PROTOCOL

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BigDye® Direct Cycle Sequencing Kit

life technologies™

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Contents

About This Guide

Purpose

The *BigDye® Direct Cycle Sequencing Kit Protocol* provides instructions and troubleshooting information for using the BigDye® Direct Cycle Sequencing Kit.

Safety information

Note: For general safety information, see this section and Appendix C, "Safety" on page 27. When a hazard symbol and hazard type appear by an instrument hazard, see the "Safety" Appendix for the complete alert on the instrument.

Safety alert words

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

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

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

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

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

SDSs

The Safety Data Sheets (SDSs) for any chemicals supplied by Life Technologies are available to you free 24 hours a day. For instructions on obtaining SDSs, see "SDSs" on page 27.

IMPORTANT! For the SDSs of chemicals not distributed by Life Technologies contact the chemical manufacturer.

BigDye® Direct Cycle Sequencing Kit

Product information

Purpose of the product

Use the BigDye® Direct Cycle Sequencing Kit to perform PCR, post-PCR clean-up, and cycle sequencing. The BigDye® Direct PCR Master Mix and the BigDye® Direct Sequencing Master Mix included in the kit are optimized specifically to work together. The BigDye® Direct Sequencing Master Mix contains BigDye® terminators. The sequencing master mix also allows you to combine post-PCR clean-up with cycle sequencing in the same reaction plate. The elimination of the post-PCR clean-up step simplifies and streamlines the sequencing workflow. The universal sequencing primers included in the kit enable you to perform sequencing runs with POP-7™ polymer and obtain high quality 5' data. With POP-7™ polymer, the electrophoresis times are shorter compared to runs using POP-6™ polymer.

Kit contents and storage

Three kit sizes are available.

Kit	Part no.
BigDye® Direct Cycle Sequencing Kit, 24 reactions	4458689
BigDye® Direct Cycle Sequencing Kit, 100 reactions	4458687
BigDye® Direct Cycle Sequencing Kit, 1000 reactions	4458688

Upon receipt, store the tubes at the recommended temperatures.

Component	Storage
BigDye® Direct PCR Master Mix	–20°C until first use, then 4°C
BigDye® Direct Sequencing Master Mix†	–15°C to –25°C
BigDye® Direct M13 Fwd Primer	–15°C to –25°C
BigDye® Direct M13 Rev Primer	–15°C to –25°C
Control DNA CEPH 1347-02, 20 µL, 50 ng/µL‡	–15°C to –25°C

† For optimal performance, freeze and thaw the BigDye® Direct Sequencing Master Mix no more than 5 times.

‡ The control DNA is provided at 50 ng/µL. Dilute to 4 ng/µL before use.

Materials and equipment required but not included

For capillary electrophoresis and sequencing analysis materials and equipment, see page 23.

Equipment

Item	Source
Thermal cycler: • Veriti® 96-Well Fast Thermal Cycler • Veriti® 96-Well Thermal Cycler • Aluminum 96-Well GeneAmp® PCR System 9700 • Gold-Plated 96-Well GeneAmp® PCR System 9700 • Silver 96-Well GeneAmp® PCR System 9700	Life Technologies • PN 4375305 • PN 4375786 • PN 4314879 • PN 4314878 • PN N8050001
Pipettors: 1- to 20-µL range, 20- to 200-µL range, 100- to 1000-µL range	Major laboratory supplier (MLS)
Multichannel pipettor	MLS
Plate vortexer	MLS
Tabletop centrifuge with microplate adapters	MLS
Vacuum centrifuge (optional)	MLS

Reagents and plastics

For the SDS of any chemical not distributed by Life Technologies Corporation, contact the chemical manufacturer. Before handling any chemicals, refer to the SDS provided by the manufacturer, and observe all relevant precautions.

Table 1 Reagents and plastics for PCR amplification and cycle sequencing

Item	Source
MicroAmp® 96-well optical reaction plates: • MicroAmp® Optical 96-Well Reaction Plate, 10 plates • MicroAmp® Optical 96-Well Reaction Plate with Barcode, 20 plates • MicroAmp® Optical 96-Well Reaction Plate with Barcode and Optical Adhesive Films, 100 plates with covers • MicroAmp® Optical 96-Well Reaction Plate with Barcode and Optical Caps, 20 plates with caps • MicroAmp® Fast Optical 96-Well Reaction Plate with Barcode, 0.1 mL, 20 plates • MicroAmp® Fast Optical 96-Well Reaction Plate with Barcode, 0.1 mL, 200 plates	Applied Biosystems • PN N8010560 • PN 4306737 • PN 4314320 • PN 403012 • PN 4346906 • PN 4366932
MicroAmp® caps strips: • MicroAmp® 8-Cap Strip, Assorted Colors, 300 strips • MicroAmp® 8-Cap Strip, 1500 strips • MicroAmp® 8-Cap Strip, 300 strips	Applied Biosystems • PN N8010835 • PN N8011535 • PN N8010535

Item	Source
MicroAmp® tubes and tube strips: • MicroAmp® Reaction Tube with Cap, 0.2 mL, 1000 tubes • MicroAmp® 8-Tube Strip, 0.2 mL, 125 strips • MicroAmp® Fast 8-Tube Strip, 0.1 mL, 125 strips • MicroAmp® Fast Reaction Tube with Cap, 0.1 mL, 1000 tubes	Applied Biosystems • PN N8010540 • PN N8010580 • PN 4358293 • PN 4358297
MicroAmp® Clear Adhesive Film, 200 covers	Applied Biosystems PN 4306311
MicroAmp® Optical Adhesive Film, 25 covers	Applied Biosystems PN 4360954
MicroAmp® Optical Film Compression Pad, 5 each	Applied Biosystems PN 4312639
Deionized water	MLS

Table 2 Reagents and plastics for purification of sequencing products

Item	Source
For the BigDye XTerminator® Purification Kit	
BigDye XTerminator® Purification Kit: • 2 ml (~100 20-µL reactions) • 20 ml (~1,000 20-µL reactions) • 50 ml (~2,500 20-µL reactions) • 800 ml	Applied Biosystems • PN 4376486 • PN 4376487 • PN 4376484 • PN 4376485
Heat Seal Film for Sequencing and Fragment Analysis Sample Plates, 1 package **Note:** Use for direct injection without a septa mat on the 3730/3730*xl* instrument.	Applied Biosystems PN 4337570
For spin column or spin plate purification	
2.2% Sodium dodecyl sulfate (SDS)	MLS
Centri-Sep™ Columns: • 100 columns • 32 columns	Applied Biosystems • PN 401762 • PN 401763
Centri-Sep™ 96-Well Plates: • 50 plates • 2 plates	Applied Biosystems • PN 4367821 • PN 4367819

Workflow

Use the BigDye Direct Cycle Sequencing Kit

Perform PCR amplification (page 10)

↓

Perform cycle sequencing (page 12)

↓

Purify the sequencing products (page 13)

↓

Perform capillary electrophoresis (page 16)

Perform PCR amplification

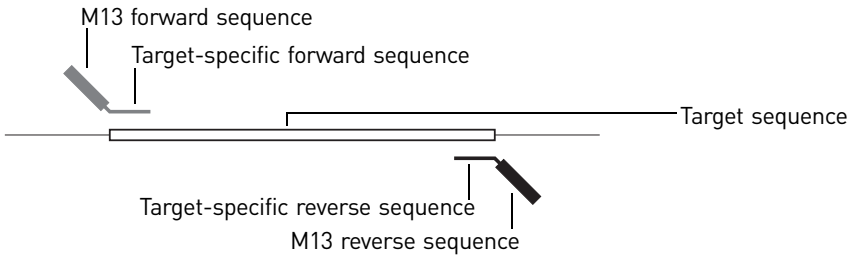
Guidelines for genomic DNA template quality

For optimal results, use high-quality gDNA with no degradation or contaminants:

- **A_{260}/A_{280} ratio**: Use gDNA with an A_{260}/A_{280} ratio between 1.7 and 1.9. A ratio outside of this range indicates contamination by protein or by organic chemicals, such as salt or phenol.
- **0.4% agarose gel**: Use gDNA that appears as a single, high-molecular-weight band (>10 kb). Smearing and low-molecular-weight bands indicate DNA degradation or RNA contamination.

PCR primer design requirements

To use the BigDye® Direct Kit, your PCR primers must include the M13 universal primer sequences as illustrated below:



- **M13 forward primer sequence**: 5′ TGTAAAACGACGGCCAGT 3′
- **M13 reverse primer sequence**: 5′ CAGGAAACAGCTATGACC 3′

Prepare and run the PCR reactions

1. For each forward or reverse reaction, add the components to an appropriate reaction plate:

Component	Volume
Genomic DNA (4 ng/µL)	1.0 µL
M13-tailed PCR primer mix (0.8 µM each primer)	1.5 µL
BigDye® Direct PCR Master Mix	5.0 µL
Deionized water	2.5 µL
Total volume for each reaction	**10.0 µL**

Note: The PCR primers must include the M13 forward and reverse sequences (page 10).

2. Pipet up and down to mix well, seal the plate with adhesive film or caps, then spin the plate briefly.

3. Run the reactions in a thermal cycler:

Stage	Veriti® thermal cyclers		9700 thermal cycler	
	Temp	Time	Temp	Time
Hold	95°C	10 min	96°C	5 min
Cycle (35 cycles)	96°C	3 sec	94°C	30 sec
	62°C	15 sec	62°C	45 sec
	68°C	30 sec	68°C	45 sec
Hold	72°C	2 min	72°C	2 min
Hold	4°C	∞	4°C	∞

4. (Optional) For replicates or controls, assess the quality and quantity of PCR products by running them on an agarose gel.

STOPPING POINT. (Optional) Store the amplified DNA at 4°C overnight or at –15°C or –25°C for long-term storage.

Perform cycle sequencing

Quantity of PCR product to use

The minimum quantity of PCR product to use for sequencing is 20 ng, as checked by running on an agarose gel.

Prepare and run the cycle sequencing reactions

IMPORTANT! You need to use the BigDye® Direct M13 forward or reverse primers in your BigDye® Direct cycle sequencing reactions.

1. Prepare a forward or reverse sequencing reaction mix in a tube on ice:

Components	Volume for each reaction
BigDye® Direct Sequencing Master Mix	2.0 µL
One sequencing primer: • BigDye® Direct M13 Fwd Primer or • BigDye® Direct M13 Rev Primer	1.0 µL
Total volume for each reaction	**3.0 µL**

2. For each sequencing reaction, add 3 µL of the sequencing reaction mix to the appropriate well in the respective forward or reverse reaction plate.
3. Seal the reaction plate with adhesive film or caps, then spin the plate briefly.
4. Run the reactions in a thermal cycler:

Stage	Veriti® thermal cyclers		9700 thermal cycler	
	Temp	Time	Temp	Time
Hold	37°C	15 min	37°C	15 min
Hold	80°C	2 min	80°C	2 min
Hold	96°C	1 min	96°C	1 min
Cycle (25 cycles)	96°C	10 sec	96°C	10 sec
	50°C	5 sec	50°C	5 sec
	60°C	75 sec	60°C	4 min
Hold	4°C	∞	4°C	∞

5. After the cycle sequencing reactions are complete, spin the plate briefly.

STOPPING POINT. (Optional) Store the reaction plate at 4°C overnight or at –15°C or –25°C for long-term storage.

Purify the sequencing products

For optimal results, completely remove unincorporated dye terminators before performing capillary electrophoresis. Excess dye terminators in sequencing products can obscure data in the early part of the sequence and interfere with basecalling.

The purification methods described here are optimized for use with the BigDye® Direct Cycle Sequencing Kit at the specified sequencing volumes and are not recommended for other BigDye® products.

Select the purification method

Select the method that works best for your application:

- BigDye XTerminator® Purification Kit (page 13)
- Spin columns (page 14)
- Spin plates (page 15)

Purify sequencing products using the BigDye XTerminator® Purification Kit

Note: This procedure is specifically optimized for use with the BigDye® Direct Cycle Sequencing Kit.

1. Spin the reaction plate at 100 *x g* for 1 minute, then remove the seal.
2. Prepare a premix with SAM™ Solution and XTerminator® Solution in an appropriately sized tube:

Component	Volume for 1 well	Volume for 96 wells
SAM™ Solution	45 µL	4752 µL
XTerminator® Solution	10 µL	1056 µL
Total volume	55 µL	5808 µL

   a. Add the SAM™ Solution to the tube using a conventional pipette tip.

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

   b. Vortex the XTerminator® Solution bulk container at maximum speed for at least 10 seconds, until the solution is homogeneous.

   c. Using a wide-bore pipette tip, aspirate the XTerminator® Solution.

      IMPORTANT! Avoid pipetting from the top of the liquid.

   d. Mix the reagents until homogeneous.

3. Add 55 µL of SAM™ Solution/XTerminator® Solution premix to each well.
4. Seal the plate using one of the following methods, then verify that each well is sealed:
   - MicroAmp® Clear Adhesive Films

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

   Note: For direct injections without a septa mat on the 3730/3730*xl* instrument, only the Heat Seal Film for Sequencing and Fragment Analysis Sample Plates are supported.

5. Vortex the reaction plate for 20 minutes, using the following conditions:

Vortexer	Speed
Digital Vortex-Genie® 2	1800 rpm
IKA MS3 Digital	2000 rpm†
IKA Vortex 3	Setting 5‡
Taitec MicroMixer E-36	Maximum
Union Scientific Vertical Shaker	Setting 100§

† Set the vortexer to Mode B.

‡ Use the maximum setting without allowing the vortexer to move across the bench.

§ Add more plates, if necessary, to meet mass requirements.

6. In a swinging-bucket centrifuge, spin the plate at 1000 × *g* for 2 minutes.

STOPPING POINT. If you plan to store the plate before proceeding with capillary electrophoresis, store the sample plates sealed with heat seal film or adhesive film for up to 48 hours at room temperature (20 to 25°C) or up to 10 days at 4°C or –20°C.

Purify sequencing products with Centri-SEP™ spin columns

1. Treat the samples with 2.2% sodium dodecyl sulfate (SDS):
   a. Prepare 2.2% SDS in deionized water. This SDS solution is stable at room temperature.
   b. Add 7 µL of water and 2 µL of 2.2% SDS to each 13 µL of completed cycle sequencing reaction to bring the final SDS concentration to 0.2%.
   c. Seal the plate, then mix thoroughly.
   d. Incubate the reaction plate in a thermal cycler:

Temp	Time
98°C	5 min
25°C	10 min

   e. Spin the plate briefly.
2. Hydrate the spin columns:
   a. Gently tap the column to cause the gel material to settle to the bottom of the column.
   b. Remove the upper end cap and add 0.8 mL of deionized water.
   c. Replace the upper end cap and vortex or invert the column a few times to mix the water and gel material.
   d. Allow the gel to hydrate at room temperature for at least 2 hours.

Note: You can store hydrated columns for a few days at 2–6°C. Longer storage in water is not recommended. Allow columns stored at 2–6°C to warm to room temperature before use.

3. Assemble the spin columns:
   a. Invert or tap the column and allow the gel to settle to remove air bubbles.
   b. Remove the upper end cap first, then remove the bottom cap.
   c. Allow the column to drain completely by gravity.

      Note: If flow does not begin immediately, apply gentle pressure to the column with a pipette bulb.

   d. Insert the column into the wash tube provided.
   e. Spin the column in a microcentrifuge at 730 *x g* for 2 minutes to remove the interstitial fluid.
   f. Remove the column from the wash tube, then insert it into a sample collection tube.
4. Purify the sequencing products using the spin columns:
   a. Carefully transfer the sequencing reaction treated with SDS from its tube to the center of the gel material.
   b. Spin the column in a microcentrifuge at 730 *x g* for 2 minutes to elute the sample into the sample collection tube.

      Note: If you are using a centrifuge with a fixed-angle rotor, place the column in the same orientation as it was in for the first spin. This is important because the surface of the gel will be at an angle in the column after the first spin.

   c. Discard the column.
5. Dry the sample in a vacuum centrifuge for 10–15 minutes without heat, or until dry.

   Note: Do not overdry.

Purify sequencing products with Centri-SEP™ spin plates

For large-scale procedures, you can use Centri-SEP™ 96-well spin plates.

1. Treat the samples with 2.2% sodium dodecyl sulfate (SDS):
   a. Prepare 2.2% SDS in deionized water. This SDS solution is stable at room temperature.
   b. Add 7 µL of water and 2 µL of 2.2% SDS to each 13 µL of completed cycle sequencing reaction to bring the final SDS concentration to 0.2%.
   c. Seal the plate, then mix thoroughly.
   d. Heat the reaction plate, then allow the plate to cool to ambient temperature in the thermal cycler:

Temp	Time
98°C	5 min
25°C	10 min

   e. Spin the plate briefly.
2. Prepare the spin plate and perform the purification according to the manufacturer's instructions.

Perform capillary electrophoresis

Refer to your instrument user guide for instructions on setting up and performing the capillary electrophoresis run.

- Use Dye Set Z and the Sequencing Install Standard, BigDye® Terminator v3.1 Kit to create the BigDye® Direct spectral calibration information to apply to the data.

  Note: Current Dye Set Z spectra information can be applied to BigDye® Direct data without rerunning spectral calibration.
- Use the BigDye® mobility and calibration files for optimal basecalling with the BigDye® Direct Cycle Sequencing Kit.

  Note: For instructions on installing the mobility and calibration files, see Appendix B, "Installing the BigDye® Direct Cycle Sequencing Kit Software v1.1" on page 25.

Capillary electrophoresis run conditions

Table 3 Run conditions for 3500/3500*xl* DNA Analyzers

Polymer	Array	Run module	Mobility file
POP-7™ polymer	50 cm	StdSeq50_POP7	KB_3500_POP7_BDTv3direct.mob
POP-7™ polymer	50 cm	FastSeq50_POP7	KB_3500_POP7_BDTv3direct.mob
POP-7™ polymer	50 cm	RapidSeq50_POP7	KB_3500_POP7_BDTv3direct.mob
POP-7™ polymer	50 cm	ShortReadSeq50_POP7	KB_3500_POP7_BDTv3direct.mob
POP-7™ polymer	50 cm	BDX_StdSeq50_POP7	KB_3500_POP7_BDTv3direct.mob
POP-7™ polymer	50 cm	BDX_FastSeq50_POP7	KB_3500_POP7_BDTv3direct.mob
POP-7™ polymer	50 cm	BDX_RapidSeq50_POP7	KB_3500_POP7_BDTv3direct.mob
POP-7™ polymer	50 cm	BDX_ShortReadSeq50_POP7	KB_3500_POP7_BDTv3direct.mob

Table 4 Run conditions for 3130/3130*xl* Genetic Analyzers

Polymer	Array	Run module	Mobility file
POP-7™ polymer	36 cm	RapidSeq36_POP7	KB_3130_POP7_BDTv3direct.mob
POP-7™ polymer	36 cm	UltraSeq36_POP7	KB_3130_POP7_BDTv3direct.mob
POP-7™ polymer	50 cm	StdSeq50_POP7	KB_3130_POP7_BDTv3direct.mob
POP-7™ polymer	50 cm	FastSeq50_POP7	KB_3130_POP7_BDTv3direct.mob
POP-7™ polymer	36 cm	BDX_RapidSeq36_POP7	KB_3130_POP7_BDTv3direct.mob
POP-7™ polymer	36 cm	BDX_UltraSeq36_POP7	KB_3130_POP7_BDTv3direct.mob
POP-7™ polymer	50 cm	BDX_StdSeq50_POP7	KB_3130_POP7_BDTv3direct.mob
POP-7™ polymer	50 cm	BDX_FastSeq50_POP7	KB_3130_POP7_BDTv3direct.mob

Table 5 Run conditions for 3730/3730*xl* DNA Analyzers

Polymer	Array	Run module	Mobility file
POP-7™ polymer	36 cm	StdSeq50_POP7	KB_3730_POP7_BDTv3direct.mob
POP-7™ polymer	36 cm	RapidSeq36_POP7	KB_3730_POP7_BDTv3direct.mob
POP-7™ polymer	36 cm	TargetSeq36_POP7	KB_3730_POP7_BDTv3direct.mob
POP-7™ polymer	50 cm	LongSeq50_POP7	KB_3730_POP7_BDTv3direct.mob
POP-7™ polymer	50 cm	FastSeq50_POP7	KB_3730_POP7_BDTv3direct.mob
POP-7™ polymer	36 cm	BDX_StdSeq50_POP7	KB_3730_POP7_BDTv3direct.mob
POP-7™ polymer	36 cm	BDX_RapidSeq36_POP7	KB_3730_POP7_BDTv3direct.mob
POP-7™ polymer	50 cm	BDX_LongSeq50_POP7	KB_3730_POP7_BDTv3direct.mob
POP-7™ polymer	50 cm	BDX_FastSeq50_POP7	KB_3730_POP7_BDTv3direct.mob

Troubleshooting

Observation	Possible cause	Recommended action
No recognizable sequence	PCR primers do not have M13 tails on the 5′ end of the gene-specific primers.	Redesign the PCR primers with the M13 tails (see page 10 for M13 sequences).
	Insufficient template.	• Quantify the DNA template before starting. • Increase the amount of DNA in the PCR reactions.
	Inhibitory contaminant in the template.	Clean up the template.
	Insufficient PCR primers.	• Quantify the PCR primers. • Increase the amount of PCR primer in the PCR reactions.
	Poor PCR primer design or incorrect primer sequence.	Redesign the PCR primers.
	Missing reagent.	Repeat the reactions, carefully following the procedures.
	Old or mishandled reagents.	Use fresh reagents.
	Thermal cycler power failure.	Repeat the reactions.
	Thermal cycling conditions incorrect.	• Calibrate the thermal cycler regularly. • Use correct thermal cycling parameters. • Use the correct tubes or plates for your thermal cycler. • Set the ramp rate to 1°C/second.
	Sequencing products lost during reaction cleanup.	Make sure you use the correct centrifugation speeds and times for BigDye XTerminator® Purification Kit and spin column procedures.
	Sequencing products are not resuspended.	Carefully resuspend the sample pellet in loading buffer.
	Electrokinetic injection failure.	Repeat the injections.
Off-scale peaks, pull-up peaks, or bleedthrough Raw data exceed the off-scale data limits: • 3500 series: >32,000 rfu • 3130 series: >8200 rfu • 3730 series: >32,000 rfu	• Amplicon has a high amplification efficiency. OR • Too much genomic DNA added to the PCR reaction.	• Reinject the affected samples and/or optimize injection parameters by reducing time or voltage (page 16). • Reduce the number of PCR amplification cycles to 32–33 cycles (page 11).
		• Quantify the genomic DNA template and the M13-tailed PCR primers.

Observation	Possible cause	Recommended action
Noisy data throughout the sequence with low signal strength	Insufficient PCR product in the sequencing reactions.	• Redesign M13-tailed PCR primers to optimal Tm. • Make sure the quality of the M13-tailed PCR primers is good. • For AT-rich difficult templates, increase PCR annealing time by increments of 5 seconds or adjust PCR annealing temperature by 5°C increments. • Increase denaturing time and temperature or adjust the PCR annealing time and temperature.
	Degraded template.	Prepare fresh DNA and repeat the reactions.
	Old or mishandled reagents.	• Use fresh reagents. • Verify the reagent storage and handling conditions.
	Thermal cycling conditions incorrect.	• Calibrate the thermal cycler regularly. • Use correct thermal cycling parameters. • Use the correct tubes or plates for your thermal cycler.
	Electrokinetic injection failure.	Repeat the injections.

Observation	Possible cause	Recommended action
Noisy data throughout the sequence with good signal strength	Inhibitory contaminant in the template	Clean up the template.
	Multiple templates in the sequencing reaction or template contamination	Examine template on an agarose gel to be sure only one PCR product is present.
	Multiple priming sites	• Make sure the M13-tailed PCR primer has only one priming site. • If necessary, redesign the M13-tailed PCR primers.
	Primer with N-1 contamination	Use HPLC-purified M13-tailed PCR primers.
	High signal saturating the detector	• Reduce the number of PCR amplification cycles by 2–3 cycles (page 11). • Use less DNA in the PCR reactions. • Optimize injection parameters by reducing time or voltage.
	Incorrect run module	Make sure you use the correct run module, Dye Set Z, and the correct BigDye® Direct mobility file (page 16).
	Incorrect analysis protocol	1. Make sure you use the BigDye® Direct mobility file (page 16). 2. Repeat the basecalling with the correct analysis settings.
Noise up to or after a specific point in the sequence	Multiple PCR products	Make sure you have only one PCR product.
	Primer-dimer contamination in PCR sequencing	• Optimize your PCR amplification. • Make sure there is no sequence complementarity between the two PCR primers. • Make sure your M13-tailed PCR primers do not overlap the sequences of the sequencing primers.
	Slippage after repeat region in template	Use the traditional PCR/sequencing workflow with the dRhodamine Dye Terminator Cycle Sequencing Ready Reaction Kit.
Poor mobility correction	Incorrect dye set/primer (mobility) file	Use the correct dye set/primer (mobility) file.
	Incorrect Peak 1 location for data analysis	Select a new Peak 1 location.

Observation	Possible cause	Recommended action
Excess dye peaks at the beginning of the sequence	Poor removal of unincorporated dye terminators	• Make sure that you thoroughly mix the SAM solution and the BigDye® XTerminator solution (page 13). • Use one of the recommended vortexers (page 14). • When preparing sequencing products for spin column or spin plate purification, increase the final SDS concentration to 0.4%. • With Centri-Sep spin columns, take care to load the sample on the center of the gel surface. **Note:** Do not touch the gel surface with the pipette tip. **IMPORTANT!** Make sure you hydrate the column for at least 2 hours.
Peak splitting or temporary poor peak resolution in the middle of the trace	Excess PCR product or template	• Reduce the number of PCR cycles by 2–3 cycles. • Reinject the affected samples. • Optimize injection parameters by reducing time or voltage.

Appendix A Ordering Information

How to order BigDye® Direct Cycle Sequencing Kits

For information on the BigDye® Direct Kits, go to the BigDye® Direct product page at **www.appliedbiosystems.com/bigdyedirect**.

Kit	Part no.
BigDye® Direct Cycle Sequencing Kit, 24 reactions	4458689
BigDye® Direct Cycle Sequencing Kit, 100 reactions	4458687
BigDye® Direct Cycle Sequencing Kit, 1000 reactions	4458688

Materials for capillary electrophoresis and sequencing analysis

Item	Source
Genetic Analyzer: • 3500/3500*xl* Genetic Analyzer • 3730/3730*xl* DNA Analyzer • 3130/3130*xl* Genetic Analyzer	Contact your local Applied Biosystems sales representative.
SeqScape® Software v2.7: • Initial License • Upgrade from v2.6	Applied Biosystems • PN 4327091 • PN 4332045
10× Genetic Analyzer Buffer with EDTA	Applied Biosystems PN 402824
Sequencing Install Standard, BigDye® Terminator v3.1 for 3500/3500xl Genetic Analyzers	Applied Biosystems PN 4404312
310/3100/3100-Avant™/3130/3130xl Genetic Analyzer Sequencing Standards, BigDye® Terminator v3.1	Applied Biosystems PN 4336935
3730/3730xl DNA Analyzer Sequencing Standards, BigDye® Terminator v3.1	Applied Biosystems PN 4336943
POP-7™ Polymer for 3500/3500*xl* Genetic Analyzers: • 384 samples • 960 samples	Applied Biosystems • PN 4393708 • PN 4393714
POP-7™ Polymer for 3130/3130*xl* Genetic Analyzers: • 3500 µL • 7000 µL	Applied Biosystems • PN 4363785 • PN 4352759

Item	Source
POP-7™ Polymer for 3730/3730*xl* DNA Analyzers: • 1 × 28 mL • 5 × 28 mL • 10 × 28 mL • 30 × 28 mL	Applied Biosystems • PN 4363929 • PN 4335615 • PN 4363935 • PN 4335611

Appendix B Installing the BigDye® Direct Cycle Sequencing Kit Software v1.1

The installer for the BigDye® Direct Cycle Sequencing Kit Software v1.1 installs the mobility (*.mob) files and calibration (*.bcc) files that the KB Basecaller requires for proper basecalling of sequencing products produced using the BigDye® Direct Cycle Sequencing Kit.

Go to **www.appliedbiosystems.com/bigdyedirectinstaller** for your free download of the BigDye® Direct Cycle Sequencing Kit Software v1.1.

Supported software versions and platforms

Operating system	Supported software versions
Windows® XP	• 3130/3130*xl* Data Collection Software v3.0, v3.1, and v3.1.1 • 3730/3730*xl* Data Collection Software v3.0, v3.1, and v3.1.1 • Sequence Analysis Software v5.3, v5.3.1, and v5.4 • SeqScape® Software v2.6 and v2.7 • Variant Reporter® Software v1.0 and v1.1
Windows® Vista	• 3500/3500*xl* Data Collection Software v1.0 • Sequence Analysis Software v5.4 • SeqScape® Software v2.7 • Variant Reporter® Software v1.1

Note: If you are using the Windows® 2000 operating system with 3130/3130*xl* Data Collection Software or 3730/3730*xl* Data Collection Software, you need to upgrade to Windows® XP to use the installer. Contact your Life Technologies service representative to upgrade your operating system.

Files that are installed

The appropriate mobility and calibration files are installed according to the data collection or analysis software installed on your computer:

- KB_3130_POP7_BDTv3direct.mob and KB_3130_POP7_BDTv3direct.bcc
- KB_3500_POP7_BDTv3direct.mob and KB_3500_POP7_BDTv3direct.bcc
- KB_3730_POP7_BDTv3direct.mob and KB_3730_POP7_BDTv3direct.bcc

Confirm installation

Refer to your instrument or software user guide for instructions on how to confirm that the mobility and calibration files for the BigDye® Direct Cycle Sequencing Kit were installed.

Note: Instructions are provided below for the 3500/3500*xl* Data Collection Software and the Variant Reporter Software.

1. Start the 3500/3500*xl* Data Collection Software, select **Library > Basecalling Protocols**, then click **Create**.
2. Expand the Mobility File section, view the contents of the **Mobility File** dropdown list, then confirm that the list contains KB_3500_POP7_BDTv3direct.
3. Start the Variant Reporter Software, create a new project, then import the *.ab1 sample file from the 3500 instrument.
4. Select **Set Analysis Properties**, then expand the Basecall and the Mobility File sections.
5. In the Mobility File section, select the **Override Mobility File for All Traces with** checkbox, select the dropdown list, then confirm that the list contains KB_3500_POP7_BDTv3direct.

Note: When you create a new BigDye® Direct assay on the 3500 instrument, the 3500 Data Collection Software displays a warning, "Selected Mobility File in Primary Analysis Protocol for Basecalling does not match the dye set. Do you want to continue?"
If you have selected Dye Set Z and the BigDye® Direct mobility file, click **OK** to create an analysis protocol that uses the BigDye® Direct mobility file.

Appendix C Safety

This appendix covers:

Chemical safety

General chemical safety

Chemical hazard warning

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

Chemical safety guidelines

To minimize the hazards of chemicals:

- 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. (See "About SDSs" on page 27.)
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing). For additional safety guidelines, consult the SDS.
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood). For additional safety guidelines, consult the SDS.
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended in the SDS.
- Comply with all local, state/provincial, or national laws and regulations related to chemical storage, handling, and disposal.

SDSs

About SDSs

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

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

Obtaining SDSs

The SDS for any chemical supplied by Life Technologies is available to you free 24 hours a day. To obtain SDSs:

1. Go to **www.appliedbiosystems.com**, click **Support**, then select **MSDS**.
2. In the Keyword Search field, enter the chemical name, product name, SDS part number, or other information that appears in the SDS 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

Note: For the SDSs of chemicals not distributed by Applied Biosystems, contact the chemical manufacturer.

Biological hazard safety

General biohazard

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

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

Additional information about biohazard guidelines is available at:

www.cdc.gov

Documentation and Support

Related documentation

The following related documents are available:

Document	Part no.
BigDye® Direct Cycle Sequencing Kit Quick Reference Card	4458017

The portable document format (PDF) version is available on our website at **www.appliedbiosystems.com**.

Obtaining support

For the latest services and support information for all locations, go to:

www.appliedbiosystems.com

At our website, you can:

- Access worldwide telephone and fax numbers to contact Life Technologies Technical Support and Sales facilities.
- Search through frequently asked questions (FAQs).
- Submit a question directly to Technical Support.
- Order Life Technologies user documents, SDSs, certificates of analysis, and other related documents.
- Download PDF documents.
- Obtain information about customer training.
- Download software updates and patches.

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