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Research Article

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Rapid Determination of Clonality by Detection of Two Closely-linked X Chromosome Exonic Polymorphisms Using Allele-specific PCR

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Abstract

We reported two specific, reproducible, and quantitative clonality assays based on detection of exonic polymorphisms of the X chromosome genes p55 and G6PD using rtPCR-LDR. These assays are inconvenient for screening purposes. This study sought to develop a simple, reproducible assay, practical for screening genomic DNA samples for p55/G6PD genotypes, rapid clonality determination, and to determine the linkage relationship between these closely related loci. The salient feature of ASPCR is the performance of two PCR rounds. The first generates template; the second, using one aliquot of first-round products in two reaction tubes, each containing one allele-specific primer, detects each allele. ASPCR and rtPCR-LDR produced identical p55/G6PD results in 91 normal female genomic DNAs, and in 12 clonal hematopoietic disorder cDNAs, confirming assay validity. 209 female and 207 male genomic DNA samples were analyzed for p55/G6PD genotype by ASPCR; 60% of females were heterozygous at one or both loci. G6PD and p55 allelic frequencies were significantly different among African-American men and women, but were not significantly different among Caucasian men and women. These loci were in linkage equilibrium among African Americans, but not among Caucasians. ASPCR is a rapid, sensitive, and specific method for screening large numbers of genomic DNAs, and for rapid clonality determination. (*J. Clin. Invest.* 1997. 99:1984–1990.) Key words: allele specific PCR • clonality • gene frequency • G6PD • p55

Introduction

Clonality assays based on the Lyon/Beutler hypothesis of random X chromosomal inactivation (1, 2) have been instrumental in broadening knowledge about normal and abnormal hematopoiesis. The first clonality assays exploited electro-

phoretically distinguishable G6PD isoenzymes present in Africans (30% of African women are heterozygous) (2). Second-generation clonality assays employ methylation-sensitive restriction endonucleases to detect methylation pattern differences between transcriptionally active versus inactive X chromosome linked polymorphic loci (i.e., PGK [3, 4], HPRT [5], m27 beta [6, 7], and HUMARA [8, 9]). These assays are limited by racial specificity, lack of quantitation, and inapplicability to nonnucleated cells such as platelets and reticulocytes. We recently developed a clonality assay based on transcriptional polymorphisms of two ubiquitously expressed X chromosome genes; G6PD (C/T at 1311) and palmitoylated membrane protein, p55 (G/T at cDNA 358) (10, 11, 12). The assay uses reverse transcription polymerase chain reaction followed by ligase detection reaction (rtPCR-LDR)¹ (10, 11, 12) to distinguish the transcriptionally active X chromosome. The assay is sensitive, specific, reproducible, quantitative, and applicable to nonnucleated cells. The screening of populations for informative subjects using polymerase chain reaction-ligase detection reaction (PCR-LDR), however, is laborious, time-consuming, expensive, and entails extended exposure to radioactive material. To overcome these disadvantages, we have modified the previously reported techniques of allele-specific oligonucleotide hybridization (ASOH) (13), PCR-ASOH (14), and PCR amplification of specific allele (PASA) (15) into a simpler assay (allele-specific PCR) for genotype screening and clonality determination based on G6PD and p55 exonic polymorphisms (16). Allele-specific PCR (ASPCR) requires two rounds of PCR. The first round generates a high concentration of DNA template (from either genomic DNA or cDNA) containing the polymorphic region of interest. The second round, using the first round products as a template, distinguishes each polymorphic allele by using allele-specific primers. Using ASPCR, 209 human female genomic DNA samples of defined racial groups were analyzed to determine the degree of heterozygosity (and thus informativeness for clonality studies) at these closely apposed X chromosome exonic polymorphic sites. 207 human male genomic DNA samples of defined racial groups were analyzed for G6PD and p55 haplotype determination, calculation of allelic frequencies, and determination of linkage equilibrium/disequilibrium. Linkage studies were conducted because multiple crossovers were noted, despite the observation that these two loci are proximate on the X chromosome. We have determined the respective allelic frequencies for both loci, and have conducted linkage analysis. In addition, we demonstrate that clonality can be determined rapidly, qualitatively, and without using radioactive material by employing rtPCR followed by ASPCR.

Yunying Liu and John Phelan contributed equally to this manuscript, and should both be considered first authors.

A portion of these data was presented in preliminary form at the 36th annual convention of the American Society of Hematology.

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1. *Abbreviations used in this paper:* ASOH, allele-specific oligonucleotide hybridization; ASPCR, allele-specific PCR; LDR, ligase detection reaction; PASA, PCR amplification of a specific allele.

Methods

Subjects. To examine large numbers of individuals for the polymorphic genotypes, peripheral blood samples from unselected individuals of three defined racial groups: Caucasians, African-American, and Asians (Chinese and Vietnamese) were used as a source of DNA.

Isolation of myeloid cells. Peripheral blood samples anticoagulated with EDTA were used. Granulocytes, mononuclear cells, platelets, and reticulocytes were separated by differential centrifugation, isopycnic density gradient separation, and dextran sedimentation using standard protocols (17).

DNA isolation and preparation. Genomic DNA samples were prepared from 5–10 ml of peripheral blood. Red blood cells were lysed utilizing a $\text{NH}_4\text{HCO}_3/\text{NH}_4\text{Cl}$ buffer system. This was followed by overnight incubation at 37°C with nuclear membrane lysis buffer, 1% SDS, and proteinase K (1 U/ml; Applied Biosystems Inc., Foster City, CA). Impurities were precipitated out with saturated NaCl (18), and DNA was extracted with 2 vol of 100% ethanol followed by 70% ethanol. After air-drying, the DNA pellet was dissolved in 200–400 μl of TE buffer or distilled/deionized water, and stored at 4°C.

Preparation of mRNA. mRNA was prepared by acid guanidium thiocyanate-phenol/chloroform extraction and ethanol precipitation from the isolated myeloid cells (19). RNA samples were dissolved in water treated with DEPC, and were stored at -80°C until analysis.

Reverse transcription of mRNA. The aliquots of mRNA purified from separated myeloid cells were transcribed to first-strand cDNA using SuperScript RT (GIBCO BRL, Gaithersburg, MD) with random hexanucleotide primers (Pharmacia Biotech, Uppsala, Sweden).

Allele-specific PCR. ASPCR consists of two rounds of PCR (see Fig. 1). In the first round, high concentration and purity products containing the region of interest are generated. 1–10 μl of genomic DNA, or 5 μl of cDNA is added to a 50- μl reaction volume composed of

PCR buffer, 2 mM MgCl_2 , 1 unit of Taq polymerase (GIBCO BRL), 0.2 mM dNTP, 25 pmol of forward primer (6J-p55 or 7g-G6PD), and 25 pmol of reverse primer (8gR-p55 or 9gR-G6PD). 35 cycles are carried out in a GeneAmp PCR System 9600 (Perkin Elmer, Emeryville, CA). Each cycle consisted of 40 s at 94°C for denaturation, 1 min at 62°C for annealing, and 1 min at 72°C for elongation. The second round was carried out in two reaction tubes. Each tube contained a 50- μl reaction volume composed of PCR buffer, 1.5 mM of MgCl_2 , 1 U of Taq polymerase (GIBCO BRL), 0.2 mM dNTP, and 10 pmol of allele-specific primer as forward primer (e.g., 1gT or 3gG for p55/3C, or 4T for G6PD), and the same reverse primer as that used in the first round of PCR (8gR for p55 or 9gR for G6PD). 5 μl of first-round products was used as a template for the second round. The second round consisted of five cycles performed in the same GeneAmp PCR system (30 s at 94°C for denaturing; 1 min at 64°C for annealing, and 1 min at 72°C for elongation). 15 μl of products (from each second-round reaction tube) were then analyzed directly on 1% agarose gel with 0.5 $\mu\text{g}/\text{ml}$ ethidium bromide. The first-round products were always visualized under UV light as the same high molecular weight band on both first- and second-round agarose gels, and thus served as an internal control. The sequences of the oligonucleotide primers employed in the ASPCR assay are depicted in Table I.

For rapid determination of clonality, the template for the first round of PCR was cDNA synthesized from total RNA by reverse transcription. Second-round PCR was conducted in an identical fashion as with genomic DNA.

Ligase detection reaction (LDR). The LDR assays of p55 and G6PD were performed using PCR products generated from genomic DNA or cDNA templates as previously reported (10–12). The 10- μl reaction volume consisted of the following: (a) 10 ng of template, (b) 20 U of cloned thermostable ligase (20) (a kind gift of Dr. Barany), (c) 5 pmol of each allele-specific oligonucleotide (as 5' oligonucleotides),

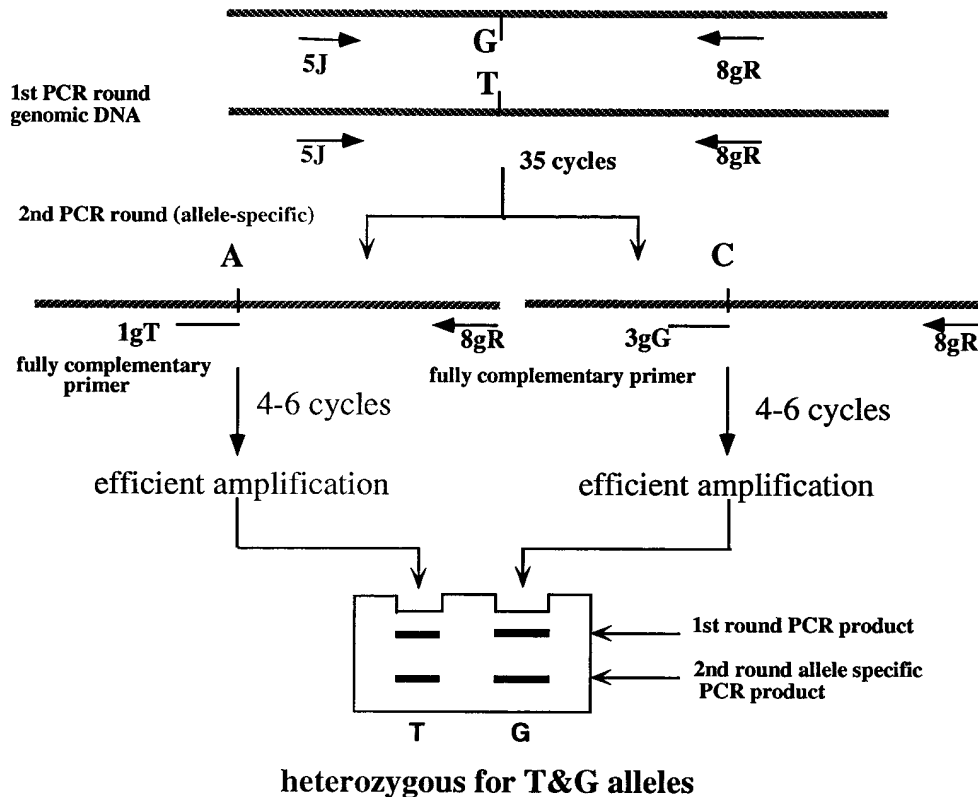


Figure 1. Outline of allele-specific PCR (ASPCR) using detection of p55 X chromosome exonic polymorphism (G/T at cDNA 358) as an example: detection of G and T alleles in a heterozygous individual. First-round PCR products serve as an internal control. Well-designated G has G-specific allele primers. Well-designated T has T-specific allele primers. Genomic DNA from homozygote individuals will generate second-round allele-specific products in only one well. Genomic DNA from heterozygous individuals will generate second-round allele-specific products in both wells. Clonal samples (using cDNA from heterozygous patients) will show second-round allele-specific products in only one well. Nonclonal samples (using cDNA from heterozygous patients) will show second-round allele-specific products in both wells. The same principles apply to procedures used for detection of G6PD X chromosome exonic polymorphism (C/T 1311) by this assay.

Table I. Primer Sequences Used in ASPCR for p55 and G6PD

Gene	Primer	Length	Sequence 5' to 3'	Temp.	Paired with
		<i>bp</i>		<i>°C</i>	
p55	6J #/@	25	TCAAGGGACAGGAGGTGCGGAAAGT	57	8gR
	9J @	25	CAGAGGCTGTATCGCATCCATTGAA	53.2	8gR
	8gR #/@	25	TTTGGTTTCTTTCATCGTCTTCTGC	54	6J/9J
	1gT#	24	TCGCCTTTATTCAGGGAATCACT	70.5	8gR
	3gG#	26	TATCGCCTTTATTCAGGGAATCACG	71	8gR
	1T@	21	TCAGAGCCCATGGGAATCACT	74	8gR
G6PD	2G@	23	TCTCAGAGCCCATGGGAATCAC	78	8gR
	7g#	21	TTTGCAGCCGTCGTCCTCTAT	47.6	9gR
	9gR#	21	TGCAGCAGTGGGGTGAATA	46.6	7g
	3C #/@	23	CTGTGAAGCTCCCTGACGCCTAC	55.7	9gR
	4T #/@	25	CTTTGTGAAGCTCCCTGACGCCTAT	55.7	9gR
	1@	20	TGATGACCAAGAAGCCGGGC	60.8	2R
	2R@	20	TGGGCTTGGGCTTCTCCAGC	62.3	1
	3@	20	CTTCAACCCCGAGGAGTCGG	59.5	4R
	4R@	20	TCTGGTGCAGCAGTGGGGTG	60.5	3

For the second PCR round, in p55 ASPCR assay, different forward primers for genomic DNA and cDNA analysis are required because of the nearness of the polymorphic site to the intron/exon boundary (# denotes allele specific primers for genomic DNA, @ for cDNA). Forward primers for genomic DNA and cDNA in G6PD ASPCR are the same, because the polymorphic site is located well within exon 11.

and (d) the common downstream ligation oligonucleotide partner (5' end-labeled with ³²P by T4 kinase). Five cycles (1 min at 94°C, 4 min at 64°C) were performed using a Thermocycler 480 (Perkin Elmer Applied Biosystems, Foster City, CA). 4 µl of the resulting reaction products plus 9 µl of formamide stop buffer were then loaded onto a 15% polyacrylamide gel with 7 M urea. After 2 h of electrophoresis at 60 W, products were visible on the autoradiogram (see Fig. 2).

Calculations of gene allele frequencies and linkage disequilibrium. Maximum likelihood methods (21) were used to calculate the gene frequencies of the different alleles given the genotypic distributions in each racial group. The z test for difference in binomial proportions (22) was used to identify any significant gene frequency differences among men and women of specified racial groups (race and gender). A significant difference among the genders indicated that the gene frequencies at these X-linked loci were not in equilibrium. To test for significant deviations from linkage equilibrium (D = 0), a Chi-square test (23–25) was applied to the male haplotype data.

Results

Specificity of ASPCR. Identical genotypes were obtained in all 91 female genomic DNA samples analyzed simultaneously with ASPCR and PCR-LDR p55 or G6PD assays. In Fig. 2, parallel data obtained by two methods of three individuals tested in p55 assay and of another three individuals tested in

G6PD assay are depicted, demonstrating that ASPCR reliably determines both G6PD and p55 exonic X chromosome polymorphic genotypes.

Determination of clonality by rtPCR-ASPCR. 12 heterozygous females known to have clonal hematopoietic disorders as defined by rtPCR-LDR assay, had cDNA of their clonal tissues reevaluated using the rtPCR-ASPCR assay. In all 12, the same clone-defining allele previously identified by rtPCR-LDR was similarly identified by rtPCR-ASPCR. This demonstrates that rtPCR-ASPCR is suitable as a qualitative clonality assay. ASPCR analysis (p55 assay) of peripheral blood platelets of patients 1 and 2 as well as that of a subject with a non-clonal hematological disorder (patient 3) is shown in Fig. 3. Similar data were obtained for G6PD (data not shown). Genomic DNA analysis, documenting p55 heterozygosity is shown in A and cDNA analysis identifying p55 clonality is shown in B.

Frequency of heterozygosity (Table II). Among the 209 human female genomic DNA samples analyzed, 48% were heterozygous in the p55 locus, and 27% were heterozygous in the G6PD locus. When both loci were considered together, 43% of African-Americans, 50% of Asians, and 44% of Caucasians were heterozygous at either locus. Moreover, 18% of African-American, 9% of Asian, and 15% of Caucasian females were

Table II. Exonic Polymorphism Genotypes and Genotype Frequencies in African-American, Asian, and Caucasian Women

	p55			G6PD			
	n	Homozygous-GG (p)	Homozygous-TT (q)	Heterozygous-G/T (pq)	n	Homozygous-CC (p)	Heterozygous-C/T (pq)
African-American	49	18 (0.37)	10 (0.20)	21 (0.43)	49	28 (0.57)	3 (0.06)
Caucasian	126	19 (0.15)	41 (0.33)	66 (0.52)	126	95 (0.75)	2 (0.02)
Asian	34	4 (0.12)	16 (0.47)	14 (0.41)	34	24 (0.71)	1 (0.03)
Total	209	41 (0.20)	67 (0.32)	101 (0.48)	209	147 (0.70)	6 (0.03)

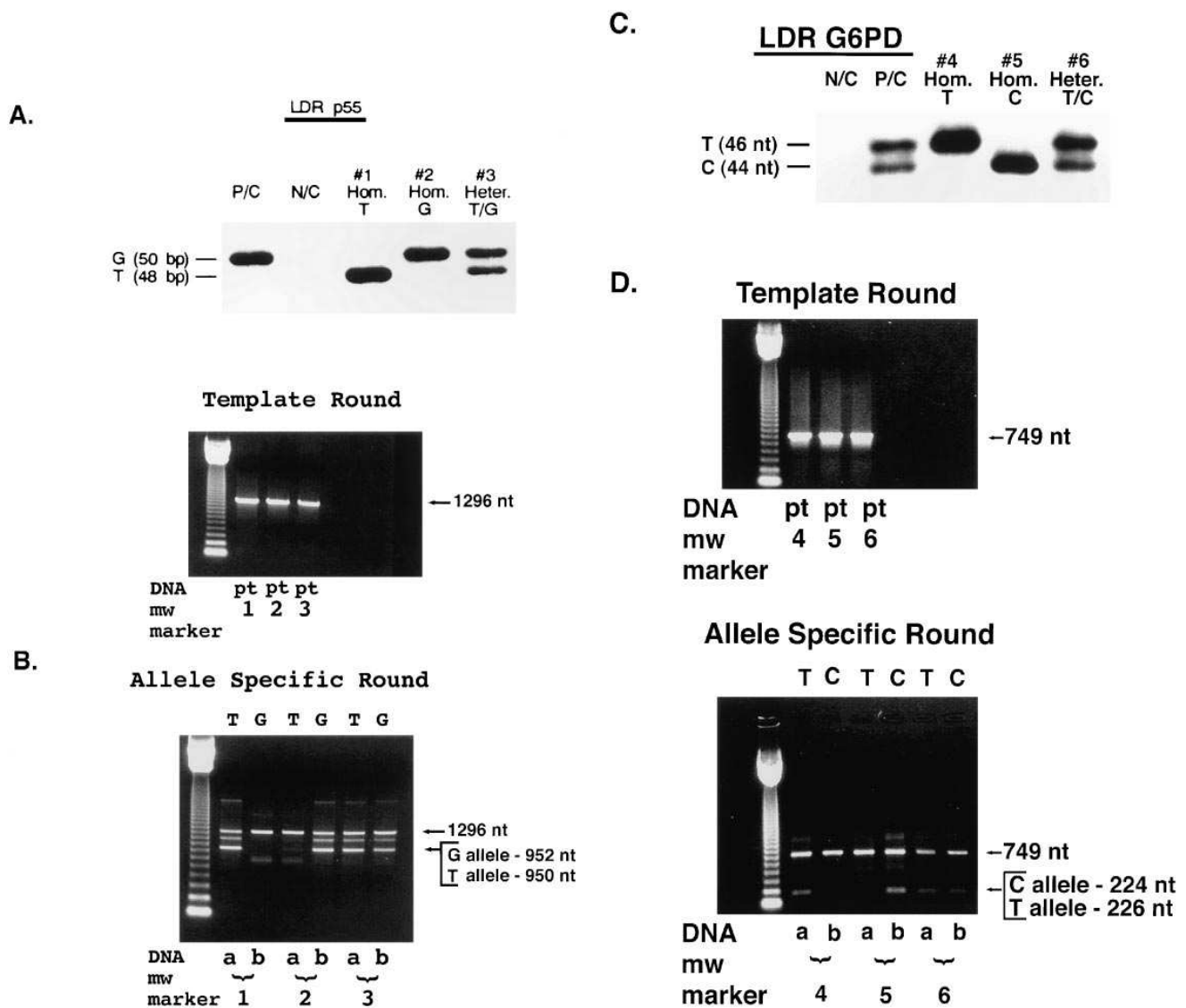


Figure 2. Specificity of ASPCR. Representative p55 exonic polymorphism genotype analysis of three human female genomic DNA samples analyzed by rtPCR-LDR (A) and ASPCR (B). (A) Representative PAGE/autoradiogram of rtPCR-LDR for p55 exonic polymorphism genotype analysis of three human female genomic DNA samples. G designates a 50-bp ligation product consisting of a G allele-specific oligonucleotide ligated to a radiolabeled common downstream oligonucleotide. T designates a 48-bp ligation product consisting of a T allele-specific oligonucleotide ligated to a radiolabeled common downstream oligonucleotide. Lane P/C represents a positive control using genomic DNA from a known G allele homozygote. Lane N/C represents a negative control wherein the genomic DNA template was substituted with water before the LDR step. Lane 1 represents analysis of individual homozygous for T allele; lane 2 represents analysis of individual homozygous for G allele; and lane 3 represents individual heterozygous for G and T alleles. (B) Representative agarose gel electrophoreses of ASPCR for p55 exonic polymorphism genotype analysis of three human female genomic DNA samples (same individuals as in A). *Template round* represents 35-cycle amplification of that portion of the gene containing the polymorphism of interest (p55 gene in this instance). The high concentration/purity product from this round serves as a template for the next round (allele-specific round). In the second (or allele-specific) round, two aliquots from each template round reaction are subjected to six cycles of PCR amplification; an allele-specific forward primer (G-specific or T-specific for the p55 locus) is used for each reaction. Under these PCR conditions, only perfectly complementary primer-template pairs will undergo amplification efficiently enough to generate products visible by agarose gel electrophoresis. Each individual tested is thus represented by two gel lanes. The topmost bright band in each lane represents first-round generated template, and serves as an internal control. The presence of a second bright band demonstrates an allele-specific PCR product. Lane 1a/b represents an individual homozygous for the p55 T allele. Lane 2a/b represents an individual homozygous for the G allele. Lane 3a/b represents an individual heterozygous for both the G and T alleles. The fainter band between the first and allele-specific round products represents heterodimer formation. (C) Representative PAGE/autoradiogram of rtPCR-LDR for G6PD exonic polymorphism genotype analysis of three human female genomic DNA samples. C designates a 46-bp ligation product consisting of a C allele-specific oligonucleotide ligated to a radiolabeled common downstream oligonucleotide. T designates a 44-bp ligation product consisting of a T allele-specific oligonucleotide ligated to a radiolabeled common downstream oligonucleotide. Lane P/C represents a positive control using genomic DNA from a known G allele homozygote. Lane N/C represents a negative control wherein the genomic DNA template was substituted with water before the LDR step. Lane 1 represents analysis of individual homozygous for T allele; lane 2 represents analysis of individual homozygous for C allele; and lane 3 represents individual heterozygous for C and T alleles. (D) Representative agarose gel electrophoreses of ASPCR for G6PD exonic polymorphism genotype analysis of three human female genomic DNA samples. *Template round* represents 35-

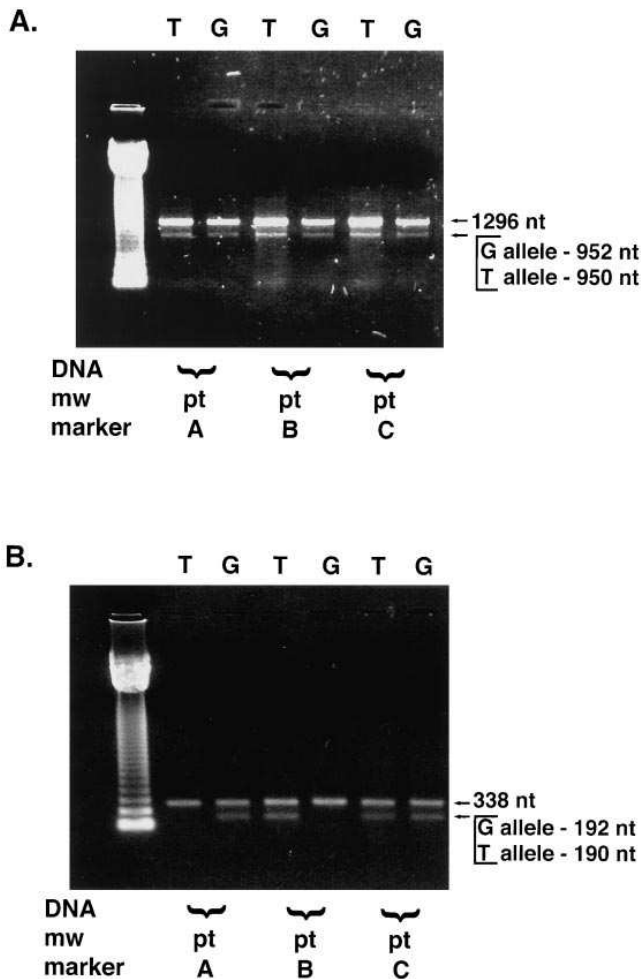


Figure 3. Determination of clonality by rtPCR-ASPCR. (A) Screening ASPCR (allele-specific round depicted) performed on genomic DNA isolated from the peripheral blood mononuclear cells of three females with hematological abnormalities. All are identified as heterozygous for the G/T exonic polymorphism at the p55 locus. Patient A was diagnosed with Fanconi anemia; patient B was diagnosed with essential thrombocythemia; and patient C was found not to have a primary hematological disorder. (B) Total RNA was isolated from peripheral blood of patients A, B, and C, subjected to reverse transcription (using p55 specific primers), and the resulting cDNAs were subjected to ASPCR. The allele-specific round is depicted here. Patients A and B, though genotypically heterozygous at the p55 locus, are here shown to transcribe only one allele, and thus demonstrate clonal hematopoiesis. Patient C demonstrates nonclonal hematopoiesis.

Table III. Exonic Polymorphism Allele Number and Allelic Frequencies in African-American and Caucasian Males and Females and Asian Females (Determined in Females by Chromosome Counting)

Allele	African-Americans		Caucasians		Asians
	Females	Males	Females	Males	Females
p55	98	105	252	102	68
G	57 (0.58)	78 (0.74)	104 (0.41)	37 (0.36)	22 (0.32)
T	41 (0.42)	27 (0.26)	148 (0.59)	65 (0.64)	46 (0.68)
G6PD	98	105	252	102	68
C	74 (0.76)	92 (0.88)	219 (0.87)	92 (0.90)	57 (0.84)
T	24 (0.24)	13 (0.12)	33 (0.13)	10 (0.10)	11 (0.16)

In 209 women and in 207 men, there are 615 alleles total.

heterozygous at each locus (doubly heterozygous). When combined, 60% of the human females analyzed were heterozygous at one or both loci, and thus were potentially informative for further clonality evaluation.

p55 and G6PD allelic frequencies. Genomic DNA samples from 209 human females of African-American, Asian, and Caucasian origin, and 207 human males of African-American and Caucasian origin (representing a total of 615 X chromosomes) were analyzed by ASPCR. Allelic frequencies for both males' and females' p55 and G6PD exonic polymorphisms are depicted in Table III. The χ^2 test for difference in binomial proportions was used to test for statistically significant allelic frequency differences between the genders in the African-American and Caucasian racial groups. The intergender p55 and G6PD allelic frequencies were significantly different in African-Americans ($P = 0.016$ and $P = 0.025$), but not in Caucasians ($P = 0.384$ and $P = 0.435$).

Linkage disequilibrium studies. The allelic frequencies of the p55 and G6PD haplotypes in male subjects of African-Americans and of Caucasians are depicted in Table III A and B. The data were subjected to linkage disequilibrium analysis, which demonstrated that the p55 and G6PD exonic loci were in linkage equilibrium among African-Americans ($D = 0.00625$, $\chi^2 = 0.1977$, $P > 0.05$), but not among Caucasians ($D = -0.04372$, $\chi^2 = 9.29$, $P < 0.005$).

Discussion

We have reported two novel clonality assays (rtPCR-LDR of the transcriptional products of the G6PD/p55 exonic polymorphisms) which reliably quantify active X chromosome transcrip-

Figure 2 legend (Continued)

cycle amplification of that portion of the gene containing the polymorphism of interest (G6PD gene in this instance). The high concentration/purity product from this round serves as a template for the next round (allele-specific round). In the second (or allele-specific) round, two aliquots from each template round reaction are subjected to six cycles of PCR amplification; an allele-specific forward primer (C-specific or T-specific for the G6PD locus) is used for each reaction. Under these PCR conditions, only perfectly complementary primer-template pairs will undergo amplification efficiently enough to generate products visible by agarose gel electrophoresis. Each individual tested is thus represented by two gel lanes. The topmost bright band in each lane represents first-round generated template, and serves as an internal control. The presence of a second bright band demonstrates an allele-specific PCR product. Lane 1a/b represents an individual homozygous for the G6PD T allele. Lane 2a/b represents an individual homozygous for the C allele. Lane 3a/b represents an individual heterozygous for both the C and T alleles.

tional products (10, 11). We have verified the reproducibility, sensitivity, and quantitative nature of rtPCR-LDR (10–12). Nonetheless, the rtPCR-LDR assays are cumbersome, labor intensive, time-consuming, and require working with radiolabeled oligonucleotides. The rtPCR-ASOH method, reported by Beutler's laboratory (26), though simpler to carry out, still requires blotting, radiolabeled oligonucleotide hybridization, and autoradiography. We believe that ASPCR, when utilized for genotype determination or as a qualitative clonality assay (as reported herein), may enjoy an advantage over ASOH by dispensing with the use of radioisotopes and time-consuming blotting, hybridization, and autoradiography steps. Furthermore, we demonstrate ASPCR specificity in that 91 genomic DNA samples analyzed concomitantly by ASPCR and PCR-LDR produced identical genotype results. We did not conduct any direct comparison between ASPCR and ASOH for minor allele sensitivity of detection.

This ASPCR assay is a modification of the previously reported ASPCR (13), PCR-ASOH (14) and PCR amplification of specific alleles (PASA) (15). The utility of PASA for this type of application is limited because the mismatched pairing which occurs at the 3' end of the primer–template duplex (i.e., T:C, T:G, T:T) is amplified by Taq polymerase as efficiently as the perfectly complementary primer–template duplexes. Xu and Hall (27) attempted to overcome this difficulty by manipulation of as many as 12 reaction variables (19). Their assay successfully analyzed the *Escherichia coli* genome; when we applied their assay to human genomic DNA, bands representing nonspecific products were always apparent on agarose gel electrophoresis. Reducing the number of cycles in the second round from > 20 to 5, and assuring the presence of high template concentration eliminates the tendency of Taq polymerase to amplify primer-template mismatches, substantially decreasing the amount of nonspecific products while retaining allele-specific products. The entire ASPCR procedure can be completed in 6 h; PCR-LDR can take as long as 2 or 3 d. ASPCR specificity is not limited by the expected variation in concentration or quality of genomic DNA or cDNA. In addition, the same PCR conditions are employed in the analysis of p55 and G6PD, allowing for simultaneous determination of both genotypes. Unlike ASOH or PCR-ASOH, performance of ASPCR for genotype determination does not require extensive restriction endonuclease digestion of sample DNA, radiolabeled oligonucleotides, or time-consuming autoradiography.

The p55 and G6PD loci are ~ 200 kb apart on the X chromosome (28). They are in linkage equilibrium among African-Americans, but not among Caucasians. Linkage disequilibrium found in the Caucasian males may result from epistatic effects, genetic hitchhiking to a selected locus, or population subdivision (combining different ethnic groups with different allele frequencies). Which of these mechanisms is responsible for this phenomenon cannot be determined from these data alone.

The allelic frequencies are significantly different between the sexes in African-Americans, but not in Caucasians. The immediate reason for this fact is unclear. Moreover, there is no statistically significant difference among the relative allelic frequencies for either the p55 or G6PD locus among the human racial groups studied to date. This suggests that these polymorphisms were evolutionarily well-established before the geographic separation of humans into currently recognized racial groups. Interestingly, examination of Table III reveals a rela-

tive paucity of the G6PD T allele in males, and of the T/T genotype in females among all racial groups analyzed.

The ASPCR methodology is suitable for automation and further simplification. The identification of other X chromosome polymorphic loci should increase the proportion of females suitable for research and clinical purposes such as rapid discrimination of malignant (clonal) from nonmalignant (polyclonal) tissue. The underlying principles of ASPCR can and have been already used (29) for rapid determination of mutations, genotypes, and polymorphisms for diverse clinical and nonclinical purposes.

Acknowledgments

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