

Development of a Workflow to Detect Sequence Variants in the *BRCA1* and *BRCA2* Genes



The oncogenetics group in the DNA Diagnostics division of the Department of Human Genetics at the Radboud University Nijmegen Medical Centre, The Netherlands, is led by Dr. Marjolijn J.L. Ligtenberg and Dr. Marcel R. Nelen. Pictured from left to right are Hicham Ouchene, Neeltje Arts, Wendy Hettema, Dr. Marjolijn Ligtenberg, and Dr. Marcel Nelen. The laboratory is one of the Dutch centers for DNA diagnostic services and is granted accreditation by CCKL, the Dutch quality assessment institute for medical laboratories. The center performs research

and molecular testing for over 100 different disorders. Their main focus areas include hereditary cancers, hereditary kidney and neuromuscular disorders, hereditary deafness and blindness, and mental retardation. Besides genetic testing, the laboratory is involved in various areas of research, including pharmacogenetics and risk factor analysis. The Department of Human Genetics uses Sanger sequencing and next-generation sequencing [Applied Biosystems SOLiD™ 3 Plus System] as separate but complementary techniques to cover all possible areas in human genetics research.

Abstract

One of the major responsibilities of a clinical genetics research laboratory is mutation scanning in one or more genes related to an inherited disease. There are many different techniques for mutation scanning, but most can be grouped into one of two broad methodologies: [1] a prescreen that detects but does not characterize variants, and [2] the industry-standard technique: direct sequence analysis of the DNA by Sanger sequencing. Prescreen approaches historically include single-strand conformation polymorphism (SSCP), chemical cleavage of mismatch, and various forms of heteroduplex analysis, of which denaturing high-performance liquid chromatography (dHPLC), denaturing gradient gel electrophoresis (DGGE), and high-resolution melt-curve analysis (HRM) are most frequently employed. After the prescreening step, aberrant samples are sequenced. However, direct sequencing is widely used for mutation scanning and allows both the detection and characterization of mutations in a single step.

As laboratories are seeking to improve the sensitivity, cost-effectiveness, and speed of their workflows, decisions about whether or not to include prescreening steps should be made with the following considerations in mind:

- Variants in some samples may be missed (false negatives), as prescreening assays can be less accurate than direct sequencing
- Neutral (nonpathogenic) polymorphisms will be detected but not characterized by prescreening techniques, and therefore need to be confirmed by direct sequencing or other techniques
- Prescreening techniques typically require shorter PCR amplicons than sequencing, increasing the number of reactions considerably
- Prescreening introduces an extra step into the workflow, and often relies on additional techniques, software, and DNA sequence confirmation of false positives; this results in more complicated workflows and more time investment

- In order to efficiently use prescreening techniques, batching of DNA samples is often needed, increasing sample handling time

In this publication we describe the development and validation of a direct resequencing workflow for mutation scanning of the *BRCA1* and *BRCA2* genes, performed by researchers at the Radboud University Nijmegen Medical Centre. This new workflow includes all steps from purified DNA to data analysis, and includes PCR for all amplicons covering both genes, PCR cleanup, cycle sequencing, electrophoresis, and data analysis. To simplify workflows and decrease the time-to-result, the Nijmegen researchers focused on a robust “one sample, one assay” approach. Key to the success of this workflow was the 96-well plate design, which contained prespotted PCR primers covering both genes and also included multiplex nontemplate controls.

The workflow was developed using a 3730xl Genetic Analyzer, and repeated by Applied Biosystems scientists using the new 3500xL Genetic Analyzer.

Introduction

BRCA1 and *BRCA2* are tumor suppressor genes. Like many other tumor suppressors, the proteins produced from these two genes are directly involved in repairing damaged DNA and preventing cells from growing and dividing too rapidly or in an uncontrolled way.

Both *BRCA1* and *BRCA2* are large genes, comprising 23 and 27 exons, respectively. Mutations in *BRCA* genes are distributed throughout the gene-coding region and have been correlated with an increased cancer risk [3]. Ongoing *BRCA1* and *BRCA2* research has highlighted the need for a simple and robust mutation detection workflow.

Automated Sanger sequencing of DNA is widely acknowledged as the gold standard for mutation detection and/or characterization. It is a highly referenced and robust technique that delivers long read lengths and the ability to sequence anywhere from a few to several hundred samples in a single day. The protocols involved are generally straightforward and the assays are cost-effective. Applied Biosystems® Genetic Analyzers are widely used in life science research laboratories for DNA sequence analysis as well as for numerous DNA fragment analysis assays.

Here we highlight the workflow used at the Radboud University Nijmegen Medical Centre for analysis of the *BRCA1* and *BRCA2* genes in a preclinical research setting (Figure 1), which was developed using 20 human DNA samples derived from whole blood.

This publication is aimed at:

- Highlighting a customer's approach to a resequencing protocol for the analysis of the *BRCA1* and *BRCA2* genes for research purposes
- Describing their method that enables *BRCA1* and *BRCA2* screening in a "one sample, one assay" manner, facilitating analysis of a sample without the need for batching
- Demonstration of data concordance

between the Applied Biosystems® 3730xl and 3500xL Genetic Analyzers

- Highlighting key benefits delivered by the 3500 Series instruments for Sanger sequencing

Materials and Methods

Blood samples from 20 human cancer subjects were collected by clinical molecular geneticists at the Radboud University Nijmegen Medical Centre and were analyzed in this blinded study. Genomic DNA was isolated from 7 mL of fresh whole blood using a Chemagen magnetic particle processor and the Chemagic kit (Chemagen, Baesweiler, Germany) according to the manufacturer's instructions. Amplification, cycle sequencing, and purification were performed by the Nijmegen group, who analyzed the reactions on the 3730xl Genetic Analyzer. The Nijmegen group also forwarded a portion of the purified reactions to the Applied Biosystems scientists for analysis on the 3500xL Genetic Analyzer.

Amplification Reactions and Conditions

Making use of the Human Genome build NCBI36 [*BRCA1*: Acc. nr: cDNA: NM_007294.3, *BRCA2*: Acc. nr: NM_000059.3], M13 tailed PCR primers were designed and optimized for 100% coverage of the coding sequences of the *BRCA1* and *BRCA2* genes, including sections of approximately 50 bp up- and downstream of each exon. All amplicons were produced and sequenced using a universal set of PCR conditions.

BRCA1 comprises 23 exons. In order to scan the exons for mutations, the gene was divided into 34 amplicons using specific primer pairs. *BRCA2* comprises 27 exons, which were divided into 47 amplicons using specific primer pairs. To enable a single sequencing approach, all forward primers include the M13 forward primer sequence and all reverse primers include the M13 reverse primer sequence. The amplifications were performed using 100 ng of the extracted DNA with AmpliTaq Gold® 360 Master Mix on a Veriti® 96-well, 0.2 mL Thermal Cycler. AmpliTaq Gold® 360 delivered good overall amplification, even in difficult-to-sequence regions such as exon 1 of the *BRCA1* gene. Using AmpliTaq Gold® 360 and producing target amplicons that varied in size from

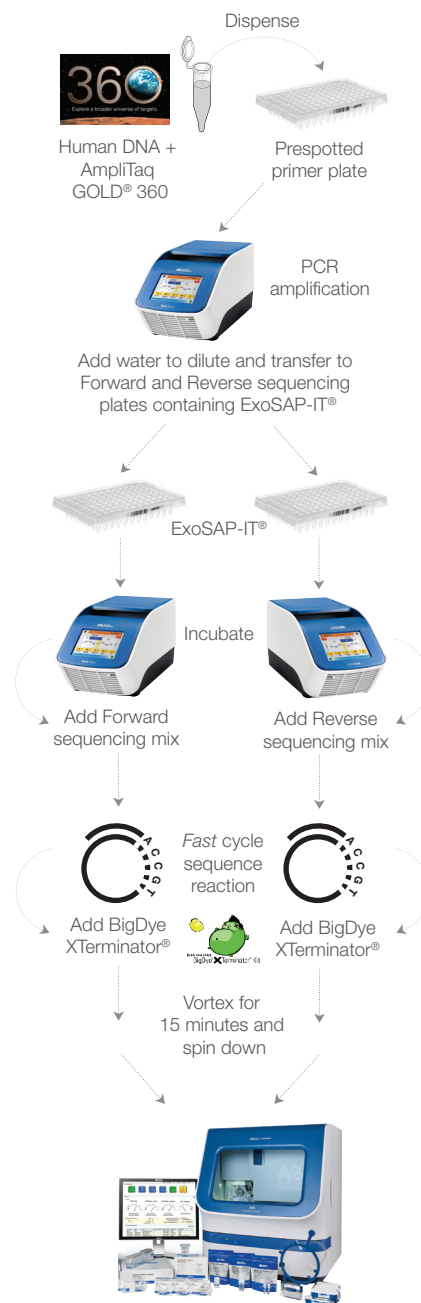


Figure 1. Direct Sequencing Workflow for *BRCA1* and *BRCA2* Analysis in Plate Format.

300 bp to 530 bp ensured that good coverage of the *BRCA1* and *BRCA2* genes was achieved (Figure 2). For more information on the protocol and primers, please contact the researchers directly at: dna@umcn.nl.

To facilitate a single DNA screening approach, primer pairs for both genes are prespotted and dried down in the wells of 96-well PCR plates (Figure 3). Multiplex nontemplate controls for every amplicon are included in the plate (Figure 3) to ensure that any amplification observed is specific. The collaborators optimized the plate workflow using the protocol presented in Figure 3, using the same amplification reactions and cycling conditions.

Results

The Department of Human Genetics at the Radboud University Nijmegen Medical Centre, The Netherlands, developed the optimized sequencing protocol presented in this publication. All 51 amplicons that comprise the *BRCA1* and *BRCA2* genes were amplified with AmpliTaq Gold® 360 and sequenced using BigDye® Terminator v1.1 chemistry. The protocol has been subsequently verified on 20 human DNA samples. Following sequence analysis by both teams, the mutations observed using the Applied Biosystems® 3730xl Genetic Analyzer were found to be identical to the results obtained by Applied Biosystems scientists running the same samples on the 3500xL Genetic Analyzer.

Variant Reporter® software v1.1 provides easy review of mutations, with clear comparison to the reference sequence and the resulting variants, which reduces the time required for data review. Quality value bars provide data quality information at a glance (blue-colored bars indicate good-quality data; yellow- and red-colored bars prompt the reviewer to manually check the data). Figures 4 and 5 show that mutations from exon 11 of the *BRCA1* and exon 23 of the *BRCA2* genes are easily identified by the user in the project view of Variant Reporter® software.

Conclusion

Using the approach described in this publication, mutation scanning of multiple exon genes by direct sequencing becomes very feasible. At the laboratory of Human

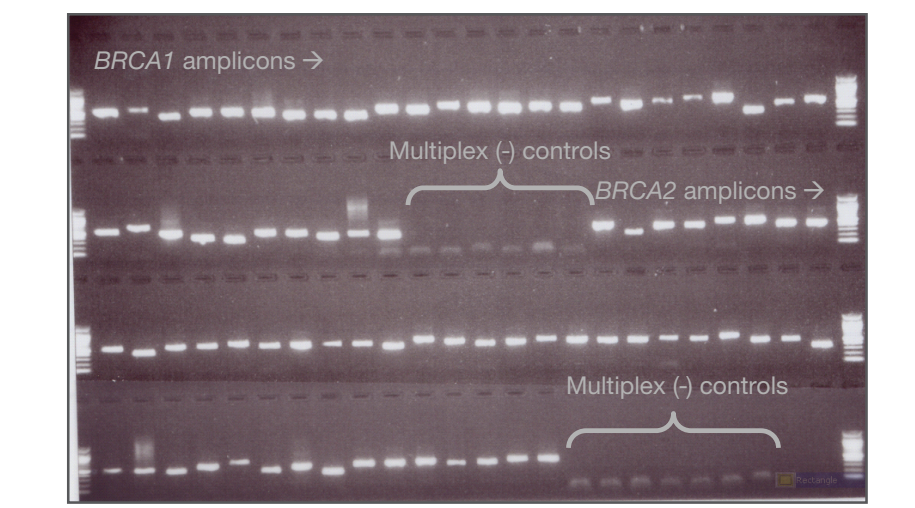


Figure 2. Agarose Gel Showing Amplification of the Amplicons for *BRCA1* and *BRCA2* Analysis.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Ex-1	Ex-10	Ex-11-8	Ex-15	Ex-23	Ex-1	Ex-10-1	Ex-11-5	Ex-11-13	Ex-14-2	Ex-22	MP-2
B	Ex-2	Ex-11-1	Ex-11-9	Ex-16	Ex-24	Ex-2	Ex-10-2	Ex-11-6	Ex-11-14	Ex-15	Ex-23	MP-3
C	Ex-3	Ex-11-2	Ex-11-10	Ex-17	MP-1	Ex-3	Ex-10-3	Ex-11-7	Ex-11-15	Ex-16	Ex-24	MP-4
D	Ex-5	Ex-11-3	Ex-11-11	Ex-18	MP-2	Ex-5	Ex-10-4	Ex-11-8	Ex-11-16	Ex-17	Ex-25	MP-5
E	Ex-6	Ex-11-4	Ex-11-12	Ex-19	MP-3	Ex-6	Ex-11-1	Ex-11-9	Ex-11-17	Ex-18	Ex-26	MP-6
F	Ex-7	Ex-11-5	Ex-12	Ex-20	MP-4	Ex-7	Ex-11-2	Ex-11-10	Ex-12	Ex-19	Ex-27-1	MP-7
G	Ex-8	Ex-11-6	Ex-13	Ex-21	MP-5	Ex-8	Ex-11-3	Ex-11-11	Ex-13	Ex-20	Ex-27-2	MP-8
H	Ex-9	Ex-11-7	Ex-14	Ex-22	MP-6	Ex-9	Ex-11-4	Ex-11-12	Ex-14-1	Ex-21	MP-1	MP-9

BRCA1 BRCA2 Multiplex nontemplate control

Figure 3. Positions of the Amplicon-Specific Primers and Controls in the Sequencing Plate.

Genetics in Nijmegen, skipping the prescreening step accelerated analysis, reduced labor time, and simplified laboratory setup.

The AmpliTaq Gold® 360 Master Mix gave reproducible and robust results. Following this protocol optimization, AmpliTaq Gold® 360 is now widely used in the routine workflows of the Nijmegen laboratory.

Below is a summary of the advantages of this direct sequencing workflow:

- Obtaining sequence data on all exons makes it possible not only to confirm known SNPs, but also to identify other possible variations
- With Sanger sequencing, amplicon length

can be designed longer than 500 bp to obtain additional sequence context information, especially in regions around exon-intron boundaries

- The instrument platform is flexible, allowing complementary studies on the same instrument (e.g., STR analysis)
- When amplicons are generated using tailed primers, a single protocol can be implemented in the lab for automated sequencing
- Samples can be processed as soon as they come into the lab, without the need for batching; this significantly increases the speed of analysis
- The primer design enables a single protocol to be used for both the PCR and sequencing steps, facilitating automated sequencing

Step-by-Step Protocol:

1. Amplification Reactions and Cycling Conditions for Each Plate

Prepare for each sample:

- Sample amplification mix:
 - 1.05 mL AmpliTaq Gold® 360 Master Mix
 - 966 µL H₂O (ultrapure)
 - 84 µL human genomic DNA (100 ng/µL)
- Nontemplate control mix:
 - 200 µL AmpliTaq Gold® 360 Master Mix
 - 200 µL H₂O (ultrapure)
- Use prespotted plate with *BRCA1* and *BRCA2* primers (see Figure 3)
- Add 25.0 µL of the sample amplification mix
- Add 25.0 µL of the nontemplate control mix

Amplification cycling protocol

Initial denaturation	95°C for 10 minutes	
Denaturation	95°C for 30 seconds	30 cycles
Annealing	60°C for 30 seconds	
Extension	72°C for 1 minute	
Final extension	72°C for 7 minutes	
Hold temperature	10°C	

2. Purification of PCR Products

Add 75 µL of H₂O to every well in the PCR plate (to make a 1:4 dilution).

- Prepare ExoSAP-IT® Mix
 - 200 µL ExoSAP-IT® Mix
 - 600 µL H₂O

Following the plate layout in Figure 3, prepare the forward and reverse sequencing plates. Transfer 1 µL of the diluted PCR product to the sequencing plate. Add 4 µL ExoSAP-IT® mix to every well.

- Process the plates on the Veriti® thermal cycler following this protocol:
 - 15 minutes at 37°C
 - 15 minutes at 80°C
 - Hold at 4°C

3. Cycle Sequencing

Prepare forward and reverse sequencing mixes:

- Forward mix:
 - 200 µL BigDye® Terminator v1.1
 - 100 µL 5X Sequencing buffer
 - 200 µL M13 forward primer (8 pmol/µL)

Add 5 µL sequencing mix to every well of the M13 forward primer PCR plate. The final volume in each well is 10 µL.

- Reverse mix:
 - 200 µL BigDye® Terminator v1.1
 - 100 µL 5X Sequencing buffer
 - 200 µL M13 reverse primer (8 pmol/µL)

Add 5 µL sequencing mix to every well of the M13 reverse primer PCR plate. The final volume in each well is 10 µL.

Perform cycle sequencing on a Veriti® thermal cycler.

Initial denaturation	96°C for 1 minutes	
Denaturation	96°C for 10 seconds	25 cycles
Annealing	50°C for 5 seconds	
Extension	60°C for 2 minutes	
Hold temperature	10°C	

4. Purification of the Sequencing Reactions

- Prepare BigDye XTerminator® mix:
 - 9 mL SAM solution
 - 2 mL bead mix

Add 55 µL of the BigDye XTerminator® mix to every well of the two sequencing plates.

Vortex for 15 minutes at 1,800 rpm. Centrifuge the plates for 2 minutes at 1,000 RCF.

5. Electrophoresis and Analysis

Purified sequencing reactions were analyzed at the two collaborating sites: at Radboud University using the Applied Biosystems® 3730xl Genetic Analyzer, and at Applied Biosystems using the 3500xL Genetic Analyzer (see *The 3500 Series Genetic Analyzers* sidebar) using the dedicated run module developed for use with the BigDye XTerminator® Purification Kit (BDX_FastSeq_50_POP7 run module).

The sequence results for each sample were analyzed independently. At the Radboud University Nijmegen Medical Centre, Sequence Pilot® software was used for data analysis. At the Applied Biosystems laboratory, Variant Reporter® Software was used.

- This automated workflow significantly reduces hands-on time and increases the speed with which results are obtained

The 3500 Series Genetic Analyzers offer the following features, which are beneficial to basic research labs and also to teams working in process-controlled environments:

- Easy instrument setup
- Ready-to-use consumables that are easy to load and run
- Simplified user interface with easy display of consumable and array usage information, quick-start functionality, and system maintenance reminders
- Performance-check functionality that simplifies how researchers verify instrument parameters as required by their operating procedures
- BigDye XTerminator®-optimized modules
- Faster run times compared to previous generations of Sanger sequencing platforms
- Security, audit trail, and electronic signature features to help process-controlled labs comply with 21 CFR Part 11 requirements
- The new instrument software reporting features make it easy for researchers to quality-control their procedures

Acknowledgment

We acknowledge Joop Theelen, Applied Biosystems Senior Field Application Specialist, Molecular Biology, for his generous contribution to this collaboration.

References

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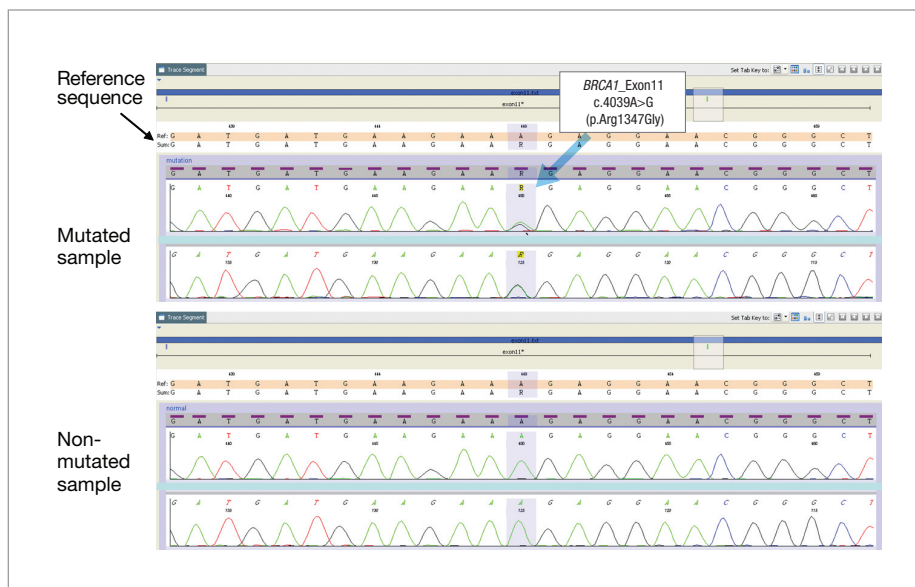


Figure 4. *BRCA1* Mutation Analysis Using Variant Reporter® Software. Through automated sequence analysis, superior basecalling, and sophisticated data visualization tools, Variant Reporter® Software produces definitive mutation analysis, as exemplified in this research study of *BRCA1*_Exon11_c.4039A>G (p.Arg1347Gly).

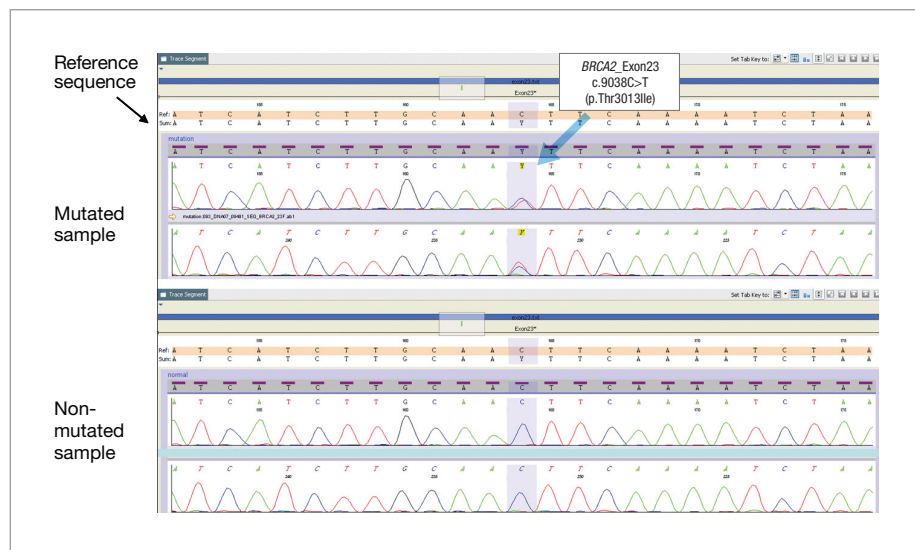


Figure 5. *BRCA2* Mutation Analysis Using Variant Reporter® Software. Through automated sequence analysis, superior basecalling, and sophisticated data visualization tools, Variant Reporter® Software v1.0 produces definitive mutation analysis, as exemplified in this research study of *BRCA2*_Exon23_c.9038C>T (p.Thr3013Ile).



The 3500 Series Genetic Analyzers

The 3500 Series Genetic Analyzers are automated fluorescence-based capillary electrophoresis systems. Samples are sequenced in less than 40 minutes on the 24-capillary 3500xL system or on the 8-capillary 3500 system, using 3500 POP-7™ polymer and the 50 cm capillary array. For convenience, the 3500 Series instruments also include a sequencing module that incorporates the BigDye X Terminator® Purification Kit workflow. After the sequence data are collected, the KB™ Basecaller software embedded in the 3500 Data Collection Software automatically processes the sequenced samples and provides read

length greater than 850 bp, with average base quality values greater than 20 (QV20). Furthermore, the 3500xL system, using the RapidSeq50_POP7 run module, can efficiently sequence up to 840 samples in a 23-hour period, generating high-quality, high-resolution data with minimal hands-on time.

Fully automated from the moment you place an 8-tube strip, 96-well, or 384-well sample plate on the instrument and start the run, the instrument provides continuous, unattended operation for every phase of the process, including polymer loading, sample injection, separation, detection, and primary data analysis. The 3500 Series Systems feature

simplified, easy-to-install consumables. The Anode and Cathode Buffer Containers are supplied with ready-to-use 1X Genetic Analysis Buffer formulations.

Easy-to-use wizards for instrument operation and maintenance ensure predictable, hassle-free performance. The majority of applications can be analyzed on a single configuration of POP-7™ polymer with a 50 cm capillary array. Integrated primary analysis and QC software includes improved data collection software, with an intuitive workflow from plate setup to primary analysis that performs base calls and applies quality control flags to alert the user to failed or low-quality samples.

ORDERING INFORMATION

Description

Genetic Analyzers

3500 and 3500xL Series Systems Packages

Learn more at www.appliedbiosystems.com/3500Series or contact your local Sales Representative for more information.

Description	Quantity	P/N
PCR Amplification		
AmpliTaq Gold® 360 PCR Master Mix	1 mL	4398876
Sequence Detection Primer (medium scale)	1 tube	4304971
Sequencing Reactions and Purification		
BigDye® Terminator v1.1 Cycle Sequencing Kit	100 rxn	4337450
BigDye XTerminator® Purification Kit	20 mL	4376487

Description

System Consumables and Reagents	Quantity	P/N
3500xL Capillary Array (50 cm)		4404689
3500 Capillary Array (50 cm)		4404685
3500 POP-7™ Polymer	960 samples	4393714
3500 POP-7™ Polymer	384 samples	4393708
Anode Buffer Container (ABC) 3500 Series		4393927
Cathode Buffer Container (CBC) 3500 Series		4408256
Septa Cathode Buffer Container 3500 Series		4410715
Conditioning Reagent 3500 Series		4393718
Hi-Di™ Formamide	1 bottle (5 mL)	4401457
BigDye XTerminator® v1.1 Cycle Sequencing Kit	100 rxns	4337450

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For those who require IVD-marked devices, the 3500 Dx and 3500xL Dx Genetic Analyzers and system accessories meet the requirements of the In Vitro Diagnostic Medical Devices Directive (98/79/EC). The 3500 Dx and 3500xL Dx systems are for distribution and use in specific European countries only. For more information about the 3500 Dx Series Systems, contact your Applied Biosystems representative.

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