Generating high-quality data for Minor Variant Finder Software using the BigDye™ Terminator v3.1 Cycle Sequencing Kit

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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:

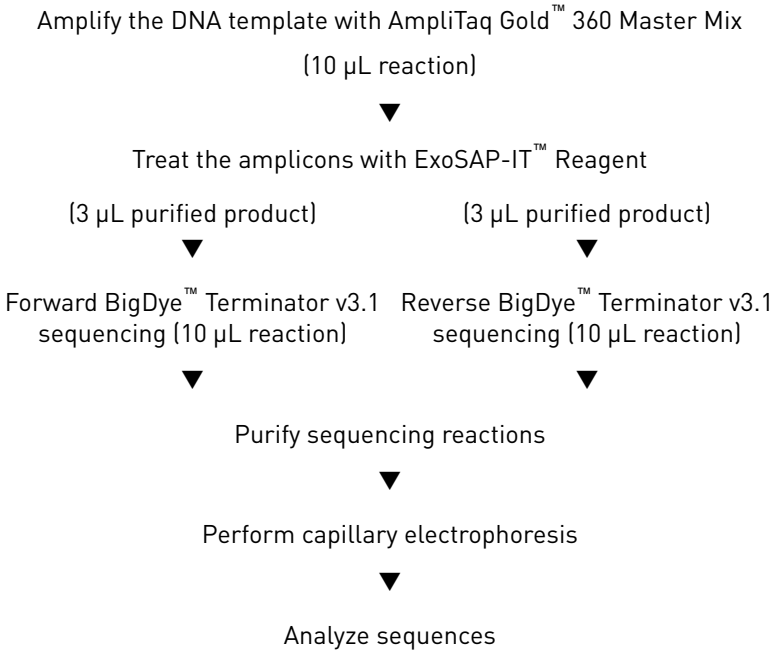
- BigDye™ Terminator v3.1 Cycle Sequencing Kit
- Applied Biosystems™ Genetic Analyzers

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. This protocol uses ExoSAP-IT™ Reagent (Affymetrix™) for purification of amplicons. Supporting documentation such as application notes may be available from Thermo

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

For sequencing short amplicons, use the alternative protocol, *Generating high-quality data using the BigDye™ Direct Cycle Sequencing Kit* (Pub. no. MAN0014436), which has been optimized to reduce loss of 5′ sequences.

Workflow



Required materials

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

Item	Source
Reagents	
BigDye™ Terminator v3.1 Cycle Sequencing Kit	4337456
AmpliTaq Gold™ 360 Master Mix	4398881
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
ExoSAP-IT™ PCR Product Cleanup	78250

Item	Source
DNA Suspension Buffer, RNAse DNAse Free (10 mM Tris/0.1 mM EDTA, pH 8.0)	Teknova, Inc. T0223
PCR and sequencing primers (HPLC-purified recommended)	Primers can be designed, chosen, and ordered with the Primer Designer™ Tool at **http://www.thermofisher.com/primerdesigner**
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, Inc. 3054-1002
Digital Vortex-Genie™ 2 or equivalent	Scientific Inducstries, Inc. SI-A536
Centrifuge with swinging bucket (with PCR plate adapter)	MLS
Compatible thermal cyclers [1]	
GeneAmp™ PCR System 9700	Contact your local sales office
Veriti™ Thermal Cycler	
Compatible genetic analyzers	
3130/3130*xl* Genetic Analyzer	Contact your local sales office
3500/3500xL Genetic Analyzer	
3730/3730*xl* DNA Analyzer [2]	

[1] If you use a different thermal cycler, you may need to optimize the thermal cycling conditions.

[2] 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 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.

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.

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 and sequencing 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 AmpliTaq Gold™ 360 Master Mix

Set up the PCR reaction

1. Completely thaw the AmpliTaq Gold™ 360 Master Mix and store on ice.

   Note: Store reagents 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]
AmpliTaq Gold™ 360 Master Mix	5 µL	528 µL
GC Enhancer	0.5 µL (5%)	53 µL
UltraPure™ DNase/RNase-Free Distilled Water	0.5 µL	53 µL
Total volume	**6 µL**	**634 µL**

[1] Includes 10% additional volume.

   Note: Store on ice until ready for use.

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	6 µL
DNA template (10ng)	1 µL
Pooled PCR primers (0.8 µM each)	3 µL

   IMPORTANT! Change pipette tips after each transfer.

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

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

Parameter	Stage/step					
	Incubate	Cycling (35 cycles)			Final extension	Hold
		Denature	Anneal [1]	Extend [2]		
Temperature	95°C	95°C	58°C	72°C	72°C	4°C
Time	10 minutes	30 seconds	30 seconds	45 seconds	7 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. For more information, refer to the *AmpliTaq Gold™ 360 DNA Polymerase Protocol*.

3. Place the plate on ice or store the plate at 4°C until ready for treatment with ExoSAP-IT™ Reagent.

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

Treat the amplicons with ExoSAP-IT™ Reagent

1. Remove the 96-well plate from the thermal cycler, then centrifuge in a swinging bucket centrifuge for 10 seconds at 1,000 x g.
2. Place the plate and the tube of ExoSAP-IT™ Reagent PCR Purification reagent on ice.
3. Remove the MicroAmp™ Clear Adhesive Film.
4. Add 4 µL of ExoSAP-IT™ Reagent to each well.
5. Label the PCR plate "+ExoSAP-IT™."
6. Seal the plate with MicroAmp™ Clear Adhesive Film.
7. Vortex the plate for 2 to 3 seconds, then centrifuge (5 to 10 seconds) at 1,000 × *g*.
8. Place the ExoSAP-IT™ Reagent-treated plate into the thermal cycler and run with the following settings.

Parameter	Stage/step		
	Digest	Heat deactivation	Hold
Temperature	37°C	80°C	4°C
Time	15 minutes	15 minutes	Hold

9. Store the treated plate on ice for immediate use or at –20°C for longer term storage.

Run sequencing reactions using the BigDye™ Terminator v3.1 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™ 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. 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	96 well plate [1]	1 reaction	96 well plate[1]
BigDye™ Terminator v3.1 Ready Reaction Mix	2 µL	211 µL	2 µL	211 µL
5x Sequencing Buffer	1 µL	106 µL	1 µL	106 µL
Deionized water (RNase/DNase-free)	3 µL	317 µL	3 µL	317 µL
M13 forward primer (3.2 µM)	1 µL	106 µL	—	—
M13 reverse primer (3.2 µM)	—	—	1 µL	106 µL
Total volume	**7 µL**	**740 µL**	**7 µL**	**740 µL**

[1] Includes 10% additional volume.

   Note: Store on ice and protected from light.

4. Vortex the tubes for 2 to 3 seconds, then centrifuge briefly (2 to 3 seconds) with a benchtop microcentrifuge.
5. Label a new 96-well reaction plate "sequencing".
6. Place the "PCR + ExoSAP-IT" plate on ice and remove the MicroAmp™ Clear Adhesive Film.

7. For each reaction, add the following, in order:

Component	Quantity
Reaction mix	7 µL
ExoSAP-IT™-purified PCR product	3 µL

IMPORTANT! Change pipette tips after each transfer.

Note: Use an 8-tip multi-channel P10 pipette, if available, to facilitate the amplicon transfer.

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

9. Vortex the plate for 2 to 3 seconds, then centrifuge in a swinging bucket centrifuge to collect contents to the bottom of the wells (5 to 10 seconds) at 1,000 × *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, then run with the following conditions:

Parameter	Stage/step				
	Incubate	Cycling (25 cycles)			Hold
		Denature	Anneal [1]	Extend	
Temperature	96°C	96°C	50°C	60°C	4°C
Time	1 minute	10 seconds	5 seconds	4 minutes [2]	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] Shorter amplicons (<500bp) can be run with shorter extension times (for example 2 minutes).

Note: Cycle sequencing will complete in 2 to 2.5 hours.

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

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.

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

- "Purify sequencing reactions with BigDye XTerminator™" on page 10
- "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. Remove the BigDye XTerminator™ bead solution from 4°C storage and place on ice.

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. Keep reagents on ice between pipetting steps.

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

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

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

5. Dispense 55 µL/well of the SAM/BigDye XTerminator™ bead working 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.

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

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

8. In a swinging bucket centrifuge, centrifuge the plate at 1,000 × *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 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.

4. Prepare the SDS heat treatment:

Component	Volume
Sequencing reaction	10 µL
UltraPure™ DNase/RNase-Free Distilled Water	10 µL
2.2% SDS	2 µL
Total volume	**22 µL**

5. Vortex the plate for 2 to 3 seconds, then centrifuge (5 to 10 seconds) at 1,000 × *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™ 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 14.

    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 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. This can cause precipitation of the EDTA.

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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.

5. Add the following in order:

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

IMPORTANT! Dispense the EDTA solution directly into the sample in each well before adding ethanol. 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 to 3 seconds, then centrifuge (5 to 10 seconds) at 1,000 × *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 there is a delay between steps, centrifuge the plate for an extra 2 minutes and 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 and carefully invert the plate onto the paper without dislodging the pellet. Centrifuge at 185 × g for 1 minute.

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

11. Add 30 µL of 70% ethanol to each well.

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

IMPORTANT! Proceed to the next step immediately. If there is a delay between steps, centrifuge the plate for an extra 2 minutes and proceed to 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 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 help with 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 14.

    Note: To store, seal the plate with MicroAmp™ Clear Adhesive Film, and store, protected from light, 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.

   Note: There is no default run module for POP-6™ when using the BigDye™ Terminator v3.1 Cycle Sequencing Kit on a 3500/3500xL Genetic Analyzer. Refer to the instrument user guide for creating run modules.

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.
6. Start the run.

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™ Terminator v3.1 Cycle Sequencing Kit User Guide	4337035	Describes the BigDye™ Terminator v3.1 Cycle Sequencing Kit hardware and software and provides information on preparing, maintaining, and troubleshooting the system.
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.
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.
Using an SDS/Heat Treatment with Spin Columns or 96-Well Spin Plates to Remove Unincorporated Dye Terminators	4330951	This protocol 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.

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- 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.

Limited product warranty

Life Technologies Corporation and/or its affiliate(s) warrant their products as set forth in the Life Technologies' General Terms and Conditions of Sale found on Life Technologies' website at **www.thermofisher.com/us/en/home/global/terms-and-conditions.html**. If you have any questions, please contact Life Technologies at **www.thermofisher.com/support**.

Manufacturer: Multiple Life Technologies Corporation manufacturing sites are responsible for manufacturing the products associated with the workflow covered in this guide.

Corporate entity: Life Technologies Corporation | Carlsbad, CA 92008 USA | Toll Free in USA 1 800 955 6288

Revision history: Pub. No. MAN0015798

Revision	Date	Description
B.0	13 June 2017	Title change and changes to compatible POP polymers.
A.0	13 April 2016	New document.

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