# An overview of the use of SNaPshot for predicting blood group antigens

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The use of SNaPshot (Applied Biosystems, Foster City, CA) for predicting blood group antigens has emerged as an alternative to hemagglutination testing and also to the current low- and high-throughput blood group genotyping methods. Several groups have developed multiplex–polymerase chain reaction SNaPshot assays to determine single nucleotide polymorphisms (SNPs) in blood group genes with the purpose of identifying clinically relevant antigens and rare alleles. The selection of SNPs is based on the population or laboratory reality and the purpose of the genotyping. Unlike high-throughput genotyping strategies that are provided as commercial platforms, the SNPs can be chosen to best meet the needs of the user, and the interpretation of the results do not depend on the manufacturer. ***Immunohematology* 2015;31:53–57.**

**Key Words:** SNaPshot, blood group alleles, single nucleotide polymorphisms, multiplex-PCR

SnaPshot (Applied Biosystems, Foster City, CA) is a minisequencing assay based on a single nucleotide primer extension capable of detecting single nucleotide polymorphisms (SNPs).[1] Minisequencing was first described in the 1990s[2–7] and was used to detect SNPs in apolipoprotein E genotyping[4] and in cystic fibrosis genotyping.[2,7] Although the minisequencing reaction differs from SNaPshot, the basic principle of the method remains the same. The first reports used assays based on an internal reaction performed with an internal primer that ends exactly at 5′ of each SNP/point mutation site and radioisotope-labeled nucleotides for a specific allele. The assays varied in format, method of detection, ability to multiplex, and complexity.[1]

Using fluorescent-labeled nucleotides and an automated sequencer, the SNaPshot method follows the same principle as minisequencing.[8–10] The steps involved in SNaPshot are illustrated in Figure 1. The approach consists of a multiplex polymerase chain reaction (PCR) containing amplicons flanking selected SNPs. In the example, four hypothetic genes are amplified with forward and reverse primers comprising all SNPs of interest. While designing multiplex PCR primers, two SNPs can be included in the same amplicon when fragment size allows, as demonstrated in Gene 2 (Fig. 1A). Following multiplex PCR, electrophoresis in agarose gel could be performed to verify amplification of all fragments. The multiplex PCR product is purified with exonuclease and alkaline phosphatase. After purification, an internal reaction is carried out with an internal primer for each SNP, dideoxynucleotides (ddNTPs) labeled with different fluorescent dyes, and a polymerase enzyme. Internal primers can be designed to anneal with both directions of DNA, but they purposely end exactly one nucleotide before the polymorphism and present repetitive nucleotide tails to ensure that they differ in size (Fig. 1B). At the end of the internal reaction, primers with different-sized tails presenting ddNTPs with different colors attached represent the alleles. In the case of a heterozygous allele, both possibilities of ddNTP incorporation appear (Fig. 1C). The reaction is denatured, size

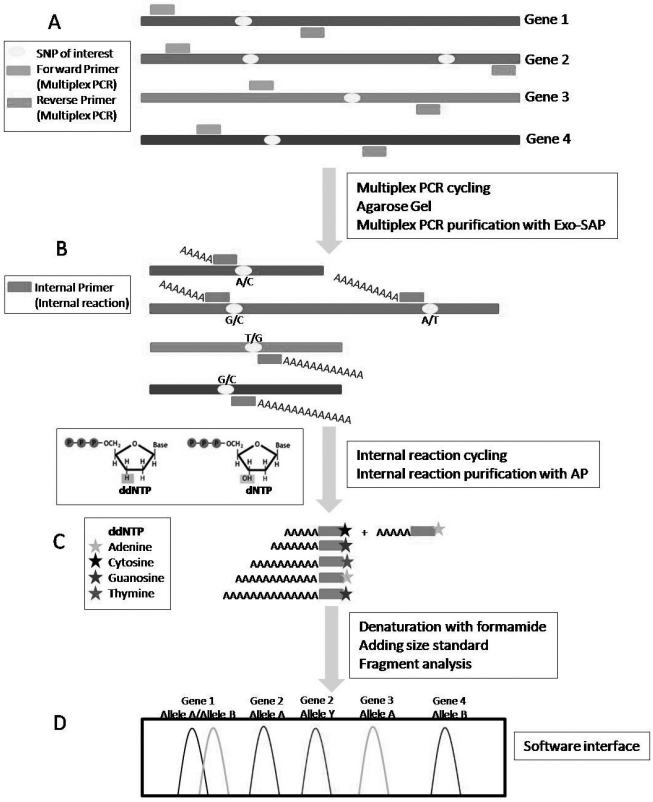


**Fig. 1.** Schematic representation of SNaPshot. (**A**) Multiplex polymerase chain reaction (PCR). (**B**) Internal primers. (**C**) Fragments after internal primer reaction. (**D**) Sample genotype using a specific software: *Gene1*A/Gene1*B, Gene2*A/Gene2*A, Gene2*Y/Gene2*Y, Gene3*A/Gene3*A, Gene4*B/Gene4*B.*

standard is added, and the fragment analysis is performed in a capillary-based sequencer. Amplicons are separated according to size, and the fluorescent dye is detected. These combinations represent all possible alleles. Fragment analysis is evaluated using specific software (Fig. 1D).

Internal or probe primers are the key point of the SNaPshot reaction, as they are responsible for the SNP identification and allele discrimination. SNPs are identified by the internal primers, as they are designed to show different sizes recognized during migration in the sequencing analyzer. These sizes are determined by a tail added to the 5′ end of each internal primer, which could be a poly(A), (C), (C), or (G), or a missense repeated sequence. While designing internal primers, their migration through capillary electrophoresis in the sequencer should be considered, as the distance among the peaks must be clear and each fluorescent dye has an individual weight that also interferes on migration. Moreover, because each internal primer ends exactly one nucleotide before the SNP, the allele will be determined when the ddNTP is incorporated. For these two reasons, internal primers are crucial in this approach.[1,11]

Another fundamental feature of SNaPshot is the use of ddNTPs instead of deoxynucleotides (dNTPs) in internal reactions because the ddNTPs lack the hydroxyl-radical. Because of this lack, when the internal primer is hybridized in the target sequence, a ddNTP is incorporated in the internal primer 3′ end where the SNP is located and the reaction stops. As each allele is characterized by a different nucleotide, the fluorescent dye present in ddNTP will identify the genotype.[1,11]

Therefore, at the end of the reaction, we obtain several amplicons with singular lengths and with different fluorescent dyes that correspond to a particular combination between migration and color. This precise combination is analyzed using specific software capable of predicting the allele. In general, this software is provided with the automated sequencer, and compares the obtained migration pattern of the sample with a prior defined panel. The better way to build this panel would be to perform the reactions with previously genotyped samples, including those heterozygous for every allele. Software interpretation will provide the genotype, and phenotype can be easily predicted.

## SNaPshot and Blood Group Genotyping

Although serology is the gold standard method used in immunohematology, it has certain limitations. These limitations include labor-intensive hemagglutination testing and data entry, the lack and high cost of commercial reagents, and a paucity of potent antisera.[12,13] Because the molecular basis of the majority of blood group antigens is known, this knowledge, together with the availability of DNA test methods, are being used to determine the genotype and to predict the phenotype of an individual.[14,15] Considering this scenario, several methods of blood group genotyping were incorporated in the clinical laboratory and SNaPshot was evaluated as a medium-throughput option.[12]

Several groups have already reported the use of the SNaPshot method to detect blood group SNPs.[11,16–24] Table 1 summarizes their findings, including alleles identified, the purpose of the studies, number of SNPs detected, number of reactions, number of tested samples, and validation features. Reviewing these, we can conclude that the choice of the approach was supported based on the limitations of serologic methods, on throughput, and on good feasibility regardless of DNA quality.

In summary, the approaches presented in Table 1 were developed with the focus on forensic identification and on genotyping of a group of alleles with specific goals, which included predicting clinically relevant Rh and low-incidence antigens. The number of investigated SNPs varied from 5 to 39 depending on the purpose and standardization of the protocols.

An additional important feature addressed in Table 1 is validation, where primer concentration and migration pattern in fragment analysis are evaluated. For that reason, testing previously genotyped samples or performing parallel genotyping with known methodologies is required.[11,16,17,21–23] Additionally, samples comprising all known alleles are necessary to inform the software where the peaks should be to build the panel of analyses. Hence, the inclusion of heterozygous samples certainly helps the validation process.[11] Blood group genotyping results obtained with SNaPshot were mainly validated by comparing them with those from previously genotyped[11,22,23] or phenotyped samples when commercial reagents were available.[16,17,21] Interestingly, sequencing results were concordant with the SnaPshot results, even when the results were discordant with those obtained with allele-specific PCR[16] or restriction fragment length polymorphism analysis of PCR-amplified fragments (PCR–restriction fragment-length polymorphism [RFLP]).[11]

## Strengths and Limitations

SNaPshot emerged as a technology capable of genotyping several polymorphisms in a single reaction. As a medium to the high-throughput method, it meets quite a few service realities. This robust approach improves the throughput of

**Table 1.** Outline of reports on SNaPshot to determine blood group antigens

| Blood group alleles | Aim | Number of tested SNPs/ Number of multiplex PCRs/Number of internal reactions | Number of tested samples | Validation method | Reference |
|---|---|---|---|---|---|
| ***KEL*3/KEL*4, KEL*6/KEL*7, DI*1/DI*2, DI*3/DI*4, YT*1/YT*2, CO*1/CO*2, DO*1/DO*2, DO*4,* and *DO*5*** | Rare donor screening | 9/1/1 | 305 | PCR-RFLP/ sequencing | Latini et al. (2014, Brazil)[11] |
| ***CO*1/CO*2, KEL*1/KEL*2, YT*1/YT*2, JK*1/JK*2, FY*1/FY*2/FY*X, DO*1/ DO*2, MNS*3/MNS*4, GYPBS_230/ GYPBS-Int5*** | Identification of SNPs responsible for clinically relevant phenotypes | 11/1/1 | 227 | Allele-specific PCR, sequencing, and serology | Di Cristofaro et al. (2010, France)[16] |
| **ABO alleles** | Forensic identification | 6/1/1 | 127 | PCR-RFLP and serology | Doi et al. (2004, Japan)[17] |
| **ABO alleles** | Forensic identification | 5/1/1 | 70 | — | Ferri et al. (2004 and 2009, Italy)[18,19] |
| **Alleles from *RHCE, DUFFY, MN,* and *KIDD*** | Forensic identification | 39/1/5 | 152 | Analysis of 16 paternity test cases and 1 personal identification | Inagaki et al. (2004, Japan)[20] |
| ***FYAB, GATA, FYX, DOA/B1, DOA/ B2, DOA/B3, DOJOA, DOHY, LWA/B, COAB, SC1/2, DIA/B, JKA/B, LUA/B, M/N, S/s, K/k*** | Genotyping common blood group systems | 17/3/3 | 29 | Previously genotyped and/or phenotyped samples | Palacajornsuk et al. (2009, United States)[21] |
| **6 variant *RHCE*ce, KEL*6/KEL*7*** | Knowledge of *RHCE* and *KEL* allele frequencies to reduce alloantibody formation | 6/1/1 | 1205 | Previously genotyped samples | Silvy et al. (2011, France)[22] |
| **13 variants of *weak D* and *DEL*** | Simultaneous detection of 14 *RHD* SNPs | 14/1/1 | 152 | Exon-specific PCR and sequencing | Silvy et al. (2011, France)[23] |
| **17 alleles in MNS, Kell, Duffy, Kidd, Cartwright, Dombrock, Indian, Colton, Diego, and Landsteiner–Wiener systems** | Description of antigen prevalence to improve transfusion practice | 21/2/2 | 599 | Genotyping of the same SNP using 2 SNaPshot protocols and previously genotyped or phenotyped samples | Mazières et al. (2013, France)[24] |

SNP = single nucleotide polymorphism; PCR = polymerase chain reaction; RFLP = restriction fragment-length polymorphism.

methodologies such as PCR-RFLP and allele-specific PCR, while maintaining the flexibility for customization, making it, like an "in-house" protocol, easily adaptable to the user's needs. Conversely to high-throughput genotyping strategies such as commercial microarrays, SNPs can be chosen to best meet the needs of patients and donors, SNPs for uncommon antigens can be included, and the interpretation of the results does not depend on the manufacturer.[11,15,25]

If indicated, the protocol can be adapted to allow for the addition of SNPS following validation. As an example, our laboratory decided to include genotyping of *SMIM1* to predict the Vel antigen in our SNaPshot standardized for identification of rare alleles, after the publication of its molecular background.[26–28] Although the event was not an SNP, but a 17-bp deletion, inclusion was possible because the internal primer was designed upstream from the deletion and the subsequent nucleotide differs if deletion is present. Figure 2A shows the internal primer location and ddNTP related to each allele. To allow fragment analysis (Fig. 2B), the internal primer was designed with a longer polyA tail. To validate the assay, we used samples previously genotyped by the described PCR-RFLP protocol.[26]

Moreover, the upgrade performed in our developed SNaPshot protocol for rare blood group alleles showed that this method is not exclusive for SNP analysis, which is another advantage of the method. Knowledge of the SNaPshot principle and the molecular bases of blood group polymorphisms allows for different applications of the approach.

Another important benefit of SNaPshot is the sensitivity of the method. Previous reports tested samples with genomic DNA concentration ranging from 4.3 to 529.0 ng/μL[11] and 0.1 ng template genomic DNA[17] with positive results. Corroborating, SNaPshot has been used in forensic genetics, showing achievement of good results with poor-quality

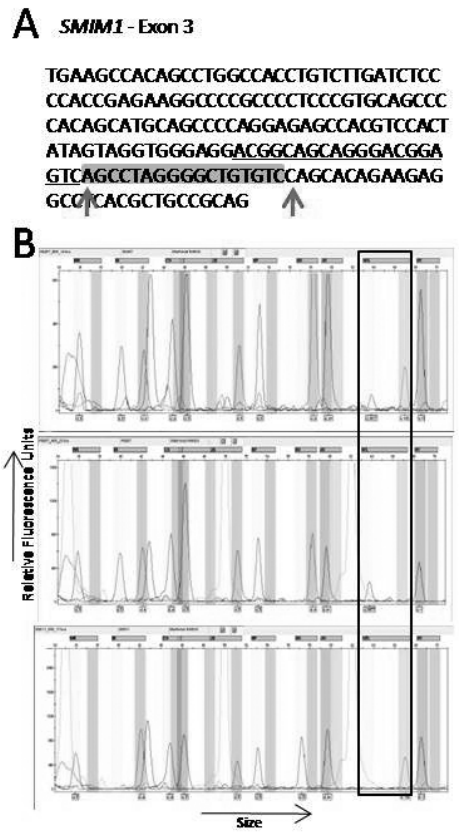


**Fig. 2.** Inclusion of genotyping to predict Vel antigen in our multiplex SNaPshot reaction for detection of 16 blood group alleles. (**A**) The 17-bp deletion in exon 3 of *SMIM1* associated with Vel– phenotype is highlighted in gray and the internal primer used is underlined. Red arrows indicate ddNTPs incorporated depending on the genotype. For Vel+ phenotype, an adenine (ddNTP) is incorporated after the internal primer, whereas for Vel– phenotype, a cytosine (ddNTP) is incorporated. Heterozygous samples present both adenine and cytosine. (**B**) Analysis (Gene Mapper, Applied Biosystems) of representative samples emphasizing genotyping to predict Vel antigen. First picture is a heterozygous sample with the presence of both alleles; in the second picture, the 17-bp deletion is present in both alleles, and therefore only cytosine (ddNTP) is detected; and in the last picture, both alleles have adenine.

samples, including formalin-fixed paraffin-embedded tissues,[29] heat-degraded samples,[17] and DNA extracted from bones, teeth, muscles, organs, nails, semen-contaminated vaginal fluid, and aged dried blood on blood type test paper.[17,20]

When SNaPshot costs are analyzed and compared with other methods, we conclude that it can be an excellent option. Depending on the reality, commercial platforms may be very expensive and in-house strategies become an option to perform genotyping.[14,25] Depending on the study, reagent cost may vary from $0.96 to $2.00 per detected SNP.[11,21] In our experience, after standardization, costs were successfully decreased because reagent volume could be reduced while maintaining quality.[11] When compared with low-throughput methods such as PCR-RFLP, for which costs were estimated to be $1.08 per SNP, required time until final results could be 14-fold reduced in SNaPshot (even though the resource costs are similar).[11] Evaluating another study that analyzed 35 red blood cell antigens, estimated cost per antigen was $0.48 using multiplex PCR and $1.97 to $2.14 using conventional serology.[30] These approaches suggest that noncommercial assays present similar costs per SNP, although they differ on throughput and turnaround time.[11,21,30] On the other hand, platforms such as microarrays may differ in cost depending on the country, but are an excellent option when considering time and throughput.[12]

Moreover, as with other genotyping strategies, automation is possible and several samples can be simultaneously analyzed. A sequencer is the required equipment for SNaPshot, in particular a capillary electrophoresis–based sequencer, and therefore the throughput depends on the equipment used. Speed is related to the number of capillaries in the sequencer and pipetting, which can range from manual (single or multichannel) and repetition to adjustable platforms.

SNaPshot is a method from Applied Biosystems, and therefore the reagents, equipment, and software are acquired from this company.

The main challenges of implementation of this approach are primer design and validation. Primer design comprises PCR multiplex and internal reaction steps. Bioinformatics tools may help avoid undesirable matches, although meticulous testing is required. Structures like hairpin, self-dimer, and heterodimers must be avoided. Concentration of pairs of primers for multiplex PCR and internal primers for internal reaction must be adjusted. Amplification of all fragments in multiplex PCR is monitored by agarose gel electrophoresis, and internal primer performance is verified by fragment analysis.

Furthermore, validation with heterozygous samples for every SNP previously genotyped by other methods is mandatory,[11] because building of the software panel for the result's analysis must include all possible alleles. This step can be problematic, as heterozygous samples could be rare if low-incidence antigens are being analyzed. The number of samples for validation depends on the protocol and laboratory requirements, but at least one heterozygous sample per SNP is mandatory. Discrepancies with other genotyping methods and phenotyping results are common during validation. When genotyping results differ, sequencing agrees with SNaPshot.[11,16] Divergence with phenotyping is more frequent, as secondary genetic alterations are described to affect protein expression, such as the GATA change that influences Duffy expression.

The success of the final protocol can be exhaustive until reaching ideal primer design/concentration and validation.

## Final Consideration

After overcoming the barriers of protocol standardization, SNaPshot can be a cost-effective, practical, and robust molecular strategy for medium- to high-throughput genotyping. Depending on the user's needs, SNaPshot allows grouping SNPs of interest, which is an excellent alternative when there is a specific purpose. In our experience, including polymorphisms related to rare blood group alleles in a single reaction was the best way to screen rare donors and supply our rare donor program.[11]

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