

Application of Kinetic Polymerase Chain Reaction and Molecular Beacon Assays to Pooled Analyses and High-Throughput Genotyping for Candidate Genes

Min Shi,¹ Diana Caprau,¹ John Dagle,¹ Lene Christiansen,² Kaare Christensen,² Jeffrey C. Murray^{1*}

¹Department of Pediatrics, University of Iowa, Iowa City, Iowa

²Epidemiology, Institute of Public Health, University of Southern Denmark, Odense University, Odense, Denmark

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BACKGROUND: The addition of DNA analysis to epidemiologic studies that have traditionally incorporated demographic and interview data can provide additional power and open new avenues for investigation. DNA can be obtained from a variety of tissues, but each has attendant variation in sample quantity, quality, and cost of acquisition. Analytic approaches for DNA genotyping are under constant development, but current applications allow small amounts (less than 2 ng per assay) of DNA to be used for genotyping. **METHODS:** In this report, we designed effective assays for a spectrum of genes using either kinetic polymerase chain reaction (PCR) or molecular beacon applications. We also investigated the extent to which DNA use and reagent cost could be minimized. Kinetic PCR assays were also applied to investigate the potential of pooled sample analysis. **RESULTS:** Our results show that small amounts of DNA can be successfully amplified in a high-throughput fashion using both kinetic PCR and molecular beacon methods. Greater than 97% of the genotype results from these two methods are consistent. In addition, error rates in allele frequency measurements using DNA pools of 100 or more samples were often less than 1% and usually less than 3%, which provides another option for substantially minimizing the costs of genotyping in studies involving large numbers of individuals. **CONCLUSIONS:** Effective assays have been designed for a spectrum of genes widely studied in birth defects, including: *MTHFR*, *NAT1*, *TGFA*, *RFC1*, *PAX9*, *EPHX1*, and *SKI*. An efficient assay has been designed for the detection of the presence of X and Y chromosomes, which can be applied to the studies of sex chromosome abnormalities or sample quality control. *Birth Defects Research (Part A) 70:65–74, 2004.*

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INTRODUCTION

For studies involving large numbers of individuals, power increases with the numbers of samples studied. However, increasing the number of samples also increases cost. When DNA is included in this analysis, the cost of genotyping can quickly become substantial in studies that might potentially benefit from the analysis of dozens, or even hundreds, of individual genes or loci. In the last two decades, a movement from Southern-blot–based restriction fragment length polymorphism (RFLP) analyses to polymerase chain reaction (PCR)–based analyses has offered the promise that thousands of samples can be reliably typed for genetic variation at hundreds of loci in a cost-effective manner. In addition, an alternative to individual genotyping is the use of pooled sample analysis, which was first introduced by Arnheim et al. (1985) and has had varying success in frequency determinations for individual alleles, as well as in linkage studies, since that time (Arn-

heim et al., 1985; Murray et al., 1987; Carmi et al., 1995; Scott et al., 1996; Barcellos et al., 1997; Germer et al., 2000; Zhou et al., 2001). We report here our experience with identifying cost-effective assays using both kinetic PCR (Germer et al., 2000; Chen et al., 2002) and molecular beacons (Tyagi et al., 1998; Marras et al., 1999) on a quantitative PCR apparatus for a variety of candidate genes valuable for studies of birth defects and other disorders. The kinetic PCR assay was also evaluated in pooled sample analyses. We also included comparisons of DNA samples

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*Correspondence to: Jeffrey C. Murray, M.D., University of Iowa, 140 Eckstein Medical Research Building, Iowa City, IA 52242. E-mail: jeff-murray@uiowa.edu

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extracted from whole blood as well as from cheek swabs or blood spots to provide for studies where sample access might be limited to one or another method. The data reported here suggest that assays that are cost-effective and can successfully amplify relatively small amounts of even poor-quality DNA can be readily developed. Samples from whole blood may be effectively used in pooled sample strategies to the extent that genome-wide linkage disequilibrium or association searches may even be entertained.

MATERIALS AND METHODS

DNA Samples

DNA samples from two major populations were used in this study. The first set of samples came from individuals native to the Philippines. The DNA was extracted from venous blood samples collected under the auspices of Operation Smile. Operation Smile is an international volunteer organization that carries out surgery on children with cleft lip and palate in underserved populations (Poe, 1994; Phillips, 1999). Patients were evaluated by a pediatrician and a geneticist, and blood samples were drawn from patients and their relatives. The blood was processed using QiaAmp DNA Blood Maxi or Midi kits (QIAGEN Inc., Valencia, CA), depending on sample volume. Blood samples from patients with nonsyndromic cleft lip, with or without cleft palate (NSCLP), were used in this study. Triads composed of the affected child and the parents can be used to carry out family-based statistical analysis, such as the transmission disequilibrium test (TDT) (Spielman et al., 1993) and the affected family-based control test (AF-BAC) (Thomson, 1995). DNA samples from 307 nuclear triads were used in this study, and those from 149 triads were used in pooled sample analyses. The second set of samples came from Denmark, a primarily native European population. DNA samples from the Danish population were purified from cheek swabs using the QIAamp DNA mini-kit as per the "buccal swabs extraction procedure" protocol. A total of 447 Danish DNA samples from 150 families with cleft lip with/without cleft palate (CLCP) or cleft palate only (CP) were used in this study. These samples consisted of 13 incomplete nuclear triad families and 137 complete triad families. Aliquots of the DNA samples were stored at -20°C for short-term storage and the rest were stored at -80°C . All samples were collected following signed, informed consent.

Pool Construction

Pooled sample analyses were carried out for both whole blood samples and cheek swab samples. DNA from whole blood was diluted to $20\text{ ng}/\mu\text{l}$ in concentration after ascertaining the DNA concentration with a UV spectrophotometer (Beckman DU 640, Fullerton, CA). A total of 31 whole blood DNA samples with equal amounts of DNA were pooled to construct the small pools, which were further pooled into larger pools. The larger pools consisted of 93, 186, and a maximum of 447 samples. Equal volumes of cheek swab DNA samples were pooled in similar ways, with smaller pools of 29 to 62 DNA samples and larger pools of 97 to 447 samples. Pool sizes were selected to represent experiments that would pool in the range of 10 to 1000 samples and for convenience of pooling and analysis.

Kinetic PCR

A kinetic allele-specific PCR assay was described by Germer et al. (2000). In brief, the specificity of the PCR reaction was conferred by placing the 3' end of the primer directly over the polymorphic sites, and primers were designed for each allele of the two polymorphic variants of each single nucleotide polymorphism (SNP). Individual samples were genotyped in two separate allele-specific PCR reactions with a common primer; the samples were used in allele-specific PCR reactions for a particular marker, and a primer specific for one of the two alleles was used in each reaction. Reactions were carried out in duplicate or triplicate. PCR reaction conditions were optimized for maximum allele specificity and consistent results by using SYBR Green Master Mix (Applied Biosystems, Foster City, CA), and reactions were performed in a total volume of $1\text{--}10\ \mu\text{l}$ with a final DNA concentration of $0.05\text{--}2\ \text{ng}/\mu\text{l}$. In each reaction, $0.2\ \mu\text{M}$ primers were used to reduce primer dimer formation.

The kinetic PCR reactions for the pooled sample experiments were conducted on an ABI PRISM 7700 sequence detection system (Applied Biosystems, Foster City, CA), in a total volume of $10\ \mu\text{l}$, with PCR profiles consisting of an initial 10-min incubation at 95°C for enzyme activation, followed by 55 two-step amplification cycles of 20 sec at 95°C for denaturation, and 20 sec for annealing and extension at the annealing temperatures shown in Table 1. Individual sample genotyping was carried out on the ABI PRISM 7900 sequence detection system (Applied Biosystems, Foster City, CA) with a 1-min annealing and extension. All the other conditions were identical to those used in the pooled sample experiments.

Primer Design

The allele-specific primers were designed by placing the 3' ends of the primers directly over the polymorphic sites, with the last 3' base corresponding to one of the polymorphic variants. The primers have lengths of 20–24 bases, with annealing temperatures in the neighborhood of 60°C . To ensure consistent results, PCR amplicons were designed to be around 100 bp long. To obtain greater allele specificity, the 3' penultimate base was changed to a mismatched base in some primers (for example, A to C or G to T).

Standard PCR

PCR fragments used as templates in direct sequencing were generated by standard PCR reactions, which were performed in a total volume of $10\text{--}25\ \mu\text{l}$ containing $20\text{--}40\ \text{ng}$ DNA, $1\times$ PCR buffer (Biolase, San Clemente, CA), $200\ \mu\text{M}$ each of dNTPs, $15\ \mu\text{M}$ MgCl_2 , $0.3\ \mu\text{M}$ each primer, and $0.5\ \text{U}$ Taq polymerase. PCR reactions were conducted with an initial 3-min denaturation at 94°C , followed by a 30-sec denaturation at 94°C , then a 30-sec annealing at appropriate temperatures, and finally a 30-sec extension at 72°C for a total of 35 cycles. The primer sequences and the reaction conditions can be found on the website: <http://genetics.uiowa.edu/publications.html>.

Molecular Beacons

Molecular beacon probes (TriLink BioTechnologies, San Diego, CA) were designed for each allele of the two SNP variants using the DNA folding website designed by Mi-

Table 1
Summary of Successful Primer Sets for Kinetic PCR

Gene name	Locus	Primers	Sequences	Annealing temperature
<i>MTHFR</i>	C677T	C allele specific T allele specific Common	5' AGCACTTGAAGGAGAAGGTGTCTGCGGGAAC 3' 5' AGCACTTGAAGGAGAAGGTGTCTGCGGGAAT 3' 5' GCGGAAGAATGTGTCAGCC 3'	64°C
<i>NAT1</i>	G560A	G allele specific A allele specific Common	5' TAGAAGACAGCAAATATCG 3' 5' CTAGAAGACAGCAAATATCA 3' 5' TGCAGGTATGTATTTCATAGACTC 3'	56°C
<i>TGFA</i>	C3296T	C allele specific T allele specific Common	5' CTCCTCTGGGCTCTTCTG 3' 5' TCCTCTCTGGGCTCTTCTA 3' 5' CTTATTTTCCCAACGTGGCC 3'	56°C
<i>TGFA</i>	C3827T	T allele specific C allele specific Common	5' GAGAAGCTGTATCCTCTAACCACGAGAAT 3' 5' AGAAGCTGTATCCTCTAACCACGAGAAC 3' 5' GGATGGTCTTCAATGTTCATG 3'	56°C
<i>PAX9</i>	T1843C	T allele specific C allele specific Common	5' GGCTGCTCGGGCTCCTGGCT 3' 5' GGCTGCTCGGGCTCCTGGCC 3' 5' GTTGAAGCCTTCACTGCATACAC 3'	64°C
<i>RFC1</i>	G80A	A allele specific G allele specific Common	5' GAAGCAAAGGTAGCACACGAGTT 3' 5' CAAAGGTAGCACACGATGC 3' 5' AGCGGTGGAGAAGCAGGTG 3'	60°C
<i>DFFRY</i>	(Detection of Y chromosome)	Y chromosome specific forward Y chromosome specific reverse	5' GAGCCCATCTTTGTCAGTTAC 3' ¹ 5' CTGCCAATTTCCACATCAACC 3' ¹	58°C
<i>UTX</i>	(Detection of X chromosome)	X chromosome specific forward X chromosome specific reverse	5' CATGTTCCCTGTAGCACATC 3' ¹ 5' CGTTCCACTTCCATTTCTG 3' ¹	
<i>EPHX1</i>	T113C	T allele specific C allele specific Common	5' GCAGGTGGAGATTCTCAACAGCT 3' 5' CAGGTGGAGATTCTCAACAGCC 3' 5' AAGGCTGTCTCATGACATAC3'	62°C
<i>SKI</i>	C391T	T allele specific C allele specific Common	5' GATGAGGTAAGGACGGGGA 3' 5' GGAGCGGCGCGTCTCC 3' 5' GGGAGCAGCGCGTCTCT 3'	62°C

Three primers were designed for each SNP marker, two allelic specific primers, and one common primer. For abbreviations, see details in the text.

¹Sequences from NCBI UniSTS database (<http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=unists>).

chael Zuker (<http://bioinfo.math.rpi.edu/~mfold/dna/form1.cgi>). In individual tubes, PCR reactions were carried out with probes for both alleles. The sequences of the probes are listed in Table 2. Reactions were carried out on the ABI PRISM 7700 sequence detection system in a total volume of 2.5–10 μ l, with a final concentration of 1 \times GeneAmp PCR Buffer II (Applied Biosystems, Foster City, CA), 4 mM MgCl₂, 0.25 mM dNTPs, 1 μ M each of the forward and reverse primers, 0.25 μ M each beacon, and 0.05 U/ μ l of Amplitaq Gold Enzyme (Applied Biosystems, Foster City, CA). PCR reactions were conducted with an initial 10-min incubation at 95°C for enzyme activation followed by 55 three-step amplification cycles consisting of 30 sec at 95°C for denaturation, 30 sec at 58°C for annealing, and 20 sec at 72°C for extension.

Genotype Analysis

Kinetic PCR. The threshold cycle (Ct) is the number of cycles elapsed before a reaction achieves a defined level of amplification. In the case of *MTHFR* C677T, for example, the difference in the Ct values between the C-allele-specific PCR and the T-allele-specific PCR (Δ Ct) provides the opportunity to distinguish between different genotypes and also serves as a mechanism by which a mixture of two different alleles can be quantified in a pooled sample. To

compensate for the differential amplification efficiencies of different allele-specific primers, the averaged Δ Ct value of heterozygous samples in the same run was subtracted from the Δ Ct measured (Germer et al., 2000); the resulting Δ Ct was designated the "corrected Δ Ct." Individual genotypes were determined after manual inspection of amplification curves for each sample. Samples with corrected Δ Ct values greater than 6 were genotyped as "TT," those with corrected Δ Ct values less than -6 were genotyped as "CC," and those with values between -2 and 2 were genotyped as "CT." Samples with corrected Δ Ct values from -2 to -6 and from 2 to 6 were considered undeterminable and subject to re-genotyping. For every primer set designed successfully, direct sequencing of at least 40 samples was conducted to confirm the genotypes generated from kinetic PCR. The Centre d'Etude du Polymorphisme Humain (CEPH) (Dausset et al., 1990) control samples, whose genotypes have been confirmed with both direct sequencing and kinetic PCR, are available on the website: <http://genetics.uiowa.edu/publications.html>.

For the pooled sample experiments, the allele frequencies were calculated as previously described (Germer et al., 2000), using the following equation:

$$\text{frequency of allele}_1 = 100 / (2^{\Delta\text{Ct}} + 1), \quad (\text{A})$$

Table 2
Sequences of Primers and Probes for Molecular Beacon Assays

Gene	Locus		Primer sequences		Probe sequences
<i>MTHFR</i>	C677T	F	5' TTGAGGCTGACCTGAAGCAC	C	5'(FAM) CGC AGC ATG AAA TCG ACT CCC GCA GCT GCG (DABCYL)3'
		R	5' ATGTCGGTGCATGCCTTCAC	T	5'(HEX) CGC AGC ATG AAA TCG GCT CCC GCA GCT GCG (DABCYL)3'
<i>TGFA</i>	TaqI	F	5' GCACTTCCCCTTTTTCATCT	+	5'(FAM) CGC AGC TCT CGA CCT TAA AAC CCT TGC TGC G (DABCYL)3'
		R	5' GGCGAAAATATCCAGGAAAC	-	5'(HEX) CGC AGC TCT CTA ATG ACC TTA AAA CCG CTG CG (DABCYL)3'
<i>PVRL2</i>	C18736T ¹	F	5' ACAGCAATTCCAAAATCATA	C	5'(FAM) CGC AAG CTT CAT AGT ACA CGT TAT AGT TGT GCT TGCG (DABCYL)3'
		R	5' CACCTGGAAAAGACATTTTA	T	5'(HEX) CGC AAG CCT CAT AGT ACA TGT TAT AGT TGT GCT TGC G (DABCYL)3'
<i>RFC1</i>	G80A	F	5' CAGCGGTGGAGAAGCA	A	5'(JOE) CGG AGG TTG GCG GCA CCT CGT GTC CTC CG (DABCYL)3'
		R	5' CGTATCTGCGCCATGAA	G	5'(FAM) CGG AGG GCG GCG CCT CGT GTG CCT CCG (DABCYL)3'
<i>CYP3A5</i>	G22893A	F	5' TCATTCTAACCATAATCTCTTT	A	5'(JOE) CGC AGC TTT GTC TTT CAA TAT CTC TTC CGC TGC G (DABCYL)3'
		R	5' TCATATGATGAAGGGTAATGT	G	5'(FAM) CGC AGC TTT GTC TTT CAG TAT CTC TTC CGC TGC G (DABCYL)3'

¹GenBank accession number: NT_011109.

where ΔCt is the average of the corrected ΔCt values for the replicate assays.

Two sources of errors are evident for pooled sample experiments: the measurement error (or error due to experimental variation) and the sampling error (or error arising from the variation in allele frequencies from sample to sample). The measurement error was calculated as previously described by Chen et al. (2002) using the equation:

$$\sigma_m = \frac{F(100-F)\ln(2)\sigma_{\Delta Ct}}{100\sqrt{M}} \quad (B)$$

where F is the frequency calculated according to Equation 1, $\sigma_{\Delta Ct}$ is the standard deviation (SD) of ΔCt , and M is the number of measurements.

Sampling error is calculated according to the following formula:

$$\sigma_s = \sqrt{F(100-F)/2n} \quad (C)$$

where n stands for the number of individuals in a pool.

The total error is calculated from the root mean square of the measurement error and the sampling error:

$$\sigma_t = \sqrt{\sigma_s^2 + \sigma_m^2} \quad (D)$$

Molecular Beacon. Molecular beacon technology utilizes the differential hybridization of fluorescently labeled oligonucleotides that are complementary to target DNA molecules possessing SNPs. Allele-specific fluorescence is generated as a result of a conformational change induced by this hybridization. Fluorescence generated during PCR amplification was recorded in real time. Following the amplification reactions, genotypes were scored by manually inspecting the amplification curves for each fluorophore (FAM and HEX or FAM and JOE) representing reactions with each allele-specific probe. These curves were manually compared to curves obtained with equal

quantities of DNA possessing known genotypes (heterozygous and both homozygous conditions). In all cases, increased fluorescence was seen from the appropriate beacon during amplification of the complementary DNA. Interestingly, we generally observed a decrease in fluorescence to less than baseline from the molecular beacon complementary to the allele not being amplified. Heterozygous samples showed significant increases in fluorescence from both probes during amplification. In contrast, amplification curves for a single fluorophore are seen in homozygous samples. A lack of significant amplification seen with both beacons was indicative of a PCR reaction failure. For each gene of interest, approximately 95 DNA samples were genotyped using molecular beacons (see Table 2). To confirm the results, all samples were genotyped by direct sequencing. An exception was the methylenetetrahydrofolate reductase (*MTHFR*) C677T polymorphism, for which an additional set of 460 DNA samples was genotyped, using both molecular beacons and kinetic PCR assays.

RESULTS

Kinetic PCR Assays

Primers for the kinetic PCR assays were designed for 13 genes, and successful primers are available for genes encoding growth factors (the transforming growth factor, alpha [*TGFA*]), transcription factors (paired box gene 9 [*PAX9*]), cell signaling molecules (SK oncogene [*SKI*]), genes involved in folate metabolism (*MTHFR* and reduced folate carrier 1 [*RFC1*]), and detoxification (the arylamine N-acetyltransferase 1 [*NAT1*] and epoxide hydrolase 1 [*EPHX1*]). A successful assay was also developed for genotyping the X and Y chromosomes, as shown in Table 1. The kinetic PCR assays were both reliable and reproducible using High-quality DNA samples isolated from whole blood samples, as well as DNA in a more dilute and less pure form, isolated from buccal swabs or blood spots. Genotypes of both homozygotes and heterozygotes were readily determined using duplicate assays and over 90% of

Table 3
Success Rates of Both Assays for Different SNPs on DNA Samples From Different Sources

Assays	Gene name	Sample types	Sample sizes	Range of success rate (%)	Average success rate (%)	
Kinetic PCR	<i>MTHFR</i> C677T	CS	447	96.9	96.9	
		WB	1924	88.1–99.3	95.9	
		Total	2371	88.1–99.3	96.1	
	<i>NAT1</i> G560A	WB	93	100.0	100.0	
		<i>PAX9</i> T1843C	WB	45	100.0	100.0
		<i>RFC1</i> G80A	CS	447	96.0	96.0
	<i>TGFA</i> C3296T	WB	1043	94.2–99.8	97.3	
		Total	1490	94.2–99.8	96.9	
		WB	631	72.9–97.4	83.5	
	<i>TGFA</i> C3827T	BS	273	96.9	96.9	
		Total	904	72.9–97.4	84.2	
		BS	547	91–95.7	91.6	
	<i>EPHX1</i> T113C	WB	617	86.8–98.9	97.7	
		Total	1164	91–98.9	94.8	
		CS	1160	95.2	95.2	
	Molecular Beacon	<i>SKI</i>	WB	273	95.0	95.0
		Summary of all genes		7500	72.9–100.0	94.6
<i>MTHFR</i> C677T		WB	500	97.3	97.3	
<i>TGFA</i> -Taq		WB	90	98	98	
<i>PVRL2</i> C18736T		WB	85	94.8	94.8	
<i>RFC1</i> G80A		WB	115	80	80	
<i>CYP3A5</i> G22893 A		WB	95	82.4	82.4	
Summary of all genes		WB	885	93.3	93.3	

samples provided accurate genotyping during a single quantitative analysis. Table 3 lists the success rates—the proportion of samples from which genotype results can be obtained—for different SNPs tested on different sources of DNA. We also investigated the extent to which DNA usage and reagent cost could be minimized. Kinetic PCR reactions are routinely carried out in a volume of 3 μ l but can be reduced to as low as 1 μ l. The use of a final DNA concentration of 0.7 ng/ μ l is reliable, and this can be minimized to as low as 0.05 ng/ μ l. With the reagents used in this study, the cost of a single genotype would then be as low as 10 cents, and the cost of genotyping for the largest pool is less than 1 cent per sample per genotype, including replications.

Molecular Beacon Analysis

As observed in the kinetic PCR reactions, genotyping using molecular beacons was both reliable and reproducible. Successful assays have been designed for SNPs in the *MTHFR*, *RFC1*, *TGFA*, and poliovirus receptor-like II (*PVRL2*) genes, and in the cytochrome p450 subfamily IIIA polypeptide 5 (*CYP3A5*) genes (Kuehl et al., 2001). Comparing the results of molecular beacon genotyping with known genotypes obtained from DNA sequencing demonstrated accuracy between 94% and 99%. More than 400 Philippine samples were genotyped on *MTHFR* C677T SNP using both kinetic PCR and molecular beacon assays with success rates of 97% and 95% for each assay, respectively. Genotyping results of 97% of the samples are replicated by these two methods.

A second goal of this study was to determine the practical limits of molecular beacon genotyping with respect to minimizing the cost per reaction. Genotyping reactions using as little as 50 pg of genomic DNA isolated from whole blood were informative, greatly expanding the possibility of large-scale genotyping when DNA is a limiting reagent. Additionally, genotyping reactions employing

volumes as low as 2.5 μ l were also successful, suggesting that a significant cost saving may be possible by scaling down from the typical 25- to 50- μ l volumes.

Pooled Sample Analysis

Pools varying in size from approximately 30 to 450 samples were successfully amplified in most cases. In one set of experiments, two of the pools, after being initially derived, appeared to be contaminated, as the allele frequency determinations from sequential replicate assays had a wide degree of variation. In other pooled samples, although there were occasionally divergent results, which were easily identified and likely secondary to reagent failure, allele frequency measurements were both reproducible and accurately determined when compared to the known frequencies based on the genotype determination of the individual samples. Table 4 shows the summary of the results from pooled sample analyses. These analyses were equally reproducible for equimolar amounts of DNA as determined by spectrophotometry and isolated from whole blood, as well as DNA samples where there was likely to be wide variation in individual DNA amounts as extracted from buccal swabs. Nonetheless, despite this likely variation in DNA amounts, pooling resulted in accurate allele frequency determinations in all but two cases. Most of the measurement errors were less than 2% (Fig. 1), which was much lower than the sampling error, indicating the high reliability of the pooled sample approach.

MTHFR and *RFC1* Genotype Analysis

The pooled sample analyses comparing the mother, father, and child allele frequencies for the *MTHFR* C677T SNP showed small differences. This result was consistent with what was observed on the individual genotyping assays, where no significant difference in allele frequencies could be identified. The TDT test for the *MTHFR* gene

Table 4
Results from the Pooled Sample Analyses

Plates	From pooled sample	From individual genotyping	ABS* value of measured and expected from difference ¹	No. of samples per pool	No. of measurements	SD of ΔC_t	Measurement error	Sampling error	Total error
1	91.8	91.9	0.1	31	9	0.72	1.26	3.46	3.68
2	92.7	93.5	0.8	31	10	0.61	0.90	3.12	3.25
3	88.3	87.1	1.2	31	5	0.43	1.38	4.26	4.47
4	91.4	90.9	0.6	93	9	0.27	0.49	2.11	2.17
5	93.3	93.5	0.2	93	10	0.20	0.27	1.80	1.82
6	89.5	89.2	0.3	93	12	0.91	1.70	2.27	2.84
7	94.9	94.1	0.8	93	11	0.45	0.46	1.73	1.79
8	90.7	89.5	1.2	93	9	0.47	0.91	2.24	2.42
9	93.7	92.0	1.7	183	11	0.57	0.71	1.42	1.58
10	64.3	63.8	0.5	29	7	0.44	2.63	6.31	6.84
11	62.9	63.2	0.4	34	7	0.21	1.25	5.85	5.98
12	69.3	70.6	1.3	34	8	0.13	0.67	5.53	5.57
13	70.7	69.1	1.6	53	8	0.66	3.36	4.49	5.60
14	69.8	70.0	0.2	57	6	0.27	1.62	4.29	4.59
15	69.3	68.4	0.9	57	7	0.29	1.61	4.36	4.64
16	77.1	75.0	2.1	58	6	0.16	0.78	4.02	4.10
17 ²	77.6	71.6	6.0	62	7	2.13	9.69	4.05	10.50
18	81.9	74.6	7.3	62	8	0.24	0.89	3.91	4.01
19 ²	87.1	71.6	15.5	62	8	1.03	2.83	4.05	4.94
20	65.5	66.0	0.5	97	8	0.16	0.87	3.40	3.51
21	70.9	69.2	1.7	167	6	0.36	2.07	2.53	3.27
22	79.8	73.7	6.1	183	7	0.24	1.03	2.30	2.52
23	69.3	70.3	1.0	447	8	0.60	3.11	1.53	3.46

¹ABS, absolute.

²Experiments 17 and 18 are replicating assays on the same set of sample.

showed a slight overtransmission of T alleles to the affected children, and the AFBAC test showed a borderline p-value, as shown in Tables 5 and 6. No significant association was identified in the G80A polymorphism in *RFC1* and orofacial clefting (results not shown).

DISCUSSION

In this report, we have analyzed several strategies for improving the throughput in epidemiologic studies involving genotyping of subjects. First, we sought to determine how readily new, cost-effective assays could be identified for a collection of 15 SNPs in 13 candidate genes already under study with respect to either birth defects or cardiovascular disease. For the kinetic PCR approach, more than 50% of SNPs had easily-identified assays that remained robust across a variety of DNA samples from different sources. Using standard guidelines describing beacon design, all of the five pairs of molecular beacons tested were successful on the first trial. This high rate of success is especially important with respect to molecular beacon technology, given the higher initial cost associated with the synthesis of modified oligonucleotides, compared to the kinetic PCR approach, which utilizes unmodified oligonucleotides.

In the second study evaluation, we showed that even small quantities of DNA (100 pg or less) could be successfully amplified, even when the DNA source is relatively impure, as was found in the buccal swab samples. Since buccal swabs can routinely give 1 to 10 μ g of DNA per individual, these samples would then be able to provide sufficient material for 1000 or more assays. The develop-

ment of the kinetic PCR and molecular beacon assays for the candidate genes in themselves is noteworthy; several of these now have high-throughput assays not previously available, which will facilitate additional studies of birth defects or other disorders. In a final set of evaluations, we have looked into whether pooled samples could be used to determine allele frequencies representative of the sum of the individual genotypes of the component samples. Although some samples obtained from cheek swabs did not appear to be of sufficient quality to allow for a consistent determination of allele frequencies, most samples from whole blood did. Error rates in allele frequencies when comparing pooled samples to individual genotypes were often below 1% and usually below 3%. Since association and linkage disequilibrium studies have often successfully identified differences in case and control populations of 5–10% or more, the reliable identification of differences of 3% or less would suggest that these pooling methodologies might be successful. Finally, specific data on the *MTHFR* variation in nonsyndromic cleft lip and palate patients are also reported.

A success rate of 50% for kinetic PCR SNP assay primer design is not impressive; however, the success rate rose with the experience of the investigators. In later studies, 10 polymorphic variants, including eight SNPs and two large deletions were investigated, and successful allele-specific primers were designed for all of these polymorphic sites (unpublished results). Since DNA variants are commonly found within haplotype blocks in most studies (Gabriel et al., 2002), many SNP sites will be available for assay design, each of which will give similar information. It follows

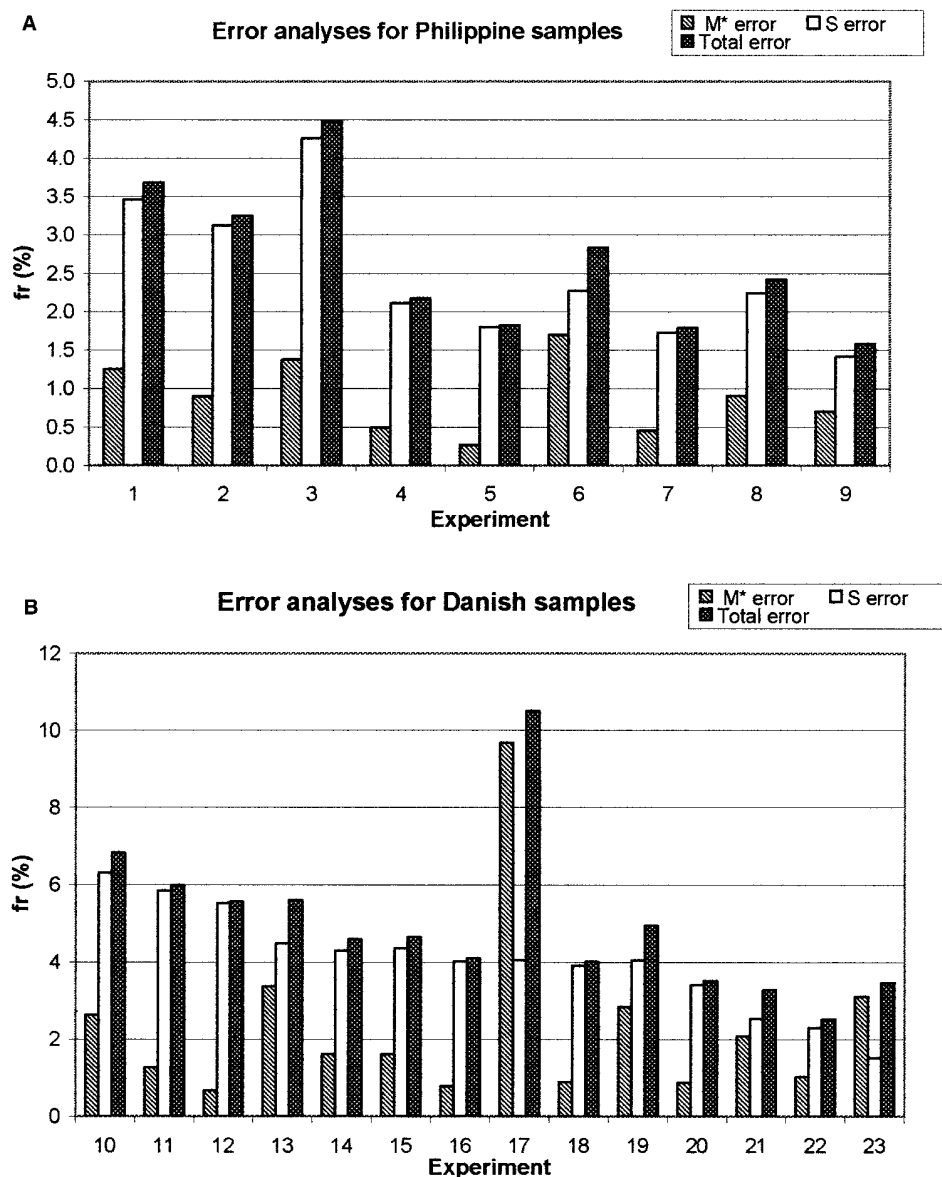


Fig. 1. Error analyses of the pooled sample experiments. The measurement errors (crossed bars), sampling errors (open bars), and total errors (dotted bars) for each pooled sample experiment are shown. Due to the baseline allele frequency difference in the Philippine (A) and Danish (B) samples, experiments for these two sample sources are plotted separately. The experiments are sorted by the number of samples in each pool.

that in most circumstances even a 50% failure rate in design will not impede useful assay development.

Once a reliable assay is designed, both kinetic PCR and molecular beacon assays have the advantages of high success rate in genotyping, cost-effectiveness, and high throughput. Molecular beacon has a higher success rate in designing reliable assays. The trade-off, however, is that the cost of the modified oligos used in molecular beacon assay is much higher than that of the unmodified oligos used in kinetic PCR assay. In addition, the design of successful molecular beacons is not always straightforward, as numerous thermodynamic parameters must be considered. However, even though the cost of modified oligos is relatively high, it accounts for only a small proportion of total cost. Once a successful assay has been designed, the cost per genotype for molecular beacon assay is comparable to that of the kinetic PCR assay. Kinetic PCR assays also

have the potential of being applied to pooled sample analysis, which is not available with the molecular beacon technology. Investigators can choose between these two genotyping methods based on their individual research interests.

Success rate not only depends on the biological sources of the DNA but also on the method of extraction and possibly the duration of storage, as evidenced by the large difference in success rates for whole blood samples on the *TGFA* C3296T SNP (ranging from 72.9 to 97.4%). The samples with a success rate of 72.9% were collected over 10 years ago and extracted using the salting out method (Grimberg et al., 1989), while the samples with a success rate of 97.4% were collected in the last two to three years and extracted using QiaAmp DNA Blood Maxi or Midi kits. Many platforms for carrying out genotypic analysis are currently available or are under development. Molec-

Table 5
Results of the AFBAC Tests for the *MTHFR* C677T SNP on the Danish Check Swab Samples

CP ¹	Transmitted	Nontransmitted	CL ¹	Transmitted	Nontransmitted
C	40	36	C	79	74
T	19	23	T	30	35
Chi-square	0.59		Chi-square	0.55	
<i>p</i> value	0.44		<i>p</i> value	0.46	
CLCP ¹	Transmitted	Nontransmitted	Total	Transmitted	Nontransmitted
C	58	70	C	177	180
T	35	23	T	84	81
Chi-square	3.61		Chi-square	0.08	
<i>p</i> value	0.058		<i>p</i> value	0.78	
CLCP (mothers) ²	Transmitted	Nontransmitted	CLCP (fathers) ²	Transmitted	Nontransmitted
C	29	32	C	24	33
T	13	10	T	17	8
Chi-square	0.54		Chi-square	4.67	
<i>p</i> value	0.46		<i>p</i> value	0.03	

¹The AFBAC test on samples from patients with CP (cleft palate only), CL (cleft lip only), and CLCP (cleft lip with cleft palate).

²AFBAC test on the CLCP samples with the mothers and the fathers separated.

ular beacon assays (Tyagi and Kramer, 1996), Taqman assays (Heid et al., 1996), MALDI-TOF (Haff et al., 1997), and others (Hoogendoorn et al., 2000; Mein et al., 2000; Cutler et al., 2001; Prince et al., 2001) are all potential winners in the race to identify forms of genotyping that will be most effective and transportable to routine laboratory analysis. The kinetic PCR approach that we report here is easily transportable to laboratories that have experience with PCR and in which the purchase of a single instrument can allow for the throughput of thousands of samples per day. Individual genotypic analysis can be carried out using currently available equipment for 384 samples in a single run. Of perhaps greater utility is the possibility of extending this analysis to a pooled sample approach. High-quality DNA samples with known concentrations can be pooled in equimolar amounts so that each individual is making an equal contribution to the template used in PCR amplification. Individual genotypic analysis can be carried out on the samples used in the pool to ensure that each analyzes equally. Pools can be derived from a variety of sources. They can consist of cases and controls, where the controls come from either matched populations or parental samples. When case pools and control pools consisting of parental samples are used in a

study, the TDT test can still be applied. However, the Hardy-Weinberg equilibrium (HWE) has to be assumed due to the absence of individual genotype information. This TDT test with the HWE assumption is less powerful than the usual TDT, but it can still achieve the power of a haplotype-based haplotype relative risk (HHRR) test (Risch et al., 1998). Pooling of the offspring samples can also be used for the usual TDT test, since individual offspring genotype information is not required to carry out the TDT test (Risch et al., 1998). While haplotype information is available when samples are typed individually, the pooled strategy limits this approach. An alternative strategy is to carry out the pooled analysis in the case and control population on a large number of candidate genes. These assays will be most effective when they focus on those DNA sequence variants that underlie alterations in either the protein sequence or in regulatory regions, such as promoters, that will affect either gene function or expression in terms of quantity or position. Alternatively, to maximize heterozygosity detection, one could build assays based on the haplotype blocks that contain the gene. When dozens or hundreds of candidate genes are analyzed in this way, the subset showing the largest frequency differences between cases and controls can then undergo individual

Table 6
Results of the TDT Tests for the *MTHFR* C677T SNP on the Danish Cheek Swab Samples

CP	Father	Mother	Total	CL	Father	Mother	Total
C-transmitted	7	8	15	C-transmitted	15	11	25
T-transmitted	7	4	11	T-transmitted	11	7	18
TDT-statistics	0.00	1.33	0.62	TDT-statistics	0.62	0.89	1.14
<i>p</i> -value	1.00	0.25	0.43	<i>p</i> -value	0.43	0.35	0.29
CLCP	Father	Mother	Total	All	Father	Mother	Total
C-transmitted	6	6	12	C-transmitted	28	25	53
T-transmitted	15	9	24	T-transmitted	33	20	53
TDT-statistics	3.86	0.60	4.00	TDT-statistics	0.41	0.56	0.00
<i>p</i> -value	0.05	0.44	0.05	<i>p</i> -value	0.52	0.46	1.00

sample analysis, in which analytic approaches, such as the TDT, can then be applied to determine the significance of the preliminary findings. Since the number of estimated genes is now in the vicinity of 30,000 (Venter et al., 2001), and since the number of electronically identified SNPs now exceeds 2,000,000, there is already an enormous amount of gene-specific variations in hand that can be applied in such studies. The development of more robust software tools to construct primers and probes that will have a high probability of success in the kinetic PCR, molecular beacon, or other quantitative PCR approaches will further strengthen these approaches. The pooled sample analysis, besides offering cost-effective opportunities, also opens the door to genome-wide linkage disequilibrium searches. Current evidence suggests that for the physical distances over which linkage disequilibrium appear to be strong, such disequilibrium frequently resides in blocks of 50–100 kb (Daly et al., 2001; Rioux et al., 2001). Thus, once markers could be identified across each of these blocks, a collection of perhaps 30,000 appropriately spaced markers would capture more than 50% of all of the expected linkage disequilibrium in the genome and would make a genome-wide approach practical. One application of this approach has already been reported for myocardial infarction (Ozaki et al., 2002). The candidate genes chosen here and their effective assays will now provide opportunities for additional investigations by other groups into their role in cleft lip and palate, neural tube defects, preeclampsia, and cardiovascular disease, as has been already undertaken.

A number of additional investigations remain. First, better algorithms for the identification of primers that can be applied to individual assays need to be determined. DNA sample quality also remains an issue, but we believe that the trade-off of buccal swab DNA, which can be reliably individually genotyped for 1000 or more assays from a single individual, affords an appropriate option when the expense or difficulty of obtaining whole blood may preclude it as a choice. This will be particularly true when small infants or fetuses are involved, in which blood volumes are minimal, and in studies where the cost and expense of the sample acquisition itself exceeds budget realities. The cost of the individual genotyping itself is also well within the means of most budgets that might involve hundreds or thousands of samples, and is likely to be further minimized as reagent costs and sample volumes decrease even further.

Modifications to the pooled sample approach to limit the number of false positives would include several considerations. First, the quality and amount of the starting DNA should be assured and is best derived from preparations of DNAs that come from large numbers of cells obtained either via culture or whole blood sampling. Although the experience with DNA extracted from cheek swabs and blood spots has proven that these can be reliable sources of DNA in individual analysis, their ability to contribute to a pooled sample analysis has only been demonstrated in our work in a preliminary fashion. Next, a metric to determine which allele frequencies are different also needs to be established and adhered to. One could either empirically work back from those samples that demonstrate the largest allele frequency differences or one could set prior cutoffs based on acceptable differences in those frequencies.

In summary, quantitative PCR can provide effective genotyping for large number of samples of low concentra-

tion and quality. Kinetic PCR may allow rapid allele frequency determination of many loci to allow a quick assessment of allele frequencies for candidate genes or loci.

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REFERENCES

- Arnheim N, Strange C, Erlich H. 1985. Use of pooled DNA samples to detect linkage disequilibrium of polymorphic restriction fragments and human disease: studies of the HLA class II loci. *Proc Natl Acad Sci USA* 82:6970–6974.
- Barcellos LF, Klitz W, Field LL, et al. 1997. Association mapping of disease loci, by use of a pooled DNA genomic screen. *Am J Hum Genet* 61:734–747.
- Carmi R, Rokhlina T, Kwitck-Black AE, et al. 1995. Use of a DNA pooling strategy to identify a human obesity syndrome locus on chromosome 15. *Hum Mol Genet* 4:9–13.
- Chen J, Germer S, Higuchi R, et al. 2002. Kinetic polymerase chain reaction on pooled DNA: a high-throughput, high-efficiency alternative in genetic epidemiological studies. *Cancer Epidemiol Biomarkers Prev* 11: 131–136.
- Cutler DJ, Zwick ME, Carrasquillo MM, et al. 2001. High-throughput variation detection and genotyping using microarrays. *Genome Res* 11: 1913–1925.
- Daly MJ, Rioux JD, Schaffner SF, et al. 2001. High-resolution haplotype structure in the human genome. *Nat Genet* 29:229–232.
- Dausset JC, Cohen H, Lathrop DM, et al. 1990. Centre d'étude du polymorphisme humain (CEPH): collaborative genetic mapping of the human genome. *Genomics* 6:575–577.
- Gabriel SB, Schaffner SF, Nguyen H, et al. 2002. The structure of haplotype blocks in the human genome. *Science* 296:2225–2229.
- Germer S, Holland MJ, Higuchi R, et al. 2000. High-throughput SNP allele-frequency determination in pooled DNA samples by kinetic PCR. *Genome Res* 10:258–266.
- Grimberg J, Nawoschik S, Belluscio L, et al. 1989. A simple and efficient non-organic procedure for the isolation of genomic DNA from blood. *Nucleic Acids Res* 17:8390.
- Haff LA, Smirnov IP. 1997. Single-nucleotide polymorphism identification assays using a thermostable DNA polymerase and delayed extraction MALDI-TOF mass spectrometry. *Genome Res* 7:378–388.
- Heid CA, Stevens J, Livak KJ, Williams PM. 1996. Real time quantitative PCR. *Genome Res* 6:986–994.
- Hoogendoorn B, Norton N, Kirov G, et al. 2000. Cheap, accurate and rapid allele frequency estimation of single nucleotide polymorphisms by primer extension and DHPLC in DNA pools. *Hum Genet* 107:488–493.
- Kuehl P, Zhang J, Lin Y, et al. 2001. Sequence diversity in CYP3A promoters and characterization of the genetic basis of polymorphic CYP3A5 expression. *Nat Genet* 27:383–391.
- Marras SA, Kramer FR, Tyagi S. 1999. Multiplex detection of single-nucleotide variations using molecular beacons. *Genet Anal* 14:151–156.
- Mein CA, Barratt BJ, Dunn MG, et al. 2000. Evaluation of single nucleotide polymorphism typing with invader on PCR amplicons and its automation. *Genome Res* 10:330–343.
- Murray JC, Shiang R, Carlock LR, et al. 1987. Rapid RFLP screening procedure identifies new polymorphisms at albumin and alcohol dehydrogenase loci. *Hum Genet* 76:274–277.
- Ozaki K, Ohnishi Y, Iida A., et al. 2002. Functional SNPs in the lymphotoxin-alpha gene that are associated with susceptibility to myocardial infarction. *Nat Genet* 32:650–654.
- Phillips DF. 1999. Operation Smile volunteers travel far to transform lives. *JAMA* 281:597–598.
- Poe D. 1994. Operation Smile International: missions of mercy. *Plast Surg Nurs* 14:225–230.
- Prince JA, Feuk L, Howell WM, et al. 2001. Robust and accurate single nucleotide polymorphism genotyping by dynamic allele-specific hybridization (DASH): design criteria and assay validation. *Genome Res* 11:152–162.
- Rioux JD, Daly MJ, Silverberg MS, et al. 2001. Genetic variation in the 5q31 cytokine gene cluster confers susceptibility to Crohn disease. *Nat Genet* 29:223–228.
- Risch N, Teng J. 1998. The relative power of family-based and case-control

- designs for linkage disequilibrium studies of complex human diseases I. DNA pooling. *Genome Res* 8:1273–1288.
- Scott DA, Carmi R, Elbedour K, et al. 1996. An autosomal recessive non-syndromic-hearing-loss locus identified by DNA pooling using two inbred Bedouin kindreds. *Am J Hum Genet* 59:385–391.
- Spielman RS, McGinnis RE, Ewens WJ. 1993. Transmission test for linkage disequilibrium: the insulin gene region and insulin-dependent diabetes mellitus (IDDM). *Am J Hum Genet* 52:506–516.
- Thomson G. 1995. Mapping disease genes: family-based association studies. *Am J Hum Genet* 57:487–498.
- Tyagi S, Kramer FR. 1996. Molecular beacons: probes that fluoresce upon hybridization. *Nat Biotechnol* 14:303–308.
- Tyagi S, Bratu DP, Kramer FR. 1998. Multicolor molecular beacons for allele discrimination. *Nat Biotechnol* 16:49–53.
- Venter JC, Adams MD, Myers EW, et al. 2001. The sequence of the human genome. *Science* 291:1304–1351.
- Zhou G, Kamahori M, Okano K, et al. 2001. Quantitative detection of single nucleotide polymorphisms for a pooled sample by a bioluminometric assay coupled with modified primer extension reactions (BAMPER). *Nucleic Acids Res* 29:E93.