Short Technical Reports

- regulation of atrial natriuretic factor gene transcription in cardiac cells. *Mol. Cell. Biol.* 14:777-790.
2. **Cheng, J., J. Yang, Y. Xia, M. Karin, and B. Su.** 2000. Synergistic interaction of MEK kinase 2, c-Jun N-terminal kinase (JNK) kinase 2, and JNK1 results in efficient and specific JNK1 activation. *Mol. Cell. Biol.* 20:2334-2342.
3. **Deacon, K. and J.L. Blank.** 1999. MEK kinase 3 directly activates MKK6 and MKK7, specific activation of the p38 and c-jun NH2-terminal kinases. *J. Biol. Chem.* 274:16604-16610.
4. **Hannan, R.D., A. Cavanaugh, W.M. Hempel, T. Moss, and L. Rothblum.** 1999. Identification of a mammalian RNA polymerase I holoenzyme containing components of the DNA repair/replication system. *Nucleic Acids Res.* 27:3720-3727.
5. **Hannan, R.D., J. Luyken, and L.I. Rothblum.** 1996. Regulation of ribosomal DNA transcription during contraction-induced hypertrophy of neonatal cardiomyocytes. *J. Biol. Chem.* 271:3213-3220.
6. **Hannan, R.D., V. Stefanovsky, L. Taylor, T. Moss, and L.I. Rothblum.** 1996. Overexpression of the transcription factor UBF1 is sufficient to increase ribosomal DNA transcription in neonatal cardiomyocytes: implications for cardiac hypertrophy. *Proc. Natl. Acad. Sci. USA* 93:8750-8755.
7. **Lu, X., S. Nemoto, and A. Lin.** 1997. Identification of c-Jun kinase (JNK)-activation kinase 2 as an activator of JNK but not p38. *J. Biol. Chem.* 272:24751-24754.
8. **Nemoto, S., Z. Sheng, and A. Lin.** 1998. Opposing effects of Jun kinase and p38 mitogen-activated protein kinases on cardiomyocyte hypertrophy. *Mol. Cell. Biol.* 18:3518-3526.
9. **Trejo, J., T. Massamiri, T. Deng, N.N. Dewji, R.M. Bayney, and J.H. Brown.** 1994. A direct role for protein kinase C and the transcription factor Jun/AP-1 in the regulation of the Alzheimer's β -amyloid precursor protein gene. *J. Biol. Chem.* 269:21682-21690.
10. **Zohar, M., A. Mesika, and Z. Reich.** 2001. Analysis of genetic control elements in eukaryotes: transcriptional activity or nuclear hitchhiking? *Bioessays* 23:1176-1179.

This research was supported by funds from the Geisinger Foundation. Address correspondence to Dr. Lawrence I. Rothblum, Henry Hood Research Program, Weis Center for Research, Geisinger Clinic, Danville, PA 17822-2618, USA. e-mail: lrothblum@geisinger.edu

Received 18 January 2002; accepted 13 March 2002.

**Qiyue Hu, Kiyofumi Suzuki,
Iwona Hirschler-Laszkiewicz,
and Lawrence I. Rothblum**
*Geisinger Clinic
Danville, PA, USA*

SNP Genotyping Using Single-Tube Fluorescent Bidirectional PCR

BioTechniques 33:80-90 (July 2002)

ABSTRACT

SNP genotyping is a well-populated field with a large number of assay formats offering accurate allelic discrimination. However, there remains a discord between the ultimate goal of rapid, inexpensive assays that do not require complex design considerations and involved optimization strategies. We describe the first integration of bidirectional allele-specific amplification, SYBR Green® I, and rapid-cycle PCR to provide a homogeneous SNP-typing assay. Wild-type, mutant, and heterozygous alleles were easily discriminated in a single tube using melt curve profiling of PCR products alone. We demonstrate the effectiveness and reliability of this assay with a blinded trial using clinical samples from individuals with sickle cell anemia, sickle cell trait, or unaffected individuals. The tests were completed in less than 30 min without expensive fluorogenic probes, prohibiting design rules, or lengthy downstream processing for product analysis.

INTRODUCTION

Although PCR was described nearly two decades ago (11), it still remains a core enabling technology for many diagnostic procedures. More recently, there has been renewed interest in the use of PCR for detecting SNPs since it has the potential to offer rapid and inexpensive diagnosis. SNP genotyping has witnessed a progression from platforms requiring secondary analysis of PCR products [e.g., RFLP (2), single-strand conformational polymorphism (8), and DNA sequencing (14)] to rapid homogeneous genotyping with sequence-specific detection (10,20). This evolution has largely been accomplished through the availability of PCR instruments that combine amplification and product detection. Continued improvement of instrumentation in terms of speed and detection sensitivity suggests that PCR may be an effective platform on which to base SNP-typing as-

says for diagnostic application.

To this end, a number of different fluorescent chemistries have been developed that provide sequence-specific probes for real-time PCR. These typically employ fluorescence resonance energy transfer (FRET) between two fluorophores, with one quenching the fluorescence of the other (3,17,21,22). The design, synthesis, and purification of these probes are both difficult and expensive, which limits their commercial suitability.

A widely used method for mutation detection that does not use fluorescent probes is allele-specific amplification (ASA) (12,15) and is based on the observation that a mismatch between template and PCR primer reduces or prevents amplification by *Taq* DNA polymerase. This process is assisted by the lack of a 3'→5' proofreading activity in the enzyme. Two allele-specific primers complementary to the wild-type and mutant sequence are used to amplify target DNA in a pairwise manner with a single common primer. The presence or absence of PCR product in each reaction mixture indicates whether the normal or mutant allele is present in the target. Typically, two reactions are required to genotype a two-allele system, and internal control amplification is necessary to provide a positive control for the PCR test (19).

More recently, we introduced a single-tube adaptation called bidirectional ASA (23), in which two allele-specific primer pairs are included in a single PCR test. Like similar methods published independently [e.g., PCR-CTPP (5), SB-ASA (6), bidirectional AS-PCR (7), bi-PASA (9), SAS-PCR (16), tetra primer PCR (27), and tetra primer ARMS PCR (28)], this strategy has benefits in terms of reducing reagents and consumable costs, yet requires a secondary analysis for product identification by gel electrophoresis or chromatography, which decreases overall throughput.

We describe a new assay format called fluorescent bidirectional PCR, which represents the first integration of bidirectional PCR with detection of specific PCR products according to their T_m . This introduces homogeneity to a previously heterogeneous system and takes advantage of contemporary PCR instruments, which offer in-

Short Technical Reports

creased speed and cost-effectiveness. The test is compatible with rapid-cycle PCR, decreasing the time taken for bidirectional PCR methods from 4 h to 35 min. In addition, specificity is improved since shorter extension times strongly disfavor mismatched primer extension (17,25). The use of a single generic DNA binding dye, in this instance SYBR Green® I, has enabled inexpensive characterization of the amplification profile, which offers a more rapid alternative to the T_m -shift genotyping assay previously described (4).

Critically, fluorescent bidirectional PCR does not require any additional primer modifications such as GC tails (4) or additional mismatches near the 3' end of the primers to improve specificity and utilizes a commercially available reaction mixture with no additional reagents to those normally employed for a standard PCR assay. Since the true cost of a genotyping test is defined by

not only reagent and consumable costs but also time and labor for design and optimization, our simplified strategy adds to overall cost-effectiveness. To optimize any new PCR effectively, a magnesium and oligonucleotide titration is usually performed, and once conditions have been established for a particular SNP, re-optimization is unnecessary.

We demonstrate the efficiency and reliability of the assay by detecting the single-base-pair mutation that causes the autosomal recessive disease, sickle cell anemia. Here, a single A→T transversion in the sequence encoding codon 6 of the human β -globin gene expresses itself as an amino acid substitution of glutamine to valine, forming a mutant globin chain (HbS) (26). In this study, a set of clinical DNA samples was genotyped to validate fluorescent bidirectional PCR as an interesting and cost-effective alternative to other SNP genotyping methodologies.

MATERIALS AND METHODS

Fluorescent Bidirectional PCR Primers

Oligonucleotides were designed using PrimerCalc® software (Q-Bio-gene, Carlsbad, CA, USA) and synthesized by Sigma-Genosys (Cambridgeshire, UK), with a reverse-phase cartridge purification step. Each primer was designed with a T_m of 60°C to allow generic PCR cycling conditions. SCF-WT (5'-ATGGTGCACCTGACT-CCTGA-3') and WT-CP86 (5'-AGGG-CCTCACCACCAACTTC-3') direct the amplification of an 86-bp product from the normal β -globin gene, with a calculated T_m of 87°C (wild-type primer set). SCF-MUT (5'-GCAGT-AACGGCAGACTTCTCCA-3') and MUT-CP112 (5'-AGGGCAGAGCC-ATCTATTGC-3') amplify a 112-bp fragment from homozygous mutant

Table 1. Primer Titration Matrix

Reaction	SCF-WT	WT-CP 86	SCF-MUT	MUT-CP112
1	A	A	A	A
2	A	A	B	B
3	A	A	C	C
4	B	B	A	A
5	B	B	B	B
6	B	B	C	C
7	C	C	A	A
8	C	C	B	B
9	C	C	C	C

Primers were aliquoted according to this matrix, where A = 0.15 μ M, B = 0.3 μ M, and C = 0.9 μ M. Inter-set primer concentrations were chosen empirically to cover a wide range.

DNA, with a calculated T_m of 85°C (mutant primer set). Product melting temperatures were predicted using PrimerCalc software, which employs nearest neighbor thermodynamic algorithms for T_m calculations (1).

Fluorescent Bidirectional PCR Assay

Reactions were performed in a total volume of 20 μ L, containing 2 μ L LightCycler™ DNA FastStart SYBR Green I mixture (*Taq* DNA polymerase,

reaction buffer, dNTP mixture, and SYBR Green I dye; Roche Applied Science, Lewes, UK), a final concentration of 3 mM $MgCl_2$ and 20 ng human genomic DNA. For primer optimization sets, oligonucleotides were aliquoted according to Table 1, with control DNA templates. For the blinded trial, optimal primer ratios, as defined by reaction 3, Table 1, were used (0.15 μ M each wild-type Primer, and 0.9 μ M each mutant primer) with 45 DNA samples of unknown genotype.

PCR Cycling and Melt Curve Analysis

PCR cycling and melt analysis protocols were performed on the LightCycler PCR instrument (Roche Applied Science). Amplifications were completed in approximately 30 min, including a 10-min *Taq* DNA polymerase activation step at 95°C, 35 PCR cycles of denatura-

Short Technical Reports

tion at 95°C for 1 s, primer annealing at 62°C for 3 s, and primer extension for 0 s at 72°C. All temperature transition rates were set to 20°C/s and a single fluorescence acquisition was made at 72°C for each cycle. Fluorescence gains (used to set sensitivity of detection) for the LightCycler were F1-2 throughout.

Product identification and specificity was confirmed by executing a melting curve analysis immediately after amplification in the same closed capillary tube. An initial denaturation at 95°C for 0 s was followed by a cooling step to 67°C (5°C above primer annealing, ensuring all fluorescence from dsDNA originates from product-to-product re-annealing) and a slow denaturation phase to 95°C at a rate of 0.1°C/s, with continual fluorescence acquisition. Product denaturation was observed as a rapid loss of fluorescence near the calcu-

lated T_m . These data were converted into melting peaks by LightCycler software, plotting the first negative derivative of fluorescence loss with respect to temperature ($-d(F1)/dT$ vs. T). The apex of the melt peak represents product T_m .

Genotype Validation by Restriction Digestion

To validate genotype scoring by fluorescent bidirectional PCR, conventional PCR was performed using the DNA samples of unknown genotype, followed by restriction enzyme digestion with *DdeI*. Each PCR was a total volume of 25 μ L, containing 20 ng template DNA, 0.5 μ M each outer primer (WT-CP86 and MUT-CP112), and 10 μ L PCR Master Mix (Promega, Madison, WI, USA) containing *Taq* DNA polymerase, dNTPs, $MgCl_2$, and

reaction buffers at optimal concentrations for efficient amplification. All reactions were performed using an MJ Thermal Cycler (PCT-100; GRI, Braintree, UK) through an initial denaturation at 95°C for 2 min, followed by 30 cycles of denaturation at 95°C for 30 s, primer annealing at 60°C for 30 s, and extension at 72°C for 35 s. A 10- μ L aliquot of the reaction was mixed with 20 U *DdeI* (Promega) and 3 μ L OnePhor-All *Plus* buffer (Amersham Pharmacia, Little Chalfont, UK), made up to 30 μ L with PCR-grade water. The solutions were incubated at 37°C for 1 h, followed by an enzyme inactivation step at 65°C for 15 min. Digestion products were separated according to size on a 2.5% agarose gel (Q-Biogene) and visualized by staining with ethidium bromide. This protocol was used for all gel-based separations.

Figure 1. Schematic of fluorescent bidirectional PCR. In a single reaction with two allele-specific primer sets, genomic DNA (■) will be amplified by either set depending on template zygosity, yielding allele-specific PCR products (■). Straight arrows indicate complementarities between primer and template, whereas upward/downward-facing arrows represent a single-base mismatch at the 3' end of the allele-specific primer. (A) Samples homozygous for the wild-type allele will be amplified by SCF-WT and WT-CP86, giving a product with a predefined T_m of 87°C. (B) Samples homozygous for the mutant allele will generate a product with a predefined T_m of 85°C. Heterozygous inheritance will amplify both allele-specific PCR products. Internal control primers are not necessary since at least one allele-specific fragment will be amplified per reaction under optimized conditions.

Figure 2. Gel images from primer titrations. Reactions 1-9 correspond to conditions described in Table 1 using (A) wild-type and (B) mutant human genomic DNA template. Samples void of template are denoted by "n". Actual product T_m values for the 86-bp product were 87.1°C ($\pm 0.3^\circ$ C), and 85.7°C for the 112-bp fragment. The 158-bp target region was not amplified to a detectable level across the reaction set.

Short Technical Reports

RESULTS AND DISCUSSION

Assay Design

The principle of the fluorescent bidirectional PCR assay is shown schematically in Figure 1. A key requirement of the test is the ability to design appropriate primer pairs that amplify allele-specific PCR products with a predicted T_m difference of 2°C, allowing adequate discrimination by melt curve analysis. This can be accomplished by defining primer T_m value and searching for outer primers that generate products between 50 and 150 bp. Smaller products are recommended, giving the widest

variation in melt temperature, as well as being the size of choice for rapid cycling protocols (18,24). The thermal profile employed in our experiments can be transferred between assays following these guidelines, and a slower, more-controlled temperature transition during melting may enhance T_m discrimination further (13).

We chose the sequence surrounding the sickle cell mutation site to demonstrate the applicability of the test, aided by the high GC content downstream of the SNP site that formed the wild-type fragment (58%) compared to the upstream sequence that formed mutant fragment (49%). As with all genotyp-

ing assays, the actual SNP position and surrounding sequence is nonvariable, meaning that under rare conditions the assay may not be appropriate. However, using the described procedure, we have successfully designed primer sets to detect a range of sequence polymorphisms using conventional primer design software.

Cycling conditions were manipulated to favor smaller products by reducing the extension time to 0 s, relying on the transitional times of the kinetic PCR cycle to amplify the shorter products (16). *Taq* DNA polymerase extends the two primers flanking the target region regardless of genotype, generating a 158-bp product with a calculated T_m of approximately 88°C. The melt peak from this larger fragment would be indistinguishable from the wild-type product, which has a slightly lower T_m of 87°C. Thus, amplification of this fragment may lead to false results by masking ASAs. Very rapid cycling combined with internal competition from smaller fragments and a high-fidelity enzyme system totally suppressed amplification of this region throughout our experiments (Figure 2).

Assay Optimization

We specify optimal discriminatory conditions as those able to amplify allele-specific products only with similar efficiency as determined by melt peak height and peak area (13). In addition, these conditions should reduce or completely remove artifactual amplification in the form of primer dimers. We used a strategy that would normally be applied to new PCR optimization, modified to accommodate oligonucleotide pairs. An initial magnesium titration was performed using "outer" primers to direct amplification. Maximum specificity and product yield was achieved with 3 mM magnesium (data not shown). Two oligonucleotide titration sets using control DNAs of known genotype under rapid cycling conditions defined optimal primer set concentrations for allelic discrimination. The ratios and interactions of reaction 3 gave optimal results, yielding single products with a T_m of 87.4°C with wild-type DNA and 85.7°C with mutant DNA. Each melt peak had a height of approximately 10 fluorescence

Figure 3. Example melting profiles from diagnostic tests representing one of each possible genotype. (A) Homozygous wild-type (sample 4); (B) homozygous mutant (sample 6); and (C) heterozygous DNA (sample 10). The three genotypes are easily identified by visual inspection of the graphs as described in text. Actual T_m values were slightly higher than calculated in most cases.

Short Technical Reports

Figure 4. Cluster analysis to determine sickle cell genotype. The x-axis represents peaks within the actual T_m range of $85.4^{\circ}\text{C} (\pm 0.3^{\circ}\text{C})$, and the y-axis represents areas within the actual T_m range of $87.3^{\circ}\text{C} (\pm 0.3^{\circ}\text{C})$. A symbol is assigned to represent homozygous DNA (▲) or heterozygous DNA (○). Points lining up along either the x- or y-axis confirm homozygous inheritance of mutant or wild-type alleles, respectively, and points lying near to the $x = y$ axis confirm heterozygous inheritance of the sickle cell mutation.

units and similar peak areas. The size of corresponding products was confirmed by gel electrophoresis (Figure 2).

SNP Genotyping

The fluorescent bidirectional PCR assay was validated using optimized discriminatory conditions to genotype 45 unknown samples for sickle cell status based on their melting profiles alone. In each case, clearly distinct profiles were achieved depending on genotype (Figure 3). Thirty-three samples generated a melt peak around $87.5^{\circ}\text{C} (\pm 0.2^{\circ}\text{C})$, five samples produced a melt peak at $85.7^{\circ}\text{C} (\pm 0.2^{\circ}\text{C})$, and seven samples amplified both allele-specific fragments, giving a dual-peak with maxima at $85.3^{\circ}\text{C} (\pm 0.3^{\circ}\text{C})$ and $87.6^{\circ}\text{C} (\pm 0.3^{\circ}\text{C})$, thus diagnosing homozygous wild-type, homozygous mutant, and heterozygous inheritance, respectively. Cluster analysis of product T_m values

Short Technical Reports

showed three unambiguous and distinct clustering regions, each corresponding to a particular genotype (Figure 4). For each test, "no template control" samples were included to assess contamination and nonspecific amplification by primer sets; no amplification occurred in these controls. If primer dimer had been formed in the reaction, then the T_m of the artifact would ensure that points were well away from profiles generated by actual template amplifications.

Experienced users of melt curve analysis will acknowledge that there can be well-to-well variation in the actual T_m of PCR products. Such variation is usually attributed to SYBR Green I concentration, salt concentration, or actual product yield (13). By using a commercial master mixture with a consistent concentration of SYBR Green I and employing optimized magnesium concentrations, this variation was minimized. In practice, the deviations observed were likely due to differences in product yield and did not cause error in our tests. Melt peak data was 100% concordant with restriction digestion band patterns (data not shown), validating our assay entirely.

In summary, fluorescent bidirectional PCR is mediated through a highly efficient kinetic process, coupled with an accurate melt curve analysis. The data presented here suggest that it has potential as a tool for screening programs of sickle cell anemia, particularly if coupled with informatics tools that would allow automation. While we appreciate that for clinical use a more thorough evaluation of the test would be appropriate, our trial has adequately demonstrated the potential of this method for use in SNP research. It provides a rapid, single-tube format, with internal control and sequence validation for SNP screening, in particular for near patient testing where the cost-effectiveness of the assay is the main driver. We believe that this will prompt a revival in an interest in allele-specific PCR for SNP detection.

REFERENCES

- Breslauer, K.J., R. Frank, H. Blocker, and L.A. Marky. 1986. Predicting DNA duplex stability from the base sequence. *Proc. Natl. Acad. Sci. USA* 83:3746-3750.
- Chang, J.C. and W.K. Kan. 1982. A sensitive new prenatal test for sickle-cell anemia. *N. Engl. J. Med.* 307:30-36.
- Crockett, A.O. and C.T. Wittwer. 2001. Fluorescein-labeled oligonucleotides for real-time PCR: using the inherent quenching of deoxyguanosine nucleotides. *Anal. Biochem.* 290:89-97.
- Germer, S. and R. Higuchi. 1999. Single-tube genotyping without oligonucleotide probes. *Genome Res.* 9:72-78.
- Hamajima, N. 2001. PCR-CTPP: a new genotyping technique in the era of genetic epidemiology. *Exp. Rev. Mol. Diagn.* 1:119-123.
- Jiang, Z., J. Shi, S. Yang, C. Zhang, H. Jiang, Z. Chen, L. Jin, D. Lu, and W. Huang. 2001. A simple and rapid new method for SNP typing by single-tube bidirectional allele-specific amplification. *Zhonghua Yi Xue Yi Chuan Xue Za Zhi* 18:306-309.
- Karhukorpi, J. and R. Karttunen. 2001. Genotyping interleukin-10 high and low producers with single-tube bidirectional allele-specific amplification. *Exp. Clin. Immunogenet.* 18:67-70.
- Lareu, R.R., N.R. Swanson, and S.A. Fox. 1997. Rapid and sensitive genotyping of hepatitis C virus by single strand conformation polymorphism. *J. Virol. Methods* 64:11-18.
- Liu, Q., E.C. Thorland, J.A. Heit, and S.S. Sommer. 1997. Overlapping PCR for bidirectional PCR amplification of specific alleles: a rapid one-tube method for simultaneously differentiating homozygotes and heterozygotes. *Genome Res.* 7:389-398.
- Livak, K.J. 1999. Allelic discrimination using fluorogenic probes and the 5' nuclease assay. *Genet. Anal. Biomol. Eng.* 14:143-149.
- Mullis, K., F. Faloona, S. Scharf, R. Saiki, G. Horn, and H. Erlich. 1986. Specific enzymatic amplification of DNA *in vitro*: the polymerase chain reaction. *Cold Spring Harbor Simple Quantitative Biol.* 51:263-273.
- Newton, C.R., A. Graham, L.E. Heptinstall, S.J. Powell, C. Summers, N. Kalsheker, J.C. Smith, and A.F. Markham. 1989. Analysis of any point mutation in DNA. The amplification refractory mutation system (ARMS). *Nucleic Acids Res.* 17:2503-2516.
- Ririe, K., R.P. Rasmussen, and C.T. Wittwer. 1997. Product differentiation by analysis of DNA melting curves during the polymerase chain reaction. *Anal. Biochem.* 245:154-160.
- Ronaghi, M., S. Karamohamed, B. Pettersson, M. Uhlen, and P. Nyren. 1996. Real-time DNA sequencing using detection of pyrophosphate release. *Anal. Biochem.* 242:84-89.
- Sarkar, G., J. Cassady, C.D.K. Bottema, and S.S. Sommer. 1990. Characterisation of polymerase chain reaction amplification of specific alleles. *Anal. Biochem.* 186:64-68.
- Sasvari-Szekely, M., A. Gerstner, Z. Ronai, M. Staub, and A. Guttman. 2000. Rapid genotyping of factor V Leiden mutation using single-tube bidirectional allele-specific amplification and automated ultrathin-layer agarose gel electrophoresis. *Electrophoresis* 21:816-821.
- Silva, D.D., A. Reiser, M. Herrmann, K. Tabiti, and C.T. Wittwer. 1998. Rapid Genotyping and quantification on the LightCycler™ with hybridization probes. *Biochemica* 2:12-15.
- Silva, D. and C.T. Wittwer. 2000. Monitoring hybridisation during polymerase chain reaction. *J. Chromatogr. B. Biomed. Appl.* 741:3-13.
- Sommer, S.S., A.R. Groszbach, and C.D.K. Bottema. 1992. PCR amplification of specific alleles (PASA) is a general method for rapidly detecting known single-base changes. *BioTechniques* 12:82-87.
- Tapp, L., L. Malmberg, E. Rennel, M. Wik, and A.C. Syvanen. 2000. Homogeneous scoring of single-nucleotide polymorphisms: comparison of the 5'-nuclease TaqMan assay and molecular beacon probes. *BioTechniques* 28:732-738.
- Thelwell, N., S. Millington, A. Solinas, J. Booth, and T. Brown. 2000. Mode of action and application of Scorpion primers to mutation detection. *Nucleic Acids Res.* 28:3752-3761.
- Tyagi, S., S.A.E. Marras, and F.R. Kramer. 2000. Wavelength-shifting molecular beacons. *Nat. Biotechnol.* 18:1191-1196.
- Waterfall, C.M. and B.D. Cobb. 2001. Single tube genotyping of sickle cell anaemia using PCR-based SNP analysis. *Nucleic Acids Res.* 29:e119.
- Wittwer, C.T. and D.J. Garling. 1991. Rapid cycle DNA amplification: time and temperature optimization. *BioTechniques* 10:76-83.
- Wittwer, C.T., B.C. Marshall, G.H. Reed, and J.L. Cherry. 1993. Rapid cycle allele-specific amplification: studies with the cystic fibrosis $\Delta F508$ locus. *Clin. Chem.* 39:804-809.
- Wu, Y., L. Ugozzoli, B.K. Pal, and R.B. Wallace. 1989. Allele-specific enzymatic amplification of β -globin genomic DNA for diagnosis of sickle cell anemia. *Proc. Natl. Acad. Sci. USA* 86:2757-2760.
- Ye, S., S. Dhillon, X. Ke, A.R. Collins, and I.N.M. Day. 2001. An efficient procedure for genotyping single nucleotide polymorphisms. *Nucleic Acids Res.* 29:e88.
- Ye, S., S. Humphries, and F. Green. 1992. Allele specific amplification by tetra-primer PCR. *Nucleic Acids Res.* 20:1152.

We thank Drs. John Old and Chris Fisher of the National Haemoglobinopathies Reference Service (Oxford, UK) for genotyping controls and unknown DNA samples. This work was supported by research grants from Molecular Sensing plc and the University of Bath. Address correspondence to Dr. Christy M. Waterfall, Molecular Sensing plc, Chalvey Business Park, Bradford Road, Melksham, Wiltshire SN12 8LH, UK. e-mail: christy.waterfall@molecular-sensing.com.

Received 21 February 2002; accepted 27 March 2002.

Christy M. Waterfall and Benjamin D. Cobb
Molecular Sensing plc
Melksham, Wiltshire, UK