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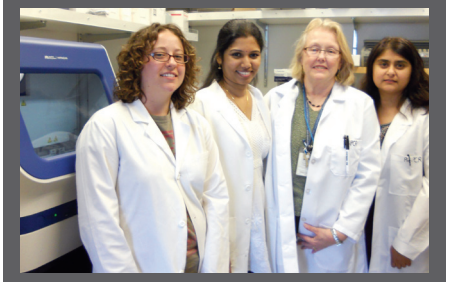
BigDye® Direct Cycle Sequencing Kit simplifies workflow for characterizing melanoma mutations in research samples



From left to right: Rebecca Terrill, BS, MB (ASCP)CM, Swapna Vemula, MS, MB(ASCP)CM, Susan Charzan, BS, CG(ASCP)CM, and Sonia Mirza, MBBS of the Dermatopathology Specialty Lab at UCSF standing alongside the Applied Biosystems® 3500 Genetic Analyzer.

Clinical scientists from the Dermatopathology Specialty Lab at the University of California, San Francisco (UCSF) have developed a sequencing-based melanoma mutation detection workflow that simplifies and increases throughput, while improving the accuracy of detection. This article describes how research incorporating the Applied Biosystems® BigDye® Direct Cycle Sequencing Kit together with the Applied Biosystems® 3500 Genetic Analyzer from Life Technologies has improved this capillary electrophoresis Sanger sequencing workflow. Through use of this workflow, the laboratory has been able to simultaneously genotype single nucleotide polymorphisms (SNPs) and find new mutations in the analysis of metastatic melanoma DNA research samples.

Featured products

- Applied Biosystems® BigDye® Direct Cycle Sequencing Kit
- Applied Biosystems® BigDye XTerminator® Purification Kit
- Applied Biosystems® 2720 Thermal Cycler
- Applied Biosystems® 3500 Genetic Analyzer

Molecular underpinnings of melanoma

Melanoma is an aggressive skin cancer with increasing incidence worldwide [1]; it is a malignant tumor arising from pigment-producing melanocytes in the skin, eye, or mucosal surfaces. Although environmental and genetic factors have been shown to play a role in the disease, the exact mechanism underlying the development of melanoma is still largely unknown.

Based on histopathological and molecular studies, melanoma has been subclassified into various categories. Melanomas arising from non-chronically sun damaged (non-CSD) skin show frequent mutations in the *BRAF* gene and no mutations in the *KIT* gene. In contrast, 20% of melanomas in chronically sun damaged (CSD) skin have mutations in the *KIT* gene and infrequently harbor mutations in the *BRAF* gene [2]. In two other categories, acral and mucosal melanomas, 10 to 40% of cancers have been shown to have mutations in the *KIT* gene or amplifications of this genomic region [3].

Genetic analysis classifies melanoma into subcategories

The Dermatopathology Specialty Lab at UCSF focuses on the molecular mechanisms of melanoma. This lab uses molecular evidence to help classify melanoma into various subcategories, providing invaluable information that the scientists and their colleagues can use in the analysis of genetic variants of the disease.

The lab uses capillary electrophoresis Sanger sequencing for genetic analysis to help researchers identify mutations. The sequencing results identify variations in the samples that help to categorize them, resulting in further targeted investigation.

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A simple, accurate, and fast workflow for melanoma mutation detection

A simple, fast workflow and good resolution of data are crucial for a laboratory to guarantee accurate and timely results. Researchers at the Dermatopathology Specialty Lab at UCSF have found that incorporating the BigDye® Direct Cycle Sequencing Kit into a melanoma mutation detection workflow speeds and simplifies the workflow, while improving the accuracy of detection.

In a disease research lab, accuracy is paramount. According to the scientists in the Dermatopathology Specialty Lab, compared to BigDye® Terminator reagents, the use of the BigDye® Direct Cycle Sequencing Kit has improved the resolution at the 5´ end (Figure 1) and eliminated the superimposed base positions (Figure 2). By sequencing across the amplicon, as opposed to interrogating just one SNP, the researchers were able to simultaneously genotype SNPs and look for new mutations as well.

Moreover, the rapid turn-around time afforded by the BigDye® Direct workflow enables researchers to provide colleagues with accurate data and information regarding the metastatic

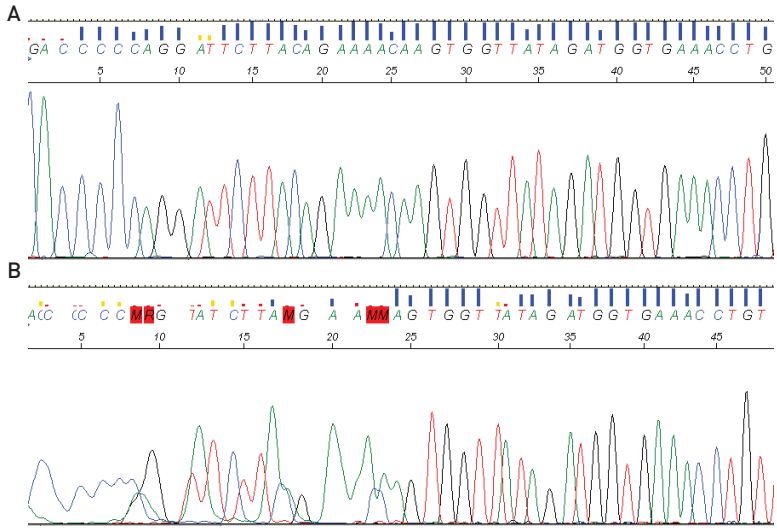

Figure 1. Comparison of 5' resolution of (A) BigDye® Direct Cycle Sequencing using POP-7™ Polymer and (B) BigDye® Terminator version 1.1 using POP-7™ Polymer.

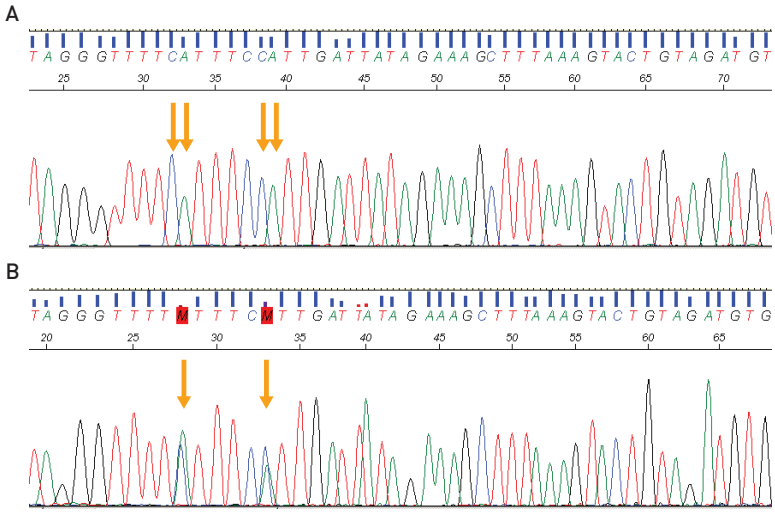

Figure 2. (A) An example of an accurate peak separation with BigDye® Direct Cycle Sequencing using POP-7™ Polymer, and (B) an example of a superimposition of two peaks using BigDye® Terminator version 1.1 with POP-7™ Polymer.

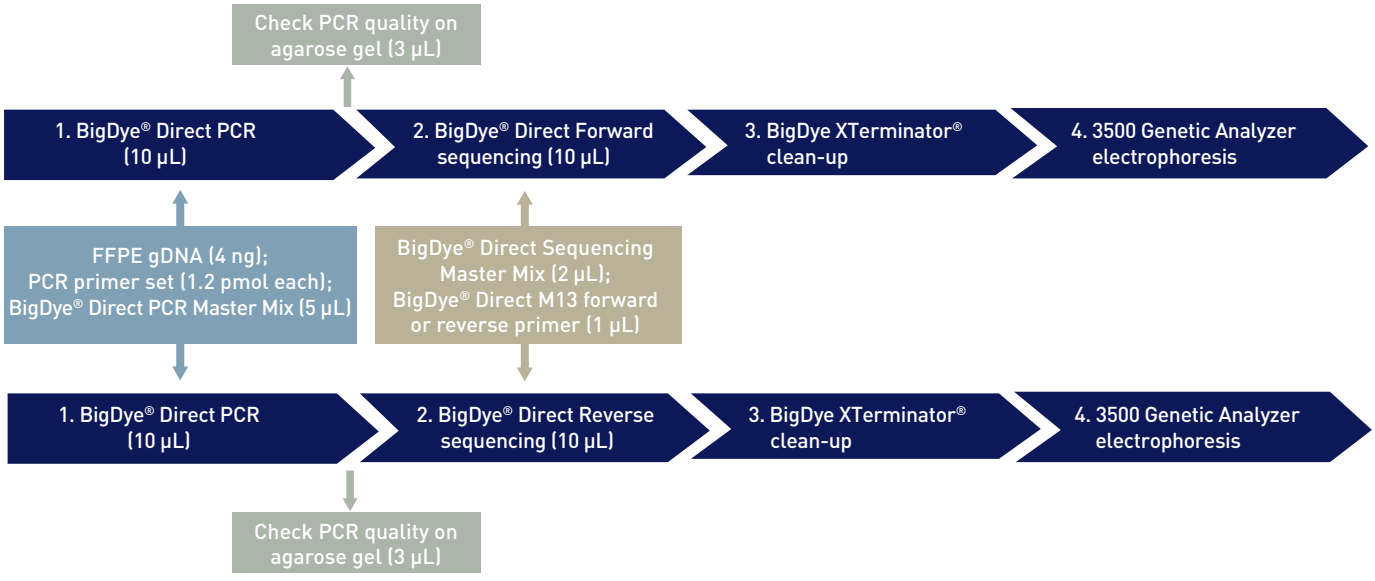

Figure 3. The BigDye® Direct PCR and sequencing workflow. The workflow consists of PCR amplification followed by sequencing, clean-up with BigDye XTerminator® reagent, capillary electrophoresis, and analysis. This workflow differs from the standard BigDye® Direct workflow in that 3 µL of PCR amplification mixture is analyzed for quality purposes on an agarose gel.

melanoma DNA research samples within a short time. In particular, the elimination of the separate PCR clean-up step and the ability to use the same plate without having to transfer samples between steps—as is required in standard sequencing workflows—reduces hands-on time and consumable costs, and decreases the number of pipetting steps, hence reducing the possibility of pipetting errors.

The BigDye® Direct PCR and sequencing workflow

Figure 3 shows the four steps in the BigDye® Direct workflow:

1. PCR amplification
2. DNA sequencing
3. Post-sequencing clean-up
4. Capillary electrophoresis and analysis

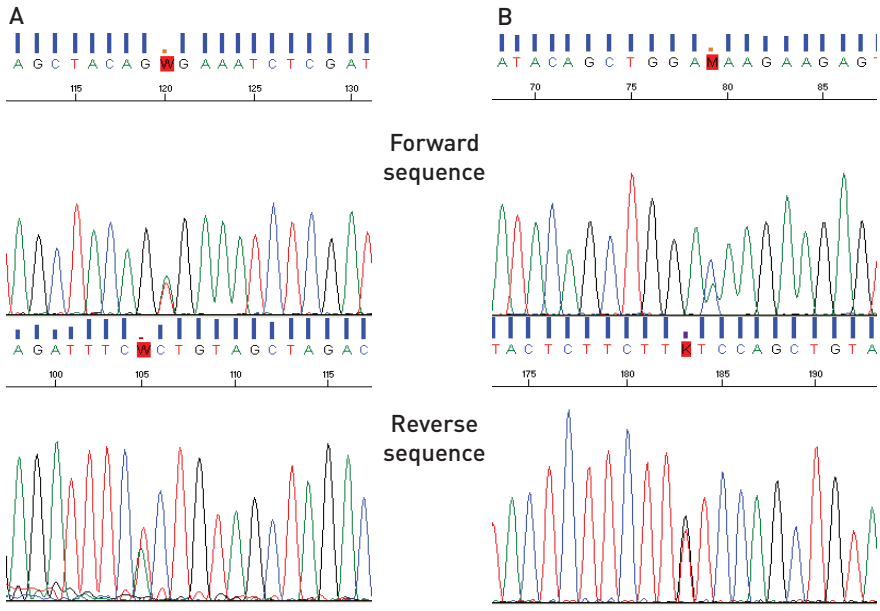

Figure 4. Forward and reverse sequences from two samples using the BigDye® Direct workflow. (**A**) Sample showing a mutation in the *BRAF* gene. (**B**) Sample showing mutation in the *NRAS* gene.

PCR amplification

DNA was obtained through microdissection from formalin-fixed paraffin-embedded (FFPE) tissue. The microdissected tumor tissue was digested overnight in buffer containing Tris EDTA, 0.5% Tween 20, and proteinase K.

Amplification primers were designed to contain M13F or M13R sequences at the 5´ end of the gene-specific PCR primers. Each 10 µL PCR amplification reaction contained 1 µL gDNA (DNA concentration of 4 ng/µL), 1.2 pmol of each primer, and 5 µL BigDye® Direct PCR Master Mix. For each amplicon, two separate PCRs were set up—one for the forward sequencing reaction and one for the reverse sequencing reaction.

The reactions were amplified using the Applied Biosystems® 2720 Thermal Cycler. Cycling conditions were:

- 95°C for 10 min
- 96°C for 3 sec
- 62°C for 15 sec
- 35 cycles of amplification at 68°C for 30 sec
- Post extension: 72°C for 2 min

After amplification, 3 µL of the PCR product was transferred to an agarose gel to check quality, or was stored for backup. The researchers made this single modification to the standard workflow to comply with the reagent validated workflow requirements for the tests the UCSF Dermatopathology lab had developed.

DNA sequencing

The BigDye® Direct workflow eliminates the additional PCR product purification step typically used in traditional sequencing. Instead, this step is incorporated into the cycle sequencing reaction as a single reaction.

BigDye® Direct sequencing reactions were performed using BigDye® Direct specific M13 Fwd or M13 Rev sequencing primers provided with the kit. Each sequencing reaction contained:

- 7 µL of PCR product
- 2 µL of BigDye® Direct Sequencing Master Mix
- 1 µL of BigDye® Direct M13 Fwd or M13 Rev sequencing primer

After an initial incubation at 37°C for 15 min and 80°C for 2 min, cycling conditions were:

- 96°C for 1 min
- 25 cycles of sequencing at:
  - 96°C for 10 sec
  - 50°C for 5 sec
  - 60°C for 75 sec

Post-sequencing clean-up

At completion of the sequencing reaction, the sequencing products were purified using the BigDye XTerminator® Purification Kit. Specifically, the BigDye® Direct sequenced products were purified by addition of 45 µL of SAM Solution and 10 µL of BigDye XTerminator® Solution. The 96-well plate was placed on IKAMK3 basic at 2,000 rpm for 20 min, followed by spin at 1,000 x *g* for 2 min.

Capillary electrophoresis and analysis

Electrophoresis was performed on the Applied Biosystems® 3500 Genetic Analyzer with POP-7™ Polymer and 3500 Genetic Analyzer Capillary Array, 50 cm using BDxShortReadSeq50_POP7 run module and KB_3500_POP7_BDTv3direct .mob. New mobility files and basecallers were installed to enhance the sequencing trace resolution and base-calling accuracy for the BigDye® Direct Cycle Sequencing Kit (free download at www.appliedbiosystems.com/bigdyedirectinstaller).

The sequencing results for each sample were analyzed by Mutation Surveyor® (SoftGenetics) or Sequencher® (Gene Codes) software to verify the sequencing results and to identify the putative mutations for each research sample (Figure 4).

Use of FFPE DNA and validation advantages

According to the researchers, this protocol provides excellent sequence quality with FFPE samples that are known to have fragmented DNA. The use of separate components, each with different lot numbers, requires revalidation of the complete workflow each time one of the components is changed due to a new lot number. By providing multiple reagents in a single kit, the BigDye® Direct Cycle Sequencing Kit reduces the time required to validate a new kit lot number. One box with one lot number requires less time to validate compared to validating several individual reagents, each of which requires revalidation every time a lot number is changed.

Conclusion

The Dermatopathology Specialty Lab at UCSF has found that the BigDye® Direct Cycle Sequencing Kit provides a streamlined workflow by eliminating the PCR clean-up step, and improves resolution of sequencing data at the 5´ end. Moreover, the BigDye® Direct PCR and sequencing workflow requires use of only one plate, without having to transfer between steps. This reduces hands-on time and improves accuracy by reducing the possibility of pipetting errors.

Acknowledgements

Special thanks to Peter Ma, Life Technologies Senior Staff Scientist, Molecular Biology, and Stephanie Schneider, Life Technologies Scientist, Molecular Biology, for their generous contributions in this collaboration.

Ordering information

Product	Quantity	Part No.
Applied Biosystems® BigDye® Direct Cycle Sequencing Kit	24 reactions	4458689
	100 reactions	4458687
	1,000 reactions	4458688
Applied Biosystems® BigDye XTerminator® Purification Kit	2 mL (~100 20 µL reactions)	4376486
	20 mL (~1,000 20 µL reactions)	4376487
	50 mL (~2,500 20 µL reactions)	4376484
	800 mL	4376485
Applied Biosystems® 2720 Thermal Cycler		4359659
Applied Biosystems® 3500 Genetic Analyzer for Sequence Typing and Fragment Analysis		4440470

References

1. Rigel DS (2010) Trends in dermatology: melanoma incidence. *Arch Dermatol* 146(3):318.
2. Curtin JA, Fridlyand J, Kageshita T et al. (2005) Distinct sets of genetic alterations in melanoma. *N Engl J Med* 353(20):2135–2147.
3. Sigrid M, Broekaert C, Ritu R et al. Genetic and morphologic features for melanoma classification. *Pigment Cell Melanoma Res* 23:763–770.

To learn more about the Applied Biosystems® BigDye® Direct Cycle Sequencing Kit, go to www.appliedbiosystems.com/bigdyedirect, or contact your sales representative for more information.

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