

# A modified PCR-RFLP genotyping method demonstrates the presence of the HPA-4b platelet alloantigen in a North American Indian population

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The diallelic HPA-4 (Pen/Yuk) platelet alloantigen system is polymorphic in Asian populations and accounts for the majority of cases of neonatal alloimmune thrombocytopenia in Japan. At the molecular level, the HPA-4a/4b dimorphism is associated with an arginine/glutamine substitution at amino acid 143 of the gene encoding platelet glycoprotein IIIa. Unlike the five other major diallelic human platelet antigen systems (HPA-1, -2, -3, -5, and -6), the nucleotide substitution corresponding to the HPA-4 antigen system does not involve a common naturally occurring restriction enzyme site. This paper describes a new genotyping method for HPA-4 (polymerase chain reaction–restriction fragment length polymorphism [PCR-RFLP]) that involves restriction enzyme digestion of PCR-amplified genomic DNA using a modified PCR primer to create an artificial *TaqI* restriction site that is present in the HPA-4a but not in the HPA-4b DNA sequence. The HPA-4 PCR-RFLP method was validated by testing a reference panel of 10 known HPA-4 genotyped Japanese individuals. Thus, genotyping by PCR-RFLP can now be performed for all six major HPA systems. Using the HPA-4 PCR-RFLP genotyping method, we determined a frequency of 2.9 percent for the *HPA-4b* allele in a North American Indian population. This finding indicates the importance of the HPA-4 antigen system as a potential cause of alloimmune thrombocytopenia in American Indians. *Immunohematology* 1997; 13:37–43.

The diallelic HPA-4 (Pen/Yuk) platelet alloantigen system was described in 1985 by Friedman and Aster<sup>1</sup> and in 1986 by Shibata et al.<sup>2</sup> Using a serologic assay, genotype frequencies of 98.3 percent, 1.7 percent, and > 0.01 percent for *HPA-4(a/a)*, *HPA-4(a/b)*, and *HPA-4(b/b)*, respectively, were reported in the Japanese population.<sup>3</sup> The low-frequency HPA-4b antigen subsequently has been reported in 1–2 percent of all Asian populations studied. HPA-4 has been localized to platelet membrane glycoprotein IIIa (GPIIIa),<sup>4</sup> and is associated with an arginine/glutamine polymorphism at amino acid 143 that results from a single base G/A substitution in the *GPIIIa* gene.<sup>5,6</sup>

Clinically, HPA-4 alloimmunization is the most common cause of neonatal alloimmune thrombocytopenia in Japanese individuals.<sup>3,7,8</sup> In Caucasians, the frequency of the less common *HPA-4b* allele is extremely low (< 1%),<sup>9,10</sup> and only one case of HPA-4b-associated neonatal alloimmune thrombocytopenia in a Caucasian family has been reported.<sup>11</sup> Posttransfusion purpura due to HPA-4a alloimmunization has been reported in a multiparous Navajo woman.<sup>12</sup>

The identification of the molecular genetic basis of the human platelet antigen systems has led to the development of various polymerase chain reaction–based genotyping assays, such as polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP). In PCR-RFLP, PCR-amplified genomic DNA containing the platelet antigen of interest is digested with a specific restriction enzyme that recognizes only one of the two alleles. Of the six major human platelet antigen systems (HPA 1–6), however, HPA-4 is the only one that does not involve a common, naturally occurring restriction enzyme site. For this reason, HPA-4 is the only platelet antigen for which a PCR-RFLP typing method is unavailable. We now describe a method for HPA-4 genotyping by PCR-RFLP that uses a modified oligonucleotide primer to introduce an “artificial” *TaqI* restriction site that encompasses the HPA-4 site within the *GPIIIa* gene and thus allows distinction between *HPA-4a* and *-4b* alleles. Because of the previous report of posttransfusion purpura due to HPA-4a alloimmunization in a Navajo woman,<sup>12</sup> we used our PCR-RFLP method to determine the HPA-4 genotype frequencies in a North American Indian population residing in western Washington state.

## Materials and Methods

### DNA samples

Genomic DNA was isolated from EDTA-anticoagulated whole blood samples from 110 healthy, unrelated Caucasians and from 105 North American Indian volunteer blood donors using the Puregene kit (Gentra Systems, Research Triangle Park, NC), according to the manufacturer's instructions. Samples from the North American Indian individuals residing in western Washington state were provided by Dr. Lakshmi Gaur (Puget Sound Blood Center Human Lymphocyte Antigen [HLA] Immunogenetics Laboratory, Seattle, WA). Tribes represented in this group of 105 unrelated American Indians include Puyallup, Nisqually, Quileute, and Stillaguamish. Ten Japanese reference DNA samples of previously determined *HPA-4* genotype were provided by Dr. Yoichi Shibata (Tokyo, Japan).

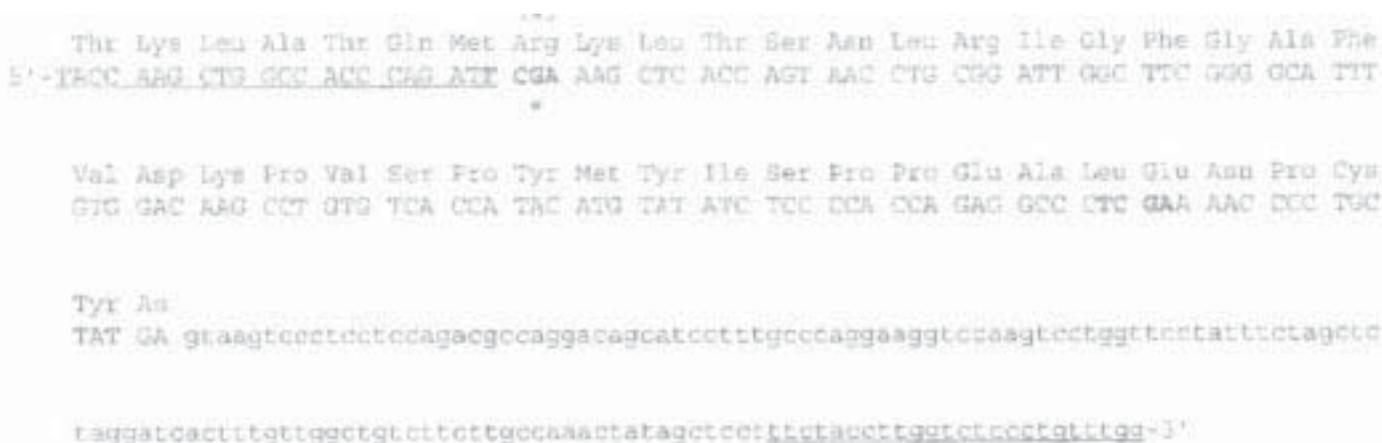
### *HPA-4* genotyping by PCR-RFLP

Two oligonucleotide primers were designed to amplify a 276 base pair (bp) region of the *GPIIIa* gene that contains the 3' portion of exon 3 as well as adjacent intronic sequence (see Fig. 1).<sup>13</sup> This region encompasses the arginine (CGA)/glutamine (CAA) dimorphism at amino acid 143 that corresponds to the *HPA-4a/4b* antigen system.<sup>5,6</sup> The forward PCR primer, 5'-TACCAAGCTGGCCACCCAGATT, includes nucleotides 13,900-13,921, while the reverse PCR

primer, 5'-CCAAACAGGGAGACCAAGGTAGAA, includes nucleotides 14,153-14,145.<sup>13</sup> The underlined 3' terminal nucleotide of the forward primer was changed from a G to a T and is two bases upstream of the *HPA-4* G/A nucleotide dimorphism. This PCR primer modification creates a *TaqI* restriction site (TCGA) in the *HPA-4a* allele but not in the *HPA-4b* allele (TCAA).

PCR amplification of 100 nanogram genomic DNA was performed in a 100  $\mu$ L reaction volume containing 1.5 mM magnesium chloride, 200  $\mu$ M dNTPs, and 20 pmoles each primer, for 30 cycles in an MJ PTC-100 thermal cycler (MJ Research, Watertown, MA), using 2.5 units of *Taq* polymerase (Perkin Elmer, Norwalk, CT) under the following conditions: denaturation at 93°C for 1 minute, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds. For all PCR amplifications, positive control DNAs of known *HPA-4* genotype as well as a negative control that did not contain template DNA were included.

A 10  $\mu$ L aliquot of the 276 bp PCR product containing the *HPA-4* site was digested with 20 units of *TaqI* restriction enzyme (New England Biolabs, Beverly, MA) in a total reaction volume of 20  $\mu$ L for 1 hour at 65°C, according to the manufacturer's instructions. To determine *HPA-4* genotype, 10  $\mu$ L of the *TaqI* digested sample was applied to a 2.0% agarose gel and visualized by ethidium bromide staining following electrophoretic separation.



**Fig. 1.** Nucleotide sequence of a 276 bp region of the *GPIIIa* gene<sup>13</sup> containing the *HPA-4* dimorphism. The DNA coding sequence from the 3' end of exon 3 is indicated by capital letters, and adjacent intron sequence by small letters. The corresponding three-letter amino acid code is indicated above the exon sequence. The nucleotides corresponding to the 5' forward and 3' reverse primers used for PCR amplification of this 276 bp segment are underlined. The 3' terminal nucleotide of the forward primer is changed from a G to a T (italicized), which creates a *TaqI* restriction site (TCGA) in the adjacent DNA sequence corresponding to the *HPA-4a* allele at Arg 143. This proximal *TaqI* site, along with a distal *TaqI* site (TCGA), are indicated by bold letters. In the *HPA-4b* allele sequence (not shown), the dimorphic nucleotide (indicated by the asterisk) is changed from a G to an A, which encodes Gln at amino acid 143 and also abolishes the proximal *TaqI* site (i.e., TCAA).

### HPA-4 genotyping by PCR-SSP

HPA-4 genotyping of PCR-amplified genomic DNA using sequence-specific primers (PCR-SSP) was performed according to a previously published method.<sup>14</sup>

### Population studies

Gene frequencies for HPA-4 alleles were calculated according to the Hardy-Weinberg equation for diallelic systems.

## Results

### HPA-4 genotyping by PCR-RFLP

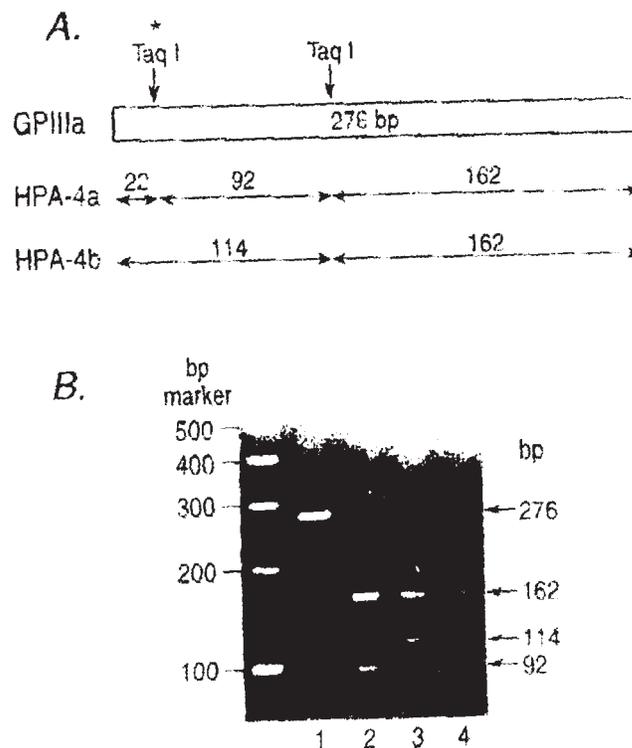
Using the modified 5' primer, PCR amplification of the HPA-4 region of the *GPIIIa* gene from human genomic DNA yields a 276 bp segment that contains two potential *TaqI* restriction sites (TCGA). The first site is created by the modified PCR primer and is present in the HPA-4a DNA sequence (TCGA) but not in the HPA-4b DNA sequence (TCAA). The second *TaqI* site is invariable and located 92 nucleotides downstream of the HPA-4 site (see Fig. 2A). The second *TaqI* site serves as an internal control for complete restriction enzyme digestion of the 276 bp PCR product. Figure 2B shows an agarose gel of PCR-amplified DNA following *TaqI* digestion. As expected, digestion of the HPA-4a allele yields fragments of 162, 92, and 22 bps, while digestion of the HPA-4b allele yields 162 and 114 bps.

### Validation of the PCR-RFLP HPA-4 genotyping method

To validate our HPA-4 PCR-RFLP typing method, we tested a panel of genomic DNA samples from 10 Japanese donors of known HPA-4 genotype. The panel contained five HPA-4(a/a), four HPA-4(a/b), and one HPA-4(b/b) individuals. In all 10 cases, our PCR-RFLP results agreed with the HPA-4 genotypes that had been previously determined by a combination of serologic and DNA typing.

### HPA-4 genotyping of Caucasian and North American Indian populations

The HPA-4 PCR-RFLP method was used to type 110 Caucasian and 105 North American Indian blood donors from the Pacific Northwest region (Washington state). HPA-4 genotype frequencies are shown in Table 1 and the calculated gene frequencies are shown in Table 2. All 110 Caucasians were HPA-4a homozygous, but 3 of 105 (2.9%) American Indians typed as HPA-4(a/b). The genotypes of the three HPA-4(a/b)



**Fig. 2.** PCR-RFLP method for HPA-4 genotyping. (A) Diagrammatic illustration of the HPA-4 PCR product containing the amino acid 143 Arg/Gln polymorphism corresponding to a G/A nucleotide substitution within the glycoprotein *IIIa* gene. Two *TaqI* restriction sites are indicated by arrows. The proximal *TaqI* site (indicated by an asterisk) is created by the 5' modified PCR primer and is present in the DNA sequence corresponding to the HPA-4a (—TCGA—) but not the HPA-4b (—TCAA—) allele. The distal *TaqI* site is invariable and serves to control for complete restriction enzyme digestion. Thus, *TaqI* digestion of the HPA-4a sequence yields fragments of 162, 92, and 22 bp, while digestion of the HPA-4b sequence yields fragments of 162 and 114 bp. (B) *TaqI* digestion of the 276 bp PCR product containing the HPA-4 polymorphism followed by gel electrophoresis on 2.0% agarose. Lane 1 contains the undigested 276 bp PCR product. Lanes 2, 3, and 4 contain *TaqI* digestion products from known HPA-4(a/a), HPA-4(a/b), and HPA-4(b/b) Japanese individuals, respectively. The 22 bp digestion product is not visible.

American Indian DNA samples, as well as a random subsample of 20 American Indian HPA-4(a/a) individuals, were confirmed using a previously reported PCR-SSP method.<sup>14</sup>

## Discussion

This paper describes a PCR-RFLP method for genotyping the HPA-4 platelet antigen system. PCR amplification of genomic DNA using a modified

**Table 1.** HPA-4 genotype frequencies

	Caucasians (n = 110)		North American Indians (n = 105)	
	Number	%	Number	%
HPA-4(a/a)	110	100	102	97.1
HPA-4(a/b)	0	0	3	2.9
HPA-4(b/b)	0	0	0	0

**Table 2.** HPA-4 calculated gene frequencies

	Caucasians (n = 110)	North American Indians (n = 105)
HPA-4a	1.000	0.986
HPA-4b	0	0.014

oligonucleotide primer creates an artificial *TaqI* restriction site that is present in the HPA-4a but not the HPA-4b DNA sequence. The modified PCR-RFLP method was validated by comparing our results with a reference panel of 10 well-characterized Japanese DNA samples that contain a representation of both *HPA-4* alleles. In addition, the accuracy of our method was confirmed by validating our results against a previously described PCR-SSP technique<sup>14</sup> on a selected sample of *HPA-4b*-positive and *HPA-4b*-negative American Indian individuals.

The use of a modified primer to introduce an artificial restriction enzyme site during PCR amplification was first described to detect single base substitutions that do not involve naturally occurring restriction enzyme recognition sites.<sup>15</sup> This method has been used successfully to detect point mutations within a variety of genes, such as those encoding coagulation proteins,<sup>16,17</sup> oncogenes,<sup>18</sup> and other intracellular proteins or enzymes.<sup>19,20</sup> This method takes advantage of the "infidelity" of Taq DNA polymerase to extend a mismatched primer/template. The method is also dependent upon the stringency of the PCR conditions as well as the specific nucleotide composition and position of the mismatched nucleotide within the PCR primer.<sup>21</sup> In our HPA-4 PCR-RFLP method, the presence of a mismatched T at the 3' terminus of the modified primer is efficiently incorporated into the PCR product.<sup>21</sup>

PCR-SSP, another DNA method used for platelet antigen typing,<sup>14,22,23</sup> also involves the use of modified PCR primers to distinguish HPA alleles.<sup>24</sup> In PCR-SSP, the PCR primer modifications are used to selectively amplify one *HPA* allele versus the other. Thus, in contrast to our method, the PCR conditions are manipulated such that the relative *inefficiency* of PCR amplification of 3'-terminus-matched versus mismatched primer/template pairs is used to distinguish *HPA* alleles.

Other PCR-based genotyping assays for the detection

of human platelet antigens have been described. These assays include allele-specific oligonucleotide hybridization,<sup>25</sup> single-stranded conformation polymorphism (SSCP),<sup>26,27</sup> reverse dot-blot hybridization,<sup>28</sup> and ligase PCR.<sup>29</sup> Each DNA typing method has its advantages and disadvantages, and a platelet-typing laboratory may prefer to use one or another of these methods based on convenience or familiarity with the technique. The overall advantages of DNA typing assays over standard platelet serologic methods for antigen typing include (1) a higher degree of accuracy and reproducibility, (2) the elimination of the need for rare reagent platelet antisera, and (3) the ability to perform typing on any nucleated cellular sample (e.g., blood, amniocytes). This latter consideration is especially important in adult patients with severe thrombocytopenia (i.e., posttransfusion purpura) or fetuses with neonatal alloimmune thrombocytopenia for whom platelets are difficult to obtain.

The HPA-1, -2, -3, -5, and -6 platelet antigen systems all involve naturally occurring restriction sites, and PCR-RFLP genotyping methods for these five systems have been described previously.<sup>30-32</sup> Although the HPA-4 system involves a *CviRI* restriction site,<sup>30</sup> this rare restriction enzyme is not commercially available. During preparation of this manuscript, a PCR-RFLP method for HPA-4 genotyping was reported by Matsuo and Reid.<sup>33</sup> In their method, a single base modification in one of the PCR primers creates an artificial *BsmI* restriction site in the HPA-4a but not the HPA-4b DNA sequence. Like our HPA-4 PCR-RFLP method, the *BsmI* PCR-RFLP method is rapid (~4 hours) and uses a modified PCR primer to create an artificial restriction site. An advantage of our method, however, is the presence of a second, invariable *TaqI* restriction site within the PCR product that serves as an internal control for complete restriction enzyme digestion, as previously described for other HPA system PCR-RFLP genotyping methods.<sup>30</sup> In addition, the cost per unit of restriction enzyme is approximately 10-fold lower for *TaqI* than for *BsmI*. With the previously described PCR-RFLP genotyping methods for HPA-1, -2, -3, -5, and -6 platelet antigen systems,<sup>30-32</sup> genotyping for all six major human platelet antigen systems can now be performed by PCR-RFLP.

The HPA-4 system is polymorphic only in non-Caucasian populations. The less frequent *HPA-4b* allele is present in approximately 2 percent of Japanese individuals<sup>3,23</sup> and also has been reported in 1.6 percent of Koreans,<sup>34</sup> 1.86 percent of Thais,<sup>35</sup> and 0.6 percent of Indonesians.<sup>36</sup> Although there has been one reported case of neonatal alloimmune thrombocytopenia in a

Caucasian woman sensitized to the HPA-4b antigen, population studies in the United States and Europe have demonstrated the virtual absence of the *HPA-4b* allele in Caucasian populations.<sup>9-11,30</sup> A recent study also demonstrated the absence of the *HPA-4b* allele in 100 African Americans.<sup>10</sup> In a population of North American Indians in western Washington state, we found a frequency of 2.9 percent for the *HPA-4b* allele. This is the first population in North America in which the HPA-4 polymorphism has been demonstrated and represents the highest frequency of the HPA-4b antigen reported in any ethnic group to date. The *HPA-4b* allele has also been reported in 0.9 percent of South American (Mapuches) Indians.<sup>37</sup> These results, taken together with a previous report of HPA-4 associated posttransfusion purpura in a Navajo woman,<sup>12</sup> suggest that the HPA-4 system should be considered as a potential cause of alloimmune thrombocytopenia in American Indians. Finally, from an evolutionary standpoint, it is interesting to note the presence of the *HPA-4b* allele in Asians and American Indians but not in Caucasian or African American populations. This may reflect a common ancestral origin, since native Americans are believed to have migrated from Asia over 10,000 years ago.<sup>38</sup>

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### References

1. Friedman JM, Aster RH. Neonatal alloimmune thrombocytopenic purpura and congenital porencephaly in two siblings associated with a "new" maternal antiplatelet antibody. *Blood* 1985;65:1412-5.
2. Shibata Y, Matsuda I, Miyaji T, Ichikawa Y. Yuk<sup>a</sup>, a new platelet antigen involved in two cases of neonatal alloimmune thrombocytopenia. *Vox Sang* 1986;50:177-80.
3. Shibata Y, Miyaji T, Ichikawa Y, Matsuda I. A new platelet antigen system, Yuk<sup>a</sup>/Yuk<sup>b</sup>. *Vox Sang* 1986;51:334-6.
4. Furihata K, Nugent DJ, Bissonette A, Aster RH, Kunicki TJ. On the association of the platelet-specific alloantigen, Pen<sup>a</sup>, with glycoprotein IIIa: evidence for heterogeneity of glycoprotein IIIa. *J Clin Invest* 1987;80:1624-30.
5. Wang L, Juji T, Shibata Y, Kuwata S, Tokunaga K. Sequence variation of human platelet membrane glycoprotein IIIa associated with the Yuk<sup>a</sup>/Yuk<sup>b</sup> alloantigen system. *Proc Japan Acad* 1991;67(B):102-6.
6. Wang R, Furihata K, McFarland JG, Friedman K, Aster RH, Newman PJ. An amino acid polymorphism within the RGD binding domain of platelet membrane glycoprotein IIIa is responsible for the formation of the Pen<sup>a</sup>/Pen<sup>b</sup> alloantigen system. *J Clin Invest* 1992;90:2038-43.
7. Matsuda T, Yonaha H, Azuma E, Sakurai M, Imai S. Neonatal alloimmune thrombocytopenic purpura associated with sensitization against the platelet-specific antigen Yuk<sup>a</sup>. *Am J Hematol* 1990;35:199-202.
8. Matsui K, Ohsaki E, Goto A, Koresawa M, Kigasawa H, Shibata Y. Perinatal intracranial hemorrhage due to severe neonatal alloimmune thrombocytopenic purpura (NAITP) associated with anti-Yuk<sup>b</sup> (HPA-4a) antibodies. *Brain & Development* 1995;17:352-5.
9. Kiefel V, Santoso S, Katzmann B, Mueller-Eckhardt C. A new platelet-specific alloantigen Br<sup>a</sup>. *Vox Sang* 1988;54:101-6.
10. Kim HO, Jin Y, Kickler TS, Blakemore K, Kwon OH, Bray PF. Gene frequencies of the five major human platelet antigens in African American, white, and Korean populations. *Transfusion* 1995;35:863-7.
11. Morel-Kopp M-C, Blanchard B, Kiefel V, Joly C, Mueller-Eckhardt C, Kaplan C. Anti-HPA-4b (anti-Yuk<sup>a</sup>) neonatal alloimmune thrombocytopenia: first report in a Caucasian family. *Transfus Med* 1992;2:273-6.
12. Simon TL, Collins J, Kunicki TJ, Furihata K, Smith KJ, Aster RH. Posttransfusion purpura associated with alloantibody specific for the platelet antigen, Pen<sup>a</sup>. *Am J Hematol* 1988;29:38-40.
13. Zimrin AB, Gidwitz S, Lord S, et al. The genomic organization of platelet glycoprotein IIIa. *J Biol Chem* 1990;265:8590-5.
14. Skogen B, Bellissimo DB, Hessner MJ, et al. Rapid determination of platelet alloantigen genotypes by polymerase chain reaction using allele-specific primers. *Transfusion* 1994;34:955-60.
15. Haliassos A, Chomel JC, Tesson L, et al. Modifi-

- cation of enzymatically amplified DNA for the detection of point mutations. *Nucleic Acids Res* 1989;17:3606.
16. Reiner AP, Thompson AR. Screening for nonsense mutations in patients with severe hemophilia A can provide rapid, direct carrier detection. *Hum Genet* 1992;89:88-94.
  17. Rabès JP, Trossaert M, Conard J, Samama M, Giraudet P, Boileau C. Single point mutation at Arg<sup>506</sup> of factor V associated with APC resistance and venous thromboembolism: improved detection by PCR-mediated site-directed mutagenesis. *Thromb Haemost* 1995;74:1379-80.
  18. Loda M. Polymerase chain reaction-based methods for the detection of mutations in oncogenes and tumor suppressor genes. *Hum Pathol* 1994;25:564-71.
  19. Chang JG, Chiou SS, Perng LI, et al. Molecular characterization of glucose-6-phosphate dehydrogenase (G6PD) deficiency by natural and amplification created restriction sites: five mutations account for most G6PD deficiency cases in Taiwan. *Blood* 1992;80:1079-82.
  20. Gasparini P, Bonizzato A, Dognini M, Pignatti PF. Restriction site generating-polymerase chain reaction (RG-PCR) for the probeless detection of hidden genetic variation: application to the study of some common cystic fibrosis mutations. *Mol Cell Probes* 1992;6:1-7.
  21. Kwok S, Kellogg DE, McKinney N, et al. Effects of primer-template mismatches on the polymerase chain reaction: human immunodeficiency virus type 1 model studies. *Nucleic Acids Res* 1990;18:999-1005.
  22. Metcalfe P, Waters AH. HPA-1 typing by PCR amplification with sequence-specific primers (PCR-SSP): a rapid and simple technique. *Br J Haematol* 1993;85:227-9.
  23. Tanaka S, Taniue A, Nagao N, et al. Simultaneous DNA typing of human platelet antigens 2, 3 and 4 by an allele-specific PCR method. *Vox Sang* 1995;68:225-30.
  24. Ugozzoli L, Wallace RB. Allele-specific polymerase chain reaction. *Methods: a companion to methods in enzymology* 1991;2:42-8.
  25. McFarland JG, Aster RH, Bussel JB, Gianopoulos JG, Derbes RS, Newman PJ. Prenatal diagnosis of neonatal alloimmune thrombocytopenia using allele-specific oligonucleotide probes. *Blood* 1991;78:2276-82.
  26. Fujiwara K, Tokunaga K, Isa K, et al. DNA-based typing of human platelet antigen systems by polymerase chain reaction-single-stranded conformation polymorphism method. *Vox Sang* 1995;69:347-51.
  27. Peyruchaud O, Nurden A, Bourre F. Non-radioactive SSCP for genotyping human platelet alloantigens. *Br J Haematol* 1995;89:633-6.
  28. Bray PF, Jin Y, Kickler T. Rapid genotyping of the five major platelet alloantigens by reverse dot-blot hybridization. *Blood* 1994;84:4361-7.
  29. Legler TJ, Köhler M, Mayr WR, Panzer S, Ohto H, Fischer GF. Genotyping of the human platelet antigen systems 1 through 5 by multiplex polymerase chain reaction and ligation-based typing. *Transfusion* 1996;36:426-31.
  30. Simsek S, Faber NM, Bleeker PM, et al. Determination of human platelet antigen frequencies in the Dutch population by immunophenotyping and DNA (allele-specific restriction enzyme) analysis. *Blood* 1993;81:835-40.
  31. Unkelbach K, Kalb R, Santoso S, Kroll H, Mueller-Eckhardt C, Kiefel V. Genomic RFLP typing of human platelet alloantigens Zw(P1<sup>A</sup>), Ko, Bak and Br (HPA-1, 2, 3, 5). *Br J Haematol* 1995;89:169-76.
  32. Tanaka S, Taniue A, Nagao N, et al. Genotype frequencies of the human platelet antigen, Ca/Tu, in Japanese, determined by a PCR-RFLP method. *Vox Sang* 1996;70:40-4.
  33. Matsuo K, Reid DM. Allele-specific restriction analysis of human platelet antigen system 4. *Transfusion* 1996;36:809-12.
  34. Han KS, Cho HI, Kim SI. Frequency of platelet-specific antigens among Koreans determined by a simplified immunofluorescence test. *Transfusion* 1989;29:708-10.
  35. Urwijitaroon Y, Barusrux S, Romphruk A, Puapairoj C. Frequency of human platelet antigens among blood donors in northeastern Thailand. *Transfusion* 1995;35:868-70.
  36. Santoso S, Santoso S, Kiefel V, Masri R, Mueller-Eckhardt C. Frequency of platelet-specific antigens among Indonesians. *Transfusion* 1993;33:739-41.
  37. Inostroza J, Kiefel V, Mueller-Eckhardt C. Frequency of platelet-specific antigens P1<sup>A1</sup>, Bak<sup>a</sup>, Yuk<sup>a</sup>, and Br<sup>a</sup> in South American (Mapuches) Indians. *Transfusion* 1988;28:586-7.

38. Fagan BF. The great journey: the peopling of the ancient Americans. London: Thames and Hudson, 1987.

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