applied biosystems

BigDye™ Terminator v3.1 Cycle Sequencing Kit

USER GUIDE

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

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For descriptions of symbols on product labels or product documents, go to thermofisher.com/symbols-definition.

Revision history: MAN1000355 A (English)

Revision	Date	Description
A	28 June 2024	New user guide for the BigDye™ Terminator v3.1 Cycle Sequencing Kit. This document replaces Pub. No. 4337035.

The information in this guide is subject to change without notice.

Contents

1 Product information

IMPORTANT! Before using this product, read and understand the information in the "Safety" appendix in this document.

Product description

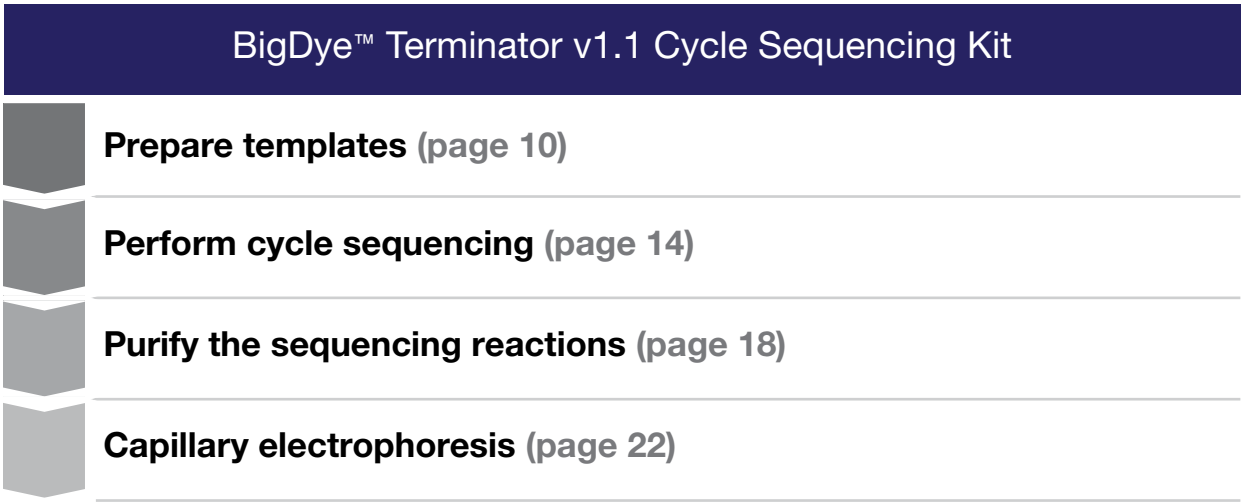
The BigDye™ Terminator v3.1 Cycle Sequencing Kit provides pre-mixed reagents for Sanger sequencing reactions.

The kit reagents are suitable for performing fluorescence-based cycle sequencing reactions on single-stranded or double-stranded DNA templates, on PCR fragments, and on large templates (for example, BAC clones).

The kit includes sequencing buffer, which is specifically optimized for use with the BigDye™ Ready Reaction mixes.

The kit has been formulated to deliver robust performance across a wide variety of DNA sequences while maximizing readlengths. When used in combination with Minor Variant Finder Software, the kit can also be used to detect variants as low as 5% in a sample (see *Minor Variant Finder Software User Guide* (Pub. No. MAN0014835).

Workflow



Kit contents and storage

Table 1 BigDye™ Terminator v3.1 Cycle Sequencing Kit

Contents	Cat. No. 4337454 (24 reactions)	Cat. No. 4337455 (100 reactions)	Cat. No. 4337456 (1,000 reactions)	Cat. No. 4337457 (5,000 reactions)	Cat. No. 4337458 (25,000 reactions)	Storage
BigDye™ Terminator v3.1 Ready Reaction Mix	1 x 192 µL	1 x 800 µL	10 x 800 µL	2 x 20 mL	10 x 20 mL	–25°C to –15°C Protect from light.
pREF-BDT™ Control DNA (200ng/µL)	1 x 30 µL	1 x 30 µL	1 x 250 µL	2 x 250 µL	4 x 250 µL	
–21 M13 Control Primers (0.8pmol/µL)	1 x 50 µL	1 x 50 µL	1 x 200 µL	2 x 200 µL	4 x 200 µL	
BigDye™ Terminator v1.1 &v3.1 5X Sequencing Buffer[1]	1 x 1 mL	2 x 1 mL	1 x 12 mL	2 x 28 mL	3 x 240 mL	4°C

[1] Shipped separately from the rest of the kit.

Usage guidelines

- Avoid excess freeze-thaw cycles (no more than 10 cycles). If needed, aliquot reagents into smaller amounts.
- Before each use of the kit, allow the frozen stocks to thaw on ice or at room temperature (do not heat).
- Keep thawed materials on ice during use. Do not leave reagents at room temperature for extended periods.
- Protect dyes from light to avoid photobleaching.

IMPORTANT! Mix each stock thoroughly, then centrifuge briefly to collect all the liquid at the bottom of each tube.

Compatible sequencing instruments

This guide provides general instructions for using the kit to generate samples for these instruments. For more detailed instructions, refer to the appropriate instrument or chemistry guide.

- SeqStudio™ Genetic Analyzer
- SeqStudio™ Flex Series Genetic Analyzer
- 3500/3500xL Genetic Analyzer
- 3730/3730*xl* DNA Analyzer

Thermal cyclers

The protocols provided in this guide were optimized using Applied Biosystems™ thermal cyclers, including the:

- GeneAmp™ PCR System 9700 Dual 96-Well
- GeneAmp™ PCR System 9700 Dual 384-Well
- Veriti™ *Fast* 96-Well Thermal Cycler
- Veriti™ 384-Well Thermal Cycler

It is possible to use a different thermal cycler, although you may need to re-optimize the thermal cycling conditions. Ramping time is very important. If the thermal ramping time is too fast (>1°/second), poor (noisy) data may result.

Required materials not supplied

Unless otherwise indicated, all materials are available through **thermofisher.com**. "MLS" indicates that the material is available from **fisherscientific.com** or another major laboratory supplier.

Catalog numbers that appear as links open the web pages for those products.

Item	Source
Reagents	
BigDye™ Terminator v3.1 Cycle Sequencing Kit	4337456
UltraPure™ DNase/RNase-Free Distilled Water	10977015
Hi-Di™ Formamide **Note:** Not required for BigDye XTerminator™ Purification Kit purification.	4311320 or 4440753
Sequencing and PCR primers (HPLC-purified recommended)	Primers can be designed and ordered with the Primer Designer™ Tool at **http://www.thermofisher.com/primerdesigner**. See “Primer Designer™ Tool” on page 46.
Laboratory supplies	
MicroAmp™ Clear Adhesive Film	4306311
MicroAmp™ Optical 96-Well Reaction Plate	N8010560
Plate Septa, 96 well	4315933
MicroAmp™ Optical 384-Well Reaction Plate	4343370
Digital Vortex-Genie™ 2 or equivalent	Scientific Industries, Inc. SI-A536
Centrifuge with swinging bucket (with PCR plate adapter)	MLS
Reagents for BigDye XTerminator™ Purification Kit purification (*optional*)	
BigDye XTerminator™ Purification Kit	4376486
Reagents for ethanol/EDTA purification (*optional*)	
0.5M EDTA, pH 8.0 for molecular biology	AM9260G
Ethanol, absolute, for molecular biology	MLS

2 Prepare templates

Control template

Include a control DNA template in every set of sequencing reactions. The results from the control help determine whether failed reactions are the result of poor template quality or sequencing reaction failure.

- pREF-BDT™ Control DNA is included with the kit as a double-stranded control (see Appendix C, “pREF-BDT™ Control DNA” for the partial sequence).

  Note: The corresponding –21 M13 forward primer is also included.

- M13mp18 is recommended as a single-stranded control.

Template preparation methods

Cycle Sequencing can be performed directly from single– or double–stranded DNA, plasmids, cosmids, BACs or purified PCR products. For high complexity DNA, PCR amplification of the target of interest before cycle sequencing is recommended.

For general guidelines on DNA isolation, see *DNA Sequencing by Capillary Electrophoresis Chemistry Guide Second Edition* (Pub. No. 4305080 or *DNA Fragment Analysis by Capillary Electrophoresis User Guide* (Pub. No. 4474504 for information on preparing single- and double-stranded templates.

DNA isolation of single- and double-stranded templates

For a comprehensive list of Thermo Fisher Scientific products available for DNA isolation, go to **https://www.thermofisher.com/us/en/home/life-science/dna-rna-purification-analysis/dna-extraction.html**.

DNA isolation of BAC templates

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

- Alkaline lysis; include extra phenol extraction and isopropanol precipitation if very clean DNA is desired
- Cesium chloride (CsCl) banding

For Thermo Fisher Scientific BAC DNA preparation products, go to:

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

DNA template amplification with PCR

PCR templates can also be used to perform reliable cycle sequencing. For optimal results, purify PCR templates before sequencing. In general, any method that removes unincorporated dNTPs and primers should work.

See **https://www.thermofisher.com/us/en/home/life-science/dna-rna-purification-analysis/dna-extraction.html** for a range of suitable kits.

See the *DNA Sequencing by Capillary Electrophoresis Chemistry Guide Second Edition* (Pub. No. 4305080 for information on sequencing PCR templates.

Template quality

DNA quality can significantly influence the length of the fragment that can be amplified and the reproducibility of amplification from one sample to another. Even if the fragment successfully amplifies, poor quality DNA can result in decreased signal or increased background fluorescent noise from the sequencing reactions.

DNA quantity can also significantly affect amplification. For recommended quantities of DNA template per reaction, see “Template quantity” on page 13.

Factors affecting template quality

- **Type and amount of source material** – Influences the effectiveness and sensitivity of PCR amplification and the quality of sequencing results. The number of sequencing targets relative to the number of primer molecules can influence the efficiency and read-length of the sequencing reaction.
- **Contamination** – Can inhibit PCR amplification and cycle sequencing. Potential contaminants include:
  - Protein, RNA, or chromosomal DNA
  - Excess PCR primers, dNTPs, enzyme, and buffer components
  - Remaining salts, organic chemicals such as phenol, chloroform, and ethanol, or detergents.
  - Heparin—can partially or completely inhibit PCR amplification and cycle sequencing. The Dynabeads™ DNA DIRECT™ Blood Kit and the QIAamp™ Blood Kit (QIAGEN™, GmbH) successfully remove heparin from heparin blood samples, leaving genomic DNA ready for PCR amplification.

Note: Use a DNA isolation kit that is specifically designed for formalin-fixed, paraffin-embedded (FFPE) tissue and ensure that amplicon sizes are appropriate for the length of DNA fragment size that can be isolated.
Smaller amplicons compatible with FFPE-fragmented DNA can be designed using the free Primer Designer™ Tool found at **http://www.thermofisher.com/primerdesigner**.

Determining template quality and quantity

Use a spectrophotometer to determine DNA quality and to check for protein contamination. Optimum absorbance ratios ($A_{260/280}$) are between 1.8 and 2.0.

If DNA and/or RNA contamination is suspected, run your sample on an agarose gel. A single band should be present for high-quality DNA.

For DNA quantification, A_{260} values can be converted into µg/µL using Beer´s Law:

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

Optical density (OD) measurements are used to determine template concentration. Highly concentrated (OD >1.0) or very dilute (OD <0.05) DNA samples can lead to inaccurate OD measurements. Dilute or concentrate the DNA if needed to obtain an OD value between 0.05 to 1.

Note: OD measurement is not a reliable method to determine template concentration following enzymatic PCR purification protocols. Instead, estimate PCR product purity and concentration using an agarose gel or a flourescence-based method like the PicoGreen™ reagent for use on the Qubit™ quantification platform.

Template quantity

The table below lists the recommended quantity of template to use in a single cycle sequencing reaction. The quantity of PCR product is optimized to maximize the number of primer binding sites for the BigDye™ reaction and is dependent upon the length and purity of the PCR product.

Note: In general, higher DNA quantities give higher signal intensities.

Table 2 Recommended DNA quantities

DNA template	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	20–50 ng
Single-stranded DNA	25–50 ng
Double-stranded DNA	150–300 ng
Cosmid, BAC	0.5–1.0 µg
Bacterial genomic DNA	2–3 µg

3 Perform cycle sequencing

Prepare the reactions

Set up the sequencing reactions

IMPORTANT! Protect dye terminators from light. Cover the reaction mix and sequencing plates with aluminum foil before use.

1. Completely thaw the contents of the BigDye™ Terminator v3.1 Cycle Sequencing Kit and your primers and store on ice.
2. Vortex the tubes for 2 to 3 seconds, then centrifuge briefly (2 to 3 seconds) with a benchtop microcentrifuge to collect contents at the bottom of the tubes.
3. Add components as indicated:

   IMPORTANT! Change pipette tips after each transfer.

   IMPORTANT! For control reactions, use 4 µL of the control primers for 20 µL and 10 µL reactions. Control primer concentration = 0.8pmol/µL.

Component	Standard reaction (20 µL)			Standard reaction (10 µL)		
	Quantity per reaction	Example Forward	Example Reverse	Quantity per reaction	Example Forward	Example Reverse
BigDye™ Terminator 3.1 Ready Reaction Mix	8 µL	8 µL	8 µL	4 µL	4 µL	4 µL
Forward primer (3.2 µM)	3.2 pmol	2 µL	—	3.2 pmol	1 µL	—
Reverse primer (3.2 µM)		—	2 µL		—	1 µL
Deionized water (RNase/DNase-free)	Varies based on template and primer volume	8 µL	8 µL	Varies based on template and primer volume	4 µL	4 µL

(continued)

Component	Standard reaction (20 µL)			Standard reaction (10 µL)		
	Quantity per reaction	Example Forward	Example Reverse	Quantity per reaction	Example Forward	Example Reverse
Template	See "Template quantity" on page 13	2 µL[1],[2]	2 µL[1],[2]	See "Template quantity" on page 13	1 µL[1], [2]	1 µL[1],[2]
Total volume	**20 µL**	**20 µL**	**20 µL**	**10 µL**	**10 µL**	**10 µL**

[1] e.g. 150-300ng/µL of dsDNA

[2] Concentration of template may affect volume, if template volume differs, adjust the volume of water in the reaction mix.

Note: Store on ice and protected from light.

4. Seal the plate with MicroAmp™ Clear Adhesive Film.

5. Vortex the plate for 2–3 seconds, then centrifuge in a swinging bucket centrifuge at 1,000 x *g* for 5–10 seconds to bring the contents to the bottom of the wells.

Note: Bubbles may be present in the wells, but they do not adversely affect the reaction.

Using BigDye™ Terminator v1.1 & v3.1 5X Sequencing Buffer to dilute sequencing reactions

Some cycle sequence reactions may be optimized using diluted BigDye™ Terminator Ready Reaction Mix. The BigDye™ Terminator Ready Reaction Mix is provided at a 2.5X concentration and can be diluted using BigDye™ Terminator v1.1 and v3.1 5X Sequencing Buffer to a final end reaction concentration of 1X.

Calculate the volume of BigDye™ Terminator v1.1 and v3.1 5X Sequencing Bufferto use:

0.5 * ((total reaction volume)/2.5) - volume of BigDye™ Terminator Ready Reaction Mix)

Note: Dilution of the BigDye™ Terminator v1.1 and v3.1 5X Sequencing Buffer without optimization, may cause deterioration of sequencing quality. We can not guarantee the performance of BigDye™ Terminator chemistry when it is diluted.

Component	Diluted reaction (0.5X)		
	Quantity per reaction	Example Forward	Example Reverse
BigDye™ Terminator 3.1 Ready Reaction Mix	4 µL	4 µL	4 µL
BigDye™ Terminator v1.1 & v3.1 5X Sequencing Buffer	2 µL	2 µL	2 µL
Forward primer (3.2 µM)	3.2 pmol	2 µL[1]	—
Reverse primer (3.2 µM)		—	2 µL[1]

(continued)

Component	Diluted reaction (0.5X)		
	Quantity per reaction	Example Forward	Example Reverse
Deionized water (RNase/DNase-free)	Varies based on template and primer volume	10 µL	10 µL
Template	See "Template quantity" on page 13	2 µL[2],[3]	2 µL[2],[3]
Total volume	**20 µL**	**20 µL**	**20 µL**

[1] The control primer is provided at 0.8pmol/µL. Use 8 µL to obtain a total primer quantity of 3.2 pmol per 20 µL reaction. If primer volume differs, adjust the volume of water in the reaction mix.

[2] e.g. 150-300ng/µL of dsDNA

[3] Concentration of template may affect volume. If template volume differs, adjust the volume of water in the reaction mix.

Perform cycle sequencing

Run the sequencing reactions

1. Place the tubes or plate(s) in a thermal cycler and set the correct volume:
   - 20 µL for microcentrifuge tubes or 96-well reaction plates
   - 10 µL for 384-well reaction plates
2. Perform cycle sequencing:

Parameter	Stage/step				
	Incubate	25 cycles			Hold
		Denature	Anneal	Extend	
Ramp rate	—	1°C/second			
Temperature	96°C	96°C	50°C	60°C	4°C
Time (mm:ss)	01:00	00:10	00:05	04:00[1]	Hold until ready to purify.

[1] Shorter extension times can be used for short templates.

3. Briefly centrifuge the reactions and proceed to Chapter 4, “Purify the sequencing reactions”.

4 Purify the sequencing reactions

Salts, unincorporated dye terminators, and dNTPs in sequencing reactions obscure data in the early part of the sequence and can interfere with basecalling.

Three different methods to purify sequencing products are provided below. For a list of the required materials for each method, see "Required materials not supplied" on page 9.

Purify sequencing reactions with BigDye XTerminator™

The following protocol takes approximately 40 minutes.

For detailed instructions, see the *BigDye XTerminator™ Purification Kit User Guide* (Pub. No. 4374408).

Note: Use disposable reagent reservoirs and an 8-channel P200 pipette, if available, to facilitate the clean-up process. Use wide bore pipet tips when pipetting the BigDye XTerminator™ reagent.

Note: If you use a 3730/3730*xl* DNA Analyzer, standard heat sealing techniques can be used. This protocol describes plate sealing with MicroAmp™ Clear Adhesive Film. The MicroAmp™ Clear Adhesive Film must be removed before loading the plate on the instrument.

1. Vortex the bottle of BigDye XTerminator™ beads for 8–10 seconds before mixing with the SAM solution.

   IMPORTANT! For effective BigDye XTerminator™ clean up, it is essential to keep the materials well mixed. Keep reagents on ice between pipetting steps.

2. Prepare the SAM/BigDye XTerminator™ bead working solution:

Component	Volume per 10 µL reaction	Volume per 20 µL reaction
SAM solution	45 µL	90 µL
BigDye XTerminator™ bead solution	10 µL	20 µL
Total volume	**55 µL**	**110 µL**

3. Remove the MicroAmp™ Clear Adhesive Film from the sequencing plate.

4. Dispense the indicated volume of bead mix (BigDye XTerminator™ bead solution and SAM solution) to each sample.

   IMPORTANT! To mix thoroughly, pipette the solution up and down 3-4 times before each transfer. Re-mix solution after each dispense step.

5. Seal the plate using MicroAmp™ Clear Adhesive Film.

6. Vortex the 96-well plate at 1,800 rpm for 20 minutes (for the Digital Vortex-Genie™ 2).

   For alternative vortex mixer manufacturers and settings, see the *BigDye XTerminator™ Purification Kit Quick Reference* (Pub. No. 4383427).

7. In a swinging bucket centrifuge, centrifuge the plate at 1,000 x *g* for 2 minutes.

   Note: To store for up to 10 days, seal the plate with MicroAmp™ Clear Adhesive Film, and store at 4°C for capillary electrophoresis (CE) preparation or at –20°C until use. BDX plates can be stored at room temperature for up to 48 hours inclusive of time on the CE instrument.

Purify the sequencing reactions with ethanol/EDTA precipitation

The following protocol takes approximately 90 minutes.

Note: This method produces a clean signal, but it can cause subtle loss of small molecular weight fragments.

IMPORTANT! Absolute ethanol absorbs water from the atmosphere, which gradually decreases its concentration and can affect sequencing results. Store appropriately and replace frequently.

1. Prepare a 125 mM EDTA solution from 0.5 M EDTA, pH 8.0.

2. Prepare 70% ethanol using absolute ethanol.

   Note: Replace every 2 weeks.

   IMPORTANT! Do NOT pre-mix 125 mM EDTA solution and absolute ethanol. Pre-mixing can cause precipitation of the EDTA.

3. Briefly centrifuge the sequencing plate in a swinging bucket centrifuge at 1,000 x *g* for 5–10 seconds.

4. Remove the MicroAmp™ Clear Adhesive Film from the plate.

5. Add the following in order:

Component	Volume	
sequencing reaction (starting volume)	10 µL	20 µL
125 mM EDTA solution	2.5 µL	5 µL
absolute ethanol	30 µL	60 µL
Total volume	**42.5 µL/well**	**85 µL/well**

IMPORTANT! Dispense EDTA directly into the sample in each well. If droplets are visible on the wall of the well, briefly centrifuge the plate to ensure that the EDTA mixes with the sequencing reactions.

6. Seal the plate with MicroAmp™ Clear Adhesive Film.

7. Vortex the plate for 2–3 seconds, then centrifuge at 1,000 x *g* for 5–10 seconds.

8. Incubate the plate at room temperature for 15 minutes.

IMPORTANT! Timing of this step is critical.

9. Centrifuge the plate in a swinging bucket centrifuge at 1,870 x *g* for 45 minutes at 4°C.

IMPORTANT! Proceed to the next step immediately. If there is a delay between steps, centrifuge the plate for an extra 2 minutes, then proceed to the next step immediately.

10. Slowly remove the MicroAmp™ Clear Adhesive Film to prevent disruption of the pellet. Place 4 layers of absorbent paper into the centrifuge, then carefully invert the plate onto the paper without dislodging the pellet. Centrifuge at 185 x *g* for 1 minute.

    Do not tip out the liquid first. Do not tap the plate to help with liquid removal.

11. Add 70% ethanol to each well.

Starting reaction volume	Volume 70% ethanol
10 µL	30 µL
20 µL	60 µL

12. Seal the plate with MicroAmp™ Clear Adhesive Film, then centrifuge at 1,870 x *g* for 15 minutes at 4°C.

IMPORTANT! Proceed to the next step immediately. If there is a delay between steps, centrifuge the plate for an extra 2 minutes, then proceed to the next step immediately.

13. Slowly remove the MicroAmp™ Clear Adhesive Film to prevent disruption of the pellet. Place 4 layers of absorbent paper into the centrifuge, then carefully invert the plate onto the paper towel without dislodging the pellet. Centrifuge at 185 x *g* for 1 minute.

Note: Do not tip out the liquid first. Do not tap the plate to help with liquid removal.

14. Allow the plate to air dry, face up and protected from light, for 5–10 minutes at room temperature.

Note: Alternatively, seal the plate with MicroAmp™ Clear Adhesive Film, then store protected from light at 4°C for CE preparation or –20°C until use.

5 Capillary electrophoresis

Capillary electrophoresis guidelines

We recommend that you verify the quality of your current matrix file or spectral calibration before proceeding. To generate a new matrix file or spectral calibration, use the appropriate matrix and/or sequencing standard for your instrument.

The existing mobility files can be used with their respective platforms.

- Resuspend purified and dried sequencing reactions in 10-µL of Hi-Di™ Formamide.

  Do not heat samples to resuspend. Run samples as soon as possible after resuspension.

  Note: It is not necessary to resuspend samples purified with the BigDye XTerminator™ Purification Kit.

- Standard heat seal consumables can only be used for the 3730/3730*xl* DNA Analyzer.
- Select the correct mobility file for your instrument, polymer and dye chemistry.

  For example: Select KB_3500_POP7_BDTv3.mob for data electrophoresed on a 3500/3500xL Genetic Analyzer with POP-7™ polymer and generated with BigDye™ Terminator v3.1 chemistry.
- Use the BDx run modules if you used the BigDye XTerminator™ Purification Kit for sequencing reaction clean up.
- If the wrong mobility file is used, this can be corrected with Sequencing Analysis Software. For more information go to *DNA Sequencing Analysis Software 6* (Pub. No. 4474239).
- Check **At PCR Stop** in the **Basecalling** tab and the **Use Mixed Base Identification** in the **Mixed Bases** tab when analyzing PCR products with Sequencing Analysis Software. Do not select these settings when performing *de novo* or plasmid sequencing.

Calibration guidelines

Matrix or sequencing standards provide a sample for multi-color spectral correction for the dye emission overlap of the BigDye™ Terminators.

Spectral calibrations for the BigDye™ Terminator v1.1 are not compatible with BigDye™ Terminator v3.1.

Perform new spectral calibrations after you install a capillary array or move capillaries in the detection areas.

See your specific instrument user guide for more information on calibration.

Dye set and spectral matrix standards

Instrument	Dye set	Standards for spectral calibration [1]
SeqStudio™ Genetic Analyzer	Z	BigDye™ Terminator v3.1 Matrix Standards Kit (Cat. No. 4336974)
SeqStudio™ Flex Series Genetic Analyzer		
3500/3500xL Genetic Analyzer		
3730/3730*xl* DNA Analyzer		N/A[2]

[1] Refer to the matrix or sequence standards product insert for instructions on performing spectral calibrations.
[2] Matrix standards are not designed for use on the 3730/3730*xl* DNA Analyzer; use sequencing standards only.

Sequencing standards

Instrument	Kit	Cat. no.
SeqStudio™ Genetic Analyzer	3500/3500xL Sequencing Standards, BigDye™ Terminator v3.1	4404312
SeqStudio™ Flex Series Genetic Analyzer		
3500/3500xL Genetic Analyzer		
3730/3730*xl* DNA Analyzer	3730/3730*xl* DNA Analyzer Sequencing Standards, BigDye™ Terminator v3.1	4336943

Electrophoresis on the 3500/3500xL Genetic Analyzer

IMPORTANT! Use BDX run modules only if you prepare samples with the BigDye XTerminator™ Purification Kit. Use non-BDX run modules for samples purified with other methods.

Note: You need to specify the mobility file in the analysis settings.

Item	Module name	Polymer	Capillary length	Mobility files
Dye set Z (BigDye™ Terminator v3.1)	RapidSeq36_POP4	POP-4™	36-cm	KB_3500_POP4_BDTv3.mob
	RapidSeq36_POP4xl	POP-4™	36-cm	
	BDxRapidSeq36_POP4	POP-4™	36-cm	
	BDxRapidSeq36_POP4xl	POP-4™	36-cm	
	RapidSeq36_POP6	POP-6™	36-cm	KB_3500_POP6_BDTv3.mob
	RapidSeq36_POP6xl	POP-6™	36-cm	
	BDxRapidSeq36_POP6	POP-6™	36-cm	
	BDxRapidSeq36_POP6xl	POP-6™	36-cm	
	StdSeq50_POP6	POP-6™	50-cm	
	StdSeq50_POP6xl	POP-6™	50-cm	
	BDxStdSeq50_POP6	POP-6™	50-cm	
	BDxStdSeq50_POP6xl	POP-6™	50-cm	
	RapidSeq50_POP6	POP-6™	50-cm	
	RapidSeq50_POP6xl	POP-6™	50-cm	
	BDxRapidSeq50_POP6	POP-6™	50-cm	
	BDxRapidSeq50_POP6xl	POP-6™	50-cm	
	FastSeq50_POP6	POP-6™	50-cm	
	FastSeq50_POP6xl	POP-6™	50-cm	
	BDxFastSeq50_POP6	POP-6™	50-cm	
	BDxFastSeq50_POP6xl	POP-6™	50-cm	
	MicroSeq50_POP6	POP-6™	50-cm	
	MicroSeq50_POP6xl	POP-6™	50-cm	
	RapidSeq36_POP7	POP-7™	36-cm	KB_3500_POP7_BDTv3.mob
	RapidSeq36_POP7xl	POP-7™	36-cm	

(continued)

Item	Module name	Polymer	Capillary length	Mobility files
Dye set Z (BigDye™ Terminator v3.1)	BDxRapidSeq36_POP7	POP-7™	36-cm	KB_3500_POP7_BDTv3.mob
	BDxRapidSeq36_POP7xl	POP-7™	36-cm	
	FastSeq36_POP7	POP-7™	36-cm	
	FastSeq36_POP7xl	POP-7™	36-cm	
	BDxFastSeq36_POP7	POP-7™	36-cm	
	BDxFastSeq36_POP7xl	POP-7™	36-cm	
	StdSeq50_POP7	POP-7™	50-cm	
	StdSeq50_POP7xl	POP-7™	50-cm	
	BDxStdSeq50_POP7	POP-7™	50-cm	
	BDxStdSeq50_POP7xl	POP-7™	50-cm	
	RapidSeq50_POP7	POP-7™	50-cm	
	RapidSeq50_POP7xl	POP-7™	50-cm	
	BDxRapidSeq50_POP7	POP-7™	50-cm	
	BDxRapidSeq50_POP7xl	POP-7™	50-cm	
	FastSeq50_POP7	POP-7™	50-cm	
	FastSeq50_POP7xl	POP-7™	50-cm	
	BDxFastSeq50_POP7	POP-7™	50-cm	
	BDxFastSeq50_POP7xl	POP-7™	50-cm	
	ShortReadSeq50_POP7	POP-7™	50-cm	
	ShortReadSeq50_POP7xl	POP-7™	50-cm	
	BDxShortReadSeq50_POP7	POP-7™	50-cm	
	BDxShortReadSeq50_POP7xl	POP-7™	50-cm	
	MicroSeq_POP7	POP-7™	50-cm	
	MicroSeq_POP7xl	POP-7™	50-cm	

Performing electrophoresis

For information on performing sample electrophoresis on the 3500/3500xL Genetic Analyzer, see:

- *3500/3500xL Genetic Analyzer with 3500 Series Data Collection Software v 3.1 User Guide* (Pub. No. 100031809)
- *3500/3500xL Genetic Analyzer Software Compatibility and User Documents Reference* (Pub. No. MAN0015980)

Electrophoresis on the 3730/3730*xl* DNA Analyzer

IMPORTANT! Use BDX run modules only if you prepare samples with the BigDye XTerminator™ Purification Kit. Use non-BDX run modules for samples purified with other methods.

Note: You need to specify the mobility file in the analysis settings.

Item	Module name	Polymer	Capillary length	Mobility files
Dye set Z (BigDye™ Terminator v3.1)	StdSeq36_POP-6	POP-6™	36-cm	KB_3730_POP6_BDTv3.mob
	BDX_StdSeq36_POP-6	POP-6™	36-cm	
	RapidSeq36_POP6	POP-6™	36-cm	
	BDX_RapidSeq36_POP6	POP-6™	36-cm	
	LongSeq50_POP-6	POP-6™	50-cm	
	BDX_LongSeq50_POP-6	POP-6™	50-cm	
	StdSeq36_POP-7	POP-7™	36-cm	KB_3730_POP7_BDTv3.mob
	BDX_StdSeq36_POP7	POP-7™	36-cm	
	RapidSeq36_POP-7	POP-7™	36-cm	
	BDX_RapidSeq36_POP-7	POP-7™	36-cm	
	TargetSeq36_POP-7	POP-7™	36-cm	
	BDX_TargetSeq36_POP-7	POP-7™	36-cm	
	FastSeq36_POP-7	POP-7™	36-cm	
	BDX_FastSeq36_POP-7	POP-7™	36-cm	
	FastSeq50_POP-7	POP-7™	50-cm	
	BDX_FastSeq50_POP-7	POP-7™	50-cm	
	LongSeq50_POP-7	POP-7™	50-cm	
	BDX_LongSeq50_POP-7	POP-7™	50-cm	

(continued)

Item	Module name	Polymer	Capillary length	Mobility files
Dye set Z (BigDye™ Terminator v3.1)	XLRSeq50_POP-7	POP-7™	50-cm	KB_3730_POP7_BDTv3.mob
	BDX_XLRSeq50_POP-7	POP-7™	50-cm	

Performing electrophoresis

For information on performing sample electrophoresis on the 3730/3730*xl* DNA Analyzer, see:

- *3730/3730xl DNA Analyzer Software/Firmware Compatibility and User Documents Reference* (Pub. No. MAN0016105)
- *Chemistry Guide, 3730/3730xl DNA Analyzer* (Pub. No. 4331467)
- *User Guide: Applied Biosystems™ 3730/3730xl DNA Analyzer* (Pub. No. 4331468)
- *Applied Biosystems™ 3730/3730xl DNA Analyzer Maintenance and Troubleshooting Guide* (Pub. No. 4359473)

Electrophoresis on the SeqStudio™ Genetic Analyzer

IMPORTANT! Use BDX run modules only if you prepare samples with the BigDye XTerminator™ Purification Kit. Use non-BDX run modules for samples purified with other methods.

Note: The mobility information is automatically selected based on the dye set that is used to collect the data.

Item	Module name	Polymer	Capillary length	Mobility files
Dye set Z (BigDye™ Terminator v3.1)	ShortSeq	N/A (Cartridge)	N/A (Cartridge)	Automatic per dye set designation
	ShortSeq_BDX	N/A (Cartridge)	N/A (Cartridge)	
	MediumSeq	N/A (Cartridge)	N/A (Cartridge)	
	MediumSeq_BDX	N/A (Cartridge)	N/A (Cartridge)	
	LongSeq	N/A (Cartridge)	N/A (Cartridge)	
	LongSeq_BDX	N/A (Cartridge)	N/A (Cartridge)	

Performing electrophoresis

For information on performing sample electrophoresis on the SeqStudio™ Genetic Analyzer, see:

- *SeqStudio™ Genetic Analyzer Instrument and Software User Guide* (Pub. No. MAN0018646)
- *SeqStudio™ Genetic Analyzer Instrument and Software Getting Started Guide* (Pub. No. MAN0018654)

Electrophoresis on the SeqStudio™ Flex Series Genetic Analyzer

IMPORTANT! Use BDX run modules only if you prepare samples with the BigDye XTerminator™ Purification Kit. Use non-BDX run modules for samples purified with other methods.

Note: The mobility information is automatically selected based on the dye set that is used to collect the data.

Item	Module name	Polymer	Capillary length	Mobility files
Dye set Z (BigDye™ Terminator v3.1)	RapidSeq36_POP7	POP-7™	36-cm	Automatic per dye set designation
	RapidSeq36_POP7xl	POP-7™	36-cm	
	BDxRapidSeq36_POP7	POP-7™	36-cm	
	BDxRapidSeq36_POP7xl	POP-7™	36-cm	
	FastSeq36_POP7	POP-7™	36-cm	
	FastSeq36_POP7xl	POP-7™	36-cm	
	BDxFastSeq36_POP7	POP-7™	36-cm	
	BDxFastSeq36_POP7xl	POP-7™	36-cm	
	RapidSeq36_POP6	POP-6™	36-cm	
	RapidSeq36_POP6xl	POP-6™	36-cm	
	BDxRapidSeq36_POP6	POP-6™	36-cm	
	BDxRapidSeq36_POP6xl	POP-6™	36-cm	
	RapidSeq36_POP4	POP-4™	36-cm	
	RapidSeq36_POP4xl	POP-4™	36-cm	
	BDxRapidSeq36_POP4	POP-4™	36-cm	
	BDxRapidSeq36_POP4xl	POP-4™	36-cm	
	ShortReadSeq50_POP7	POP-7™	50-cm	
	ShortReadSeq50_POP7xl	POP-7™	50-cm	
	BDxShortReadSeq50_POP7	POP-7™	50-cm	
	BDxShortReadSeq50_POP7xl	POP-7™	50-cm	
	RapidSeq50_POP7	POP-7™	50-cm	
	RapidSeq50_POP7xl	POP-7™	50-cm	

(continued)

Item	Module name	Polymer	Capillary length	Mobility files
Dye set Z (BigDye™ Terminator v3.1)	BDxRapidSeq50_POP7	POP-7™	50-cm	Automatic per dye set designation
	BDxRapidSeq50_POP7xl	POP-7™	50-cm	
	FastSeq50_POP7	POP-7™	50-cm	
	FastSeq50_POP7xl	POP-7™	50-cm	
	BDxFastSeq50_POP7	POP-7™	50-cm	
	BDxFastSeq50_POP7xl	POP-7™	50-cm	
	StdSeq50_POP7	POP-7™	50-cm	
	StdSeq50_POP7xl	POP-7™	50-cm	
	BDxStdSeq50_POP7	POP-7™	50-cm	
	BDxStdSeq50_POP7xl	POP-7™	50-cm	
	RapidSeq50_POP6	POP-6™	50-cm	
	RapidSeq50_POP6xl	POP-6™	50-cm	
	BDxRapidSeq50_POP6	POP-6™	50-cm	
	BDxRapidSeq50_POP6xl	POP-6™	50-cm	
	FastSeq50_POP6	POP-6™	50-cm	
	FastSeq50_POP6xl	POP-6™	50-cm	
	BDxFastSeq50_POP6	POP-6™	50-cm	
	BDxFastSeq50_POP6xl	POP-6™	50-cm	
	StdSeq50_POP6	POP-6™	50-cm	
	StdSeq50_POP6xl	POP-6™	50-cm	
	BDxStdSeq50_POP6	POP-6™	50-cm	
	BDxStdSeq50_POP6xl	POP-6™	50-cm	
	MicroSeqID50_POP6	POP-6™	50-cm	
	MicroSeqID50_POP6xl	POP-6™	50-cm	

Performing electrophoresis

For information on performing sample electrophoresis on the SeqStudio™ Flex Series Genetic Analyzer, see *SeqStudio™ Flex Series Genetic Analyzer with Instrument Software v1.1.1 User Guide* (Pub. No. 100104689).

A Troubleshooting Sanger sequencing data

Troubleshooting poor Sanger sequencing quality

Common sources of noise	How to recognize the source	Page Number
No signal	Little to no raw signal.	32
Low signal intensity	The raw signal is below the recommended minimum relative fluorescence units (RFU).	33
Mixed sequence throughout	Mixed sequence content throughout the length of the trace.	36
Mixed sequence up to or after a point	Mixed sequence content starting at a specific point.	37
Poor mobility correction	Peaks overlapped and unevenly spaced	38
Dye blobs	Large broad peak normally seen at 85–90 bp or 125–130 bp.	39
Signal saturation	The raw signal exceeds the recommended maximum RFU. **Note:** Excessive raw signal causes pull-up peaks in the analyzed data, which can incorrectly be identified as mixed bases.	41
Poor 5′ sequencing quality	Poor quality sequence in the first 35 nucleotides of the trace.	44

For more information on Sanger sequencing symptoms and troubleshooting, see the *DNA Sequencing by Capillary Electrophoresis Chemistry Guide Second Edition* (Pub. No. 4305080).

Recommended raw signal ranges

Different instruments have different recommended fluorescence ranges and levels at which signal is saturated. Data quality can be compromised when signals do not fall in the appropriate range. The subsequent troubleshooting sections will help you to recognize and correct issues relating to raw fluorescent signals.

Instrument	Recommended raw signal range	Fluorescence saturation
SeqStudio™ Genetic Analyzer	150–10,000 RFU	32,000 RFU
SeqStudio™ Flex Series Genetic Analyzer	150–10,000 RFU	32,000 RFU
3500/3500xL Genetic Analyzer	150–10,000 RFU	32,000 RFU
3730/3730*xl* DNA Analyzer	175–10,000 RFU	32,000 RFU

The use of controls

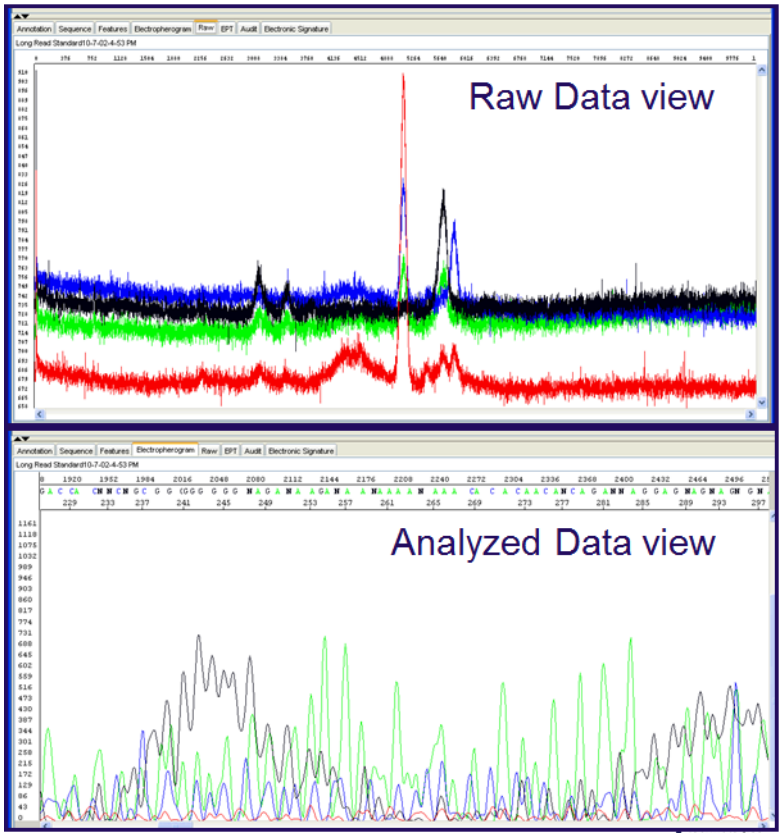
The use of controls are recommended to simplify troubleshooting.

- pREF-BDT™ Control DNA—Results can help you determine whether failed reactions are caused by poor template quality or sequencing reaction failure. See “Control template” on page 10.
- Big Dye Terminator Sequencing standards—Results can help you distinguish between chemistry problems and instrument problems.
- Use of Hi-Di™ Formamide only injection *(Optional)*—Results can help distinguish sample problems and instrument problems related to contamination.

No signal

Lack of signal can be determined by looking at the scale of signal produced in the raw data view. Signal should be above the minimum recommended RFU. See “Recommended raw signal ranges” on page 31. Lack of signal can be caused by many factors. These include problems in the sequencing reaction (template quantity/quality), thermal cycler malfunction (plate failure) and capillary electrophoresis failure (failing laser, air bubbles in lines, etc.).

Example of no signal



No signal due to hardware failure or a failed reaction

No signal: possible causes and recommended actions

Possible cause	Recommended action
Insufficient template	Quantitate the DNA template.
	Increase the amount of DNA in the sequencing reactions. See “Template quantity” on page 13.

(continued)

Possible cause	Recommended action
Inhibitory contaminant in the template	Clean up the template. See Chapter 2, “Prepare templates”
Insufficient primer	Quantitate the primer and increase the amount of primer in the sequencing reactions if needed.
Primer has no annealing site	Use a primer that is complementary to the template.
Poor primer design or incorrect primer sequence	Review the primer design and if needed redesign the primer.
Missing reagent	Repeat the reactions, carefully following the protocol. Use pREF-BDT™ Control DNA to confirm reagent workflow performance.
Old or mishandled reagents	Use fresh reagents.
Thermal cycler malfunction	Test the thermal cycler per the manufacturer's instruction and repeat the reactions.
Thermal cycling conditions incorrect	Calibrate the thermal cycler regularly.
	Use the correct thermal cycling parameters.
	Use the correct tubes or plates for your thermal cycler.
	Set the ramp rate to 1°C/second.
Extension products lost during reaction cleanup	Use the correct centrifuge speeds and times for the precipitation procedures and the spin column or spin plate procedures.
	Check that the ethanol concentration is correct for the precipitation protocols.
Extension products not resuspended	Carefully resuspend the sample pellet in Hi-Di™ Formamide.
Electrokinetic injection failure	Run the Sequencing standard to confirm the performance of the instrument and capillary array. Confirm the BDX run module is being used for BigDye XTerminator™-purified samples. Confirm the correct volume is in the well. Confirm that the sample plate does not have a bubble at the bottom of the well. Briefly centrifuge to remove bubble.

Low signal

Low signal intensity can be caused by many factors including thermal cycler malfunction (in the case of an entire plate failure) and insufficient sequencing template quantity/quality.

Examples of low signal intensity

The examples below show severely low signal traces.

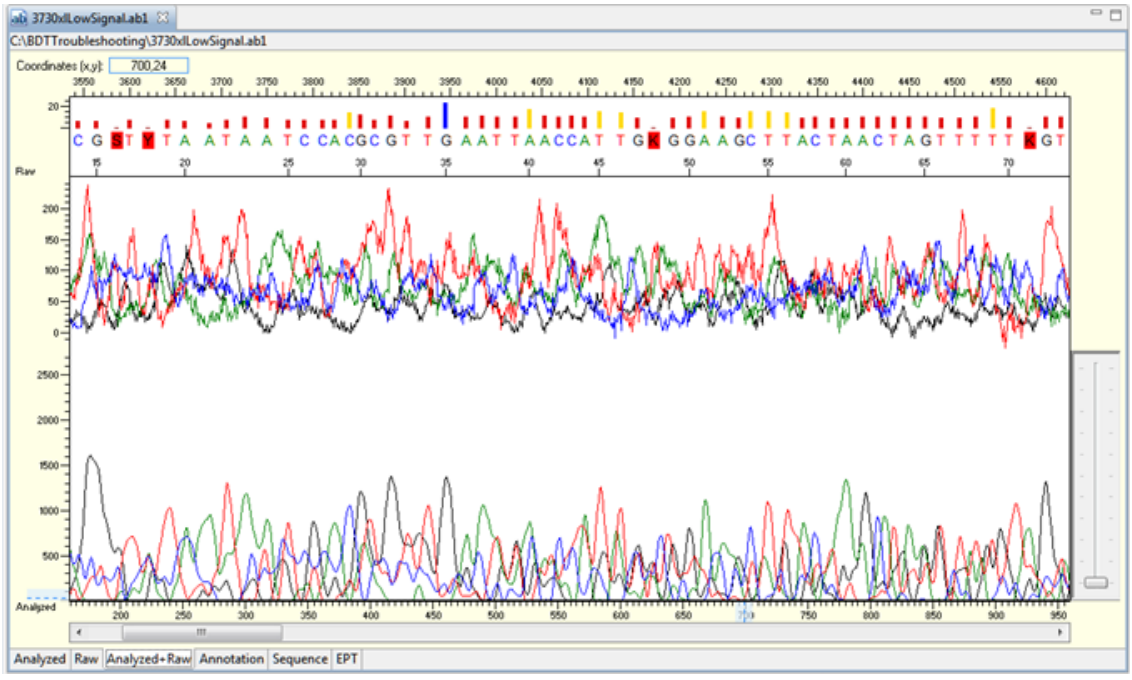


Figure 1 Severely low signal intensity

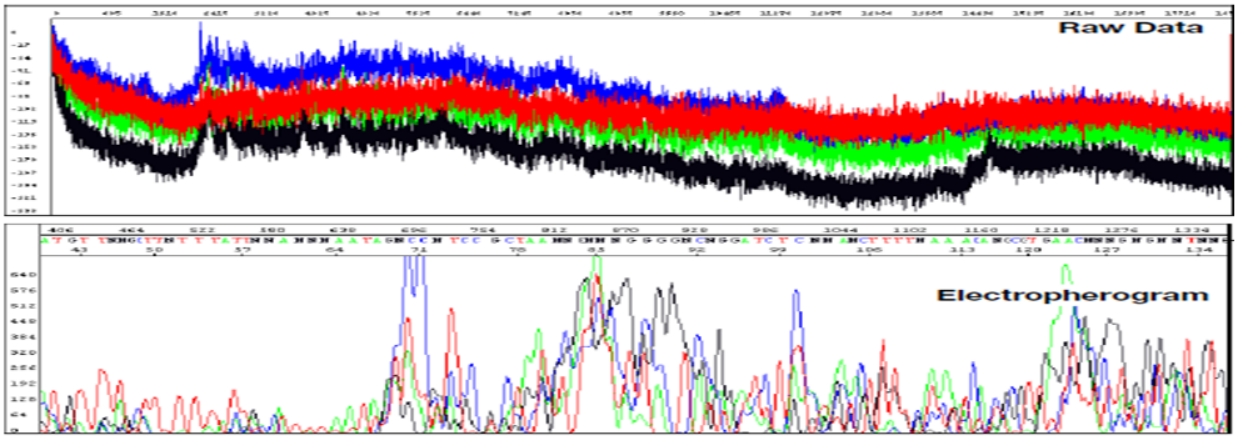


Figure 2 Severely low signal intensity due to hardware failure or a failed reaction

More severe signal issues are often related to poor injection, failed reaction, or a blocked or broken capillary.

Low signal possible causes and recommended actions

Note: When sequencing signal is weak, increasing the injection time (re-injecting sample) or increasing primer and/or template in the cycle sequencing reactions can improve signal strength if DNA quality, PCR purification, and sequencing reaction purification steps have been performed properly.

Possible cause	Recommended action
Insufficient DNA in the sequencing reactions	Increase DNA quantity in the sequencing reactions. See "Template quantity" on page 13.
	Load or inject more of the resuspended sequencing reactions by modifying the run module and increasing the injection time. See the appropriate instrument User Guide for additional information..
Poor primer quality or quantity	Prepare a fresh working stock of primer or order new primer. Use 3.2pmol in the final reaction.
Degraded template	Prepare fresh DNA and repeat the reactions.
Poor PCR cleanup	Purify PCR products before use. See "DNA template amplification with PCR" on page 11.
Old or mishandled reagents	Use fresh reagents.
Thermal cycling conditions incorrect	Calibrate the thermal cycler regularly.
	Use the correct thermal cycling parameters.
	Use the correct tubes or plates for your thermal cycler.
	Set the ramp rate to 1°C/second.
Insufficient Ready Reaction Mix in the reactions	Sequencing chemistry was diluted. See "Prepare the reactions" on page 14 for recommended procedures. **Note:** Thermo Fisher Scientific can not support diluted reactions or guarantee the performance of diluted BigDye chemistry.

Mixed sequence throughout the electropherogram

Example of mixed sequence throughout

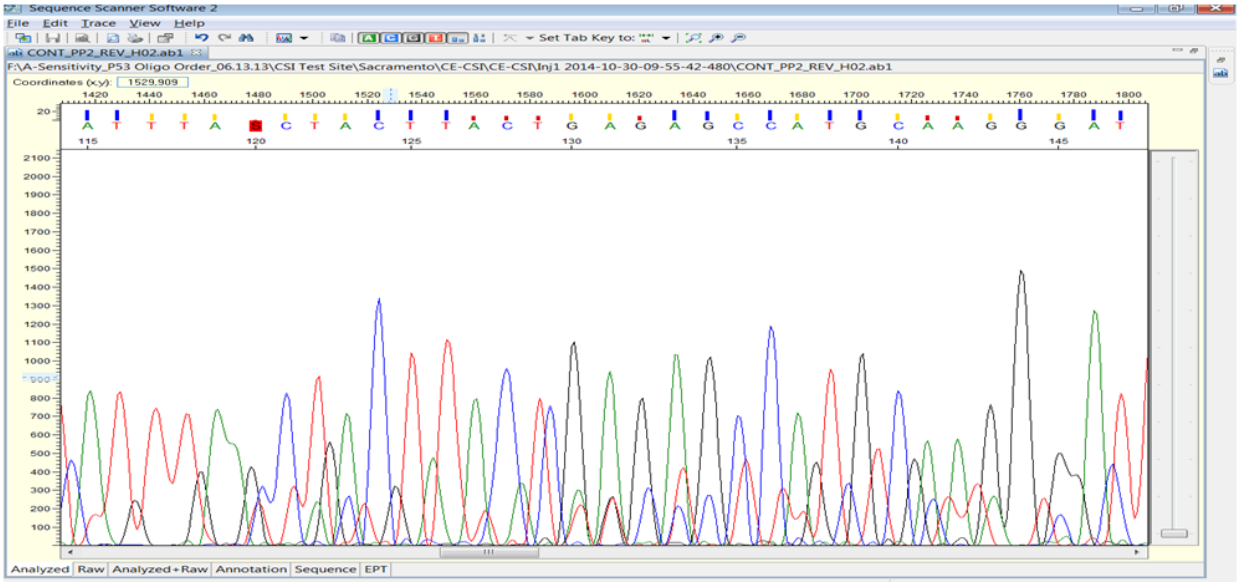


Figure 3 Secondary sequence contamination caused by well-to-well contamination of one sample into another

Mixed sequence throughout possible causes and recommended actions

Possible cause	Recommended action
Inhibitory contaminant in the template	Clean up the template.
Multiple templates in the sequencing reaction	Examine the template on an agarose gel to be sure that only one template is present.
Multiple priming sites	Verify that the primer has only one priming site.
	If needed, redesign the primer. See Appendix B, “Select sequencing primers”
Multiple primers when sequencing PCR products	Purify the PCR template to remove excess primers.
Primer with N-1 contamination	Use an HPLC-purified primer
High signal saturating the detector	Use less DNA in the sequencing reactions. See “Template quantity” on page 13.
	Load or inject less of the resuspended sequencing reactions by modifying the run module and decreasing the injection time. See the appropriate instrument User Guide for additional information.

(continued)

Possible cause	Recommended action
Incorrect run module	Use a default run module.
Incorrect instrument (matrix) file	Use the correct instrument file for BigDye™ Terminator chemistry.

Mixed sequence up to or after a certain point

Example of mixed sequence after a point

Figure 4 Example of mixed sequence content following a heterozygous insertion or deletion. Mixed sequence content is seen in both forward and reverse traces.

Mixed sequence up to or after a certain point possible causes and recommended actions

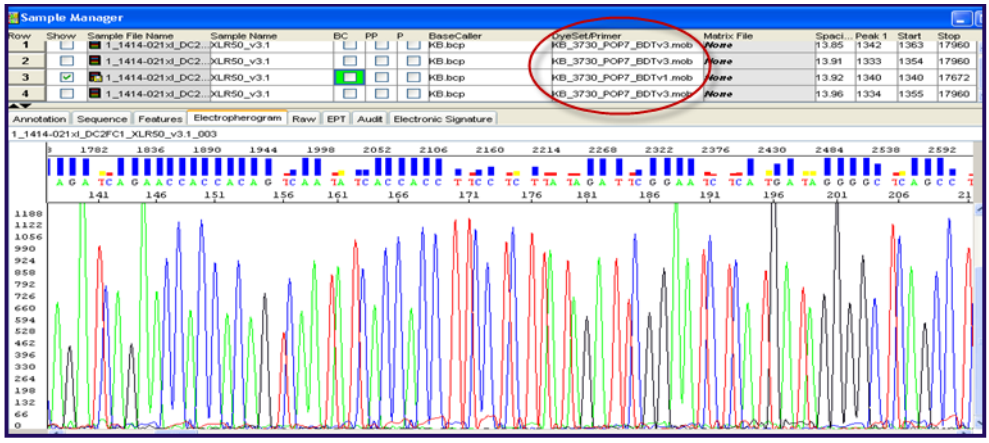
Possible cause	Recommended action
Mixed plasmid separation	Be sure that you have only one template.
Multiple PCR products	
Primer-dimer contamination in PCR sequencing	Optimize your PCR amplification.
	Be sure that there is no sequence complementarity between the two PCR primers.

(continued)

Possible cause	Recommended action
Primer-dimer contamination in PCR sequencing	Be sure that your sequencing primer does not overlap the sequences of the PCR primers.
	Use a Hot Start technique, such as with AmpliTaq Gold™ DNA Polymerase.
Slippage after repeat region in template	Try an alternative sequencing chemistry.
	Use an anchored primer. Some customers have gotten past poly(A) regions using a mixture of oligo dT18 primers with either a C, A, or G as the 3′ terminal dinucleotide or 2-base anchors. See the *DNA Sequencing by Capillary Electrophoresis Chemistry Guide Second Edition* (Pub. No. 4305080) for more information.
Heterozygous insertion or deletion mutation (HIM)	Obtain forward and reverse sequence data and assemble using SeqScape™ Software or Variant Reporter™ Software. • SeqScape™ Software lists HIMs in the Mutations Report. Clicking the **Base Change** in the **Mutations Report** to view the mutation in the **Project** view. • Variant Reporter™ Software lists HIMs in the **Project Summary Report**.

Poor mobility correction

Example of poor mobility correction



Example electropherogram with the wrong mobility file selected.

Poor mobility correction possible causes and recommended actions

Possible cause	Recommended action
Incorrect dye set/primer (mobility) file	Use the correct dye set/primer file.
Incorrect Peak 1 location for data analysis	Select a new Peak 1 location.

Dye blobs

Dye blobs are caused by unincorporated dye terminators remaining in solution after purification of the cycle sequencing reactions. Unincorporated dye terminators from the BigDye™ Terminator v3.1 Cycle Sequencing Kit and BigDye™ Direct Cycle Sequencing Kit are most commonly seen to co-migrate with the ~ 85–90 bp labeled fragments. In more severe instances, these blobs can also be detected at ~ 60–65 bp and within 125–140 bp regions. Dye blobs are typically seen as broad “C” or “T” peaks, but can also show up as “G” blobs. Dye blobs are more common when first testing new sequencing purification methods.

Example of dye blobs

Figure 5 shows severe dye blobs in the 60–65bp, 85–100bp, and 125–140bp regions. Although the sequence quality appears high, the blobs obscure nearly 40 bp of the 100 bases displayed. This would make the sequence unsuitable for variant detection.

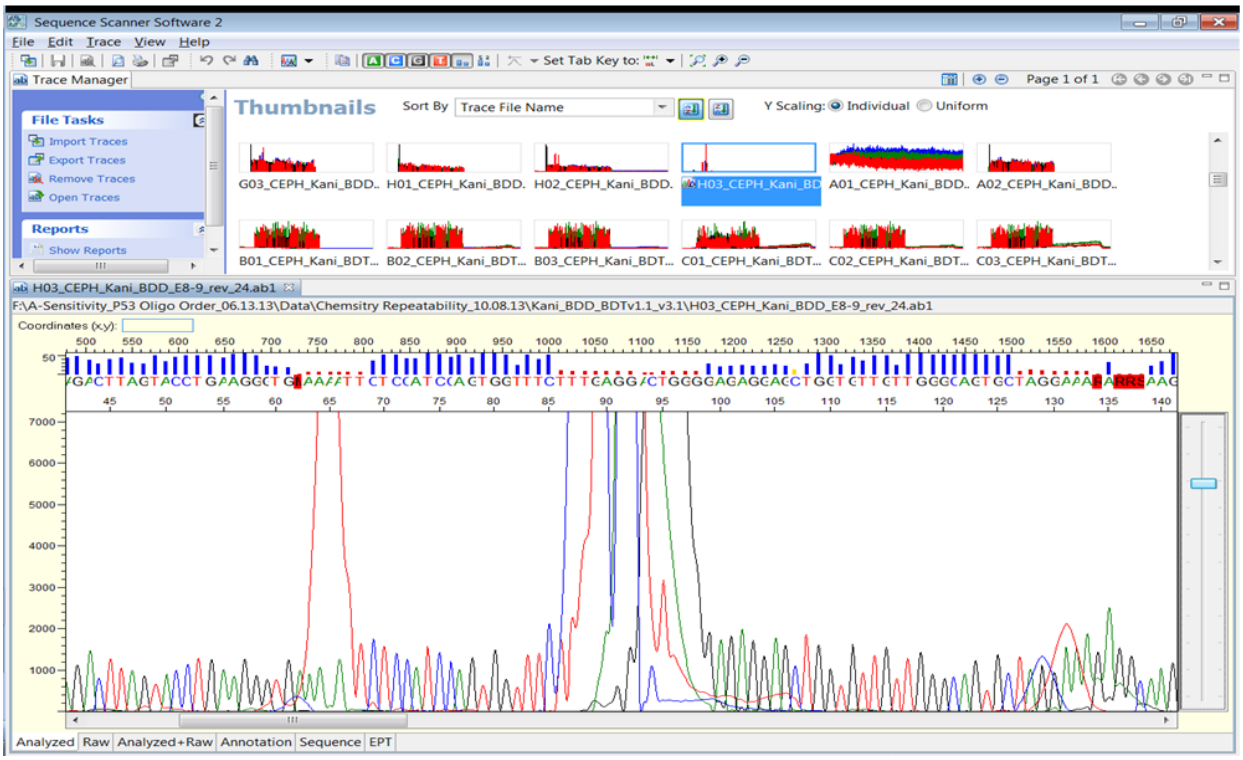


Figure 5 Severe dye blobs in the 60–65bp and 125–140bp regions

Dye blobs: possible causes and recommended actions

Possible cause	Recommended action
Sample bypassed the purification material when using spin columns/spin plates for sequencing clean-up.	Ensure transfer of the sample to the center of the purification material without the pipet tip touching the purification material. Sample dispensed along the walls of the clean-up column may bypass the purification material. Use a single channel pipette and/or position the tip directly above the spin column/plate while dispensing at low speed.

(continued)

Possible cause	Recommended action
Ethanol concentration is too high during ethanol precipitation. This leads to unincorporated dye terminators and salts precipitating with the sequencing product.	Repeat procedure with correct ethanol concentration.
Incorrect ratio of BigDye XTerminator™ reagents.	Vortex theBigDye XTerminator™ Solution bulk container at maximum speed for at least 10 seconds before dispensing. Use wide bore tips when pipetting Xterminator solution. If you pre-mix the SAM/BDX solution, ensure that the solution is well mixed before each sample well dispense step to maintain the appropriate ratio of reagents.
Insufficient mixing during the vortexing step when using the BigDye XTerminator™ Purification Kit.	Verify that the plate is firmly attached to the vortexer. Follow the protocol for vortexing. See *BigDye XTerminator™ Purification Kit Quick Reference* (Pub. No. 4383427) for recommended vortexers.

Signal saturation

High sample signal causes saturation of the CCD camera. Signal saturation causes pull-up spectral peaks that cannot be corrected by spectral calibration. Extreme signal saturation will appear as mixed sequence.

Note: The 3500 Data collection software flags .ab1 files with off-scale peaks. You must manually check for off-scale peaks from data generated with the SeqStudio™ Genetic Analyzer, the SeqStudio™ Flex Series Genetic Analyzer, or the 3730/3730*xl* DNA Analyzer platforms.

Examples of signal saturation

The following figure shows examples of signal saturation. The red line indicates the maximum raw signal recommended.

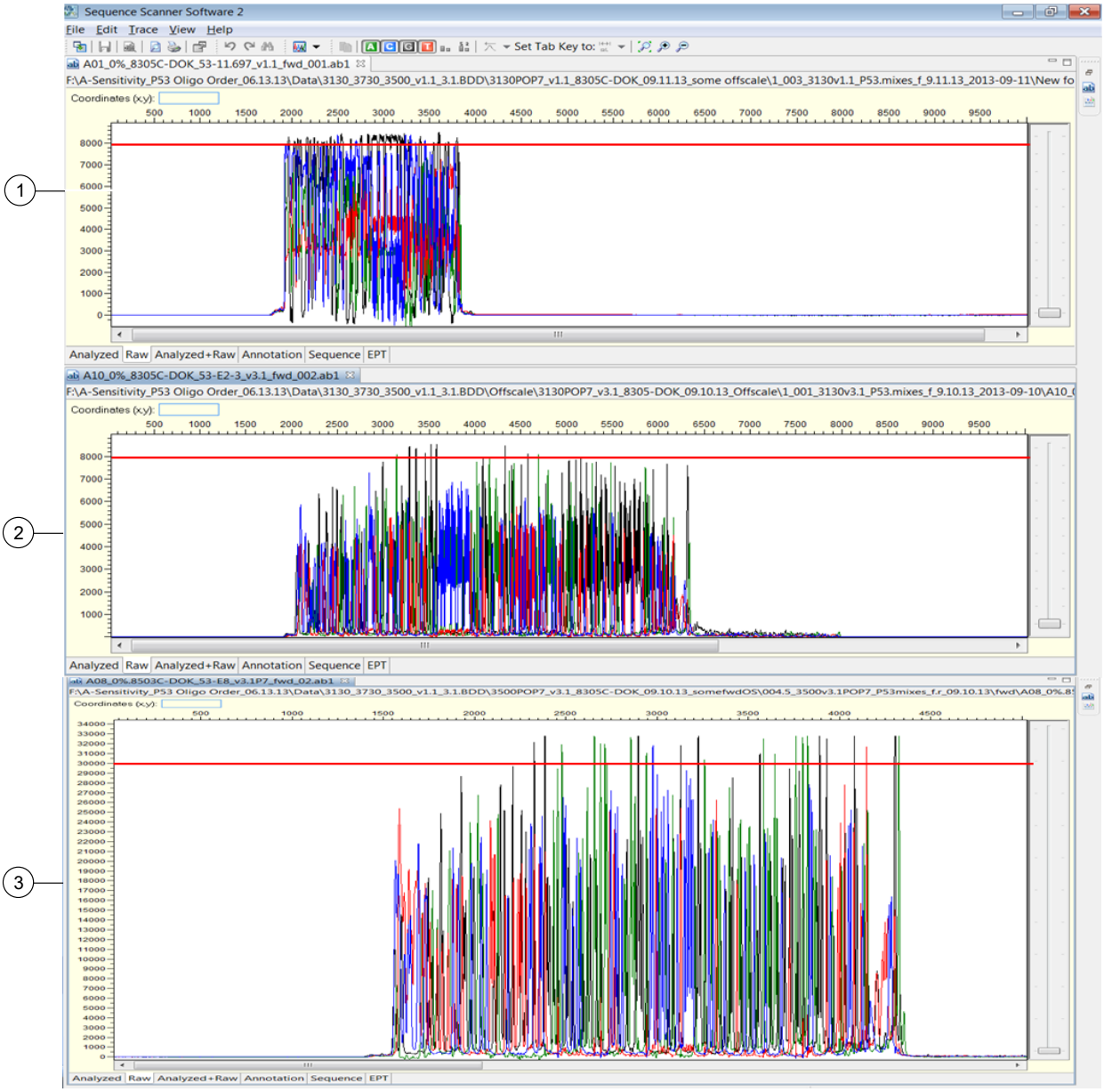
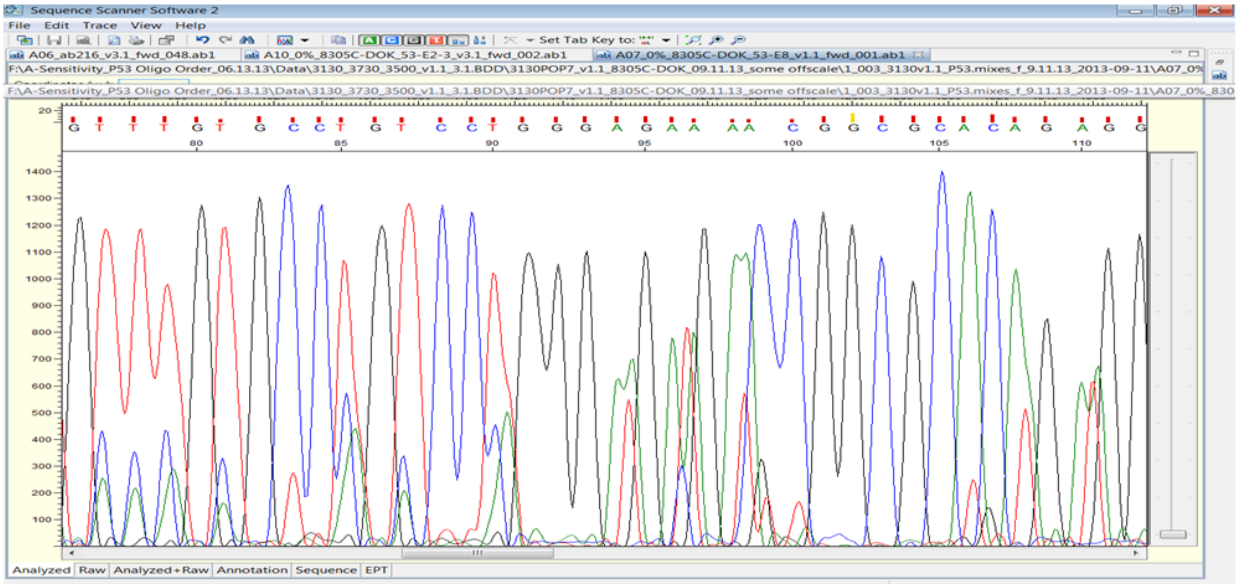


Figure 6 Signal saturation – Raw data view of minor signal saturation on a 3500 Genetic Analyzer

Example of extreme signal saturation



Mixed sequence in the **Analyzed** view. Mixed sequence can be due to extreme signal saturation caused by pull-up peaks. A quick review of the raw data can help diagnose a scenario such as this; the raw data view of the analyzed sample shown here is shown in the top panel in Figure 6.

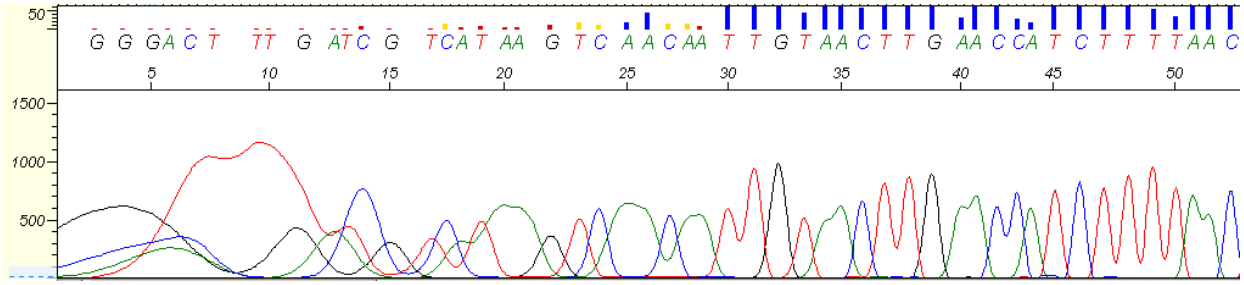
Signal saturation: possible causes and recommended actions

Possible cause	Recommended action
Too much template was used in the sequencing reaction resulting in too much sequencing product.	If the sample has been on instrument <24 hours, reduce injection time in run module, then re-inject the sample.
	If the sample is purified with the BigDye XTerminator™ Purification Kit and has been on instrument <24 hours, carefully remove 10 µL of sample off the BigDye XTerminator™ beads in the plate, then add 10 µL of 0.1 mM EDTA to dilute the sample. Re-inject the sample using a standard run module (non-BigDye XTerminator™ module). Decrease the injection voltage and injection time to match the BDX run module.
	Repeat the sequencing reaction using less template.
Water was used as the injection solution.	Use Hi-Di™ Formamide or a 0.1 mM EDTA injection solution for samples. **Note:** Using water as an injection solution causes highly variable quantities of DNA to be injected, because there is no competition for the charged DNA/salts.

Poor 5′ sequencing quality

Although v3.1 and POP-7™ provide robust read lengths, properties of the dyes and polymer limit the ability to resolve the first 20–25-bp in sequences. If a reading close to the first base following the sequencing priming region is required, an alternative approach would be to try v1.1 in combination with POP-6™ polymer or using the BigDye™ Direct Cycle Sequencing Kit.

Example of poor 5′ sequencing quality



Poor 5′ sequencing quality causes and actions

Possible cause	Recommended action
This is normal within the first 35 bp when using POP-7™ polymer and v3.1 chemistry.	To obtain sequence close to the primer, the use of POP-6™ polymer and the BigDye™ Terminator v1.1 Cycle Sequencing Kit, or the BigDye™ Direct Cycle Sequencing Kit is recommended.
BigDye XTerminator™ purification reagents exposed to temperature over 25°C.	Keep BigDye XTerminator™ purification reagents on ice if working with reagents for more than 30 minutes.

B

Select sequencing primers

Primer considerations

The following factors can have a significant effect on the quality of the sequencing data obtained in dye terminator cycle sequencing reactions with this kit:

- Choice of sequencing primer sequence
- Approach to primer purification

These decisions are particularly important when sequencing is done on capillary electrophoresis systems where signal strength is critical. Some of the guidelines provided below are based on information that is general knowledge, while others are based on practical experience gained by our scientists.

Primer guidelines

The method of primer purification and choice of M13 tailed- or non-tailed sequencing primers can have a significant effect on the ease of reaction set up and the quality of the sequencing data that is obtained in dye terminator cycle sequencing reactions.

- Use HPLC-purification for all primers to minimize cycle sequencing noise and provide longer sequencing reads.
- Use M13 sequencing primers to simplify the sequencing workflow when sequencing multiple PCR products and to reduce the loss of valuable 5′ unresolvable bases. With M13 sequencing primers, you make single forward and reverse reaction mixes, instead of multiple, primer-specific reaction mixes.

Note: The M13 forward or reverse sequence must be incorporated at the 5′ end of the PCR primer to use the M13 sequencing primers.

Optimize primer selection

Follow these guidelines to optimize primer selection:

- Primers should be at least 18 bases long to:
  - Ensure good hybridization
  - Minimize the chance of having a secondary hybridization site on the target DNA
- Avoid primers that have secondary structure or that can hybridize to form dimers.

  Several computer programs for primer selection are available. These programs help identify potential secondary structure problems and determine if a secondary hybridization site exists on the target DNA.

- Avoid runs of an identical nucleotide, especially guanine, where runs of four or more Gs should be avoided.
- Keep the G–C content in the 30–80% range.
- For cycle sequencing, primers with melting temperatures (T_m) above 45°C produce better results than primers with lower T_ms.
- For primers with a G–C content < 50%, it may be necessary to extend the primer sequence beyond 18 bases to keep the T_m > 45°C.

Primer Designer™ Tool

Primer Designer™ Tool is a free online tool to search for the appropriate PCR/Sanger primer pair from a database of >650,000 pre-designed primer pairs for resequencing the human exome.

Go to: **http://www.thermofisher.com/primerdesigner** for more information, including a direct link to purchase the designed primers online.

Primers ordered through the tool are free of known SNPs and primer-dimers, highly target-specific, and used under universal PCR conditions. Primers can be ordered unmodified, M13-tailed, HPLC-purified or desalted. The primers are checked by mass spectrometry and strict bioinformatics metrics, with bench validation showing a >95% success rate.

C

pREF-BDT™ Control DNA

The 1,000 bp pREF-BDT™ Control DNA reference sequence includes the –21 M13 forward primer.

TGTAAAACGACGGCCAGT (–21 M13 forward primer)				
GAATTGTAAT	ACGACTCACT	ATAGGGCGAA	TTCGAGCTCG	40
GTACCCGGGG	ATCCTCTAGA	GTCGACCTGC	AGGCATGCAA	80
GCTTGAGTAT	TCTATAGTGT	CACCTAAATA	GCTTGGCGTA	120
ATCATGGTCA	TAGCTGTTTC	CTGTGTGAAA	TTGTTATCCG	160
CTCACAATTC	CACACAACAT	ACGAGCCGGA	AGCATAAAGT	200
GTAAAGCCTG	GGGTGCCTAA	TGAGTGAGCT	AACTCACATT	240
AATTGCGTTG	CGCTCACTGC	CCGCTTTCCA	GTCGGGAAAC	280
CTGTCGTGCC	AGCTGCATTA	ATGAATCGGC	CAACGCGCGG	320
GGAGAGGCGG	TTTGCGTATT	GGGCGCTCTT	CCGCTTCCTC	360
GCTCACTGAC	TCGCTGCGCT	CGGTCGTTCG	GCTGCGGCGA	400
GCGGTATCAG	CTCACTCAAA	GGCGGTAATA	CGGTTATCCA	440
CAGAATCAGG	GGATAACGCA	GGAAAGAACA	TGTGAGCAAA	480
AGGCCAGCAA	AAGGCCAGGA	ACCGTAAAAA	GGCCGCGTTG	520
CTGGCGTTTT	TCCATAGGCT	CCGCCCCCCT	GACGAGCATC	560
ACAAAAATCG	ACGCTCAAGT	CAGAGGTGGC	GAAACCCGAC	600
AGGACTATAA	AGATACCAGG	CGTTTCCCCC	TGGAAGCTCC	640
CTCGTGCGCT	CTCCTGTTCC	GACCCTGCCG	CTTACCGGAT	680
ACCTGTCCGC	CTTTCTCCCT	TCGGGAAGCG	TGGCGCTTTC	720
TCATAGCTCA	CGCTGTAGGT	ATCTCAGTTC	GGTGTAGGTC	760
GTTCGCTCCA	AGCTGGGCTG	TGTGCACGAA	CCCCCCGTTC	800
AGCCCGACCG	CTGCGCCTTA	TCCGGTAACT	ATCGTCTTGA	840
GTCCAACCCG	GTAAGACACG	ACTTATCGCC	ACTGGCAGCA	880
GCCACTGGTA	ACAGGATTAG	CAGAGCGAGG	TATGTAGGCG	920

(continued)

TGTAAAACGACGGCCAGT (–21 M13 forward primer)				
GTGCTACAGA	GTTCTTGAAG	TGGTGGCCTA	ACTACGGCTA	960
CACTAGAAGG	ACAGTATTTG	GTATCTGCGC	TCTGCTGAAG	1000

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Safety

WARNING! GENERAL SAFETY. Using this product in a manner not specified in the user documentation may result in personal injury or damage to the instrument or device. Ensure that anyone using this product has received instructions in general safety practices for laboratories and the safety information provided in this document.

- Before using an instrument or device, read and understand the safety information provided in the user documentation provided by the manufacturer of the instrument or device.
- Before handling chemicals, read and understand all applicable Safety Data Sheets (SDSs) and use appropriate personal protective equipment (gloves, gowns, eye protection, and so on). To obtain SDSs, visit **thermofisher.com/support**.

Chemical safety

WARNING! GENERAL CHEMICAL HANDLING. To minimize hazards, ensure laboratory personnel read and practice the general safety guidelines for chemical usage, storage, and waste provided below. Consult the relevant SDS for specific precautions and instructions:

- Read and understand the Safety Data Sheets (SDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. To obtain SDSs, see the "Documentation and Support" section in this document.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing).
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with sufficient ventilation (for example, fume hood).
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer cleanup procedures as recommended in the SDS.
- Handle chemical wastes in a fume hood.
- Ensure use of primary and secondary waste containers. (A primary waste container holds the immediate waste. A secondary container contains spills or leaks from the primary container. Both containers must be compatible with the waste material and meet federal, state, and local requirements for container storage.)
- After emptying a waste container, seal it with the cap provided.
- Characterize (by analysis if needed) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure that the waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.
- **IMPORTANT!** Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.

WARNING! HAZARDOUS WASTE (from instruments). Waste produced by the instrument is potentially hazardous. Follow the guidelines noted in the preceding General Chemical Handling warning.

WARNING! 4L Reagent and Waste Bottle Safety. Four-liter reagent and waste bottles can crack and leak. Each 4-liter bottle should be secured in a low-density polyethylene safety container with the cover fastened and the handles locked in the upright position.

Biological hazard safety

WARNING! Potential Biohazard. Depending on the samples used on this instrument, the surface may be considered a biohazard. Use appropriate decontamination methods when working with biohazards.

WARNING! BIOHAZARD. Biological samples such as tissues, body fluids, infectious agents, and blood of humans and other animals have the potential to transmit infectious diseases. Conduct all work in properly equipped facilities with the appropriate safety equipment (for example, physical containment devices). Safety equipment can also include items for personal protection, such as gloves, coats, gowns, shoe covers, boots, respirators, face shields, safety glasses, or goggles. Individuals should be trained according to applicable regulatory and company/ institution requirements before working with potentially biohazardous materials. Follow all applicable local, state/provincial, and/or national regulations. The following references provide general guidelines when handling biological samples in laboratory environment.

- U.S. Department of Health and Human Services, *Biosafety in Microbiological and Biomedical Laboratories (BMBL)*, 6th Edition, HHS Publication No. (CDC) 300859, Revised June 2020
  www.cdc.gov/labs/pdf/CDC-BiosafetymicrobiologicalBiomedicalLaboratories-2020-P.pdf
- Laboratory biosafety manual, fourth edition. Geneva: World Health Organization; 2020 (Laboratory biosafety manual, fourth edition and associated monographs)
  www.who.int/publications/i/item/9789240011311

Documentation and support

Related documentation

Document	Publication number	Description
DNA Sequencing by Capillary Electrophoresis Chemistry Guide Second Edition	4305080	This chemistry guide is designed to familiarize you with Applied Biosystems™ genetic analyzers for automated DNA sequencing by capillary electrophoresis, to provide useful tips for ensuring that you obtain high-quality data, and to help troubleshoot common problems.
Generating high-quality data using the BigDye™ Terminator v3.1 Cycle Sequencing Kit	MAN0014628	This user bulletin provides guidance for generating high-quality data using the *BigDye™ Terminator v3.1 Cycle Sequencing Kit User Guide* for analysis with Minor Variant Finder Software.
Troubleshooting Sanger sequencing data	MAN0014435	This document provides guidance for the review of your data and troubleshooting tips for improving sequencing data quality.
BigDye XTerminator™ Purification Kit User Guide	4374408	Describes protocols for BigDye XTerminator™purification
SeqStudio™ Genetic Analyzer Instrument and Software User Guide	MAN0018646	This manual provides instructions for the set-up, operation, and maintenance of the SeqStudio™ Genetic Analyzer. It also provides the instructions for setting up the Data Collection software for a run and contains additional information for instrument troubleshooting.
SeqStudio™ Flex Series Genetic Analyzer with Instrument Software v1.1.1 User Guide	100104689	This manual provides instructions for the set-up and operation of the SeqStudio™ Flex Series Genetic Analyzer. It provides the instructions for setting up the Data Collection software, autoanalysis, monitoring and viewing data for a run.

(continued)

Document	Publication number	Description
3500/3500xL Genetic Analyzer with 3500 Series Data Collection Software v 3.1 User Guide	100031809	This manual provides instructions for the set-up, operation, maintenance and troubleshooting of the 3500/3500xL Genetic Analyzer. It also provides the instructions for setting up the Data Collection software, analysis, monitoring and viewing data for a run.
User Guide: Applied Biosystems™ 3730/3730xl DNA Analyzer	4331468	This manual provides instructions for the set-up and operation of the 3730/3730*xl* DNA Analyzer. It provides the instructions for setting up the Data Collection software, autoanalysis, monitoring and viewing data for a run.

Portable document format (PDF) versions of this guide and the documents listed above are available at **thermofisher.com**.

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  - Software, patches, and updates
  - Training for many applications and instruments
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- Product documentation
  - User guides, manuals, and protocols
  - Certificates of Analysis
  - Safety Data Sheets (SDSs; also known as MSDSs)

  Note: For SDSs for reagents and chemicals from other manufacturers, contact the manufacturer.

Limited product warranty

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