APPLICATION INFORMATION

CEQ Series

MULTIPLEX MUTATION GENOTYPING FOR HUMAN DISEASES: BREAST CANCER AND FAMILIAL MEDITERRANEAN FEVER

Zhiming Jiang[1], Milhan Telatar[2], Wayne Grody[2]
[1]Beckman Coulter, Inc.
[2]University of California, Los Angeles

Introduction

Understanding human genetic variation is currently believed to reveal the cause of individual susceptibility to disease and the large variation observed in response to treatment. Single nucleotide polymorphisms (SNPs) are the subject of many novel technology development activities because of their high genome density and association with human diseases. The ideal SNP genotyping assay will be very accurate, inexpensive, easy to perform, and capable of high throughput.

Because all SNPs can be analyzed with high specificity at the same reaction conditions, primer extension is a promising reaction principle for multiplex high-throughput genotyping assays. In the primer extension reaction, a DNA polymerase is used specifically to extend a primer that anneals immediately adjacent to the polymorphic position with a single labeled dye terminator complementary to the nucleotide at the variant site. The reaction allows highly specific multiplexing detection of point mutations and SNPs.

The CEQ™ 8000 Genetic Analysis System, CEQ DNA Size Standard 80, and CEQ SNP-Primer Extension Kit were developed to provide users an accurate, inexpensive, simple, and robust solution for multiplex SNP scoring and validation based on primer extension technology[1]. Our previous study[1] reported that single-base primer extension and the CEQ 8000 Genetic Analysis System provided a novel way to screen factor V mutation in Venous Thrombosis disease population with high degrees of throughput, automation, and accuracy. Here we demonstrate the multiplexing analysis of two additional sets of human disease mutations, Ashkenazi Jewish BRCA1/2 mutations and MEFV mutations (Familial Mediterranean Fever), using the CEQ 8000 Genetic Analysis System. The assays exhibit high accuracy and improved throughput through automation.

Materials and Methods

Template Amplification

Templates were amplified from human genomic DNA by polymerase chain reaction (PCR*). PCR conditions, thermal cycling conditions, and primer sequences are shown in Table 1, Figure 1, and Figure 3. The unincorporated PCR primers and dNTP were cleaned up by SAP and *Exo*I digestion[1,2] (Table 1). Prior to primer extension, templates were quantitated carefully by quantitative electrophoresis or laser-induced fluorescence using the Agilent* 2100 Bioanalyzer (Cat. No. G2938A).

Primer Extension

Commercial software packages are available to design primers for multiplexing SNP analysis[3]. For successful SNP analysis, lengths of the adjacent primers for the same multiplexing reaction need to have a space of at least 5 nt. Regardless of the lengths, the Tms of all primers are within the range of 60° to 76°C—too great a difference in Tm would lead to large variations in the amount of extended products generated by different primers, therefore causing imbalanced signals in the multiplexing result. Adding a Poly (A) or Poly (T) tail to the 5´

end of primers longer than 25 nt is suggested to help minimize the Tm variations. All SNP primers targeting the BRCA1, BRCA2, or MEFV mutations are listed in Figure 1, Figure 3, and Table 2.

Primer extension was performed following the procedures presented previously[1,2]. Each primer was tested individually prior to multiplexing. As shown in Table 2, primer concentrations were adjusted to achieve the best signal balance in multiplexing reactions. The remaining dye terminators were hydrolyzed from the extended products by SAP digestion[1,2]. Extended products were then diluted in the CEQ™ sample loading solution (SLS) at a ratio of 1:10, added to CEQ size standard 80/SLS mixture[1,2] and finally loaded onto the CEQ 8000[1,2].

Data Analysis

Data were analyzed using the SNP analysis feature of the CEQ 8000 software. SNP genotypes were summarized and reported in fragment list through automated SNP locus tag assignment[1].

Results and Discussion

BRCA Mutations

The BRCA1 mutations (185delAG and 5382insC) and the BRCA2 mutation (6174delT) have been detected in a significant proportion of Ashkenazi Jewish women with early-onset breast cancer (Figure 1). In the Jewish Ashkenazi population, the overall frequency of these mutations is as high as 2.5%. Based on clinical and family history data in Ashkenazi individuals at risk, the results of BRCA molecular testing are appropriately anticipated in most cases. In order to screen the BCRA mutations using multiplexing primer extension, co-amplification of all three BRCA exons (two in BRCA1 gene and one in BRCA2 gene) was performed in a single PCR reaction (Table 1 and Figure 1). SNP primers that are 23 nt, 33nt, or 43 nt long, respectively, were designed to hybridize one base pair adjacent to the corresponding mutation sites (Table 2 and Figure 1). Primer extension was performed, and all three primers were able to detect the wild type or the mutant allele of each mutation locus with a high degree of accuracy. To achieve the best

Table 1. PCR Conditions for Template Amplification

Gene	PCR Reaction		Cycling Condition	Template Cleanup
BRCA1 and BRCA2 (co-amplification of three exons)	DNA	100 ng	94°C 1 min	To 30 µL PCR product:
	BRL buffer (10X)	3 µL		10 Unit SAP
	MgCL2 (50 mM)	3.9 µL	30 cycles:	5 Unit ExoI
	dNTP (10 mM)	0.3 µL	94°C 30 sec	37°C 1 hour
	Each primer (100 µM)	0.1 µL	58°C 30 sec	75°C 15 min
	Platinum Taq	0.4 µL	72°C 3 min	
	H2O	q.s.		
	Total Volume	30 µL		
MEFV (co-amplification of three exons)	DNA	100 ng	94°C 1 min	To 30 µL PCR product:
	BRL buffer (10X)	3 µL		10 Unit SAP
	MgCL2 (50 mM)	3 µL	30 cycles:	5 Unit ExoI
	Betaine (5 mM)	12.6 µL	94°C 30 sec	37°C 1 hour
	dNTP (10 mM)	0.4 µL	60°C 30 sec	75°C 15 min
	Each primer (100 µM)	0.1 µL	72°C 50 sec	
	Platinum Taq	0.4 µL		
	H2O	q.s.	72°C 3 min	
	Total Volume	30 µL		

Table 2. SNP Primers Designed for Each Disease Mutation Site

Disease	Gene	Primer Name	Primer Length (nt)	Mutation Identity	Amount Used for Each Multiplexing SNP Reaction	Tm (°C)	Apparent Size (nt)
Breast Cancer	BRCA1	P33 185delAG	33	185delAG	0.8 pmol	61.5	19
	BRCA1	P23 5382InsC	23	5382InsC	2.0 pmol	68.8	31
	BRCA2	P43 6174delT	43	6174delT	2.0 pmol	61.5	41
Familial Mediterranean Fever	MEFV	P23 M680I	23	M680I	1.0 pmol	67.5	20
	MEFV	P55 M694V	55	M694V	1.0 pmol	65.0	57
	MEFV	P37 M694I	37	M694I	0.5 pmol	66.7	37
	MEFV	P47 V726A	47	V726A	0.5 pmol	69.9	47
	MEFV	P28 E148Q	28	E148Q	1.0 pmol	76.0	24

Figure 1A

BRCA1: 185delAG mutation

PCR primer (Forward)

CTTCGCGTTG AAGAAGTACA AAATGTCATT AATGCTATGC

Deletion

AGAAAATCTT **AG** AGTGTCCC ATCTGGTAAG TCAGCACAAG

AGTGTATTAA TTTGGGATTC CTATGATTAT CTCCTATGCA

A: P33 185delAG

AATGAACAGA ATTGACCTT**ACATACTAGGG AAGAAAAGAC A**

PCR primer (Reverse)

Figure 1B

BRCA1: 5382InsC mutation

PCR primer (Forward)

TGGAAGAAACCACCAAGGTC CAAAGCGAGC AAGAGAAT

P23 5382InsC T:

Insertion C

CCC **X** AGGACAGAA AGGTAAAGCT CCCTCCCTCA AGTTGACAAA

AATCTCACCC C**ACCACTCTG TATTCCACTC CCC**

PCR primer (Reverse)

Figure 1c

BRCA2: 6174delT

PCR primer (Forward)

GGGAAGCTTC ATAAGTCAGT CTCATCTGCA AATACTTGTG

Deletion

GGATTTTTAG CACAGCAAG **T** GGAAAATCTG TCCAGGTATC

AGATGCTTCA TTACAAAACG CAAGACAAGT GTTTTCTGAA

T: P43 6174delT

A TA**GAAGATA GTACCAAGCA AGTC**

PCR primer (Reverse)

Figure 1. *Sequences of templates, PCR primers, and SNP primers for the BRCA mutations. (The PCR primers are bolded and underlined. Each SNP primer name contains the corresponding mutation identity and the length of the primer.) 185delAG locus (SNP primer in green) 5382insC locus (SNP primer in orange) 6174delT locus (SNP primer in purple).*

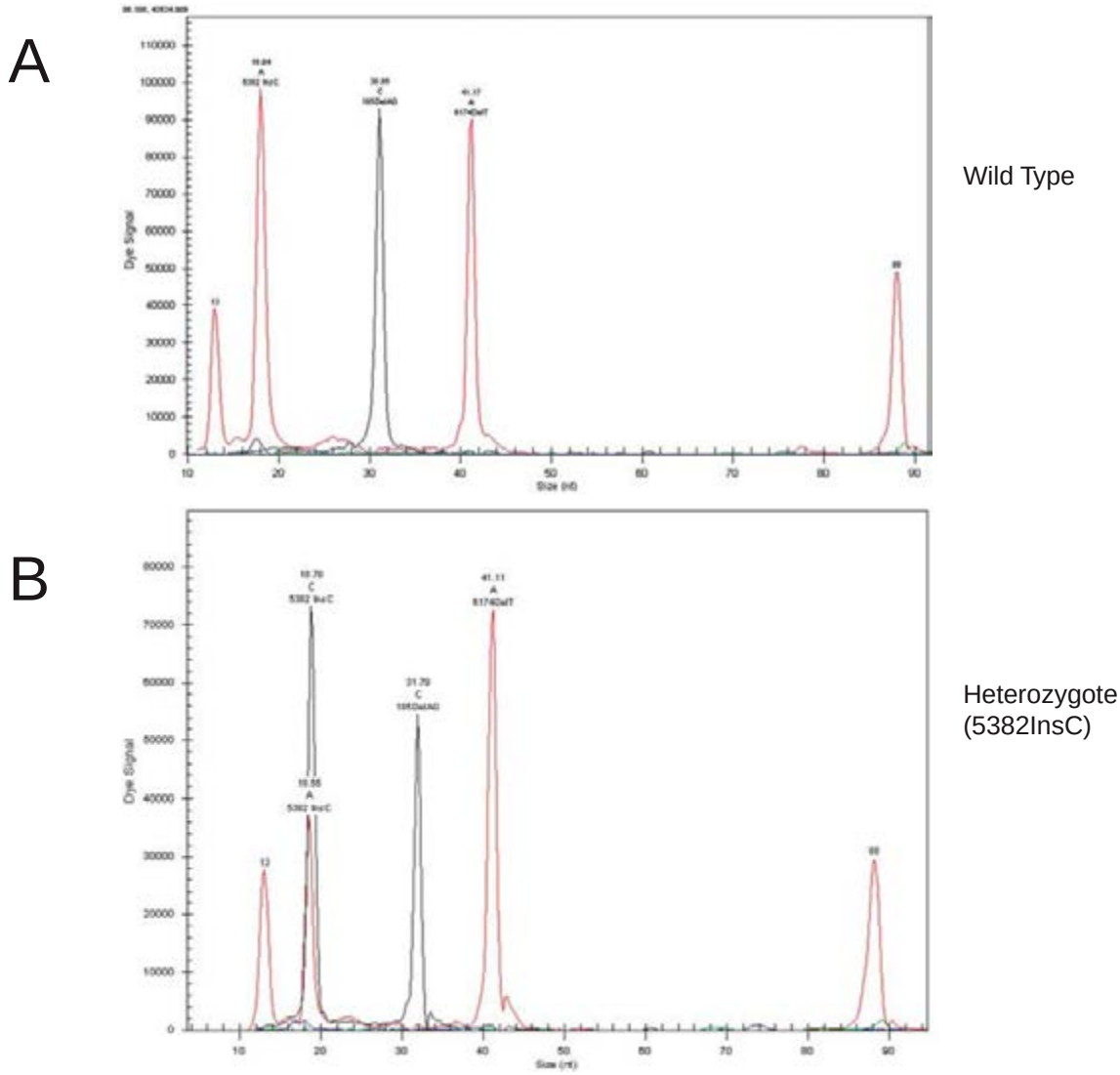

Figure 2. *Typical multiplexing genotyping results for BRCA mutations (the SNP locus tag was named after the mutation identity). Red peaks (13 and 88) are size standards.*

multiplexing results, primers were tested individually prior to multiplexing. Primer concentrations were adjusted to balance signal levels generated by the different primers in the same multiplexing result (Table 2). The extended products were cleaned up prior to separation on the CEQ™ 8000 Genetic Analysis System[1]. SNP data were analyzed using the fragment analysis module of the CEQ 8000 Genetic Analysis software[1]. Three locus tags were created according to the apparent sizes of SNP products (Table 2). For heterozygous loci, two allele IDs with the same locus name were labeled. Figure 2 exhibits typical examples of the analyzed BRCA data from a wild-type person and a person heterozygous for the 5382InsC locus. Overall, multiplexing primer extension and the CEQ 8000 system were able to detect BRCA mutations with a high degree of accuracy and minimal optimization in both chemistry and software.

MEFV Mutations

Familial Mediterranean Fever (FMF) is an autosomal recessive disorder characterized by recurring attacks of fever and serositis. Five sequence alterations (M694V, V726A, M680I, M694I, and E148Q) in the MEFV gene account for the majority of FMF cases. The MEFV gene, located on the short arm of chromosome 16, codes an anti-inflammatory protein, called marenostrine or pyrin. As shown in Figure 3, four out of the five mutations (M694V, V726A, M680I, and M694I) are located on exon10 of the MEFV gene, whereas the fifth mutation (E148Q) is on exon 2 of the MEFV gene. Since exon 2 contains a high percentage of GC nucleotides, betaine (a final concentration of 2.1 mM) was added to help co-amplification of both exon 10 and exon 2 templates in one PCR reaction (Table 1). PCR products were cleaned and

Figure 3A

MEFV Exon 10: M680I, M694V, M694I, and V726A

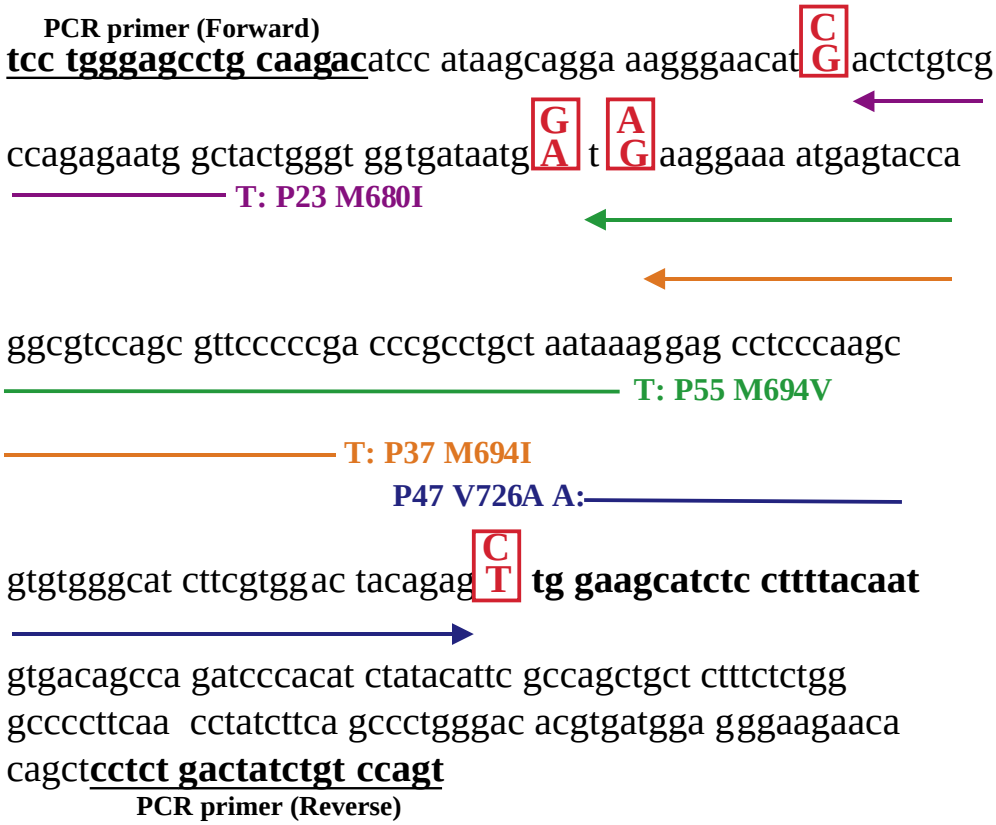

Figure 3B

MEFV Exon 2: E148Q

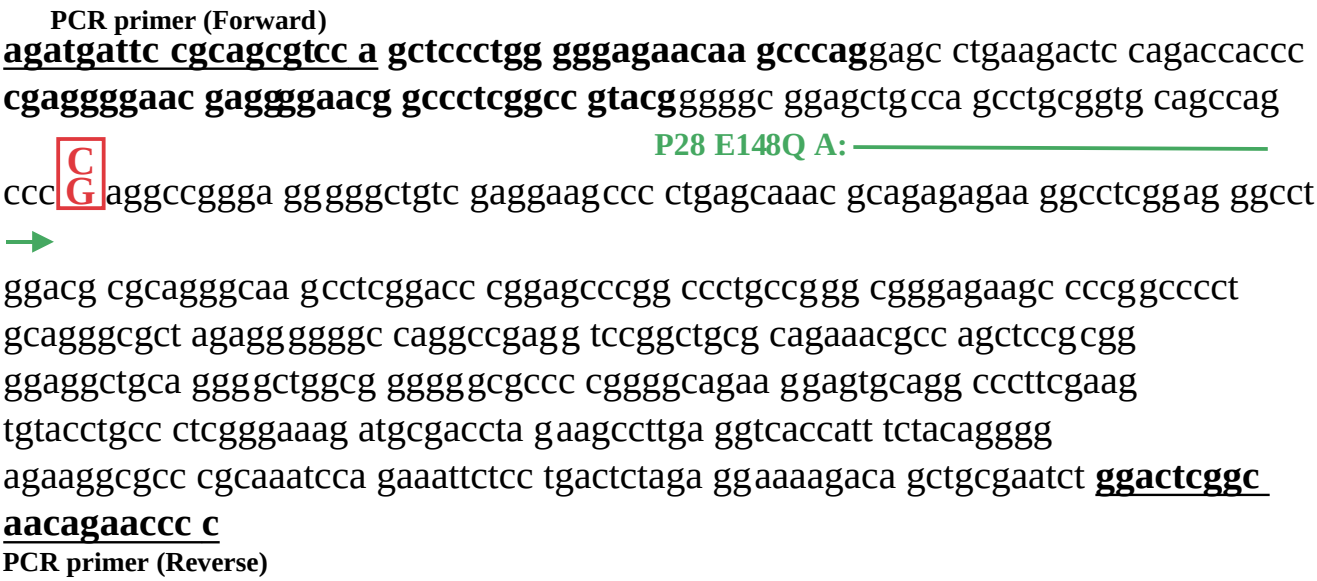

Figure 3. *Sequences of templates, PCR primers, and SNP primers for the MEFV mutations. (The PCR primers are bolded and underlined. Each SNP primer name contains the corresponding mutation identity and the length of the primer.) Four mutations in Exon10: M680I (primer in purple), M694V (primer in green), M694I (primer in orange), and V726A (primer in blue); One mutation in Exon2: E148Q (primer in green).*

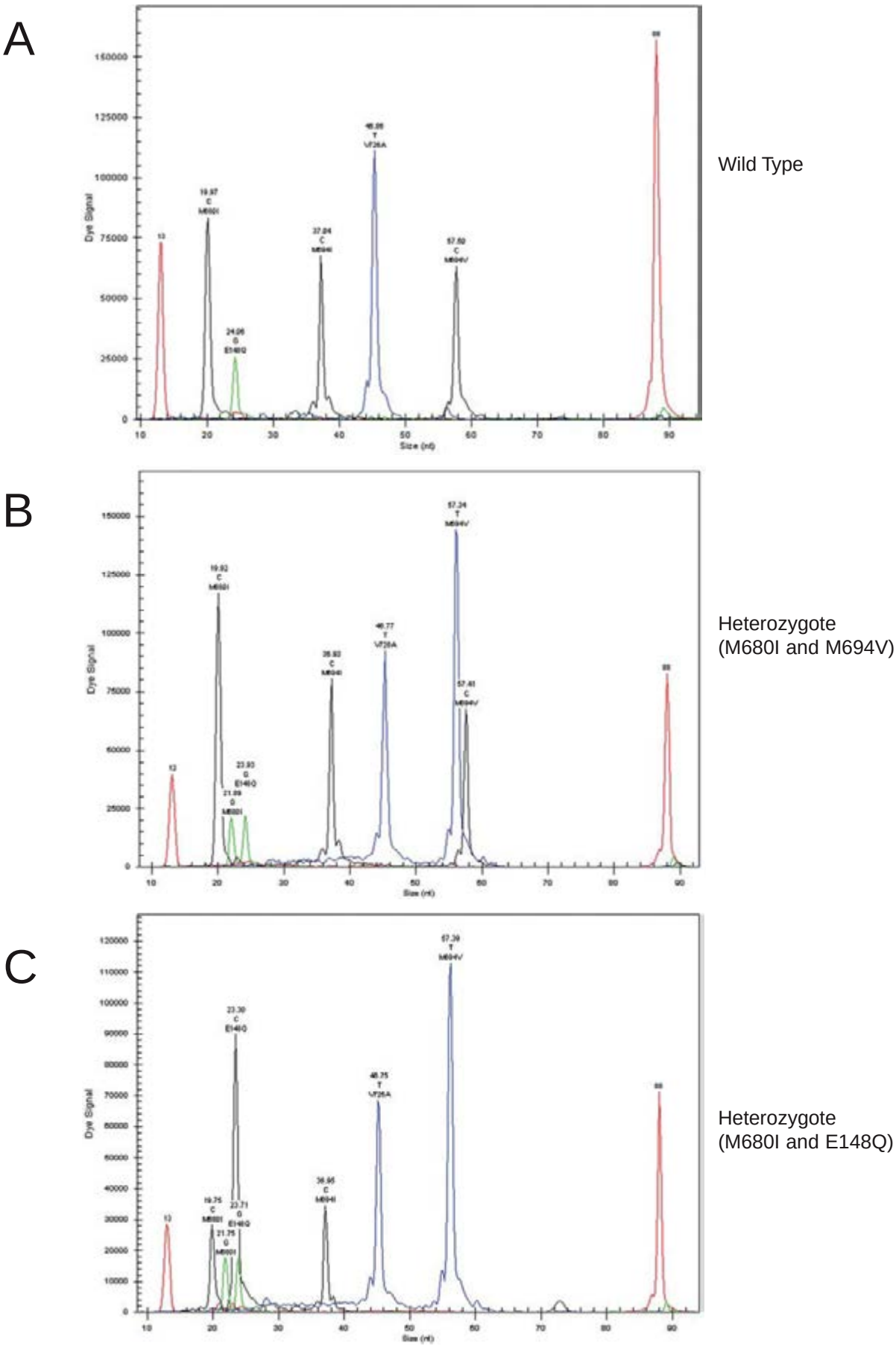

Figure 4. *Typical multiplexing genotyping results for BRCA mutations (the SNP locus tag was named after the mutation identity). Red peaks (13 and 88) are size standards.*

quantitated prior to primer extension as described in "Materials and Methods." Five SNP primers (23, 55, 37, 47, 28 nt long, respectively) were designed to score five MEFV mutations correspondingly (Table 2 and Figure 3). Within the exon10 template, two mutations (M694V and M694I) are physically only one base pair apart. Two overlapping primers (55 nt and 37 nt) were proven to be able to score these two mutations respectively (Table 2 and Figures 3 and 4). A relatively short primer (28 nt) was used to screen the E148Q mutation in the GC-rich template. As shown in Figure 4, multiplexing SNP analysis using primer extension and the CEQ™ 8000 Genetic Analysis System was capable of genotyping individuals that are wild type, heterozygous, or homozygous at all five FMF loci. Primer extension templates from each individual were co-amplified in one single PCR reaction. Five loci were screened by one multiplexing primer extension.

Conclusion

The CEQ 8000 Genetic Analysis System, CEQ DNA Size Standard 80, and CEQ SNP-Primer Extension Kit enable validation and scoring of multiple sets of disease mutations (BRCA and MEFV) with high degrees of accuracy, throughput, and automation.

The CEQ 8000 Genetic Analysis System was able to handle problematic loci with minimal requirements in assay optimization.

Both template amplification and primer extension assay are amenable to future automation with no requirement for equipments other than common lab centrifuges and vacuum manifolds.

References

1. Jiang, Zhiming, Telatar, Milhan, Grody, Wayne. Multiplex SNP Analysis: Screening Factor V R506Q (Leiden) Mutations. *Application Information Bulletin A-1928A,* Beckman Coulter, Inc.
2. Jiang, Zhiming, Oda, Rene M., Boyer, Scott K. Multiplex SNP Genotyping: Primer Design and Chemistry Optimization. *Application Information Bulletin* (in press), Beckman Coulter, Inc.
3. Rychlik, Wojciech. *Oligo Primer Analysis Software User Manual.* Molecular Biology Insights, Inc.

* *PCR is covered by U.S. patents owned by Hoffmann-La Roche, Inc. Agilent is a registered trademark of Agilent Technologies Corporation. All other trademarks are the property of their respective owners.*

I.S. EN ISO 9001

For Research Use Only. Not for use in diagnostic procedures.

SCIEX

www.sciex.com/ce

View SCIEX products at www.sciex.com
Find your local office at www.sciex.com/offices

AB SCIEX Headquarters
500 Old Connecticut Path | Framingham, MA 01701 USA
Phone 508-383-7700
www.absciex.com