Certificate of Registration

QUALITY MANAGEMENT SYSTEM - ISO 9001:2015

This is to certify that:

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Seattle
Washington
98103
USA

Holds Certificate No: **FM 705363**

and operates a Quality Management System which complies with the requirements of ISO 9001:2015 for the following scope:

The Design, Development, Manufacture and test of Raman spectroscopy systems and accessories for commercial distribution.

For and on behalf of BSI:

Carlos Pitanga, Chief Operating Officer Assurance – Americas

Original Registration Date: 2019-05-07
Effective Date: 2022-05-07
Latest Revision Date: 2022-04-01
Expiry Date: 2025-05-06

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Applied Biosystems

BigDye® Terminator v3.1 and v1.1 Cycle Sequencing Kits

- **Enhanced robustness improves success rates, particularly with challenging templates**
- **Comprehensive chemistry solution for today's wide range of sequencing applications**
- **Improved peak-height uniformity and optimized signal balance for longer, higher quality reads**
- **Enable completion of sequencing projects more quickly and economically**

Improved Performance

BigDye® Terminator v3.1 and v1.1 chemistries provide a variety of benefits over earlier versions of BigDye chemistry. The new kits offer improved performance in sequencing difficult templates, successfully reading through dinucleotide repeats and other challenging sequence motifs. Both formulations are also designed to offer improved robustness with a wide range of template types and qualities. In addition, v3.1 and v1.1 kits generate data that has greater peak-height uniformity, which enhances basecalling accuracy and mixed-base detection. Overall, v3.1 and v1.1 kits enable longer sequencing reads and higher success rates, which lead to reduced project costs.

New Chemistries to Address Your Sequencing Needs

Like its predecessor (ABI PRISM® BigDye Terminator v3.0 Cycle Sequencing Kit), the new BigDye® Terminator v3.1 Cycle Sequencing Kit is optimized for the majority of DNA sequencing applications. The BigDye® Terminator v1.1 Cycle Sequencing Kit, which is based on the original ABI PRISM® BigDye Terminator chemistry (v1.0), is formulated for specialty applications. Together, these two new powerful and versatile chemistries meet the demands of the wide range of sequencing applications performed today.

Chemistry Options

Applications	BigDye® Terminator v3.1 Kit	BigDye® Terminator v1.1 Kit
de novo sequencing	+	✔
Resequencing	+	✔
Sequencing difficult templates	+	+
Long-read sequencing	+	✔
Sequencing across all template types (plasmids, PCR products, BACs, and fosmids)	+	✔
Mixed-base detection	+	✔
Sequencing short PCR products using rapid electrophoresis run modules	✔	+

+ Recommended ✔ Satisfactory

Table 1. Chemistry Options

Easy Integration

The dyes in the new BigDye Terminator v3.1 and v1.1 kits are the same as those in the v3.0 and v1.0/v2.0 kits respectively, and thus, no new software or instrument recalibration is required for data analysis. Therefore, researchers can easily integrate both new versions into their workflow and take advantage of the benefits these new chemistries provide.

BigDye® Terminator v3.1 Chemistry

The BigDye Terminator v3.1 Cycle Sequencing Kit is a robust, highly flexible chemistry, designed for the majority of applications, including *de novo* sequencing and resequencing. The BigDye Terminator v3.1 kit generates data with uniform peak heights and optimized signal balance to produce long, high-quality reads. Improved peak patterns also contribute to more accurate base assignments for heterozygote and mutation detection. The chemistry's robust formulation is successful with a wide variety of templates, including PCR products, plasmids, and large insert clones, such as fosmids and bacterial artificial chromosomes (BACs). The BigDye Terminator v3.1 kit provides researchers with a higher success rate than the BigDye Terminator v3.0 kit, particularly with difficult to

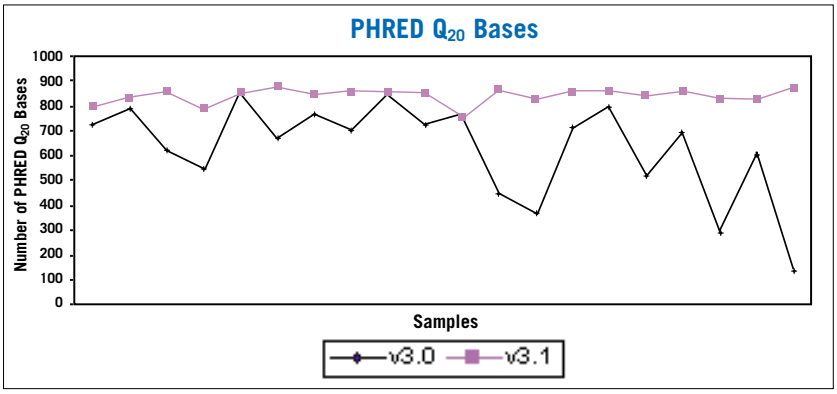

Figure 1. Longer, higher quality reads with the BigDye® Terminator v3.1 kit. The BigDye Terminator v3.1 kit generates data with an improved average number of PHRED Q_{20} bases. A library of "difficult" templates was sequenced in a customer's laboratory according to their standard protocol using the 3730*xl* DNA Analyzer. Samples using BigDye® Terminator v3.0 chemistry generated on average 629 Q_{20} bases, while samples using BigDye Terminator v3.1 chemistry generated on average 840 Q_{20} bases. (Data courtesy of Agencourt.)

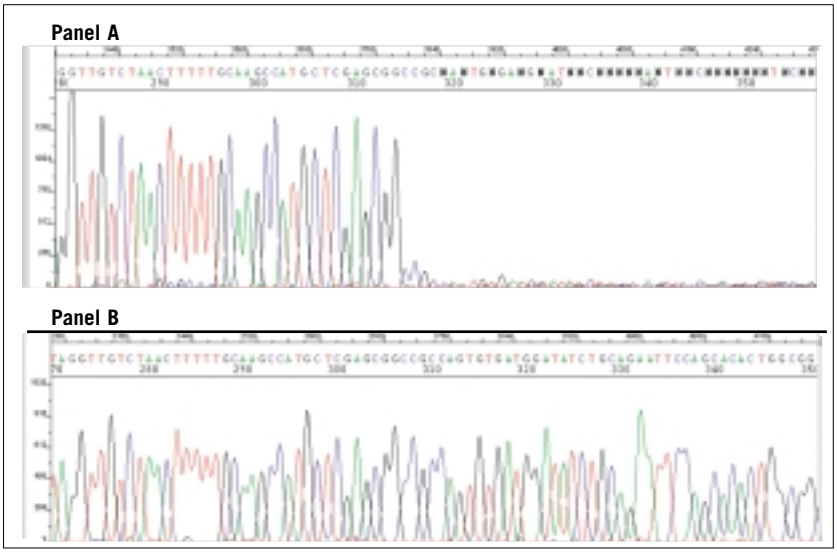

Figure 2. Improved Performance on Difficult Templates with BigDye® Terminator v3.1. A sample was run in a customer's laboratory according to their standard protocol. With the BigDye® Terminator v3.0 kit the reaction is terminated by an unknown sequence context (Panel A), while the reaction prepared with the BigDye Terminator v3.1 kit continues to read through the sample (Panel B). (Data courtesy of Agencourt.)

sequence templates, and requires only minimal changes to the current BigDye Terminator v3.0 kit protocol.

BigDye Terminator v1.1 Chemistry

The BigDye Terminator v1.1 Cycle Sequencing Kit is designed for specialty applications that require optimal basecalling adjacent to the primer. The v1.1 chemistry is an excellent choice for sequencing short PCR product templates with rapid electrophoresis run modules. With better peak-height uniformity than its v1.0 predecessor, the new v1.1 kit provides very good mixed-base detection. Like the v3.1 chemistry, the v1.1 chemistry is designed for superior robustness and provides dependable, reproducible results with a wide variety of templates. The new v1.1 protocol recommends only minimal changes to the v1.0 version.

Choosing the Right DNA Sequencing Chemistry

BigDye Terminator v3.1 and v1.1 kits allow researchers to choose the optimal chemistry for a wide range of applications. Table 1 provides guidelines for selecting the appropriate cycle sequencing kit. If your laboratory is interested in the most robust, flexible chemistry that will generate the longest reads, then you would prefer the v3.1 kit. If your lab is primarily sequencing short PCR fragments using rapid electrophor-

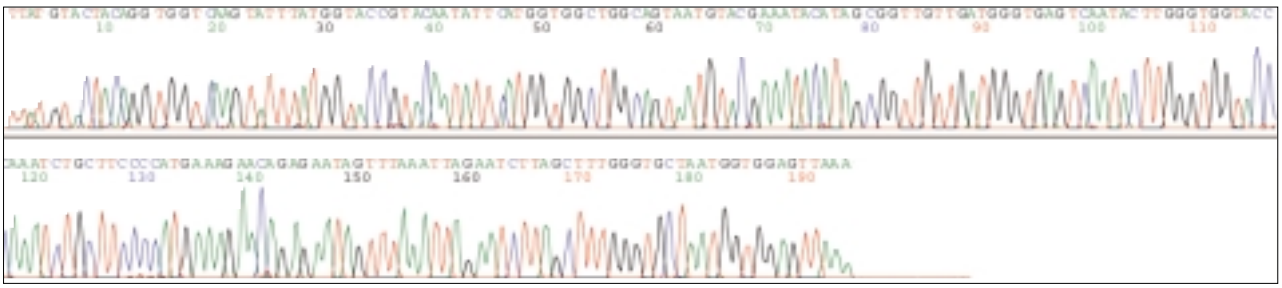

Figure 3. Short PCR Product Sequencing with BigDye® Terminator v1.1. The v1.1 kit successfully sequences a short PCR product generated from human mitochondrial DNA. The PCR product shows 100% basecalling accuracy beginning with the first base adjacent to the primer. The sample was run on the 3100 Genetic Analyzer using POP-6™ Polymer.

esis run modules, then you would prefer the v1.1 kit. Many variables contribute to DNA sequencing data quality, including template type, instrument module, total signal, peak-height uniformity, and mobility shift. All should be taken into consideration when selecting the most appropriate chemistry.

Guaranteed Performance

All BigDye sequencing reagents are tested twice for quality—first for correct formulation and then for consistent, reliable performance on our sequencing systems. Additionally, Applied Biosystems expert field and telephone support teams are readily available to answer your questions and provide whatever assistance you require.

Specifications

BigDye® Terminator v3.1 and v1.1 Cycle Sequencing Kits include all required reagents for sequencing 24, 100, 1,000, 5,000, or 25,000 single-stranded (ss) or double-stranded (ds) DNA templates. The reagents in each kit are optimized for use with the ABI PRISM® 310, 3100, and 3100–*Avant* Genetic Analyzer; the 3700, 3730, and 3730*xl* DNA Analyzer, and the 377 DNA Sequencer.

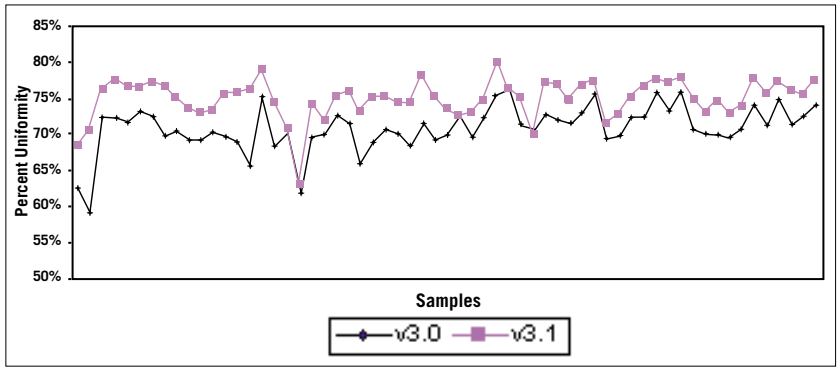

Figure 4. Improved uniformity of peak heights. BigDye® Terminator v3.1 chemistry generates data with improved peak height uniformity in customer samples. Peak height uniformity is defined as local peak height consistency of analyzed data. 100% peak height uniformity represents an idealized situation where all analyzed data peaks are of equivalent height. Improved uniformity contributes to longer, higher quality reads and more accurate mixed base detection.

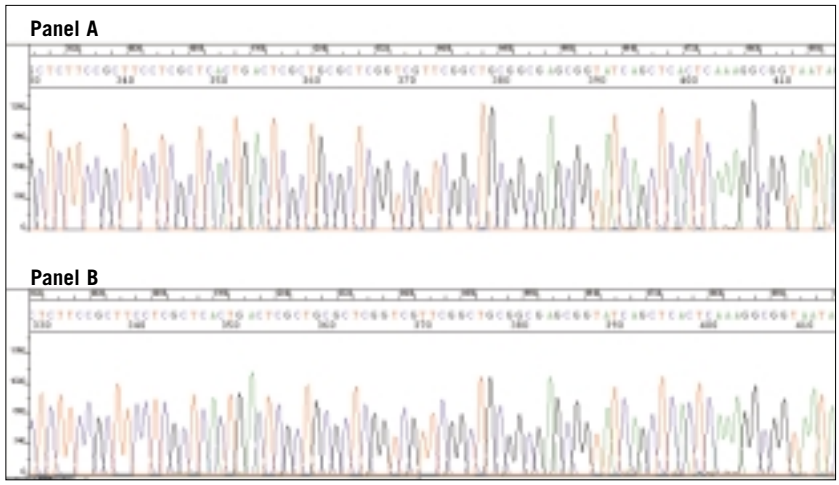

Figure 5. Improved uniformity of peak heights with BigDye® Terminator v3.1 kit. Panel A shows data from a sample sequenced using the BigDye® Terminator v3.0 kit. Panel B shows the same sample run under identical conditions with the BigDye Terminator v3.1 kit. The uniformity of the data produced with the v3.0 sample is 72% whereas the uniformity for the v3.1 sample is 76%.

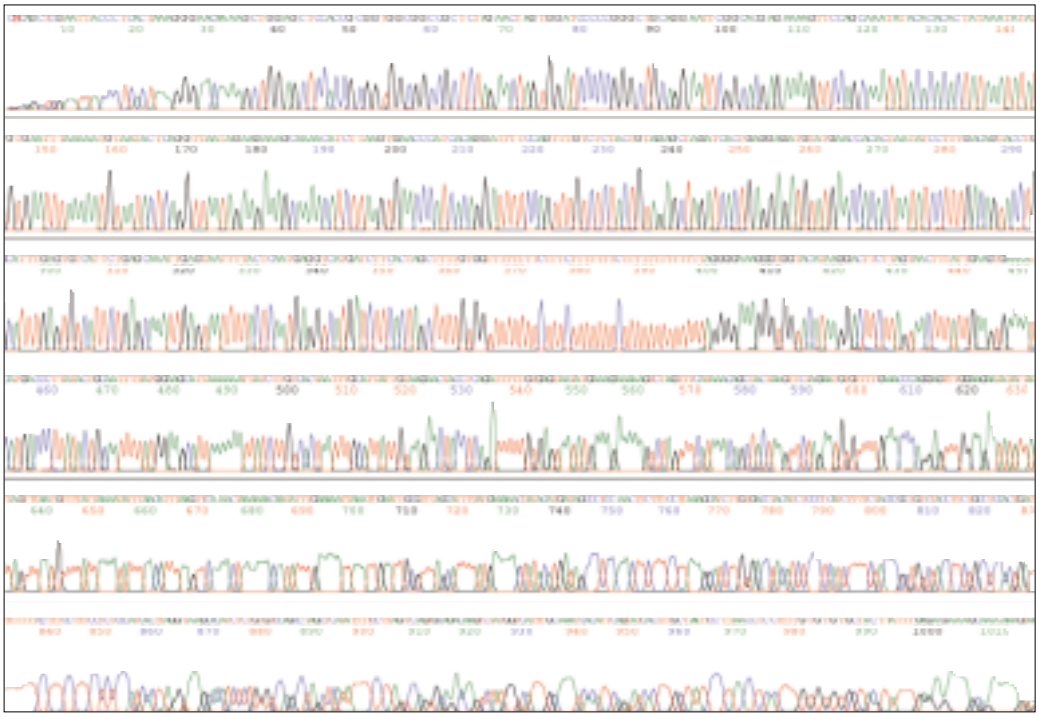

Figure 6. Longer Read Lengths with BigDye® Terminator v3.1 on the 3100 Genetic Analyzer. This figure shows accurate basecalling for more than 1,000 bases; the first ambiguity is not seen until base 1,040. The sample was run on the ABI PRISM® 3100 Genetic Analyzer with an 80 cm array using POP-4™ Polymer and the standard run module.

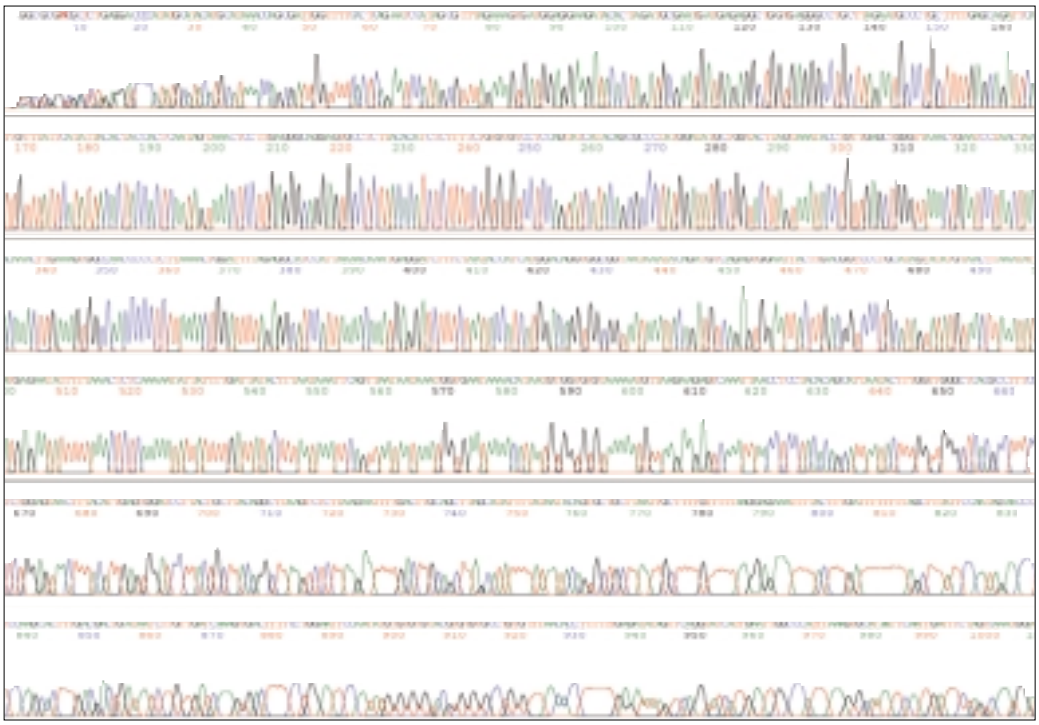

Figure 7. Longer Read Lengths with BigDye® Terminator v3.1 on the 3730*xl* Genetic Analyzer. The plasmid insert, beginning at base 23, shows accurate basecalling for more than 1,000 bases. The first ambiguity occurs at base 1,031. The sample was run on an Applied Biosystems 3730*xl* Genetic Analyzer with a 50 cm array using POP-7™ Polymer and the standard run module.

Ordering Information

BigDye® Terminator v3.1 Cycle Sequencing Kit

Ready Reactions	P/N
24	4337454
100	4337455
1,000	4337456
5,000	4337457
25,000	4337458

BigDye® Terminator v1.1 Cycle Sequencing Kit

Ready Reactions	P/N
24	4337449
100	4337450
1,000	4337451
5,000	4337452
25,000	4337453

BigDye® Terminator v3.1 Sequencing and Matrix Standards*

Description	P/N
BigDye® Terminator v3.1 Sequencing Standard	4336935
3700/3730 BigDye® Terminator v3.1 Sequencing Standard	4336943
310/377 BigDye® Terminator v3.1 Matrix Standards	4336948
3100 BigDye® Terminator v3.1 Matrix Standard	4336974
3700/3730 BigDye® Terminator v3.1 Matrix Standard	4336975

*Spatial/Spectral recalibration is not required to use v3.1 chemistry if currently using v3.0 files

BigDye® Terminator v1.1 Sequencing and Matrix Standards*

Description	P/N
BigDye® Terminator v1.1 Sequencing Standard	4336791
3700/3730 BigDye® Terminator v1.1 Sequencing Standard	4336799
310/377 BigDye® Terminator v1.1 Matrix Standards	4336805
3100 BigDye® Terminator v1.1 Matrix Standard	4336824
3700/3730 BigDye® Terminator v1.1 Matrix Standard	4336825

*Spatial/Spectral recalibration is not required to use v1.1 chemistry if currently using v1.0 or v2.0 files

BigDye® Terminator v1.1/v3.1 Sequencing Buffer (5X)

Quantity	Description	P/N
1 mL	BigDye® Terminator v1.1/v3.1 Sequencing Buffer (5X)	4336697
28 mL	BigDye® Terminator v1.1/v3.1 Sequencing Buffer (5X)	4336699
233 mL	BigDye® Terminator v1.1/v3.1 Sequencing Buffer (5X)	4336701

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Applied Biosystems

Using ExoSAP-IT™ *Express* PCR Product Cleanup to generate high quality BigDye™ Terminator v3.1 Cycle Sequencing Kit Data

Publication Number MAN0017063 **Revision** A.0

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

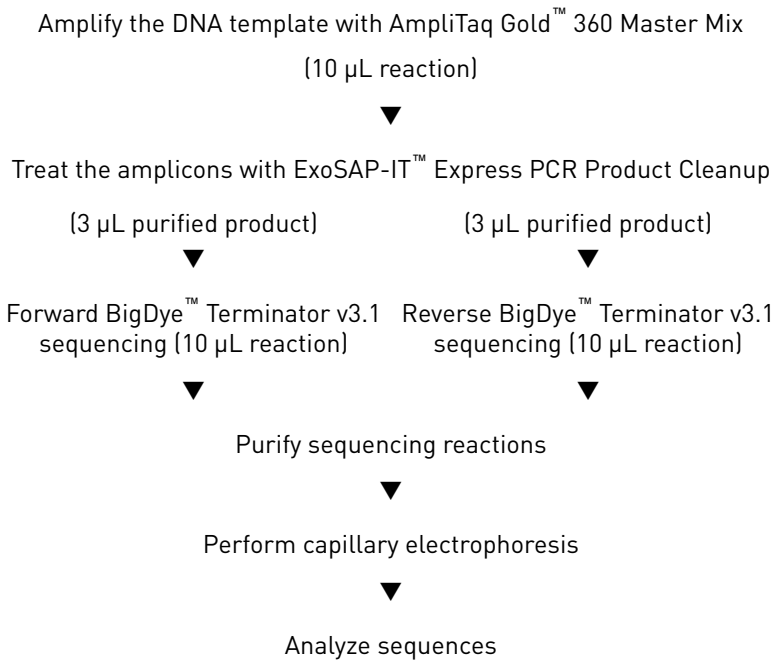
This document provides a protocol for generating high-quality sequence data using:

- BigDye™ Terminator v3.1 Cycle Sequencing Kit
- Applied Biosystems™ Genetic Analyzers
- ExoSAP-IT™ *Express* PCR Product Cleanup

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.

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™ *Express* PCR Product Cleanup	75001
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*)	

Item	Source
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 at 100 µM concentration in 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
UltraPure™ DNase/RNase-Free Distilled Water	1 µL	106 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™ *Express* PCR Product Cleanup.

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

Treat the amplicons with ExoSAP-IT™ *Express* PCR Product Cleanup

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™ *Express* PCR Product Cleanup on ice.
3. Remove the MicroAmp™ Clear Adhesive Film.
4. Transfer 5 µL of each PCR product to a new 96-well plate.
5. Add 2 µL of ExoSAP-IT™ *Express* PCR Product Cleanup to each well.

   IMPORTANT! Change pipette tips between wells.

6. Label the plate "+ExoSAP-IT."
7. Seal the +ExoSAP-IT plate with MicroAmp™ Clear Adhesive Film.
8. Vortex the plate for 2 to 3 seconds, then centrifuge (5 to 10 seconds) at 1,000 × *g*.

9. Incubate the +ExoSAP-IT plate:

Parameter	Stage/step	
	Digest	ExoSAP-IT™ Inactivation
Temperature	37°C	80°C
Time	4 minutes	1 minute

10. 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, then 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", then 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	—	—

Component	Quantity			
	Forward reaction mix		Reverse reaction mix	
	1 reaction	96-well plate [1]	1 reaction	96-well plate[1]
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 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 a new 96-well reaction plate "sequencing".

6. Place the "PCR + ExoSAP-IT" plate on ice, then remove the MicroAmp™ Clear Adhesive Film.

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

Component	Quantity
Reaction mix	7 µL
Purified PCR product (ExoSAP-IT™ *Express* PCR Product Cleanup)	3 µL

IMPORTANT! Change pipette tips after each transfer.

Note: Use an 8-tip multi-channel P10 pipette, if available, for 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 11
- "Purify the sequencing reactions with ethanol/EDTA precipitation" on page 13

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.

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.

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.

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.

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.
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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Revision	Date	Description
A.0	13 June 2017	New document.

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