applied biosystems — USER BULLETIN

Generating high-quality data using the BigDye™ Direct Cycle Sequencing Kit

Demonstrated Protocol

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WARNING! Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. Safety Data Sheets (SDSs) are available from **thermofisher.com/support**.

Overview

Effective minor variant detection with Minor Variant Finder Software requires high-quality sequencing data with minimal noise.

This document provides a demonstrated protocol for generating high-quality data for use in minor variant detection using:

- BigDye™ Direct Cycle Sequencing Kit
- Applied Biosystems™ Genetic Analyzers with POP-7™ polymer

We have tested and used this protocol extensively, but have not validated it for all of the instrument systems specified. Certain components of the protocol workflow such as reagent kits and other protocols for preparation of reagents may not be available through Thermo Fisher Scientific. Supporting documentation such as application

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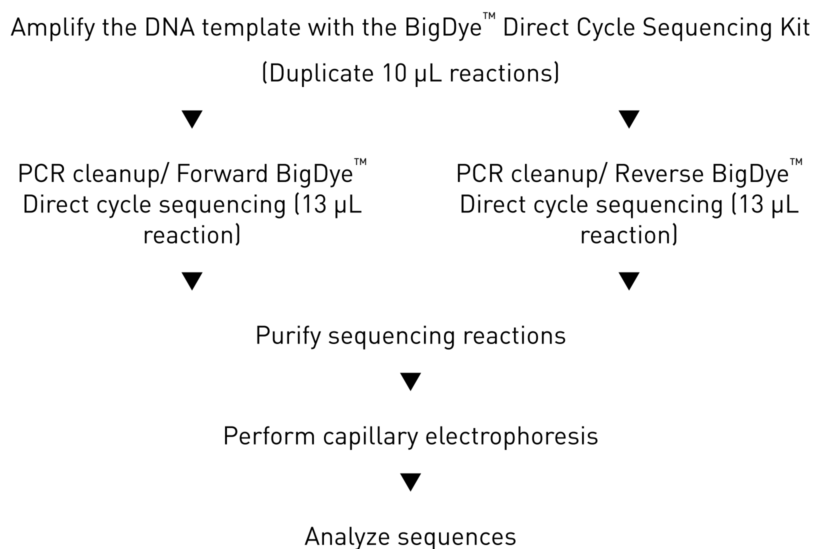
notes may be available from Thermo Fisher Scientific and/or third parties. Limited support is available from Thermo Fisher Scientific.

Workflow



Required materials

Unless otherwise indicated, all materials are available through **thermofisher.com**.

Item	Source
Reagents	
BigDye™ Direct Cycle Sequencing Kit	4458687
AmpliTaq™ 360 Buffer, $MgCl_2$, and 360 GC Enhancer [1]	4398848
BigDye XTerminator™ Purification Kit	4376486
UltraPure™ DNase/RNase-Free Distilled Water	10977-015
Hi-Di™ Formamide **Note:** Not required for BigDye XTerminator™ Purification Kit purification.	4311320 or 4440753
PCR primers (HPLC-purified recommended; M13-tailed required)	Design, choose, and order primers with the Primer Designer™ Tool at http://www.thermofisher.com/primerdesigner.
DNA Suspension Buffer, RNAse DNAse Free (10mM Tris/0.1mM EDTA, pH 8.0)	Teknova™ T0223
Reagents for Centri-Sep™ purification (*optional*)	
Sodium Dodecyl Sulfate (SDS)	15525-017
Centri-Sep™ 96-Well Plates	4367819
Reagents for ethanol/EDTA purification (*optional*)	
0.5M EDTA, pH 8.0 for molecular biology	AM9260G
Ethanol, absolute, for molecular biology	Major Laboratory Suppliers (MLS)
Laboratory supplies	
MicroAmp™ Clear Adhesive Film	4306311
MicroAmp™ Optical 96-Well Reaction Plate	8010560
Plate Septa, 96 well	4315933
25 mL Reagent Reservoir, Pyrogen-free, RNase/DNase certified, sterile	VistaLab Technologies™ 3054-1002
Digital Vortex-Genie™ 2 or equivalent	Scientific Industries, Inc. SI-A536
Centrifuge with swinging bucket (with PCR plate adapter)	MLS
Compatible thermal cyclers [2]	
GeneAmp™ PCR System 9700	Contact your local sales office
Veriti™ Thermal Cycler	
Compatible sequencing instruments	

Item	Source
3130/3130*xl* Genetic Analyzer	Contact your local sales office
3500/3500xL Genetic Analyzer	
3730/3730*xl* DNA Analyzer [3]	

[1] Only the GC enhancer is required for this protocol.
[2] If you use a different thermal cycler, you may need to optimize the thermal cycling conditions.
[3] Standard heat seal consumables can be used in place of MicroAmp™ plates and film for these instruments.

DNA and primer requirements

Input DNA requirements

The quality of the DNA 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.

For optimal results, use 10 to 20 ng/µL of template DNA with spectrophotometer absorbance ratios ($A_{260/280}$) between 1.8 to 2.0.

Factors affecting DNA quality

- **Type and amount of source material** – Influences the effectiveness and sensitivity of PCR amplification and the quality of sequencing results.
- **Heparin** – Can weaken or completely inhibit PCR amplification. It is difficult to reverse the effect of heparin. However, 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.
- **Paraffin-embedded tissue** – Use a DNA isolation kit specifically designed formalin-fixed, paraffin-embedded (FFPE) tissue.
  The following factors can affect PCR amplification:
  - Fixative used
  - Fixation length
  - Age of the block
  - Yield of DNA obtained
  - Amount of DNA degradation in the paraffin
  - Presence of PCR inhibitors in the isolated DNA

Optimizing DNA 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.

If poor DNA template quality is suspected, use Centricon™ or Microcon™ centrifugal filters (EMD Millipore) to clean the sample and remove excess salts.

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

- Concentration of single-stranded DNA = A_{260} × 33 mg/µL.
- Concentration of double-stranded DNA = A_{260} × 50 mg/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 be inaccurate. Dilute or concentrate the DNA as needed to obtain an OD value between 0.05 to 1.

Primer guidelines

- Amplicon-specific PCR primers MUST be tailed with the following M13 sequences:

M13 Forward Sequence	M13 Reverse Sequence
5′-TGT AAA ACG ACG GCC AGT-3′	5′-CAG GAA ACA GCT ATG ACC-3′

  Note: The use of M13 sequencing primers reduces the loss of valuable 5′ unresolvable bases.

- HPLC-purification of all primers is recommended to minimize cycle sequencing noise and to provide longer sequencing reads.
- HPLC-purified M13 forward and reverse sequencing primers are provided with the BigDye™ Direct Cycle Sequencing Kit at the correct concentration and do NOT need to be ordered separately.
- The sequencing reaction will fail if the M13 sequencing primers provided with the kit are not used.

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.

Prepare and store primers

1. Resuspend all PCR primer stocks in 100 µM DNA buffer (10 mM Tris/0.1 mM EDTA, pH 8.0) and store them at –20°C.

2. Create individual amplicon-specific PCR primer pools of 0.8 µM PCR primers using UltraPure™ DNase/RNase-Free Distilled Water to minimize excess salt contribution that can inhibit subsequent reactions. Store working solutions at –20°C.

Amplify the DNA template with the BigDye™ Direct Cycle Sequencing Kit

Set up the PCR reaction

1. Completely thaw and store the BigDye™ Direct Cycle Sequencing Kit reagents and primers on ice.

   Note: Reagents are stored at 4°C after first use.

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. Prepare the reaction mix:

   IMPORTANT! Change pipette tips after each transfer to avoid contamination of reagents, specimen or amplicons.

Component	Quantity (1 well)	Quantity (96 well plate) [1]
BigDye™ Direct PCR Master Mix	5 µL	528 µL
360 GC Enhancer	0.5 µL (5%)	53 µL
UltraPure™ DNase/RNase-Free Distilled Water	2 µL	211 µL
Total volume	**7.5 µL**	**792 µL**

   [1] Includes 10% additional volume.

   Note: Store on ice.

4. Vortex the tubes for 2 to 3 seconds, then centrifuge briefly (2 to 3 seconds) with a benchtop microcentrifuge.

5. Label a plate “PCR plate” and add the following, in order:

Component	Quantity
Reaction mix	7.5 µL
DNA template (10-20 ng/µL)	1 µL
Pooled PCR primers (0.8 µm each)	1.5 µL

   IMPORTANT! Create duplicate PCR reactions for use in the forward and reverse sequencing reactions.

   IMPORTANT! Change pipette tips after each transfer.

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

7. Vortex the plate for 2 to 3 seconds, then centrifuge briefly in a swinging bucket centrifuge to collect contents to the bottom of the wells (5 to 10 seconds) at 1,000 x g.

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

Run the PCR

1. Place the plate in a thermal cycler and set the volume.

2. Run the PCR with the following settings:

Thermal cycler	Parameter	Stage/step					
		Incubate	Cycling (35 cycles)			Final extension	Hold
			Denature	Anneal [1]	Extend [2]		
Veriti™	**Temperature**	95°C	96°C	58°C	72°C	72°C	4°C
	Time	10 minutes	3 seconds	15 seconds	30 seconds	2 minutes	Hold until ready to purify.
9700	**Temperature**	95°C	96°C	58°C	72°C	72°C	4°C
	Time	5 minutes	30 seconds	45 seconds	45 seconds	2 minutes	Hold until ready to purify.

[1] If your primer annealing temperatures are not between 60°C and 65°C, annealing conditions may need optimization. See "Primer Designer™ Tool" on page 5 for more information.

[2] Extension times may need to be lengthened for sequences over 700 bp.

3. Place the plate on ice or store the plate at 4°C until you are ready to set up the sequencing reactions.

   Note: Place plates at –25°C to –15°C for longer-term storage.

Perform sequencing reactions with the BigDye™ Direct Cycle Sequencing Kit

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™ Direct Cycle Sequencing Kit 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. Label microcentrifuge tubes "forward" and "reverse" and add the following components to each tube:

   IMPORTANT! Change pipette tips after each transfer.

Component	Quantity			
	Forward reaction mix		Reverse reaction mix	
	1 reaction	48 reactions [1,2]	1 reaction	48 reactions [1,2]
BigDye™ Direct Sequencing Mix	2 µL	106 µL	2 µL	106 µL
BigDye™ M13 Forward primer	1 µL	53 µL	–	–
BigDye™ M13 Reverse primer	–	–	1 µL	53 µL
Total volume	**3 µL**	**159 µL**	**3 µL**	**159 µL**

[1] Includes 10% additional volume
[2] 48 samples is the maximum number of sequencing reactions you can prepare if you want both forward and reverse sequencing reactions in the same plate.

   Note: Store on ice and protect from light.

4. Vortex the tubes for 2 to 3 seconds, then centrifuge briefly (2 to 3 seconds) with a benchtop microcentrifuge.

5. Label the PCR plate "sequencing".

6. Place the labelled PCR plate on ice and remove the MicroAmp™ Clear Adhesive Film.

7. Pipette 3 µL of the "forward"-labeled or 3 µL of the "reverse"-labeledBigDye™ Direct Sequencing Mix to the appropriate wells within the PCR plate.

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

9. Vortex the plate for 2 to 3 seconds, then centrifuge briefly in a swinging bucket centrifuge to collect contents to the bottom of the wells (5 to 10 seconds) at 1,000 x g.

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

Run the sequencing reactions

1. Place the prepared sequencing plate into the thermal cycler, set the reaction volume, and run at the following conditions:

Thermal Cycler	Parameter	Stage/step						
		Post PCR clean-up [1]	Post PCR deactivation	Denature	Cycling (25 cycles)			Hold
					Denature	Anneal	Extend	
Veriti	**Temperature**	37°C	80°C	96°C	96°C	50°C	60°C	4°C
	Time	15 minutes	2 minutes	1 minute	10 seconds	5 seconds	75 seconds	Hold
9700	**Temperature**	37°C	80°C	96°C	96°C	50°C	60°C	4°C
	Time	15 minutes	2 minutes	1 minute	10 seconds	5 seconds	4 minutes	Hold

[1] The BigDye™ Direct Cycle Sequencing Kit has the PCR/amplicon clean-up step built in, thereby eliminating the need for a separate PCR/amplicon clean-up step.

2. Place the plate on ice or store at 4°C until ready to purify the reactions.

Purify the sequencing reactions

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

The following methods are recommended for clean-up of cycle sequencing reactions:

- "Purify sequencing reactions with BigDye XTerminator™" on page 9
- "Purify the sequencing reactions with Centri-Sep™ plates" on page 10
- "Purify the sequencing reactions with ethanol/EDTA precipitation" on page 12

Purify sequencing reactions with BigDye XTerminator™

The following protocol takes approximately 40 minutes.

Note: Use disposable reagent reservoirs and an 8-channel P200 pipette, if available, to facilitate the clean-up process.

Note: If you use a 3730 DNA Analyzer, either MicroAmp™ Clear Adhesive Film or standard heat sealing techniques can be used.
This protocol describes plate sealing with MicroAmp™ Clear Adhesive Film.

1. Allow the BigDye XTerminator™ bead solution to reach room temperature.

   Note: Remove from 4°C storage approximately 1 hour before use.

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

   IMPORTANT! For effective BigDye XTerminator™ clean-up, it is essential to keep the materials well mixed.

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

Component	Volume per well	Volume per 96-well plate[1]
SAM solution	45 µL	4.75 mL
BigDye XTerminator™ bead solution	10 µL	1.06 mL
Total volume	**55 µL/ well**	**5.81 mL**

[1] Includes 10% additional volume. This is very important when you use a multi-channel pipette (electronic pipette) and a reagent reservoir.

4. Remove MicroAmp™ Clear Adhesive Film from the sequencing plate.
5. Transfer 55 µL/well of the bead mix (BigDye XTerminator™ bead solution and SAM solution) to each sample.

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

6. Seal the plate using MicroAmp™ Clear Adhesive Film.
7. If you are using the Digital Vortex-Genie™ 2, vortex the 96-well plate for 20 minutes at 1,800 rpm. For alternative vortex mixer manufacturers and settings, refer to the *BigDye XTerminator™ Purification Kit Quick Reference Card* (Pub. no. 4383427).
8. In a swinging bucket centrifuge, centrifuge the plate at 1,000 x g for 2 minutes.

   Note: To store, seal the plate with MicroAmp™ Clear Adhesive Film, and store at 4°C for CE preparation or –20°C until use.

Purify the sequencing reactions with Centri-Sep™ plates

The following protocol takes approximately 45 minutes (~25 minutes for purification and ~20 minutes for drying).

IMPORTANT! Do NOT skip the drying step in this procedure. Running samples that have not been dried will affect sequencing results.

Note: Individual Centri-Sep™ Spin columns can be used if few sequencing reactions need to be purified. Centri-Sep™ Spin columns must be hydrated for approximately 2 hours before use. Refer to the *DNA Sequencing by Capillary Electrophoresis Chemistry Guide* (Pub. no. 4305080) for more information.

1. Prepare 2.2% SDS (sodium dodecyl sulfate) in standard deionized water.

   Note: Store 2.2% SDS at room temperature. The SDS will precipitate at 4°C or below.

2. Briefly centrifuge the sequencing plate in a swinging bucket centrifuge (5 to 10 seconds) at 1,000 x g.
3. Remove the MicroAmp™ Clear Adhesive Film and add 7 µL of UltraPure™ DNase/RNase-Free Distilled Water.

4. Add 2 µL of 2.2% SDS to each sample (0.2% final SDS concentration), then seal the plate with MicroAmp™ Clear Adhesive Film.

5. Vortex the plate for 2 to 3 seconds, then centrifuge briefly (5 to 10 seconds) at 1,000 x g.

6. Perform the SDS heat treatment.

Parameter	Stage/step		
	Denature	Incubate	Hold
Temperature	98°C	25°C	4°C
Time	5 min	10 min	Hold

7. Prepare the Centri-Sep™ 96-well plate:

   Note: The Centri-Sep™ 96-well plates come pre-hydrated. The initial centrifugation step removes the hydration solution.

   a. Allow the plate to equilibrate to room temperature.

   b. Place the Centri-Sep™ 96-well plate in an empty 96-well plate.

   c. Centrifuge for 2 minutes at 1,500 x g to remove the hydration solution from the plate.

   d. Discard the plate with flow-through hydration solution.

   e. Place a new MicroAmp™ Optical 96-Well Reaction Plate beneath the prepared Centri-Sep™ 96-well plate to collect purified BigDye™ Direct sequencing reaction product.

8. Briefly centrifuge the SDS heat-treated extension product plate in a swinging bucket centrifuge (5 to 10 seconds) at 1,000 x g and remove the MicroAmp™ Clear Adhesive Film.

9. Dispense 20 µL SDS heat-treated extension product to the corresponding Centri-Sep™ well. Dispense slowly into the center of the well (e.g. electronic pipette setting 4). Do not touch the sides of the well or the gel material.

10. Place a new 96-well collection plate beneath the Centri-Sep™ plate. Using a swinging bucket centrifuge, centrifuge the Centri-Sep™ plate containing the SDS heat treated sample for 2 minutes at 1,500 x g to collect purified sample.

11. Dry the sample in a vacuum centrifuge without heat or in low heat for 10 to 15 minutes or until dry.

12. Go to “Resuspend purified sequencing reactions“ on page 13.

    Note: To store, seal the plate with MicroAmp™ Clear Adhesive Film, and store at 4°C for CE preparation or –20°C until use.

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.

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1. Prepare a 62.5 mM EDTA solution, diluted from 0.5 M EDTA solution pH 8.0.
2. Prepare a 70% ethanol solution using absolute ethanol.

   Note: Replace every 2 weeks.

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

3. Briefly centrifuge the sequencing plate in a swinging bucket centrifuge (5 to 10 seconds at 1,000 x g).
4. Remove the MicroAmp™ Clear Adhesive Film from the plate and add 2 µL of the 62.5 mM EDTA solution to each well.

   Note: 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.

5. Add 30 µL of room temperature absolute ethanol to each well. Seal the plate with MicroAmp™ Clear Adhesive Film and mix by vortexing for 5 to 10 seconds.
6. Seal the plate with MicroAmp™ Clear Adhesive Film.
7. Vortex the plate for 2 to 3 seconds, then centrifuge briefly (5 to 10 seconds) at 1,000 x g.
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 (4°C) for 45 minutes.

   IMPORTANT! Proceed to the next step immediately. If this is not possible, then centrifuge the plate for 2 minutes before performing the next step.

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

    Note: Do not tip out liquid first. Do not tap plate to facilitate liquid removal.

11. Add 33 µL of 70% ethanol to each well. Seal the plate withMicroAmp™ Clear Adhesive Film.

12. Centrifuge the plate 1,870 × g (4°C) for 15 minutes.

    IMPORTANT! Proceed to the next step immediately. If this is not possible, then centrifuge the plate for 2 minutes before performing the next step.

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

    Note: Do not tip out liquid first. Do not tap plate to facilitate liquid removal.

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

15. Go to "Resuspend purified sequencing reactions" on page 13.

    Note: To store, seal the plate with MicroAmp™ Clear Adhesive Film, and store at 4°C for CE preparation or –20°C until use.

Resuspend purified sequencing reactions

Resuspend samples purified with the Ethanol/EDTA and Centri-Sep™ methods.

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

1. Remove the MicroAmp™ Clear Adhesive Film.

2. Resuspend dried samples in 10 µL of Hi-Di™ Formamide, then cover with MicroAmp™ Clear Adhesive Film.

   Note: Do not heat samples to resuspend.

3. Vortex thoroughly (5 to 10 seconds), then centrifuge in a swinging bucket centrifuge (5 to 10 seconds) at 1,000 x g.

   Note: Run samples as soon as possible after resuspension.

Run capillary electrophoresis

1. Remove the MicroAmp™ Clear Adhesive Film and replace with a 96-well plate septa.

   IMPORTANT! Plates sealed with heat seal film can be placed directly into the 3730/ 3730xl instruments. All other instruments require 96-well plate septa.

2. Load plates into the genetic analyzer.

3. Select the capillary length, number of capillaries and polymer type.

   IMPORTANT! Use POP-7™ only.

4. Select or create an appropriate run module according to your specific instrument user guide.

   IMPORTANT! Select a run module with a BDx prefix if you purified your sequencing reactions with BigDye XTerminator™. If your instrument does not contain BDx run modules, download them. Refer to the *BigDye XTerminator™ Purification Kit User Bulletin* (Pub. no. 4483510).

5. Select the injection time. Refer to your specific instrument user guide for information on using default settings or changing injection times. Alternatively, use injection times listed in "Suggested injection times for samples generated with the BigDye™ Direct Cycle Sequencing Kit and Ethanol/ EDTA purification" on page 14.

6. Start the run.

Suggested injection times for samples generated with the BigDye™ Direct Cycle Sequencing Kit and Ethanol/ EDTA purification

Select the run module based on your target amplicon length. For more information, refer to the Instrument User Guide for the instrument being used.

Genetic Analyzer	Run Module	Suggested Injection Time (seconds) [1]
3500xL Genetic Analyzer	FastSeq50_POP-7™xl	8
	RapidSeq50_POP-7™xl	8
	ShortReadSeq50_POP-7™xl	8
	StdSeq50_POP-7™xl	8
3500 Genetic Analyzer	FastSeq50_POP-7™	5
	RapidSeq50_POP-7™	5
	ShortReadSeq50_POP-7™	5
	StdSeq50_POP-7™	5
3730*xl* DNA Analyzer	FastSeq50_POP-7™xl	8
	LongSeq50_POP-7™xl	8
	StdSeq36_POP-7™xl	8
	RapidSeq36_POP-7™xl	10
	TargetSeq36_POP-7™xl	10
3730 DNA Analyzer	FastSeq50_POP-7™	8
	LongSeq50_POP-7™	8

Genetic Analyzer	Run Module	Suggested Injection Time (seconds) [1]
3730 DNA Analyzer	StdSeq36_POP-7™	8
	RapidSeq36_POP-7™	10
	TargetSeq36_POP-7™	10
3130*xl* Genetic Analyzer	FastSeq50_POP-7™xl	11
	StdSeq50_POP-7™xl	11
	RapidSeq36_POP-7™xl	10
	UltraSeq36_POP-7™xl	12
3130 Genetic Analyzer	FastSeq50_POP-7™	4
	StdSeq50_POP-7™	4
	RapidSeq36_POP-7™	4
	UltraSeq36_POP-7™	6

[1] The suggested injection times are based on results from a wide variety of samples. However, further optimization may be required.

Sequence analysis tools

Minor Variant Finder Software

The Minor Variant Finder Software is a simple, easy-to-use desktop software designed for the accurate detection and reporting of minor variants (<25% of a major peak) or 50:50 mixtures as found in a germline heterozygous positions by Sanger Sequencing.

By comparing test specimen and control traces, the software generates a noise-minimized electropherogram for confirmation of minor variants in forward and reverse sequences. The software can detect variants (SNPs or SNVs) with a Limit of Detection (LOD) of 5% with high-quality data in amplicons of lengths 150 to 500 bp. LOD is defined as the lowest level at which sensitivity >= 95% and specificity >= 99% within the overlapping region of forward and reverse test and control .ab1 files.

Note: LOD was determined using 5% mixtures that were experimentally created with physical mixtures of molecules, and is not based on peak height ratios in electropherograms.

The software also includes an optional NGS confirmation function.

The Minor Variant Finder Software runs in a web browser window, but does not require connection to the internet in order to run. Data is secure on your desktop computer.

Sequence Scanner Software

Sequence Scanner 2 is free software for viewing electropherograms. It provides an easy way to perform a high-level sequencing data quality check or general data review that includes summary tables and electropherograms as well as a general .ab1 file raw/analyzed data view.

To obtain the software, go to: **http://resource.thermofisher.com/pages/WE28396/**.

Next-generation confirmation (NGC) module

The Applied Biosystems™ Analysis Module Next-Generation Confirmation (NGC) is CE Sanger sequencing software hosted on the Thermo Fisher Cloud environment. The software allows you to examine variants from a CE electropherogram to confirm the variants detected by Next Generation Sequencing (NGS) platforms. The software analyzes CE sequencer-generated .ab1 files and performs SNP detection and analysis, SNP discovery and validation, and sequence confirmation, all on the cloud. NGC software can automatically retrieve reference sequences from genomic databases, report variants in genomic coordinates, and report genomic annotations for SNPs. The software analyzes NGS variant .vcf files and analyzes NGS variants and Sanger variants in the same alignment view. The software can also generate a Venn diagram, allowing you to visually compare and confirm variants generated from NGS. In addition, the NGC software generates and exports variants in standard variant call format (VCF).

Variant Reporter™ Software

This software performs comparative sequencing, also known as direct sequencing, medical sequencing, PCR sequencing, and resequencing with DNA sequencing files. The software is designed for reference-based and non-reference-based analysis such as mutation detection and analysis, SNP discovery and validation, and sequence confirmation. The robust algorithms will call SNPs, mutations, insertions, deletions, and heterozygous insertions or deletions for data generated using the Applied Biosystems™ genetic analyzers.

To obtain the software, go to: **https://www.thermofisher.com/order/catalog/product/4475006**.

Related documentation

Document	Publication number	Description
BigDye™ Direct Cycle Sequencing Kit Protocol	4458040	TheBigDye™ Direct Cycle Sequencing Kit Protocol provides instructions and troubleshooting information for using the BigDye™ Direct Cycle Sequencing Kit.
Troubleshooting Sanger sequencing data	MAN0014435	This document provides guidance for the review of your data and troubleshooting tips for improving sequencing data quality.
DNA Sequencing by Capillary Electrophoresis Chemistry Guide	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.

Document	Publication number	Description
BigDye XTerminator™ Purification Kit User Bulletin	4483510	This user bulletin provides: • A list of BigDye XTerminator™ Purification Kit run modules • Instructions for downloading and running the BDX Updater Utility to install the run modules • Instructions for running the BDX Updater Utility after you recalibrate the autosampler
BigDye XTerminator™ Purification Kit Quick Reference Card	4383427	This quick reference card provides instructions for BigDye XTerminator™ purification. In particular, it includes information on compatible plate vortexers and heat seal information for 3730 users.
User Bulletin: Using an SDS/Heat Treatment with Spin Columns or 96-Well Spin Plates to Remove Unincorporated Dye Terminators	4330951	This user bulletin provides instructions for adding an SDS/heat treatment to the spin column and spin plate purification methods. This SDS/heat treatment effectively eliminates unincorporated dye terminators from your cycle sequencing reactions.

Customer and technical support

Visit **thermofisher.com/support** for the latest in services and support, including:

- Worldwide contact telephone numbers
- Product support, including:
  - Product FAQs
  - Software, patches, and updates
- Order and web support
- Product documentation, including:
  - 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.

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