to precipitate the extension products.

To precipitate half-volume (10 µL) reactions in 384-well plates: *(continued)*

7.	Place the reaction plate in a centrifuge with a plate adaptor and spin at the maximum speed, which must be ≥1400 × *g* but <3000 × *g*: • 1400 to 2000 × *g*: 45 min • 2000 to 3000 × *g*: 30 min **Note:** The reaction plate can withstand 3000 × *g* for 30 min. **IMPORTANT!** Proceed to the next step immediately. If this is not possible, then spin the reaction plate for 2 min immediately before performing the next step.
8.	Discard the supernatant as follows: a. Without disturbing the precipitates, remove the adhesive tape. b. Invert the reaction plate onto a paper towel folded to the size of the plate. c. Place the inverted reaction plate and paper towel into the centrifuge and spin up to 185 × *g*. **IMPORTANT!** The supernatants must be removed completely, as unincorporated dye terminators are dissolved in them. The more residual supernatant left in the wells, the more unincorporated dye terminators will remain in the samples.
9.	Remove the reaction plate from the centrifuge and discard the paper towel. **Note:** Pellets may or may not be visible.
10.	To avoid residual terminator peaks, before drying: a. Rinse the pellets with 35 µL of 70% ethanol. b. Seal the wells as in step 4, then invert the reaction plate a few times or vortex for 15 sec to mix. c. Place the reaction plate in the centrifuge and spin at 1650 × *g* for 15 min. **Note:** The reaction plate can withstand 3000 × *g* for 30 min. d. Repeat step 8.
11.	Dry the samples by: • Placing in a Speed-Vac for 15 min or • Air drying at room temperature for 1 h **IMPORTANT!** Make sure the samples are protected from light while they are drying.
12.	Proceed to Chapter 4, "Preparing for Electrophoresis," if you are loading the samples immediately on the 3730/3730xl DNA Analyzer. Otherwise, seal the wells as in step 4 for storage and keep in the dark at −15 °C to −25 °C.

Spin Plate Purification for BigDye Terminator v1.1 Chemistry

About Spin Plate Purification

The efficiency of removing all excess dye terminators depends on the spin plate purification kit. There are several commercially available purification kits. If not used properly, these commercially available kits can lead to dye blobs appearing in the sequencing data. To remove excess dye terminators efficiently when mixing spin plates, Applied Biosystems recommends the following protocol.

Preparing Extension Products with SDS/Heat Treatment

Use this procedure to prepare extension products for both spin column and 96-well spin plate purification.

To prepare extension products:

1.	Prepare 2.2% SDS (sodium dodecyl sulfate) in deionized water. This SDS solution is stable at room temperature. **WARNING CHEMICAL HAZARD. Sodium dodecyl sulfate (SDS)** may cause an allergic respiratory reaction. It is harmful if inhaled, swallowed, or absorbed through the skin. Exposure causes eye, skin, and respiratory tract irritation. Please read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.
2.	Add an appropriate amount of the 2.2% SDS solution to your sample to bring the final concentration of SDS to 0.2%. For example: Add 2 µL of 2.2% SDS to each 20-µL completed cycle sequencing reaction.
3.	Seal the tubes with caps and mix thoroughly.
4.	Heat the tubes to 98 °C for 5 minutes, then allow the tubes to cool to ambient temperature before proceeding to the next step. A convenient way to perform this heating/cooling cycle is to place the tubes in a thermal cycler and set the thermal cycler as follows: 98 °C for 5 minutes 25 °C for 10 minutes
5.	Spin down the contents briefly.
6.	Continue with the manufacturer's recommended protocol for spin plate purification.

Recommended Spin Plates

For large-scale procedures, you can use the commercially available spin plates listed below, or an equivalent one of your choice.

- 96-Well spin plates, Gel Filtration Kit (Edge Biosystems, PN 94880)

IMPORTANT! When using the Edge Biosystems gel filtration kit, centrifuge at 850 × *g* for 2 min.

Preparing for Electrophoresis 4

In This Chapter

This chapter includes the following topics:

Preparing Samples for Injection

Resuspending the Samples in Injection Solution

Before the samples and standards can be placed on Applied Biosystems 3730/3730xl DNA Analyzers, they must be resuspended in injection solution. Applied Biosystems recommends formamide, as follows:

- Hi-Di™ formamide (PN 4311320), or
- Your own prepared deionized formamide

See Appendix A for a discussion of formamide and preparation procedures.

WARNING **CHEMICAL HAZARD**. **Formamide** is harmful if absorbed through the skin and may cause irritation to the eyes, skin, and respiratory tract. It may cause damage to the central nervous system and the male and female reproductive systems, and is a possible birth defect hazard. Please read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

Resuspension Volumes for Reaction Plates

IMPORTANT! Always stay within the volume range specified below.

For correct delivery of samples to the capillary array tips, the sample volumes in the reaction plate wells must remain within the ranges specified in the table below.

Reaction Plate*	Minimum Volume (µL)		Maximum Volume (µL)†		Recommended Volume (µL)
	Film	Septa	Film	Septa	
96-well	10	10	200	150	10–30
384-well	5	5	20	15	5–15

*For ABI Prism reaction plates listed in “Compatible Reaction Plates” on page 2-8.
†The maximum volume is to ensure that the septa or film does not touch the sample and cause cross-contamination.

Covering the Resuspended Samples Using Septa

Samples in injection solution are subject to degradation at room temperature. Exposure to heat, humidity, or light will accelerate this process. The longer the samples are exposed to air, the more likely this problem will occur.

To avoid degradation, Applied Biosystems recommends immediately covering the reaction plates using plate septa, as follows:

Reaction Plate	Plate Septa
96-well	3100 Genetic Analyzer Plate Septa (PN 4315933) **Note:** These septa can be used on both the 3100 and the 3730/3730xl analyzers.
384-well	3100 Genetic Analyzer Plate Septa (PN 4315934) **Note:** These septa can be used on both the 3100 and the 3730/3730xl analyzers.

Covering the Resuspended Samples Using Heat-Seal Film

Samples in injection solution are subject to degradation at room temperature. Exposure to heat, humidity, or light will accelerate this process. The longer the samples are exposed to air, the more likely it is this problem will occur.

To avoid degradation, Applied Biosystems recommends immediately covering the reaction plates using heat-seal film.

Reaction Plate	Film
96-well	Optical Heat-Seal Film (PN 4337570)
384-well	

Use a thermal plate sealer set at 150 °C for 1.5 seconds. The 3-mil thick plastic heat seal film is 1-mil thick after heating.

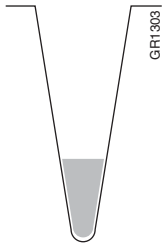
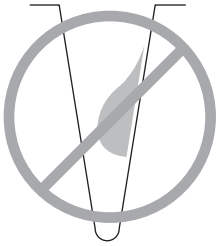
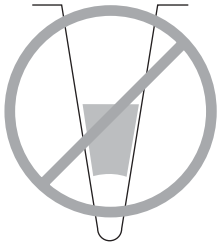
CAUTION Sealing this film for longer times or high temperatures may melt the reaction plate rim. Overly melted rims may interfere with piercing needles on the 3730/3730xl instruments.

CAUTION Do not use metallized heat seal film. It may damage the instrument's piercing needles.

Centrifuging the Samples

Before placing the reaction plates in the instrument's stacker, you must centrifuge them to bring the samples down to the bottom of the wells. Failure to centrifuge the reaction plates properly will result in the samples not being injected into the capillary array.

To centrifuge the samples:

<table>
<tr><td>1.</td><td>Centrifuge the 96- or 384-well reaction plates at 2000 x g for 1 min.</td></tr>
<tr><td>2.</td><td>Hold the plates up to the light and examine them carefully to make sure that every sample is positioned at the bottom of the tube or well. Before you place your plates in the stacker, the samples in your tubes or wells should:
<table>
<tr><th>Look like this...</th><th>Not look like this...</th><th>Not look like this...</th></tr>
<tr><td> 
</td><td>  </td><td>  </td></tr>
<tr><td>The sample is positioned correctly in the bottom of the tube or well.</td><td>The sample lies on the side wall because the plate or tube was not centrifuged.</td><td>An air bubble lies at the bottom of the tube or well because the sample was not:
• Centrifuged with enough force, OR
• Centrifuged for enough time</td></tr>
</table></td></tr>
<tr><td>3.</td><td>If one or more samples are not positioned correctly, repeat step 2.</td></tr>
<tr><td>4.</td><td>Your samples are now ready to be placed in the stacker.</td></tr>
</table>

Optimizing Electrokinetic Injection

Optimizing electrokinetic injection can greatly improve data quality and run-to-run reproducibility. The goal is to inject sufficient DNA to yield peaks of adequate height (*i.e.*, data with a good signal-to-noise ratio) while maintaining resolution and read length.

The DNA Analyzer run modules have preset values for injection times and voltages. These values are adequate for most applications. However, you should consider modifying the injection parameters when:

- The signal is too strong.
- The signal is too weak.
- The resolution is poor.

IMPORTANT! For information on setting electrokinetic injection values, please refer to the *Applied Biosystems 3730/3730xl DNA Analyzers User Guide* (PN 4331468).

Signal Too Strong

If the signal is too strong:

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

Signal Too Weak Using 50-cm Capillary Array

If the signal is too weak when using the 50-cm capillary array:

- Increase the injection time to increase the total electrokinetic injection (EKI) product. See the table below.

Injection Time (Seconds) @1.5 KV	Volts/cm	Total EKI Product (V-Sec/cm)	Description
15	25	375	Default setting for injection time
45	25	1125	Maximum injection time recommended with minimal effect on resolution **Note:** May degrade the resolution if the template load is high.
75	25	1875	Signal strength increases at this injection time, but at the expense of resolution

- Reduce the amount of salt in the sample.

IMPORTANT! Negative ions (*e.g.*, EDTA and acetate) compete with DNA for injection. To reduce the amount of salt in a sequencing reaction, use column purification. (Refer to “Spin Plate Purification for BigDye Terminator v3.0 Chemistry” on page 3-39.)

- Increase the concentration of the DNA extension products.
- Do not increase the voltage. Increasing the voltage increases the signal, but may reduce resolution across the capillary array. It is better to adjust the injection time in order to increase signal. If changes are made to both the injection time and voltage, calculate the EKI product to estimate changes to the size of the injection zone.

Signal Too Weak Using 36-cm Capillary Array

If the signal is too weak when using the 36-cm capillary array:

- Increase the injection time. See the table below.

Injection Time (Seconds)	Volts/cm	Total EKI Product (V-Sec/cm)	Description
15	25	375	Default setting for injection time
40	25	1000	Maximum injection time recommended with minimal effect on resolution **Note:** May degrade the resolution if the template load is high.
60	25	1500	Signal strength increases at this injection time, but at the expense of resolution

- Reduce the amount of salt in the sample.

  IMPORTANT! Negative ions (*e.g.*, EDTA and acetate) compete with DNA for injection. To reduce the amount of salt in a sequencing reaction, use column purification. (Refer to "Spin Plate Purification for BigDye Terminator v3.0 Chemistry" on page 3-39.)

- Increase the concentration of the DNA extension products.

Note: Applied Biosystems does not recommend increasing the voltage. Increasing the voltage increases the signal, but may reduce resolution across the capillary array. It is better to adjust the injection time in order to increase signal.

Poor Resolution

If the resolution needs to be improved:

- Decrease the injection time.

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

Optimizing Electrophoresis Conditions

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

- Read length desired
- Required degree of resolution

Determining or Changing the Required Run Time

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

Changing Run Time for 50-cm Array – Long Read Module

The Long Read Module is currently programmed to collect 1100 bases. Figure 4-1 illustrates the collection time that elapses before DNA fragments traveling through a 50-cm capillary array using the Long Read Module reach the fluorescence detector.

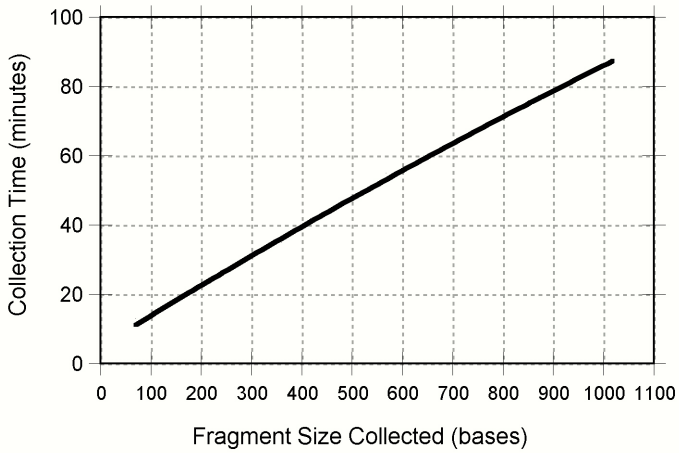


Figure 4-1 Collection time for 50-cm capillary array and Long Read Module

The graph assumes the following information:

- The run module is programmed for 28 minutes of run protocol events that occur before data collection starts.
- The run voltage is 8.5 kV.
- The separation distance is 50 cm length-to-detector (LTD) with a total capillary array length of 61 cm.
- The run temperature is 60 °C.

Changing Run Time for 36-cm Array – Rapid Run Module

The quality of the separation with the Rapid Run Module is adequate to run longer as well as shorter collection times. The default Rapid Module is set to collect 600 bases. Figure 4-2 illustrates the collection time that elapses before DNA fragments traveling through a 36-cm capillary array using the Rapid Run Module reach the fluorescence detector.

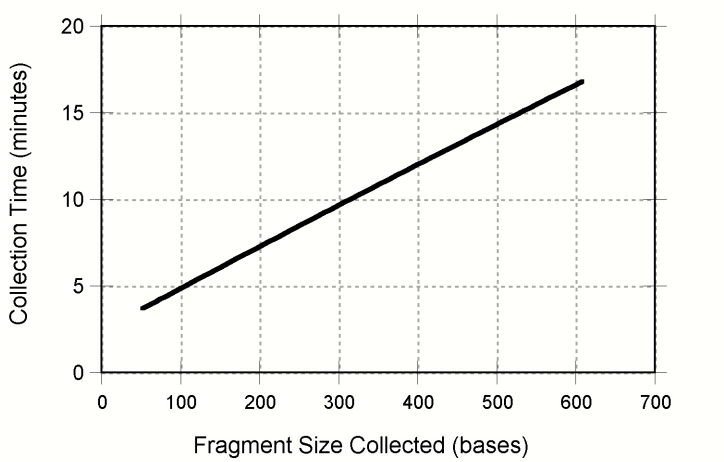


Figure 4-2 Collection time for a 36-cm capillary array and Rapid Run Module

The graph assumes the following information:

- The run module is programmed for 17 minutes of run protocol events that occur before data collection starts.
- The separation voltage is 13.2 kV.
- The separation distance is 36 cm LTD with a total capillary array length of 47 cm.
- The run temperature is 60 °C.

Changing Run Time for 36-cm Array – Standard Run Module

You can shorten the data collection time if you only need information about short extension products (*e.g.*, in PCR sequencing). The default Standard Run Module is set to collect approximately 850 bases. Figure 4-3 illustrates the collection time that elapses before DNA fragments traveling through a 36-cm capillary array using the Standard Run Module reach the fluorescence detector.

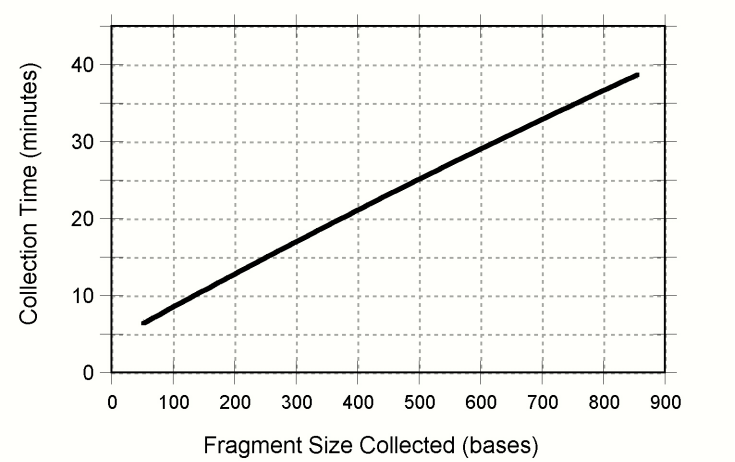


Figure 4-3 Collection time for a 36-cm capillary array and Standard Run Module

The graph assumes the following information:

- The run module is programmed for 20 minutes of run protocol events that occur before data collection starts.
- The separation voltage is 8.5 kV.
- The separation distance is 36 cm LTD with a total capillary array length of 47 cm.
- The run temperature is 60 °C.

Run Temperature and Run Voltage

Run Temperature

Protocols for sequencing applications with POP-7™ polymer specify a 60 °C electrophoresis temperature.

Run Voltage

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

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

Laboratory Temperature and Humidity

The laboratory temperature should be maintained between 15 and 30 °C. It should not fluctuate more than ±2 °C during a run for optimal results.

The DNA Analyzer can tolerate up to 80% non-condensing relative humidity. Avoid placing the instrument near heaters, cooling ducts, windows, or back-to-back with another instrument.

For More Information

For information on setting electrophoresis parameters, please refer to the *Applied Biosystems 3730/3730xl DNA Analyzers User Guide* (PN 4331468).

Purchasing or Preparing Formamide A

In This Appendix

This appendix includes the following topics:

About Formamide

Formamide is used to denature the DNA samples before placing them on the Applied Biosystems 3730/3730*xl* DNA Analyzers.

Option to Purchase or to Make

There are two ways to obtain formamide for use with the DNA Analyzer:

- Prepare it yourself, using a mixed-bed (anionic and cationic) ion-exchange resin, as described in the procedures beginning on page A-4.
- Purchase Hi-Di™ Formamide from Applied Biosystems.

Purchasing Hi-Di Formamide

Hi-Di Formamide is suitable for use with the DNA Analyzer. It is available from Applied Biosystems in 25-mL bottles (PN 4311320).

Problems with Commercial Formamide

Formamide purchased from commercial suppliers is often supplied in glass bottles, which can produce contamination from minerals. In addition, formamide purchased from commercial suppliers is often contaminated with variable amounts of water and undesirable organic and inorganic ions. Water reacts slowly with formamide to produce formic acid (methanoic acid) and ammonia. The ionic products of this reaction cause two problems:

- They compete significantly with the larger DNA ions for injection into the capillary array, resulting in weaker signals.
- They react with the DNA, causing degradation of the sample.

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.

Figure A-1 on page A-3 shows the effect of formamide exposure to the air on electropherogram data. The top panel shows electropherogram data from samples incubated for 51 hours with a lid. The bottom panel shows electropherogram data from samples incubated for 48 hours with no lid.

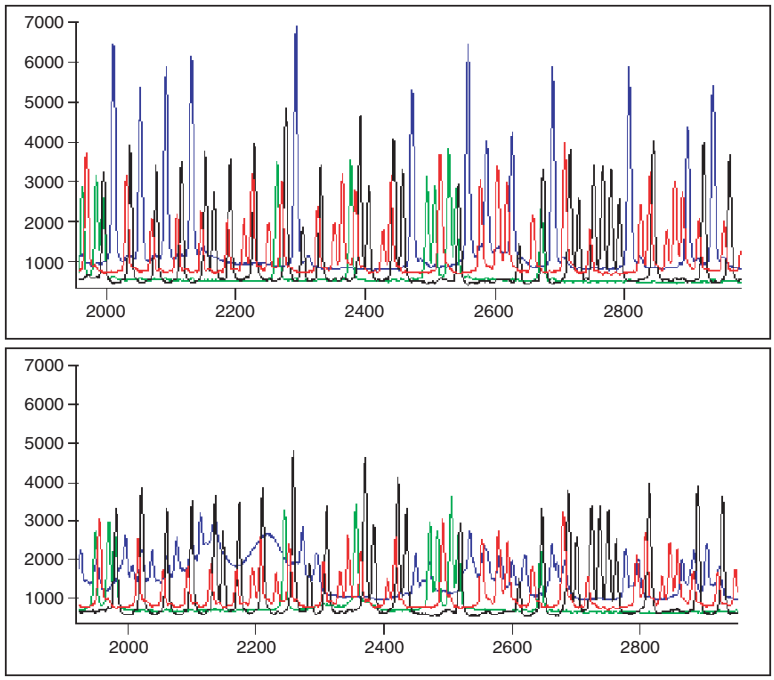


Figure A-1 Effects of exposing formamide-resuspended samples to air

Recommended Materials

The following materials are recommended for this procedure:

Material	Description
Formamide	The raw formamide (prior to deionization) should be: • 99.5% purity or greater, with low water content • Packed under an inert gas • Have a conductivity of approximately 100 ∝Siemens/cm or less **Note:** Siemens, formerly called mho, are the units of measurement for specific conductance or conductivity.
Ion-exchange resin	• Mixed-bed resin containing the following strong ion exchange functional groups: – $R\text{-}SO_3^-$ (as H^+ form) (cation) – $R\text{-}CH_2N^+(CH_3)_3$, (as OH^- form) (anion) These groups are attached to a styrene divinylbenzene matrix with 8% cross-linkage. • The minimum wet capacity is 1.5 meq/mL with 20–50 dry mesh size (AG501 X8, molecular biology grade mixed-bed resin) • Available from Bio-Rad Laboratories (PN 143-6424) or equivalent
Conductivity meter	A commercial conductivity meter, or pH meter with an external conductivity cell, is sufficient to measure the conductivity of formamide.
Na_2EDTA	• Dihydrate (M_r 372.2) • ACS reagent, 99% purity or greater • Available from Sigma (PN E4884) or equivalent
Container for storing formamide	Use a polypropylene screw-cap container **Note:** Glass containers are not recommended because of potential contamination from minerals.

Ion-Exchange Resin

The raw formamide is deionized with cationic and anionic mixed resins to remove impurities such as ammonium and formate ions. Deionization occurs at a slow mass-transfer rate in the equilibrium ion exchange kinetics due to:

- Physical changes in the resin in the presence of formamide
- Differences in molecular size and selectivity between the impurity ions and the H^+ and OH^- counterions

Therefore, the conductivity of formamide must be monitored over time to determine the extent of deionization by the resin.

Calibrating the Conductivity Meter

A conductivity meter and cell are needed to measure the effectiveness of the deionization process. The more deionized the formamide, the lower its conductivity.

Within the range or measurement, the conductivity meter should be routinely calibrated (to 50 µSiemens/cm or less). Calibrate the meter using standard solutions that are traceable to the National Institute of Standards and Technology (NIST). Because temperature affects conductivity, samples must be brought to room temperature before measuring the conductivity.

Preparing EDTA

Alkaline EDTA (ethylenediaminetetraacetic acid) is added to the deionized formamide to stabilize it and to facilitate the electrokinetic injection of DNA. To minimize the amount of water added to the formamide, a concentrated (200-mM) stock solution of the EDTA is added.

To prepare the 200-mM EDTA stock solution:

1.	Add 7.44 g of Na_2EDTA to 70 mL of deionized water and stir. **CAUTION CHEMICAL HAZARD. EDTA** may cause eye, skin, and respiratory tract irritation. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.
2.	While stirring, slowly adjust to pH 8.0 to 8.8 by dropwise addition of a concentrated solution of sodium hydroxide. **DANGER CHEMICAL HAZARD. Sodium hydroxide (NaOH)** causes severe eye, skin, and respiratory tract burns. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. **Note:** The process of adding sodium hydroxide helps the EDTA to dissolve over time, because the EDTA has a limited solubility until the pH is increased.
3.	Dilute to 100 mL with deionized water.
4.	Store at 4 °C.

Purifying and Using Formamide

Purifying Formamide

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

IMPORTANT! There is not a stopping point in this procedure. Complete the procedure from resin washing to freezing the formamide, without interruption.

To purify formamide:

1.	Calibrate the conductivity meter cell and rinse the cell with distilled water.
2.	In a polypropylene screw-cap container, wash 10 g of Bio-Rad AG501 X8 ion-exchange resin by swirling the sample with 10 to 20 mL of formamide for 1 min.
3.	Either decant off or filter through a course nylon or teflon filter, and discard the formamide.
4.	Repeat steps 2 and 3 twice.
5.	Add 100 mL of formamide to the washed resin.
6.	Cap the mixture, ensuring that it is well sealed.
7.	Stir the mixture rapidly with a magnetic stirrer, or mix with an electric shaker, ensuring that the resin is suspended and mixes thoroughly with the formamide. Stir at room temperature for approximately 2 h.
8.	Stop stirring and allow the resin to settle for 5 min.
9.	Remove a small aliquot of the mixture, and measure the conductivity at room temperature.
10.	Rinse the conductivity cell with distilled water.
11.	<table><tr><th>If the conductivity is...</th><th>Then...</th></tr><tr><td>>5 µSiemens/cm</td><td>Return to step 7, stirring for an additional 30 min.</td></tr><tr><td><5 µSiemens/cm</td><td>Continue with “Using the Formamide” on page A-7.</td></tr></table> **Note:** If the conductivity is not <5 µSiemens/cm after about 4.5 h of mixing, repeat the entire procedure using a new lot of formamide and new resin. **Note:** Starting formamide with a higher purity and lower conductivity deionizes more efficiently.

To purify formamide: ***(continued)***

12.	Vacuum-filter the deionized formamide using a 0.2-µm nylon or teflon filter.
13.	Measure the final volume of deionized formamide.
14.	Add the required volume of 200-mM EDTA to the deionized formamide to achieve a final concentration of approximately 0.3-mM EDTA. **CAUTION** **CHEMICAL HAZARD. EDTA** may cause eye, skin, and respiratory tract irritation. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. **Note:** After adding the EDTA, the final conductivity of the formulation is increased to approximately 25 µSiemens/cm. Use the equation below to calculate the volume of EDTA to add. $V_{EDTA\ (\mu L)} = 1.5V_{\ Form\ (mL)}$ Where, $_{VEDTA(\mu L)}$ = volume of EDTA to add in microliters $V_{FORM(mL)}$= measured volume of formamide in milliliters Sample calculation with a final volume of 90-mL formamide: $V_{EDTA(\mu L)} = 1.5 \times 90 = 135\ \mu L$
15.	Immediately aliquot the formamide into smaller polypropylene tubes and store at –15 to –20 °C for up to about 6 months.

Using the Formamide

When ready for use, thaw and completely use one tube at a time before opening and exposing another. Store the tubes at 4 °C during the day for intermittent use. Otherwise, refreeze them. Minimize the number of freeze-thaw and exposure cycles for each tube.

Troubleshooting

B

Troubleshooting Table

Some common observations associated with the chemistries used on the Applied Biosystems 3730/3730*xl* DNA Analyzers are listed below.

Observation	Possible Cause	Recommended Action
Poor data resolution	Clogged capillary array caused by an excess of protein, template, other sample impurities, or dried polymer	Replace the array.
	• Degradation of samples in formamide • Degradation due to formamide exposed to air	Re-prepare the samples.
	Overloading of the sample	Dilute the sample and adjust the injection parameter. Refer to "Optimizing Electrokinetic Injection" on page 4-5.
Weak signal	Quantity of template or primers in the sequencing reaction or the quantity of sample injected too low	Refer to "Template Quantity" on page 2-5 for a description of recommended template quantities.
		If possible, resuspend the template in a smaller volume.
		Increase injection time. Refer to "Optimizing Electrokinetic Injection" on page 4-5.
	Excess salt present in the sample	Clean up the sample using a spin column or a 70% ethanol wash.
	Bad post-reaction clean-up	Repeat sample preparation.
High background	Dirty template, bad primers, bad post-reaction clean-up	Refer to the documents listed on page vii of the Preface section for a description of how to clean up dirty templates.
Top-heavy data	Amount of template in the sequencing reaction too high, creating an excess of short fragments that are preferentially injected into the capillary array	Refer to "Template Quantity" on page 2-5 for a description of recommended template quantities.
	Concentration of extension products too high	Dilute the sample or decrease the injection time.
	Diluted reactions	Use more BigDye reagent.

Observation	Possible Cause	Recommended Action
Blank lanes or no signal	Cycle sequencing reaction failed	Repeat the cycle sequencing reaction, adjust primer and template concentration.
	Bad post-reaction clean-up	Repeat sample preparation.
	Blocked capillary array caused by an excess of protein, template, or other impurities, or by dried polymer	Replace the capillary array.
Failed injection	Reaction plate not centrifuged prior to injection, air bubbles in the sample wells	Centrifuge the reaction plate.
Breakdown of BigDye® G nucleotide	Formamide degradation caused by exposure to the air	Refer to "Problems with Commercial Formamide" on page A-2. Use formamide as recommended in Appendix A.
		Cover reaction plates with septa or film.
Abrupt signal loss	Reactions too dilute	Repeat run using more BigDye reagent.
	Poor quantitation of primer and/or template, leading to top-heavy data	Adjust concentrations and repeat reactions.
Poor template quality	Residual salts or organic chemicals carried over from template preparation	Precipitate the template with ethanol and resequence. Refer to Chapter 3, "Purifying the Extension Products."
	Incomplete removal of cellular components such as RNA, proteins, polysaccharides, and contaminating chromosomal DNA	
	Degradation of DNA in storage	
	More than one template DNA in the sequencing reaction	
Inhibition of the sequencing reaction	Various types of contaminates present during template preparation	Precipitate the template with ethanol and resequence. Refer to Chapter 3, "Purifying the Extension Products."
Multiple, overlapping sequences in the data (PCR templates)	More than one template present in the reaction (*i.e.,* secondary PCR products) due to lack of specificity	The majority of cleanup procedures for PCR products are designed to remove unincorporated nucleotides and residual PCR primers, not secondary PCR products. Use agarose gel electrophoresis to detect the presence of secondary PCR products.
		Optimize the PCR conditions and/or use a Hot Start method.
		Purify the PCR products using a gel before sequencing.

Observation	Possible Cause	Recommended Action
Multiple, overlapping sequences in the data (cloned DNA templates)	More than one sequence present in the reaction due to mixed plaques or colonies	Re-isolate the DNA from a pure colony and re-sequence.
		When picking bacterial colonies for growth and DNA isolation, choose a colony that is well isolated.
		With M13 plaques, use fresh plates for plaque picking.
		Check the DNA purity by running it on an agarose gel.
Multiple peaks in the same position at some points (*pull-up peaks* or *bleed-through*)	Very strong signals saturating the instrument's detector, causing the signals to be truncated The Sequencing Analysis software underestimates the amount of signal at these positions, therefore underestimating the amount of spectral overlap to correct.	Very strong signals are common when sequencing short PCR fragments, because the sequencing reaction is often very efficient. You may need to load less of this type of sample to compensate for the increased signal.
Excess dye peaks	Incomplete removal of unincorporated, fluorescently labeled ddNTPs during alcohol precipitation	Use only room-temperature alcohol. Cold alcohol will also precipitate unincorporated dye terminators.
		Do not use denatured alcohol. Denatured alcohol has inconsistent quality. The concentration of the alcohol and purity of the additives can vary.
		Use the concentration of alcohol recommended in the precipitation procedures.
		Use a precipitation method appropriate for your sequencing chemistry.

Observation	Possible Cause	Recommended Action
Difficulty sequencing GC-rich templates, resulting in weak signal	The DNA is melting at a higher temperature due to the high proportion of GC base pairs **Note:** Even a template that has a fairly average base composition overall can have a very GC-rich region that affects its ability to be sequenced.	Increase the denaturation temperature.
		Add DMSO to a final concentration (v/v) of 5%.* **Note:** Adding a mixture of 5% DMSO and 5% glycerol has also been used successfully for some templates.
		Incubate the reaction at 96 °C for 10 min before cycling.
		Add betaine to a final concentration of 1 M.†
		Double all reaction components and incubate at 98 °C for 10 min before cycling.
		Add 5 to 10% formamide or 5 to 10% glycerol to the reactions.
		Linearize the plasmids with a restriction enzyme.
		Shear the insert into smaller fragments (<200 bp) and subclone.
Secondary structure in the template, making it difficult to obtain good sequencing data beyond the region of secondary structure	Self-annealing DNA	Increase the denaturation temperature.
		Add DMSO to a final concentration (v/v) of 5%.‡ **Note:** Adding a mixture of 5% DMSO and 5% glycerol has also been used successfully for some templates.
		Incubate the reaction at 96 °C for 10 min before cycling.
		Add betaine to a final concentration of 1 M.§
		Double all reaction components and incubate at 98 °C for 10 min before cycling.
		Add 5 to 10% formamide or 5 to 10% glycerol to the reactions.
		Linearize the plasmids with a restriction enzyme.
		Shear the insert into smaller fragments (< 200 bp) and subclone.

Observation	Possible Cause	Recommended Action
Slippage in the region of the homopolymer (DNA sequencing reactions)	Long homopolymer T (or A) regions	Use an anchored primer (*i.e.,* a sequencing primer that is polyT containing an A, C, or G base at the 3´ end of a polyA region). The 3´ base will anchor the primer into place at the end of the homopolymer region.
	Use of dUTP in the deoxynucleotide mixture	
	Unknown	

*Burgett *et al.,* 1994; Landre *et al.,* 1995
†Henke *et al.,* 1997; Baskaran *et al.,* 1996
‡Burgett *et al.,* 1994; Landre *et al.,* 1995
§Henke *et al.,* 1997; Baskaran *et al.,* 1996

Index

Numerics

A

B

C

D

E

F

H

I

L

M

P

R

S

T

U

V

W

Headquarters
850 Lincoln Centre Drive
Foster City, CA 94404 USA
Phone: +1 650.638.5800
Toll Free (In North America): +1 800.345.5224
Fax: +1 650.638.5884

Worldwide Sales and Support
Applied Biosystems vast distribution and service network, composed of highly trained support and applications personnel, reaches 150 countries on six continents. For sales office locations and technical support, please call our local office or refer to our Web site at www.appliedbiosystems.com.

www.appliedbiosystems.com

Applied Biosystems

Applera Corporation is committed to providing the world's leading technology and information for life scientists. Applera Corporation consists of the Applied Biosystems and Celera Genomics businesses.

Printed in the USA, 11/2002
Part Number 4331467 Rev. B

an **Applera** business