Demonstration of SNP Genotyping Using a Single Nucleotide Extension Method and the 3500 Series Genetic Analyzer

Introduction

This publication describes the collaboration between researchers at the Okayama University Graduate School and at Applied Biosystems (Foster City, CA, USA), aimed at demonstrating a SNaPshot® Multiplex System genotyping protocol on the Applied Biosystems® 3500xL Genetic Analyzer capillary electrophoresis (CE) instrument.

Genotyping to determine ABO blood group status is a valuable tool in forensic applications where routine serological typing is not feasible. Therefore, laboratories that are developing ABO blood group typing applications appreciate workflows that provide fast, accurate, and reliable results, particularly if the assay delivers data for samples that contain very low concentrations of gDNA or degraded gDNA. In this publication, we present a simple, rapid, and robust workflow for ABO genotyping using the AmpliTaq Gold® Fast PCR Master Mix, Applied Biosystems® SNaPshot® Multiplex Kit, and the Applied Biosystems® 3500 Series Genetic Analyzer.

The Department of Legal Medicine, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences develops research methodologies for crime detection techniques that utilize DNA analyses, including the discovery of new polymorphic DNA loci and tools to identify gender from sample material. Visiting fellow Dr. Doi is involved in an ongoing effort to refine DNA-based blood typing techniques and is also developing a DNA type identification method that is useful on composite samples. Pictured are Dr. Yusuke Doi (top right); Dr. Yuji Yamamoto (top left); Mayumi Okashita (bottom right); and Hiroko Ide (bottom left).

ABO Genotyping

Identification of specific combinations of single nucleotide polymorphisms (SNPs) in the ABO locus on chromosome 9 can be used to determine ABO blood type. The Department of Legal Medicine, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences previously described the development of the SNaPshot® Multiplex System protocol [1], which allows for the simultaneous detection of six SNP sites within the ABO gene (corresponding to nucleotides 261, 297, 681, 703, 802, and 803). These six sites are sufficient for accurate determination of ten common genotypes in the Japanese population (AA, AO^A, AO^G, BB, BO^A, BO^G, O^AO^A, O^GO^G, O^AO^G, and AB). The rapid, simple, and robust SNaPshot® protocol is preferable to other methods of ABO genotyping that may be either too

time-consuming or complex for routine laboratory use (for example, PCR-RFLP or SSCP).

The SNaPshot® Multiplex System is a single-base extension/termination reagent for labeling that enables detection of SNPs. SNaPshot® chemistry is based on the dideoxy single-base extension of an unlabeled primer (Figure 1). When combined with Applied Biosystems® 3500xL Genetic Analyzer and analysis software, the SNaPshot® Multiplex System delivers a consistent and accurate blood group typing solution for forensic applications.

A Two-Step Multiplex Amplification and Primer Extension Reaction

Researchers at the Okayama University Graduate School modified the protocol previously published by Dr. Doi [1] to include PCR conditions that were developed to specifically optimize the amplification of three shorter ABO gene amplicons (Figure 2) instead of two longer ones. This change was introduced because forensic researchers are often required to analyze old or degraded DNA samples, and amplifying shorter fragments is generally a more successful approach with this type of starting material.

The initial PCR template was genomic DNA extracted from peripheral (circulating) blood (1 ng per reaction). The Group at Okayama University led by Dr. Doi and Dr. Yamamoto used AmpliTaq Gold® Fast PCR Master Mix with three sets of primers for the ABO gene to amplify a 102 bp region of exon 6, as well as a 141 bp and a 121 bp region of exon 7 (Table 1). Following PCR amplification, they carried out a single-base primer extension reaction using the reagents in the Applied Biosystems® SNaPshot® Multiplex Kit and six unique primers (one primer for each SNP of interest in the ABO gene, Table 2). After primer extension, samples were forwarded to the Applied Biosystems team for CE analysis.

At the Applied Biosystems facility (Foster City, CA, USA), labeled fragments generated for each sample using the SNaPshot® multiplex kit were subsequently separated by electrophoresis using the Applied Biosystems® 3500xL Genetic Analyzer. An aliquot of each sample (0.5 µL) was combined with 0.5 µL of GeneScan™ 120 LIZ™ Size Standard (or GeneScan™ 600 LIZ™ Size Standard v2.0 when the 3500xL data normalization function was used; see Signal Normalization) and 9 µL Applied Biosystems® Hi-Di™ formamide. Samples were denatured for five minutes at 95°C and immediately chilled on ice before analysis on the Applied Biosystems® 3500xL Genetic Analyzer using POP-7™ polymer and the SNaPshot50_POP7xl run module.

Results were analyzed by GeneMapper® v4.1 Software using a modified SNaPshot® Project and Report Manager to automate genotyping.

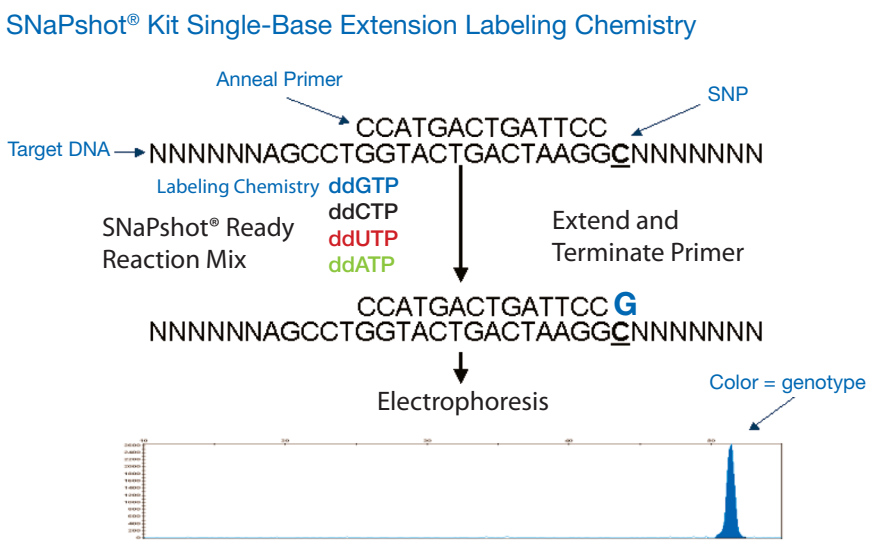


Figure 1. SNaPshot® Labeling Chemistry Relies on Single-Base Extension and Termination. The SNaPshot® Multiplex Kit uses a single-tube reaction format to interrogate SNPs at known locations. The chemistry is based on the dideoxy single-base extension of an unlabeled oligonucleotide primer (or primers). Each primer binds to a complementary template in the presence of fluorescently labeled ddNTPs and DNA polymerase. The polymerase extends the primer by one nucleotide, adding a single ddNTP to its 3′ end. The fluorescence color readout reports which base was added.

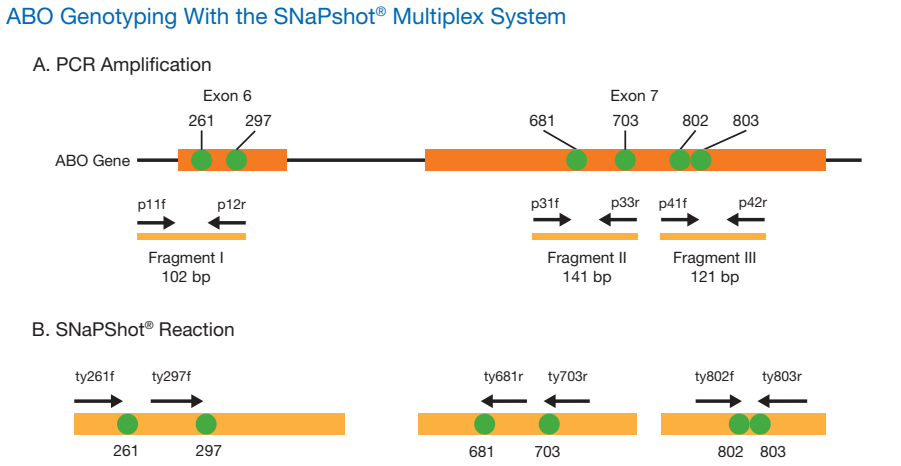


Figure 2. ABO Genotyping With the SNaPshot® System. Panel A: Regions from exon 6 and exon 7 of the ABO gene are amplified using PCR. Panel B: PCR products serve as templates in a multiplex SNaPshot® reaction. Six primers targeting unique SNPs are used to interrogate the six single-base substitutions in the fragments (261, 297, 681, 703, 802, and 803).

Analysis of Assay Sensitivity

The limit of detection is an important consideration when only very small quantities of gDNA are available, which can be the case with ABO blood typing. We examined the lower detection limit of this ABO genotyping protocol, assessing its potential to accurately genotype trace amounts of DNA using the Applied Biosystems® 3500 Series Genetic Analyzer.

Analysis of reactions using input gDNA quantities of 2 ng, 1 ng, 0.1 ng, 0.05 ng, and 0.025 ng from samples of AB and BO[A] blood types showed accurate allele calling using this workflow down to 0.025 ng for AB samples (Figure 3) and 0.05 ng for BO[A] samples (data not shown).

Automated ABO Genotyping

Automated genotyping using GeneMapper® v4.1 Software Report Manager was achieved by assigning each allele a numerical value, and then using the calculation functionality within Report Manager to convert the numerical values assigned to each allele to designate an individual genotype (Figure 4) based on each unique collection of alleles. Each allele at every nucleotide position was assigned a value (Table 3), a particular genotype corresponds to a specific set of alleles, in the case of AB and BO genotypes these differ by the allele assigned the number 2 (Tables 4 and 5).

Using the calculation functionality within the GeneMapper® software Report Manager, the group of six rows (corresponding to six nucleotide positions interrogated) for each sample was summed for each column. The total value, unique for each genotype, was decrypted and reported as the genotype in the Report Manager table: a total of 60 corresponds to the AB genotype (Table 4), whereas a total of 62 corresponds to the BO[A] genotype (Table 5).

When we defined the parameters of the automated genotyping report, we took particular care with samples whose genotypes differed by only one allele, especially if the allele was the minor contributor at a heterozygous nucleotide position. For example, in Figure 5, the AA and AB profiles differ from the AO[A] and BO[A] profiles, respectively, by the presence of the A nucleotide at position 261 in AO[A] and BO[A]. With low amounts of input

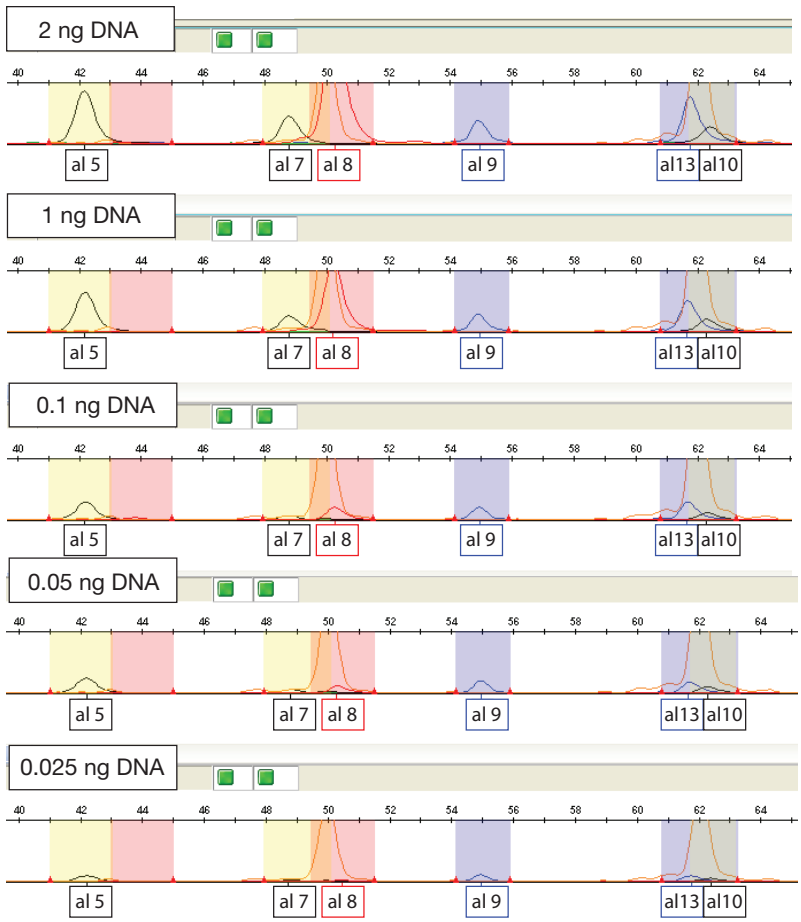


Figure 3. The SNaPshot® Workflow Enables Accurate Allele Calling Down to 0.025 ng Input DNA. Decreasing amounts of input DNA (2 ng to 0.025 ng) from an ABO sample (type AB) were analyzed using the SNaPshot® Multiplex System workflow and the 3500xL Genetic Analyzer. The genotype was accurately called for all dilutions (based on a recommended minimum peak threshold of 175 RFU).

Sample File	GQ	Marker	Allele 1	Allele 2	Sample Name	UD1
_H02_23_SNaPshot50_POP7xl_E5:	0.9961	261	1	2	16	
_H02_23_SNaPshot50_POP7xl_E5:	0.9961	297	4		16	
_H02_23_SNaPshot50_POP7xl_E5:	0.9961	681	5	6	16	
_H02_23_SNaPshot50_POP7xl_E5:	0.9961	703	7	8	16	
_H02_23_SNaPshot50_POP7xl_E5:	0.9961	802	9		16	
_H02_23_SNaPshot50_POP7xl_E5:	0.9961	803	13	10	16	BO(G)
_F03_18_SNaPshot50_POP7xl_E5:	0.9963	261	1	2	22	
_F03_18_SNaPshot50_POP7xl_E5:	0.9963	297	3		22	
_F03_18_SNaPshot50_POP7xl_E5:	0.9963	681	5		22	
_F03_18_SNaPshot50_POP7xl_E5:	0.9963	703	7		22	
_F03_18_SNaPshot50_POP7xl_E5:	0.9963	802	9		22	
_F03_18_SNaPshot50_POP7xl_E5:	0.9963	803	10		22	AO(A)
_A06_03_SNaPshot50_POP7xl_E5:	0.9963	261	1	2	41	
_A06_03_SNaPshot50_POP7xl_E5:	0.9963	297	4	3	41	
_A06_03_SNaPshot50_POP7xl_E5:	0.9963	681	5		41	
_A06_03_SNaPshot50_POP7xl_E5:	0.4982	703	7	8	41	
_A06_03_SNaPshot50_POP7xl_E5:	0.9963	802	9		41	
_A06_03_SNaPshot50_POP7xl_E5:	0.9963	803	13	10	41	BO(A)
_G02_20_SNaPshot50_POP7xl_E5.	0.9964	261	1	2	15	
_G02_20_SNaPshot50_POP7xl_E5.	0.9964	297	4	3	15	
_G02_20_SNaPshot50_POP7xl_E5.	0.9964	681	5		15	
_G02_20_SNaPshot50_POP7xl_E5.	0.4982	703	7	8	15	
_G02_20_SNaPshot50_POP7xl_E5.	0.9964	802	9		15	
_G02_20_SNaPshot50_POP7xl_E5.	0.9964	803	13	10	15	BO(A)
_E02_14_SNaPshot50_POP7xl_E5:	0.9965	261	1	2	13	
_E02_14_SNaPshot50_POP7xl_E5:	0.9965	297	4	3	13	
_E02_14_SNaPshot50_POP7xl_E5:	0.9965	681	5	6	13	
_E02_14_SNaPshot50_POP7xl_E5:	0.9965	703	7		13	
_E02_14_SNaPshot50_POP7xl_E5:	0.9965	802	9		13	
_E02_14_SNaPshot50_POP7xl_E5:	0.9965	803	10		13	AO(G)

Figure 4. A Report Generated by GeneMapper® 4.1 Software Shows the Automatically Identified Genotype for Each Sample. The report displays critical data in a single-page format: Sample File = file name of the resultant .fsa file; GQ = genotyping quality score; Marker = the marker or nucleotide position being queried; Allele 1 and Allele 2 = numerical scoring of the allele; Sample Name = reports the sample as it was named at the start of the experiment; UD1 = the identified genotype (based on the numerical calculation of each allele).

Table 1. Reaction Setup and Cycling Conditions for PCR Amplification.

Reaction Setup	
Component	**Volume**
AmpliTaq Gold® Fast PCR Master Mix, UP (2X)†	10 µL
10X PCR Primer Mix (see below for primer sequences)	2 µL
Template DNA (1 ng) + water	8 µL
Total	20 µL

Cycling Conditions	
	Temperature (°C), Time
	95°C, 10 minutes
33 cycles	96°C, 3 seconds
	65°C, 3 seconds
	68°C, 5 seconds
	72°C, 10 seconds
	10°C, hold

PCR Primers for Amplification Reaction			
Fragment No.	**Primer**	**Sequence**	**Final Concentration in PCR Reaction**
Fragment 1	p11f	5'-TCTCCATGTGCAGTAGGAAGGATGT-3'	0.5 µM (12.5 pmol)
Fragment 1	p12r	5'-TGAACTGCTCGTTGAGGATGTCGAT-3'	
Fragment 2	p31f	5'-GGCGCTTCCTCAGCGAGGTGGATT-3'	1 µM (25 pmol)
Fragment 2	p33r	5'-TGAAGGCCTCCCGGCTGCTTCCGT-3'	
Fragment 3	p41f	5'-TCCCCAAGGACGAGGGCGATTTCTA-3'	
Fragment 3	p42r	5'-ATGCCGTTGGCCTGGTCGACCATCA-3'	

† Applied Biosystems does not support the performance of AmpliTaq Gold® Fast PCR Master Mix for SNP or fragment analysis applications. Applied Biosystems only supports the performance of AmpliTaq Gold® Fast PCR Master Mix when Applied Biosystems® protocols, reagent formulations, and kit storage recommendations are followed.

Table 2. Primers for Primer Extension Using the SNaPshot® Multiplex Kit.

SNaPshot® Typing Primers for Single-Base Primer Extension	
Primer	**Sequence**
ty261f	5'-AAGGATGTCCTCGTGGT-3'
ty297f	5'-TTTTGGCTCCCATTGTCTGGGAGGGCAC-3'
ty681r	5'-TTTTTTTTTAGCCGGGGTGCAGGGTGCCGAACAG-3'
ty703r	5'-TTTTTTTTTTTTTTTTTTTGCCTCCCGGCTCCTTCCGTAGAAGC-3'
ty802f	5'-TTTTTTTTTTTTTTTTTTTTTTTTTTTCGAGGGCGATTTCTACTACCTGGGG-3'
ty803r	5'-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTCTCTTGCACCGACCCCCCGAAGAAC-3'

Table 3. Assigned Numerical Values to Each Allele at a Nucleotide Position.

Nucleotide Position	Allele 1	Allele 2
261	1	2
297	4	3
681	5	6
703	7	8
802	9	
803	13	10

Table 4. The Numerical Assignment of the AB Genotype.

Nucleotide Position	Allele 1	Allele 2
261	1	
297	4	3
681	5	
703	7	8
802	9	
803	13	10
Total: 39 + 21 = 60	**39**	**21**

Table 5. The Numerical Assignment of the BO[A] Genotype.

Nucleotide Position	Allele 1	Allele 2
261	1	2
297	4	3
681	5	
703	7	8
802	9	
803	13	10
Total: 39 + 23 = 62	**39**	**23**

DNA, the contribution from this minor allele could be too low to detect (i.e., peak height or peak area falls below the threshold set in GeneMapper® software), resulting in a miscalled genotype. In such situations, the GeneMapper® Report Manager was customized to flag the output genotype for further examination if the genotype was AB or BO^A and the peak height was less than 100 RFU. Additionally, if pairs of alleles at heterozygous sites do not fall within expectations for allele ratios (for example, in the case of a mixed DNA sample), the output genotype is similarly flagged for closer examination. (Note: mixed samples are not part of the study discussed in this publication.)

Signal Normalization

To minimize signal variation and improve data accuracy in the 3500 Series Genetic Analyzers, Applied Biosystems researchers have improved the optical system qualification processes and incorporated analysis software algorithm features that utilize the optional normalization reagent (GeneScan™ 600 LIZ™ Size Standard v2.0) in fragment analysis workflows. The GeneScan™ 600 LIZ™ Size Standard v2.0 may be used as a size standard for DNA fragment sizing applications and as a normalization standard. When used with the 3500xL instrument (and specific normalization features of the software), the GeneScan™ 600 LIZ™ Size Standard v2.0 minimizes instrument-to-instrument, run-to-run, and capillary-to-capillary variation. The improvements in the instrument, software algorithms, and size standard provide a significant reduction in the range of signal peak heights obtained across multiple 3500 Series Genetic Analyzers.

GeneMapper® 4.1 software can be used to apply the normalization factor calculated for each sample file by the 3500 Data Collection v1.0 software; this proved to be extremely valuable for accurate data analysis when the amount of input DNA was low. For example, we enabled the normalization feature of GeneMapper® 4.1 software to correct for capillary-to-capillary differences in electrokinetic injection differences. This allowed us to call the missed allele in sample 44 (Figure 6). In this way, the normalization feature helps ensure that samples with less than 1 ng input DNA are consistently and reliably called.

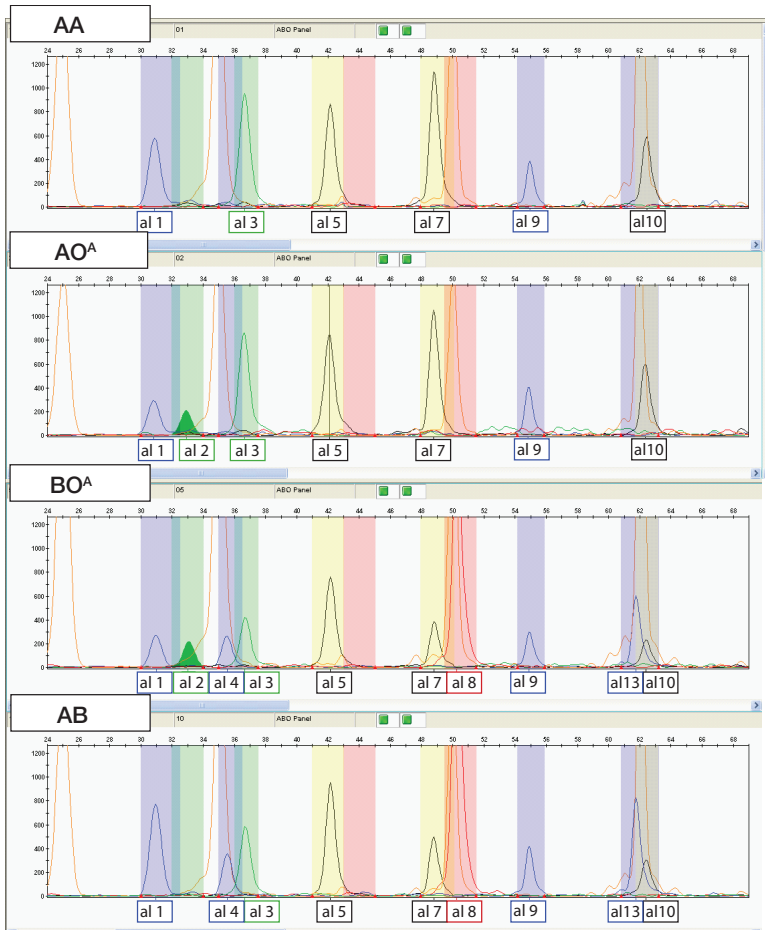
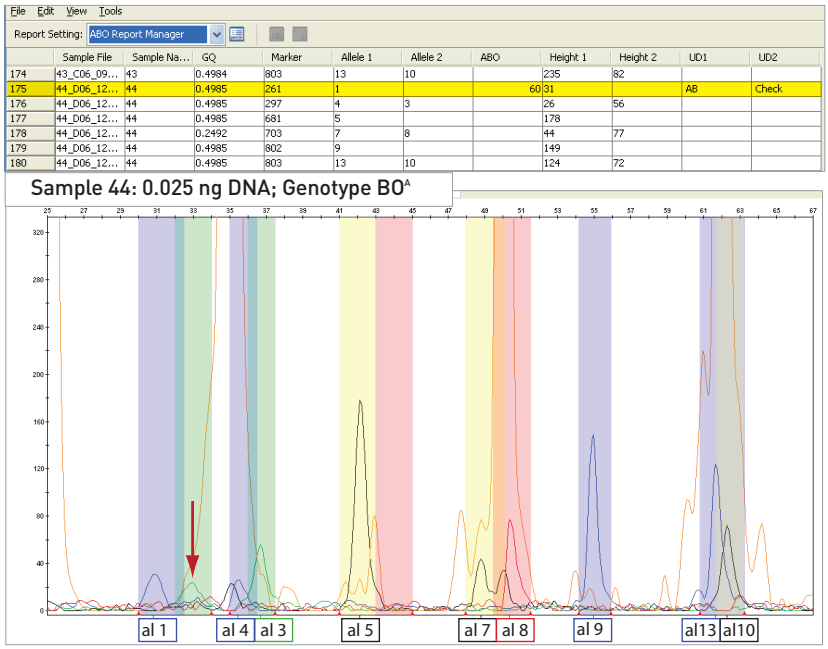


Figure 5. The SNaPshot® Multiplex Kit Distinguishes Between Alleles That Differ by One Nucleotide. Alleles that differ by a single nucleotide, such as AA and AO^A, or BO^A and AB, can be particularly difficult to genotype using an automated workflow. The software's Report Manager feature incorporates warnings for genotypes, such as those that differ by a single nucleotide, and may require manual examination of the electropherograms.



	Sample File	Sample Na...	GQ	Marker	Allele 1	Allele 2	ABO	Height 1	Height 2	UD1	UD2
174	43_C06_09...	43	0.4984	803	13	10		235	82		
175	44_D06_12...	44	0.4985	261	1		60	31		AB	Check
176	44_D06_12...	44	0.4985	297	4	3		26	56		
177	44_D06_12...	44	0.4985	681	5			178			
178	44_D06_12...	44	0.2492	703	7	8		44	77		
179	44_D06_12...	44	0.4985	802	9			149			
180	44_D06_12...	44	0.4985	803	13	10		124	72		

Figure 6. A Genotype Call Flagged by GeneMapper® Report for Further Analysis. An output genotype has been flagged in the genotyping report for further examination (see yellow highlighting). The associated trace shows that an allele present at a very low level (red arrow, corresponds to marker 261) has not been called, resulting in the flag.

Conclusion

Through this collaborative research effort between scientists at the Okayama University Graduate School and at Applied Biosystems, we have accurately genotyped samples containing as little as 0.05 to 0.025 ng of input DNA. This publication describes a number of ABO blood genotyping assay refinements designed to improve data quality and accuracy. These include using optimized amplification and SNaPshot® SNP genotyping reactions, incorporating a reliable size standard, and employing the normalization feature of the 3500xL Genetic Analyzer and GeneMapper® 4.1 software. The workflow presented here can easily be adapted to include any number of unique genotypes to enable quick, reliable, semi-automated genotyping of DNA.

Learn More

The 3500 Series Genetic Analyzers were designed for versatility and robust performance for fragment analysis workflows such as the ABO genotyping study presented here and for *de novo* sequencing and resequencing applications as well. Learn more about the advantages of the 3500 Series at www.appliedbiosystems.com/3500.

References

1. Doi Y, Yamamoto Y, Inagaki S et al. (2004) A new method for ABO genotyping using a multiplex single-base primer extension reaction and its application to forensic casework samples. *Leg Med (Tokyo)* 6(4):213–223.

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