

Nested Polymerase Chain Reaction With Sequence-Specific Primers Typing for HLA-A, -B, and -C Alleles: Detection of Microchimerism in DR-Matched Individuals

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It is widely accepted that donor leukocytes survive within the recipient periphery after blood transfusion or solid organ transplantation. The significance of this microchimerism remains unclear, partially because of the insecurity of assays used to detect the donor-derived material. The techniques used to detect donor-derived DNA within recipient peripheral blood rely largely on major histocompatibility complex class II polymorphism. We and others have shown that the sensitivity of polymerase chain reaction with sequence-specific primers (PCR-SSP) typing for HLA class II alleles can be increased 100-fold by the addition of a primary amplification step (nested PCR-SSP). We have now extended this technique to encompass typing for HLA class I alleles,

thereby adding flexibility to microchimerism testing by enabling testing of recipients HLA-DR matched with their donors. However, the high level of sensitivity achieved with the technique (1:100,000) leads to a concomitant decrease in the specificity that results in the amplification of unexpected products, a phenomenon we encountered in the development of our nested PCR-SSP typing system for HLA class II alleles. We describe here how it is possible to compensate for these anomalies by including multiple testing of a pre-transfusion sample that acts as a specificity control, establishing a rigorous baseline for subsequent analysis.

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IT IS WIDELY ACCEPTED that donor leukocytes can survive within a recipient after transplantation¹⁻⁴ or blood transfusion,⁵⁻⁸ yet the relevance of this microchimerism remains a contentious issue.^{4,9-13} Further controlled studies are required to achieve an improved understanding of the functional importance of microchimerism.

The majority of assays used to detect donor-derived material in recipient blood exploit sex mismatching between donor and recipient^{1,14-16} or the polymorphism of the HLA-DR region of the major histocompatibility complex (MHC).^{2,4,9,10,17} The most commonly used techniques based on HLA-DR polymorphism are the polymerase chain reaction with either sequence-specific primers (PCR-SSP) or sequence-specific oligonucleotides (PCR-SSO). These methods are commonly used to HLA type donors and recipients before transplantation,^{18,19} but may be applied to the detection of microchimerism. The sensitivity of detection of such techniques is variable and can range from 0.5% to 0.01%,^{3,20,21} but the sensitivity of PCR-SSP for

HLA-DR alleles has been increased by the introduction of a preliminary PCR (nested PCR-SSP), amplifying exon 2 of HLA-DRB1.^{4,17,22,23} It has been demonstrated that the sensitivity of nested PCR-SSP typing for HLA-DR alleles is at least 100-fold higher than that of standard PCR-SSP typing.^{17,22} Nevertheless, the use of nested PCR-SSP typing for HLA-DR alleles is not applicable when the donor and recipient are HLA-DR matched. In such cases, it would be useful to be able to increase the power of detection of the technique by applying the recently developed molecular typing methods for HLA class I alleles.^{19,24-26}

We describe here the development of a nested PCR-SSP typing method for the detection of HLA class I alleles. During the development of this method, we detected bands on the gel that were not indicative of donor or recipient HLA type, entirely consistent with our previous findings when developing a nested PCR-SSP typing system for HLA-DR alleles.²² These nonspecific bands do not arise from contamination, but result from the nonspecific amplification of recipient DNA with certain primer sets. The application of the technique to clinical samples is described, emphasizing the importance of rigorous controls for this type of assay system.

MATERIALS AND METHODS

Clinical material. Anticoagulated peripheral blood was obtained from selected HLA-typed healthy volunteers for the sensitivity experi-

Table 1. Nested PCR-SSP First-Round Primers

Primer	Binding Site	Sequence
HLA-A forward	Intron 1: 21-46	5' GAA ACS GCC TCT GYG GGG AGA AGC AA
HLA-A reverse	Intron 3: 66-89	5' TGT TGG TCC CAA TTG TCT CCC CTC
HLA-B forward	Intron 1: 36-57	5' GGG AGG AGC GAG GGG ACC SCA G
HLA-B reverse	Intron 3: 37-59	5' GGA GGC CAT CCC CGG CGA CCT AT
HLA-C forward	Intron 1: 42-61	5' AGC GAG GKG CCC GCC CGG CGA
HLA-C reverse	Intron 3: 12-35	5' GGA GAT GGG GAA GGC TCC CCA CT

Sequences of HLA-A, -B, and -C specific primers used in first-round PCR amplification (purchased from Genosys Europe, Cambridge, UK). These primers amplify a 979-bp region of HLA-A, a 940-bp region of HLA-B, and a 909-bp region of HLA-C.²⁸ Redundancies (bolded) are identified using the International Union of Biochemistry Group Codes, where K = G/T, S = C/G, and Y = C/T.

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Table 2. Primer Mix Composition for HLA Class I PCR-SSP Typing

Lane	PM	Primer	Size (bp)	Alleles amplified
1	151	286 + 431	629	A*0101-4N
2	3	296 + 302	489	A*0201-27
3	4	291 + 299	628	A*0301-4
4	5	284 + 302	464	A*2301,A*2402-14
5	6	292 + 249	557	A*2301,A*2413
6	9	193 + 298	440	A*2501-2,A*2601-11N,A*3401-2,A*6601-3
7	10	294 + 298	400	A*2501-2
8	12	174 + 298	442	A*4301
9	13	239 + 451 + 168	170	A*2609,A*3401-2
10	14	290 + 475 + 298	419	A*2502,A*3401-2,A*6601-2
11	225	290 + 303 + 167	520/552	A*1101-4,A*2502,A*6601
12	152	174 + 300	465	A*2901-3
13	18	295 + 301	561	A*3001-6
14	184	434 + 486	198	A*31012
15	153	173 + 234	259	A*3201-2
16	154	434 + 429	200	A*3301/3
17	226	193 + 302	468	A*6801-8,A*6901
18	24	290 + 171	383	A*6901
19	34	193 + 221	619	B*0702-9,B*8101
20	155	195 + 220	606	B*0801-3
21	160	202 + 272 + 285 + 393	480/546	B*1804,B*3519,B*4003/9/18,B*4402-7/9
22	42	192 + 220	605	B*4101-3
23	44	192 + 247	465	B*1533,B*4001-6/9-12/14-18,B*4101-3,B*4801/3/4
24	45	272 + 218	627	B*4001-4/6-11/14/16-18,B*4701-3
25	46	280 + 229	607	B*4001/7
26	47	243 + 215	486	B*1301-3
27	48	197 + 127	389	B*1401-4
28	49	205 + 232	182	B*1402-3/5,B*3904
29	50	207 + 217	507	B*3901-12,B*6701
30	172	208 + 435 + 217	498/508	B*3801-2
31	56	194 + 213	374	B*5801-2
32	55	194 + 224	351	B*5701-4
33	57	187 + 214	458	B*1801-5
34	58	209 + 236	422	B*3906,B*5501-3/5,B*5601,B*5901,B*7301
35	59	242 + 215	383	B*4501,B*5001-2,B*5401,B*5501-3/5,B*5601-2/4,B*8201
36	60	203 + 238	551	B*5601-4
37	61	395 + 236	421	B*5401
38	62	280 + 281 + 282	149/150	B*2701-11
39	64	192 + 392	422	B*3701,B*3902/8
40	65	192 + 228	414	B*3702,B*4701-3
41	67	203 + 220	594	B*4021-2
42	69	194 + 225	516	B*1516-17
43	70	240 + 241	460	B*4601
44	72	243 + 250	124	B*1501-2/4-8/11-12/14-15/19-21/25-28/30-35/38-40
45	73	192 + 214	421	B*1304,B*1501/3-7/12/14/19-20/24-27/32-35/38-40,B*4802
46	79	193 + 223	369	B*1502/13/21,B*3501-4/6-9/11-12/15/18-21/23-24,B*4406,B*5104,B*5301-2
47	80	188 + 237	128	B*1522,B*1801-5,B*3501-13/15-18/20-24,B*7801-2
48	81	195 + 213 + 277	389/390	B*3501-9/11/15/17-19/21/23-24,B*5301-2
49	82	207 + 216	400	B*1509,B*7801-2
50	84	193 + 216	451	B*1509,B*5101-9/11N,B*7801-2
51	85	192 + 216	440	B*5201
52	86	368 + 315	340	Cw*0102-3
53	87	366 + 145	522	Cw*0202-3,Cw*1701-2
54	88	368 + 389	564	Cw*0302-6/8-9
55	89	366 + 143	331	Cw*0401-5,Cw*1801-2
56	90	366 + 379	564	Cw*0501
57	91	367 + 127	297	Cw*0602-4
58	93	313 + 184	516	Cw*0701/6/7
59	94	367 + 183	302	Cw*0702/3/10
60	275	271 + 143	500	B*8201,Cw*0708,Cw*1801-2
61	96	367 + 379	536	Cw*0704
62	182	165 + 166 + 317	160/625	Cw*0801-4
63	99	159 + 389	523	Cw*0303
64	201	130 + 160 + 389	206/522	Cw*0302/4-6/8-9
65	245	507 + 215	407	Cw*1502
66	102	369 + 126	538	Cw*1202
67	103	368 + 157	446	Cw*1203/6
68	104	371 + 388	541	Cw*1402-3
69	106	366 + 223	318	Cw*1502-6
70	107	366 + 382	502	Cw*0203,Cw*0404,Cw*0604,Cw*0707,Cw*1502-6,Cw*1701-2
71	109	368 + 146	513	Cw*1601
72	110	366 + 146	503	Cw*1602
73	111	366 + 377	502	Cw*0202,Cw*0602-3,Cw*1204-5

All primer mixes have previously been described in Bunce et al.¹⁹
Abbreviations: PM, primer mix; Size, size of PCR product.

ments. To validate the technique, peripheral blood samples were obtained from patients at the Oxford Transplant Centre (Oxford, UK) awaiting a renal transplant who had received planned transfusions of HLA-typed blood. Each patient received 50 mL of freshly isolated blood (<36 hours from donation) from 2 healthy donors. Blood samples were obtained at 2 time points before the transfusion and at defined time points after transfusion.

DNA isolation. Genomic DNA was isolated from leukocytes obtained from anticoagulated blood using a salting out procedure,²⁷ was precipitated with ethanol, and was resuspended in sterile water. The genomic DNA was quantitated and purity was assessed by spectroscopic absorbance at 260 and 280 nm.

Nested PCR-SSP typing. Two hundred nanograms of DNA was initially amplified in a buffer containing 67 mmol/L Tris, pH 8.8; 16.6 mmol/L NH_4SO_4 ; 200 $\mu\text{mol/L}$ of each dATP, dCTP, dGTP, and dTTP; 1.0 mmol/L MgCl_2 ; 0.5 $\mu\text{mol/L}$ forward and reverse primer (Table 1); and 0.25 U BioTaq polymerase (Bioline, London, UK) for 30 cycles according to Cereb et al²⁸ in a PTC200-96v thermal cycler (Genetic Research Instrumentation, Essex, UK). The resultant PCR product was diluted 1:500 in water before PCR-SSP typing. Extreme care was taken to avoid contamination at any stage.²⁹ Filter tips were used when pipetting (BioExpress UK Ltd, London, UK), and the work was performed in a clean air cabinet (Heto-Holten UK Ltd, Camberley, UK).

PCR-SSP typing. Allele-specific primers (0.5 $\mu\text{mol/L}$) designed on the basis of published sequences^{19,30} (Table 2) were used in multiple amplification reactions consisting of diluted first-round PCR product (1/500); 67 mmol/L Tris, pH 8.8; 16.6 mmol/L NH_4SO_4 ; 200 $\mu\text{mol/L}$ of each dATP, dCTP, dGTP, and dTTP; 2.0 mmol/L MgCl_2 ; and 0.25 U BioTaq polymerase. PCR amplifications were performed in a PTC200-96v thermal cycler, and the products were subsequently analyzed by agarose gel electrophoresis using the conditions exactly as previously described.¹⁹

Sensitivity of nested PCR-SSP typing. Primer mixes 151 (A*0101-4N), 9 (A*2501-2, A*2601-11N, A*3401-2, A*6601-3), 155 (B*0801-3), 47 (B*1301-3), 86 (Cw*0102-3), and 106 (Cw*1502-6) (Table 2) were randomly selected to determine the sensitivity of nested PCR typing. The experiments were performed as previously described and repeated on three occasions.²² Briefly, decreasing amounts of DNA from selected HLA-typed healthy volunteers were mixed with DNA of known HLA type, both at a starting concentration of 200 ng/ μL , to give final concentrations of 1%, 0.1%, 0.01%, and 0.001% (vol/vol). The

mixtures were then subjected to nested PCR-SSP typing. All products were subsequently analyzed by agarose gel electrophoresis.

RESULTS

Sensitivity of nested PCR-SSP typing for HLA class I alleles.

The sensitivity of detection of 6 of the HLA class I primer mixes (151, 9, 155, 47, 86, and 106; see Table 2) was determined by performing mixing experiments, and the results are shown in Fig 1. Each primer mix was capable of reproducibly detecting DNA to a level of 0.001% (equivalent to a dilution of DNA of 1:100,000), demonstrating that the sensitivity of this technique is comparable to that of other nested PCR-SSP techniques.^{17,22}

Application of nested PCR-SSP typing to the detection of microchimerism. Samples were investigated from a patient who had received planned HLA-typed blood transfusions mismatched for HLA class I alleles. Samples obtained before and 2 days after blood transfusion were compared (Fig 2). All recipient alleles were detected in both samples (arrowed) and, in the posttransfusion samples, additional bands corresponding to the HLA alleles of both blood transfusion donors were evident (asterisked). However, nonspecific products also appeared in certain lanes on the gels, a finding consistent with our results of nested PCR-SSP typing for HLA-DR.²² The bands were not compatible with the known HLA type of the donors or the recipient. The pattern of bands generated by the nested PCR-SSP typing of the pretransfusion sample remains consistent in the posttransfusion samples and, thus, by inference, is recipient HLA-type dependent.

To control for the presence of the nonspecific products resulting from mispriming, a specificity control in the form of a pretransfusion sample is required. Rigorous analysis of this sample establishes a baseline for the analysis of posttransfusion samples. Therefore, nested PCR-SSP typing is routinely performed 5 times on 2 samples obtained before transfusion and on samples after transfusion. The results from one such analysis of the HLA-A locus are shown in Fig 3.

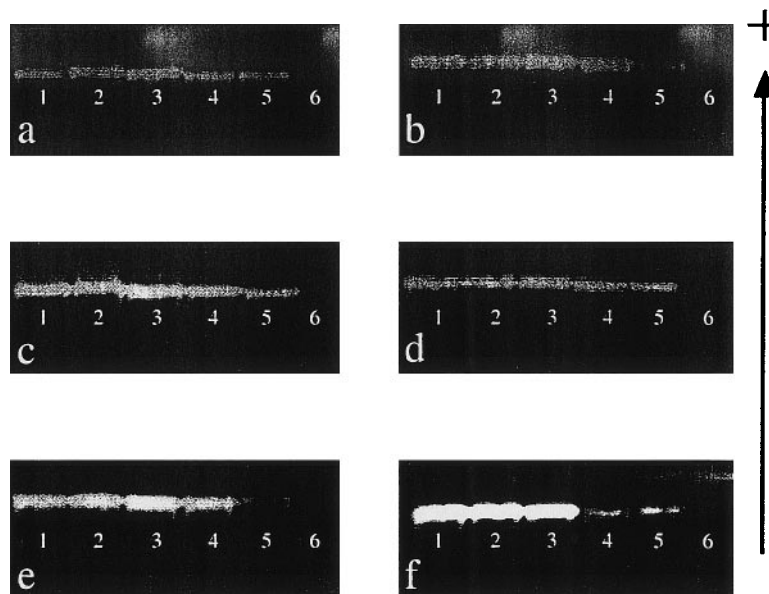


Fig 1. Sensitivity of nested PCR-SSP typing for HLA class I alleles. (a) Primer mix (PM) 151 (A*0101-4N); (b) PM 9 (A*2501-2, A*2601-11N, A*3401-2, A*6601-3); (c) PM 155 (B*0801-3); (d) PM 47 (B*1301-3); (e) PM 86 (Cw*0102-3); and (f) PM 106 (Cw*1502-6). DNA of known HLA type (200 ng/ μL) was mixed with an irrelevant DNA (200 ng/ μL) to give relative final concentrations of 10% (lane 1), 1% (lane 2), 0.1% (lane 3), 0.01% (lane 4), and 0.001% (lane 5). Lane 6 is a specificity control in which the irrelevant DNA was amplified on its own. Each mix was then subject to a primary amplification with the appropriate set of first-round primers.²⁸ The resultant product was diluted 1:500 in water before PCR-SSP typing using the method of Bunce et al.¹⁹ The gel is run from negative (-) to positive (+). Sensitivity experiments were performed on 3 occasions, and each primer mix was reproducibly capable of detecting DNA at a final concentration of 0.001%.

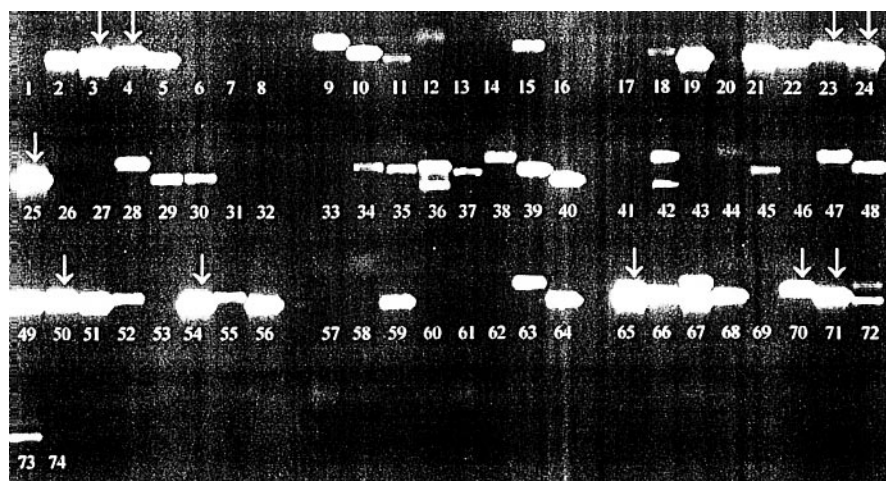
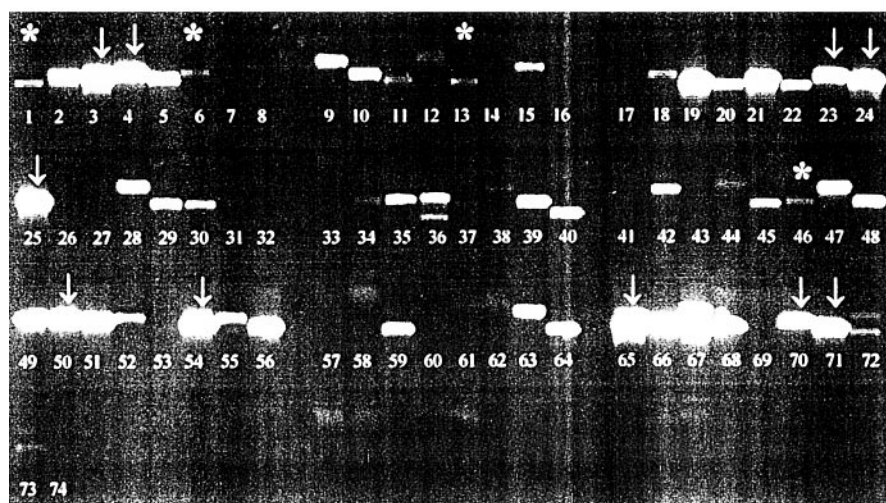
A**B**

Fig 2. Detection of microchimerism using nested PCR-SSP typing. (A) Pretransfusion DNA sample (recipient HLA type: A*0301, A*2402; B*4001, B*51011; Cw*0304, Cw*1502, all recipient bands arrowed; other bands are nonspecific). (B) Posttransfusion DNA sample (blood donor 1 HLA type: A*0101; B*0801, B*4402; Cw*0701, Cw*0704; blood donor 2 HLA type: A*2501, A*3002; B*3501, B*5501; Cw*0303, Cw*0401). Asterisked bands are those donor alleles detected; the pattern of recipient alleles and nonspecific bands is the same as that of a pretransfusion sample. Patient DNA was isolated from peripheral blood leukocytes and used in a primary amplification using HLA-A, -B, or -C primers.²⁸ The resultant products were diluted 1:500 and used in a PCR-SSP typing system.¹⁹ The gel is run from negative (-) to positive (+).

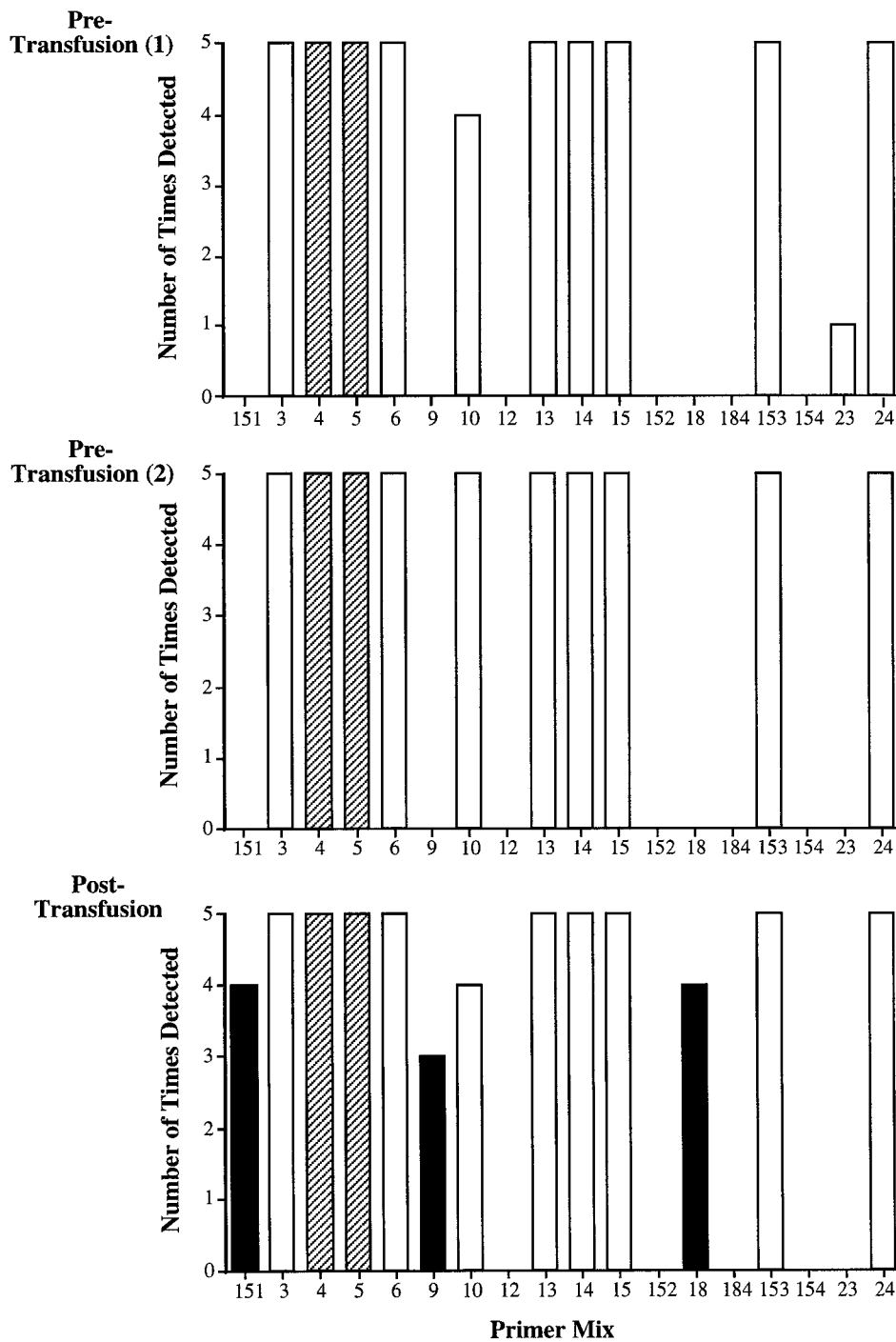
The recipient HLA type (A*0301, A*2402) is present in all repeats of the pretransfusion and posttransfusion samples (PM 4 and 5; dark shading). Potentially informative reactions are those reactions that do not yield a band in the pretransfusion samples (in this case, primer mixes 151, 9, 12, 152, 18, 184, and 154). The blood donors were typed as HLA-A*0101 (donor 1) and HLA-A*2501, A*3002 (donor 2), and these alleles are recognized by primer mixes 151 (A*0101), 9 (A*2501), 10 (A*2501), and 18 (A*3002). All of these primer mixes gave rise to a band when the posttransfusion sample was analyzed, but in the case of primer mix 10, a band was also evident in both pretransfusion samples. The reaction with primer mix 10 is therefore termed a noninformative reaction. Furthermore, there were other primer mixes that gave rise to bands in the pretransfusion samples that were also present in posttransfusion samples (PM 3, 6, 13, 14,

15, 153, and 24). The presence of these nonspecific products is dependent on the recipient HLA type.

DISCUSSION

In this report, we have described a method for the detection of HLA class I donor alleles after blood transfusion using the nested PCR-SSP technology previously developed for HLA-DR alleles.^{17,22,31} The technique is finely tuned to achieve maximum sensitivity while retaining specificity; for example, increased sensitivity can be achieved by increasing the amount of DNA in the first-round amplification, but such a modification will result in a decrease in specificity. The sensitivity of the technique has been demonstrated using mixing experiments and DNA can be detected to a level of at least 0.001% (equivalent to a dilution of

Fig 3. Validation of nested PCR-SSP typing. Recipient 01 (HLA-A type: A*0301, A*2402) was transfused with fresh blood from 2 healthy, HLA-typed volunteers (donor 1: A*0101; donor 2: A*2501, A*3002). The bar graph shows the results of amplification of 2 pretransfusion samples and a posttransfusion sample by primer mixes for the HLA-A locus alleles used in this system (Table 2). Analysis of both pretransfusion samples produces a similar pattern of amplification in which primer mixes 4 and 5 represent recipient alleles (A*0301) and (A*2402), respectively (dark shading). Potentially informative primer mixes are those that do not give rise to nonspecific products in any of the multiple tests of the pretransfusion samples. In this case, these are primer mixes 151, 9, 12, 152, 18, 184, and 154. Analysis of the posttransfusion samples shows additional amplification with primer mix 151, which is indicative of donor 1 (A*0101), and primer mixes 9 and 18, which are both indicative of donor 2 (A*2501 and A*3002, respectively; hatched shading). Primer mixes 3, 6, 10, 13, 14, 15, 153, 23, and 24 detect nonspecific products in the pretransfusion samples and thus are noninformative in the analysis of posttransfusion samples (light shading). The requirement for testing pretransfusion samples is demonstrated by the results with primer mix 10, which potentially should amplify A*2501 from donor 2. In this example, although there was amplification in the posttransfusion sample, the reaction has to be excluded from the analysis because of the nonspecific amplification present in the pretransfusion samples.



1:100,000), comparable to the techniques developed for the detection of HLA-DR alleles.^{17,22}

The high level of sensitivity achieved with the technique leads to a concomitant decrease in specificity. When the nested PCR-SSP typing was applied to the detection of donor-type microchimerism in a patient receiving an HLA-mismatched blood transfusion, nonspecific products appeared that did not correspond to the donor or recipient HLA type. This finding is

entirely consistent with our previous results obtained while developing a similar system for the detection of HLA-DR alleles. In the previous study, we sequenced several of these nonspecific products and determined that they did not result from contamination but rather from mispriming events that led to amplification of recipient-derived HLA alleles or associated pseudogenes.²² It is therefore possible that some of the nonspecific products seen in the HLA class I nested PCR-SSP typing

system result from the amplification of HLA class I-associated pseudogenes, of which 6 have been currently identified.^{32,33} Therefore, to use nested PCR-SSP typing to analyze clinical samples, a means of carefully controlling the system is required.

In the system we have devised, a pretransfusion blood sample is analyzed to act as a baseline and specificity control. Multiple analysis of this sample with nested PCR-SSP typing allows a rigorous baseline to be established for all subsequent analyses (Figs 2 and 3). If such a sample is not included in the analysis, then amplification appearing in posttransfusion samples may be wrongly assumed to be indicative of donor alleles. For example, in Fig 2, primer mix 10, which amplifies the donor allele HLA-A*2501, yields a band in the posttransfusion sample suggesting the presence of microchimerism. However, this band is also detected in the pretransfusion sample; therefore, the amplification in the posttransfusion samples is noninformative. The requirement for a pretransfusion/transplant sample has also been shown by Elwood et al,⁴ who, by using nested PCR-SSP typing for HLA-DR alleles, demonstrated that false-positive results for microchimerism would have been obtained in 17% of patients had a recipient pretransplant DNA sample not been included in the analysis.

The inclusion of HLA-A, -B, and -C alleles in the nested PCR-SSP system significantly increases the power of this type of analysis where donors and recipients are frequently matched for HLA-DR alleles. The technique is flexible and widely applicable to the detection of microchimerism after blood transfusion or solid organ transplantation and may provide the means for understanding the true relevance of microchimerism.

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