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# ABO Glycosyltransferase Genotyping by Polymerase Chain Reaction Using Sequence-Specific Primers

By C. Gassner, A. Schmarda, W. Nussbaumer, and D. Schönitzer

**Serological typing for the classical ABO blood groups is routinely performed using anti-A and anti-B antisera of polyclonal or monoclonal origin, which are able to distinguish four phenotypes (A, B, AB, and O). Modern molecular biology methods offer the possibility of direct ABO genotyping without the need for family investigations. Typing can be done with small amounts of DNA and without detection of blood group molecules on the surface of red blood cells. We developed a system of eight polymerase chain reactions (PCR) to detect specific nucleotide sequence differences between the ABO alleles $O^1$, $O^2$, $A^1$, $A^2$, and B. PCR amplification using sequence-specific primers and detection of amplification products by agarose gel electrophoresis is one of the fastest genotyping methods and is easy to handle. With our method we tested the $A^{1,2}BO^{1,2}$ genotypes of 300 randomly chosen persons out of a pool of platelet donors and found the results to be consistent with ABO glycosyltransferase phenotypes. We also identified a presumably new ABO allele, which may be the result of a crossing-over event between alleles $O^1$ and $A^2$.**



THE CODING DNA sequence of glycosyltransferase $A^1$ has been cloned and sequenced.[1] It has a length of 1,062 bp, encoding a protein of 41 kD. The currently described blood group genes coding for the other ABO phenotypes are closely related to this DNA sequence with only a few base substitutions or single deletions (Table 1). A to B transferase alleles differ in seven nucleotides at positions 297, 526, 657, 703, 796, 803, and 930, which result in four amino acid substitutions caused by nucleotide exchanges at positions 526 (C to G: Arg to Gly), 703 (G to A: Gly to Ser), 796 (C to A: Leu to Met), and 803 (G to C: Gly to Ala).[2] Stroncek et al[3] reported an exception to these results in the case of an A phenotype person with a B-specific nucleotide at position 526 in a defined A allele, which is referred to as "$A^{Stroncek}$" in this publication. The human blood group $A^2$ transferase coded by the $A^2$ allele is characterized by a single base substitution at nucleotide 467 (C to T: Pro to Leu) and a single cytosine deletion at position 1,059. This deletion is thought to be critical and results in an additional domain at the carboxyl terminal of the mature protein.[4]

O phenotype individuals have one of the three possible genetic combinations of the two known O-specific alleles, which are referred to here as "$O^1$" and "$O^2$". Allele $O^1$ has a single nucleotide deletion in the coding region of $A^1$ at nucleotide position 261 (deletion of G) causing a frame shift which creates a stop codon at nucleotides 352-354.[2] The other O phenotype allele, $O^2$, is distinguishable from $A^1$ at three nucleotide positions: 297, 526, and 802.[5] Only the substitutions at 526 (C to G: Arg to Gly) and 802 (G to A: Gly to Arg) produce amino acid changes. $O^2$ substitutions at positions 297 and 526 are the same as in allele B. The nucleotides in this work are numbered in accordance with the latest publication by Yamamoto et al.[6]

Until now all published genetic ABO typing techniques discriminate the different ABO alleles by time-consuming postamplification procedures, eg, restriction endonuclease cleavage, or the use of (radio)labeled DNA probes.[3,7-9] Additionally, none of these methods considers the second O phenotype allele ($O^2$) and therefore runs the risk of interpreting allele $O^2$ as allele $A^1$.

Polymerase chain reaction using sequence-specific primers (PCR-SSP) have proven to be a powerful tool for detecting particular nucleotides in known DNA sequences.[10-15] This simple, fast, and accurate technique allows us to distinguish all possible genetic variations of the alleles $O^1$, $O^2$, $A^1$, $A^2$, and B.

## MATERIALS AND METHODS

Determination of ABO phenotype by serological methods was done using commercially available test systems according to the manufacturer's instructions (DiaMed-ID Micro Typing System, DiaMed AG, Cressier, Switzerland; Gamma Biologicals Inc, Houston, TX; Biotest AG, Dreieich, Germany). In cases of $A^1$ / $A^2$ discrepancies between serologically and genetically determined blood groups, ABO blood group serotyping was repeated using additional test sera.

*Isolation of genomic DNA.* After obtaining informed consent, 10 mL of blood from platelet donors was collected in EDTA. Subsequently, the DNA was isolated using a modified salting-out method described by Miller et al.[16] The blood was centrifuged at 2,000*g* for 20 minutes, and the buffy coat (1 mL) was separated from red blood cells. After the addition of 10 mL lysis buffer I (0.3 mol/L sucrose, 10 mmol/L Tris/HCl pH 7.5, 5 mmol/L $MgCl_2$, 1% Triton X-100 [Sigma, St Louis, MO], 4°C ) to the buffy coat, we continued according to Miller et al.[16] DNA was also prepared from minor amounts of probe material (eg, from 50 μL whole blood, or 500 μL saliva) using the QIAamp blood kit (QIAGEN Inc, Chatsworth, CA).

*Allele-specific PCR amplification.* The approximate exon-intron borders for the published $A^1$ cDNA sequence were calculated from data given by Yamamoto et al.[4,6] PCR primer selection was performed with a computer program (MacVector, version 4.5.3; Kodak, New Haven, CT). Oligonucleotide primers used for $A^{1,2}BO^{1,2}$ genotyping, their combination and amplification product lengths are shown in Table 2. For analysis of each blood group we used three primers, two of which are allele-specific. The 3′ base of each allele-specific primer corresponds to the nucleotide that defines polymorphism. The third primer named "all" of each group is nonspecific and was used in two separate PCR reactions together with one of the two other allele-specific primers. The same consensus primer was used for alleles $O^2$ and B.

In each PCR reaction control-human growth hormone (C-hgh)

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**Table 1. DNA Sequence Data Are Shown for the Last Two Exons of the Various ABO Alleles So Far Identified**

| | 261 Deletion 261 | 297 | 467 C to T: Pro to Leu | 526 C to G: Arg to Gly | 646 T to A: Phe to Iso | 657 | 681 | 703 G to A: Gly to Ser | 771 | 796 C to A: Leu to Met | 802 G to A: Gly to Arg | 803 G to C: Gly to Ala | 829 | 871 G to A: Asp to Asr | 930 | 1054 C to T: Arg to Trp | 1059 Deletion 1059 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 0- 239 | 240- | 374 | 375- | | | | | | | | | | | | | | 1062 |
| Exon 1-5 | Exon 6 | | | | | | | | | Exon 7 | | | | | | | |
| $A^1$ | G | A | C | C | T | C | G | G | C | C | G | G | G | G | G | C | C |
| $A^2$ | G | A | T | C | T | C | G | G | C | C | G | G | G | G | G | C | – |
| $A^3$ | G | A | C | C | T | C | G | G | C | C | G | G | G | A | G | C | C |
| $A^x$ | G | A | C | C | A | C | G | G | C | C | G | G | G | G | G | C | C |
| $A^{Stroncek}$ | G | A | C | G | T | C | G | G | C | C | G | G | G | G | G | C | C |
| ABcis | G | A | T | C | T | C | G | G | C | C | G | C | G | G | G | C | C |
| $B^{(A)}$ | G | G | C | G | T | C | G | G | C | A | G | C | G | G | A | C | C |
| B | G | G | C | G | T | T | G | A | C | A | G | C | G | G | A | C | C |
| $B^3$ | G | G | C | G | T | T | G | A | C | A | G | C | G | G | A | T | C |
| $O^1$ | – | A | C | C | T | C | G | G | C | C | G | G | G | G | G | C | C |
| $O^1$ variant | – | G | C | C | A | C | A | G | T | C | G | G | A | G | G | C | C |
| $O^2$ | G | G | C | G | T | C | G | G | C | C | A | G | G | G | G | C | C |

Numbers on top reveal positions of base exchanges in the $A^1$ glycosyltransferase sequence. Only where indicated, do nucleotide differences produce different sequences in the expressed proteins among the set of ABO alleles. The broken line below schematically presents the coding sequence of $A^1$ glycosyltransferase including presumptive exon-intron borders for exons 6 and 7. Nucleotides (white on black background) at positions 261, 802, 803, and 1059 are detected in the PCR reactions using the appropriate primer (given in Table 2).

oligonucleotides were used to amplify a 434 bp PCR fragment from the human growth hormone locus position 5559 to 5992,[17] which served as a positive amplification control. In the case of a specific $A^{1,2}BO^{1,2}$ amplification, the 434 bp control fragment may disappear in the respective PCR reactions, because of competition.

Concentration of the detection primers was 0.2 $\mu$mol/L except for that in reactions "$O^2$" and "non$O^2$" where it was 0.16 $\mu$mol/L. Concentration of the control primers was 0.03 $\mu$mol/L in the amplifications "$O^1$," "non$O^1$," "B" and "nonB," and 0.025 $\mu$mol/L in the amplifications "$O^2$," "non$O^2$," "$A^2$," and "non$A^2$".

Amplification was performed in a final volume of 10 $\mu$L, containing 50 mmol/L KCl, 10 mmol/L Tris/HCl (pH 8.3), 0.01% gelatin, 200 $\mu$mol/L of each dNTP, 100 ng genomic DNA (UV-quantitated, or 2 $\mu$L of QIAamp spin column eluate) and 0.4 U of *Thermus aquaticus* polymerase (Perkin-Elmer Cetus Corp, Norwalk, CT). The reactions also contained 1.5 mmol/L $MgCl_2$ except for the "$O^2$" and "non$O^2$" amplifications, where the $MgCl_2$ concentration was dropped to 1.3 mmol/L.

To facilitate $A^{1,2}BO^{1,2}$ genotyping, all PCR reactions were triggered to work under the same thermocycling conditions on a Perkin-

**Table 2. Primers Used for $A^{1,2}$ $BO^{1,2}$ Genotyping**

| Allele | Name of Oligonucleotide | Sequence of Primers | Rct. No. PCR Product (bp) |
|---|---|---|---|
| $O^1$ | 345-all-as | 5′-*a*T*a*T*a*t ATG GCA AAC ACA GTT AAC CCA ATG-3′ | |
| | 261-$O^1$-s | 5′-tT*a* *a*GT GGA AGG ATG TCC TCG T*c*G TA-3′ | 1/139 |
| | 261-non$O^1$-s | 5′-T*a* aGT GGA AGG ATG TCC TCG T*c*G TG-3′ | 2/137 |
| $O^2$ | 649-all-s | 5′-*a*GT GGA CGT GGA CAT GGA GTT CC-3′ | |
| | 802-$O^2$-as | 5′-*t*C GAC CCC CCG AAG AA*g* CT-3′ | 3/194 |
| | 802-non$O^2$-as | 5′-C GAC CCC CCG AAG AA*g* CC-3′ | 4/193 |
| B | 649-all-s | 5′-*a*GT GGA CGT GGA CAT GGA GTT CC-3′ | |
| | 803-B-as | 5′-*at*C GAC CCC CCG AAG A*g*C G-3′ | 5/195 |
| | 803-nonB-as | 5′-CC GAC CCC CCG AAG A*g*C C-3′ | 6/194 |
| $A^2$ | 1191-all-as | 5′-*gg*G TGT GAT TTG AGG TGG GGA C-3′ | |
| | 1059-$A^2$-s | 5′-*g*AG GCG GTC CGG AA*g* CG-3′ | 7/169 |
| | 1060-non$A^2$-s | 5′-*g*AG GCG GTC CGG AAC *a*CG-3′ | 8/170 |
| | 5580 C-hgh-s | 5′-TGC CTT CCC AAC CAT TCC CTT A-3′ | /434 |
| | 5967 C-hgh-as | 5′-CCA CTC ACG GAT TTC TGT TGT GTT TC-3′ | |

The oligonucleotides are named after the position of their 3′ end on the cDNA sequence, the detected specificity and "s" (=sense), or "as" (=antisense). The first primer named "all" of each group is non-specific and is used in two separate PCR reactions together with one of the two other allele-specific primers. The Control-human growth hormone (C-hgh) primers are used for positive amplification control in each PCR reaction. Capital letters indicate hybridizing nucleotides, small type indicates mismatches with the $A^1$ cDNA sequence. Allele-specific nucleotides are underlined.

**Table 3. Possible Patterns Detected With $A^{1,2}$ $BO^{1,2}$ PCR-SSP and Statistical Results of Genotyping of 300 Randomly Chosen Individuals**

| Reaction No. | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | | | No. of Individuals Found | Calculated Genotype Frequency |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Reaction Name: | $O^1$ | non $O^1$ | $O^2$ | non $O^2$ | B | non B | $A^2$ | non $A^2$ | Genotype | Phenotype | (n = 300) | (%) |
| $O^1$ positive | + | − | − | + | − | + | − | + | $O^1$ $O^1$ | O | 107 | 34.03 |
| | + | + | + | + | − | + | − | + | $O^1$ $O^2$ | O | 11 | 3.31 |
| | + | + | − | + | + | + | − | + | $O^1$ B | B | 32 | 13.03 |
| | + | + | − | + | − | + | − | + | $O^1$ $A^1$ | $A_1$ | 74 | 25.08 |
| | + | + | − | + | − | + | + | + | $O^1$ $A^2$ | $A_2$ ($A_{int}$) | 19 | 7.19 |
| $O^2$ positive | − | + | + | − | − | + | − | + | $O^2$ $O^2$ | O | 0 | 0.08 |
| | − | + | + | + | + | − | − | + | $O^2$ B | B | 1 | 0.63 |
| | − | + | + | + | − | + | − | + | $O^2$ $A^1$ | $A_1$ | 5 | 1.22 |
| | − | + | + | + | − | + | + | + | $O^2$ $A^2$ | $A_2$ | 0 | 0.35 |
| B positive | − | + | − | +* | + | − | − | + | B B | B | 5 | 1.25 |
| | − | + | − | + | + | + | − | + | B $A^1$ | $A_1$ B | 22 | 4.80 |
| | − | + | − | + | + | + | + | + | B $A^2$ | $A_2$ B | 2 | 1.38 |
| $A^2$ positive | − | + | − | + | − | + | + | + | $A^2$ $A^1$ | $A_1$ | 8 | 2.65 |
| | − | + | − | + | − | + | + | − | $A^2$ $A^2$ | $A_2$ ($A_{int}$) | 4 | 0.38 |
| All "non" positive | − | + | − | + | − | + | − | + | $A^1$ $A^1$ | $A_1$ | 10 | 4.62 |

The 15 existing genotypes resulting from the alleles $O^1$, $O^2$, B, $A^1$, and $A^2$ together with their calculated genotype frequencies and their affiliation with ABO phenotypes are shown in this Table. To interpret the results of the $A^{1,2}$ $BO^{1,2}$ PCR-SSP, the block of the first positive allele-specific reaction must be chosen (first column of Table). Filling in the remaining positive reactions gives a clear genotype. Two individuals were serologically typed $A_{int}$, both possessed at least one allele containing the $A^2$-specific deletion at nucleotide 1059.

* Denotes weak specific amplification.

Elmer DNA Thermal Cycler (PCR System 9600; Perkin-Elmer Cetus Corp). The conditions were as follows: initial denaturation step of 120 seconds at 95°C, 5 incubation cycles for 30 seconds at 95°C, 150 seconds at 61°C, and 15 seconds at 72°C; 30 incubation cycles for 30 seconds at 95°C, 60 seconds at 64°C, and 15 seconds at 72°C.

PCR fragments were size-separated on a 2% agarose gel containing 0.5 μg/mL of ethidium bromide and visualized with ultraviolet light.

## RESULTS

Our $A^{1,2}BO^{1,2}$ PCR-SSP contains eight independent PCR reactions, four of them being allele-specific, the others nonallele-specific: "$O^1$," "non$O^1$," "$O^2$," "non$O^2$," "B," "nonB," "$A^2$," and "non$A^2$". In the case of haplotypes, a positive allele-specific reaction excludes a corresponding positive nonallele-specific reaction and vice versa. Allele $O^1$ is distinguished from the non $O^1$ alleles at nucleotide 261, $O^2$ from non $O^2$ at nucleotide 802, B from non B at position 803 and $A^2$ from non $A^2$ at nucleotide 1059. Allele $A^1$ can only be detected by exclusion of the other known alleles. A summary of all possible patterns obtainable with $A^{1,2}BO^{1,2}$ PCR-SSP is given in Table 3. Agarose gel electrophoresis of eight detected genotypes is shown in Fig 1.

*$A^{1,2}BO^{1,2}$ PCR-SSP genotyping of 300 test persons.* From a pool of randomly chosen Caucasian platelet donors 300 DNAs were tested with $A^{1,2}BO^{1,2}$ PCR-SSP. The detected genotypes together with their frequencies are listed in Table 3. The observed allele frequencies were: 0.61 for O (0.58 for $O^1$, and 0.03 for $O^2$), 0.27 for A (0.21 for $A^1$ and 0.06 for $A^2$ ), and 0.11 for B, from which phenotype frequencies were calculated to be 37.3% for O, 41.7% for A, 14.8% for B, and 6.1% for AB.

$A^{1,2}BO^{1,2}$ PCR-SSP results were compared to serologically determined blood group phenotypes. In 98.7% of the individuals (296 out of 300 persons) the results correlated precisely; 1.3% (4 out of 300) revealed discrepancies between serologically and genetically obtained results. These discrepancies could only be found within the two phenotype subgroups $A_1$ and $A_2$ and were as follows (determined genotypes in brackets): $A_1$ ($A^2A^2$), $A_1$ ($A^2O^1$), $A_1$ ($A^2O^1$), and $A_2$ ($A^1O^1$). A second serological determination confirmed the genetically obtained results in two cases. However, one of the $A^2O^1$ individuals and the $A^2A^2$ homozygote were serotyped $A_{intermediate}$ ($A_{int}$).

*Description of a possible new ABO allele.* One of the serotype O individuals (internal control number "568") displayed an "impossible" pattern of positive reactions: In addition to a positive non B and non $A^2$ reaction, this individual was $O^1$-positive, non-$O^1$-negative, $O^2$-negative, and non-$O^2$-positive, all consistent with group $O^1$ homozygosity. However, we also found a positive $A^2$ band, indicating an $A^2$-specific mutation in one of the $O^1$ alleles. The pattern is shown in Fig 1 (see genotype $O^{1\text{-}A2}O^1$) and discussed later.

## DISCUSSION

There are three major facts to be considered when genotyping ABO. Firstly, the principal requirement that nucleotide substitutions are clearly related to specific phenotypes, must be met, eg, the identification of a variant gene coding for blood group A transferase by Stroncek et al[3] makes genotyping at nucleotide 523 unreliable. In our $A^{1,2}BO^{1,2}$ PCR-SSP we focused on nucleotide position 803 to discriminate between alleles A and B. Base substitutions at this position and at position 796 change glycosyltransferase amino acid sequence and are thought to be critical in determining their sugar specificity.[18]

Secondly, assessment of PCR-SSP is impossible for some rare blood groups because information about their alleles is incomplete. Alleles of rare blood groups present their own

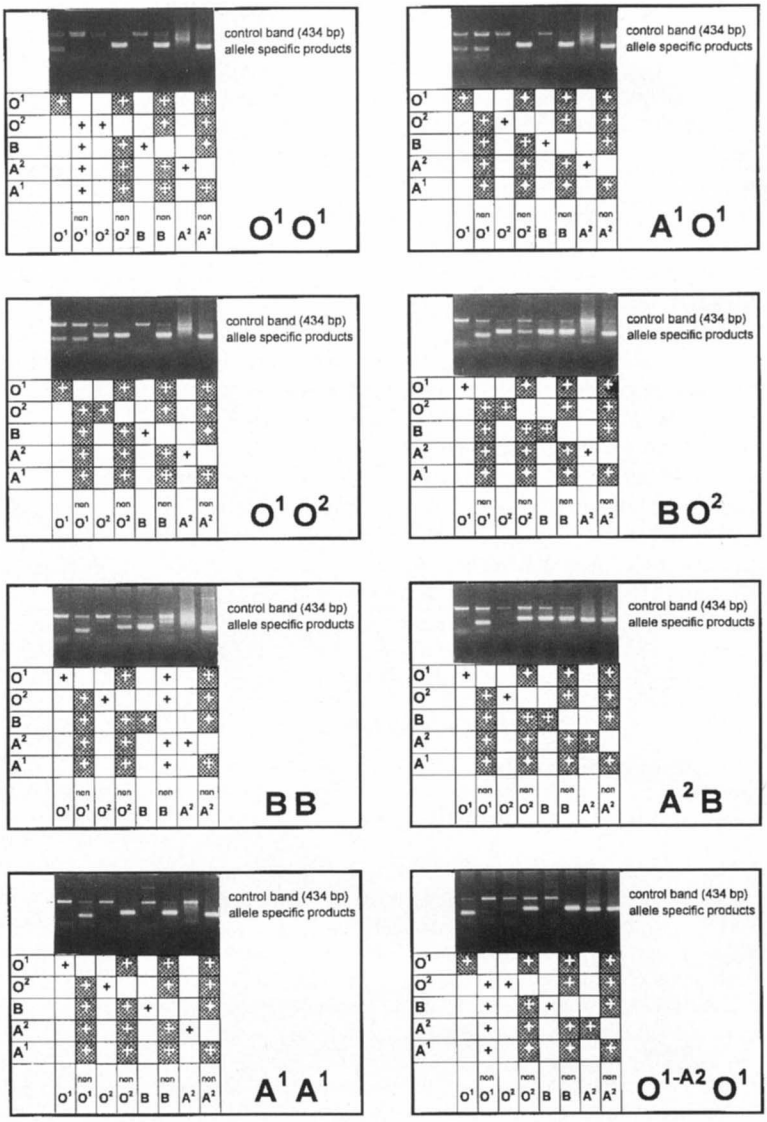


**Fig 1. Agarose gel electrophoresis and evaluation of selected $A^{1,2}BO^{1,2}$ genotypes. The names of the eight reactions for each determination are given at the bottom of the respective block. All necessary positive amplification reactions defining one special allele are marked "+." For gel electrophoresis, the real positive reactions are underlaid in gray. The resulting genotype is shown in the lower right corner of each block. The lowest right block exhibits an "impossible" reaction pattern, representing an as yet undefined ABO allele: $O^{1-A2}$.**

specific DNA sequences and therefore may give indistinct or even false results when typed by PCR-SSPs. Considering presently known DNA sequences, the possible reaction patterns in our $A^{1,2}BO^{1,2}$ PCR-SSP are as follows: presently known allelic sequences of $B^3$, cisAB, and $B^{(A)}$ exhibit the same pattern as a common blood group B allele, $A^3$, $A^x$, and $A^{Stroncek}$ give the same results as a common blood group $A^1$ allele, and a recently published variant $O^1$ is detected as $O^1$.[3,19-22]

Thirdly, allele $A^1$ can not be detected directly using our method or any other published method. Because there is no nucleotide solely specific for $A^1$, its determination is always linked to exclusion of other known alleles. Therefore, it is of extreme importance to consider the $O^2$ allele, eg, $O^1O^2$ heterozygous individuals (phenotype O) would present a false $A^1O^1$ genotype (phenotype $A_1$) without specific typing for $O^2$.

After considering these facts and after assessing our $A^{1,2}BO^{1,2}$ PCR-SSP the results revealed 100% correlation with serologically determined phenotypes in 300 investigated cases within the classic ABO blood groups. This indicates the statistical relevance of the published allelic DNA sequences and proves our method to be reliable and accurate. Among these 300 cases, 13 out of 15 possible $A^{1,2}BO^{1,2}$ allele combinations were found. However, until now, we have failed to find $A^2O^2$ heterozygous and $O^2O^2$ homozygous individuals. This can be explained by the low statistical occurence of such persons (see Table 3). Subgroups $A_1$ and $A_2$ showed 98.3% (118 out of 120) correlation between serologically and genetically determined groups. However, two cases exhibited serotype $A_{int}$, for which no allele-specific sequence data are yet available. Due to positive $A^2$ amplifications in these cases it might be postulated that the allele responsible for the $A_{int}$ serotype contains its own $A^{int}$-specific mutation(s) beside the $A^2$-specific deletion at nucleotide 1059.

This method also allowed us to interpret an unexpected result and thus, led to the probable identification of a new

ABO allele. One individual tested displayed a typical $O^1O^1$ homozygous reaction pattern, confirming the serologically determined O phenotype, but also revealed a positive $A^2$ signal. The unexpected positive $A^2$ amplification concurrent with an $O^1$ signal could be the result of an $O^1$-$A^2$ hybrid allele as a product of an earlier cross-over event. We decided to name this new ABO allele "$O^{1\text{-}A2}$," carrying an $O^1$-specific deletion at nucleotide 261 and another $A^2$ specific deletion at nucleotide position 1059. This "$O^{1\text{-}A2}$" should code for the same truncated protein as $O^1$ and is therefore serologically indistinguishable from $O^1$.

The presented typing strategy offers some advantages inaccessible with routine serological methods. The fact that the method requires no red blood cells and only a small amount of sample material for $A^{1,2}BO^{1,2}$ genotyping favors this method for pre-natal diagnosis and forensic investigations. The technique also allows ABO status to be determined independent of blood group molecules, thereby supplementing serological results. This is of interest because it confirms an acquired B phenotype as already reported by Fischer et al,[7] further elucidates the expression of incompatible A carbohydrate antigens in adenocarcinoma cells and, simply, gives additional information on unclear serological results.[7,23]

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