© 1999 Nature America Inc. • http://biotech.nature.com

Detection of PCR products using self-probing amplicons and fluorescence

David Whitcombe[1]*, Jane Theaker[1], Simon P. Guy[1], Tom Brown[2], and Steve Little[1]

*[1]AstraZeneca Diagnostics, Gadbrook Park, Rudheath, Northwich, Cheshire CW9 7RA, UK. [2]Department of Chemistry, University of Southampton, Highfield, Southampton SO17 1BJ, UK.*Corresponding author (e-mail: david.whitcombe@diagnostics.zeneca.com)*

Received 18 January 1999; accepted 21 May 1999

Molecular diagnostics is progressing from low-throughput, heterogeneous, mostly manual technologies to higher throughput, closed-tube, and automated methods. Fluorescence is the favored signaling technology for such assays, and a number of techniques rely on energy transfer between a fluorophore and a proximal quencher molecule. In these methods, dual-labeled probes hybridize to an amplicon and changes in the quenching of the fluorophore are detected. We describe a new technology that is simple to use, gives highly specific information, and avoids the major difficulties of the alternative methods. It uses a primer with an integral tail that is used to probe an extension product of the primer. The probing of a target sequence is thereby converted into a unimolecular event, which has substantial benefits in terms of kinetics, thermodynamics, assay design, and probe reliability.

Keywords: Homogeneous assays, fluorescent probes, diagnostics, PCR, ARMS, single-tube genotyping, multiplexing

© 1999 Nature America Inc. • http://biotech.nature.com

The PCR is widely used in nucleic acids-based research and is increasingly applied in clinical molecular diagnostics. Closed-tube (homogeneous) methods have a number of advantages for practical diagnostic assays. For example, the absence of downstream analysis reduces the time needed to obtain results; in addition, the closed reaction tube minimizes the potential for cross-contamination, and the assay output is numerical rather than qualitative, allowing appropriate diagnostic statistics to be applied.

Homogeneous assay methods can be characterized as specific or nonspecific. Nonspecific methods detect the presence or absence of amplicon but provide no information about the amplified product. Examples of this type of method include the use of an intercalating agent[1–4], or Sunrise primers[5]. These methods are prone to "false positives" in that undesired products such as primer dimers and other spurious amplicons can increase fluorescence. Although it is possible to minimize the amplification of side products by careful primer design, and to prevent primer dimer accumulation by amplicon tailing methods[6], it is desirable for human diagnostics to use specific methods that probe the amplification product. Examples of such methods include fluorescence polarization[7], TaqMan[8–10], and Molecular Beacons[11,12].

We have devised a new method based on a primer with a tail attached to its 5′ end by a linker that prevents copying of the 5′ extension[13]. The probe element is designed so that it hybridizes to its target only when the target site has been incorporated into the same molecule by extension of the tailed primer (Fig. 1).

There are several consequences of this difference in probe–amplicon interaction. In particular, the appearance of signal is rapid and reliable, because probe–target binding is kinetically favored over duplex reannealing and thermodynamically favored over intrastrand secondary structures. The speed of these unimolecular binding events makes this signaling technology highly suitable for rapid assays in which equilibration times are short, giving it an advantage over bimolecular methods in which the rate of the PCR is reduced[14,15].

Results

Amplicon detection: allele-specific hybridization. A tailed primer (which we call Scorpions primer) was applied to the analysis of a region of exon 10 of the *BRCA2* gene that carries a frequent polymorphism[16]. Primer B2098 and unlabeled opposing primer R187-98, which flank the polymorphic base, were used to amplify this region. The tail portion of B2098 was designed to hybridize specifically to the C variant of the amplicon, while the stem of the signaling portion was designed to be thermodynamically more stable than the mismatch target–probe hybrid (see design rules below). The fluorescence output was monitored at each of the anneal/extend segments and plotted against cycle number (Fig. 2A). All variants were equally well amplified when products were examined on an agarose gel (results not shown). The fluorescence increase over time was readily detected for the matched product, and only a marginal increase in fluorescence was observed for the singly mismatched product. Heterozygous samples yielded half the final fluorescence of the homozygous matched targets. We extended this observation by the use of admixtures of starting template from 100% A target to 100 % C. The final fluorescence was plotted against the proportion of C target in the admixture, and a direct correlation was observed (Fig. 2B).

Comparison to a bimolecular approach. To illustrate some of the functional differences between a unimolecular approach and the corresponding bimolecular system, two sets of amplifications were performed. In the first, two PCR primers (R186-98 and R187-98) were used to amplify a region of the *BRCA2* gene; also included was Molecular Beacon probe Z3702, which can hybridize to the amplicon thus produced. The second set of amplifications contained the same two primer sequences and probe sequence; however, the probe was synthesized attached to primer R186-98 (Scorpions primer B2098). The two amplifications were monitored in real time at each 60°C hold, and the results are compared in Figure 3.

Mechanism of signal generation. To investigate the mechanism of signaling we examined exon 1 of the human K-*ras* gene using TaqMan, Molecular Beacons, and Scorpions methods[17]. For the molecular beacons and TaqMan assays, primers R015-98 and R844-97 were used, with MB001-98 and KR-TaqMan, respectively; for the Scorpions method, R844-97 was replaced with C2101. The reactions were cycled under standard conditions and fluorescence monitored throughout the anneal/extend step. Figure 4 plots the fluorescence

© 1999 Nature America Inc. • http://biotech.nature.com

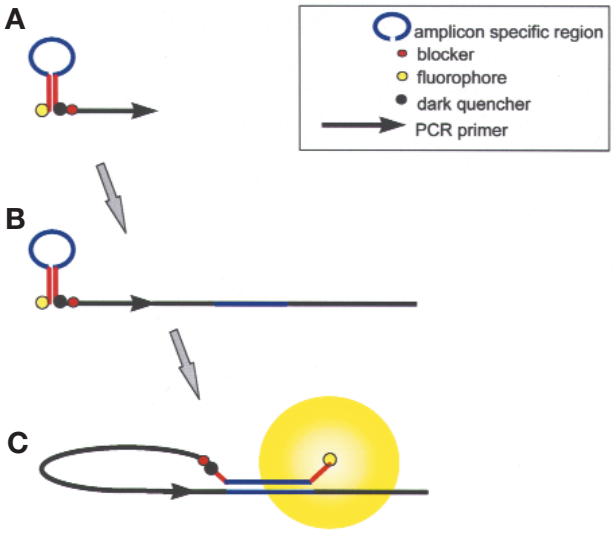

Figure 1. Detection of PCR products with self-probing amplicons. (A) A Scorpions primer carries a 5′ extension comprising a probe element, a pair of self-complementary stem sequences, and a fluorophore/quencher pair. The extension is "protected" from copying by the inclusion of a blocking HEG monomer. (B) After a round of PCR extension from the primer, a newly synthesized target region is now attached to the same strand as the probe. (C) Following a second round of denaturation and annealing, the probe and target hybridize.

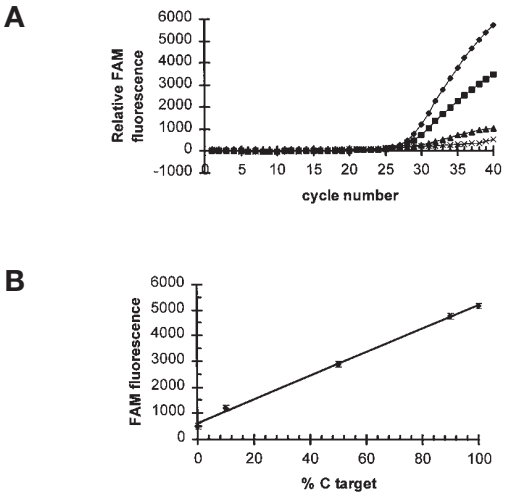

Figure 2. Amplicon detection and allelic specificity. (A) The *BRCA2* Scorpions primer B2098 was used in amplifications containing DNAs with *A/A* (▲), *A/C* (■) and *C/C* (◆) genotypes. A negative control (water) was also included (×). (B) Admixtures of homozygous (*A/A* or *C/C*) DNAs were generated at known ratios. The final fluorescence of these reactions was plotted against the proportion of *C* allele in the admixtures.

for each method for cycles 21–40, during which the product yield was growing efficiently. It is clear that each of the signaling methods has different characteristics when examined in this time frame.

Single-tube genotyping (STG). Because Scorpions primers probe in a strand-specific manner, multiplexing of reactions is straightforward, with little or no crosstalk between amplicons. In particular, by combining Scorpions with amplification refractory mutation system (ARMS) assays, both allelic variants can be tested in a single tube. The assays can be formatted in two different ways: overlapping primers (format 1, Fig. 5A) and opposing primers (format 2, Fig. 5B). We designed ARMS assays for a polymorphic site in the gene for interleukin 1A[18]. The test was in format 1: Scorpions D0680 and D0681 and primer R913-98 were each included at 500 nM. Samples of each genotype (homozygotes for each variant and heterozygotes) were tested in real time, on the ABI Prism 7700. 6-Carboxyfluorescein (FAM) reactions were specific for the *G* allele, and the *T* allele was monitored with tetrachloro-6-carboxyfluorescein (TET). The results, shown in Figure 6, demonstrate that the reactions were highly specific for the alleles in question. We have also performed STG with equal success in format 2 (results not shown).

Discussion

We have developed and tested a new method for the efficient homogeneous detection of PCR amplicons, using fluorescent molecules that combine the primer and probe functions. The major consequence of this design is that the production of probe–target hybrid from an extended Scorpion primer is concentration independent (zero order), resulting in a number of interesting functional properties.

The method works well for the detection of amplicon (Fig. 2A): large fluorescence increases were observed in the presence of amplicon, but not in the unamplified controls. Furthermore, the detection was highly specific, down to the level of single base changes. The use of stems in the probe element offers two advantages: first, background signals are minimal because signals from unincorporated Scorpions primers are switched off; second, the stem can be designed to be thermodynamically favored over the binding of probe to mismatch target. The allele-specific hybridization approach was extended to other allelic ratios, and we found that the magnitude of the allele-specific signal was proportional to the relative copy numbers of the variants (Fig. 2B).

The interaction between probe and target is efficient (see Fig. 3). Identical probe and amplicon sequences showed very different characteristics in a real-time assay. The bimolecular version of the assay did produce increased fluorescence, but the unimolecular version was much stronger (>20-fold). In addition to illustrating the efficiency of the unimolecular approach, these data show that the Scorpion probes act almost exclusively by the self-probing mechanism and do not substantially act as bimolecular probes.

The nature of the probe–target interaction was illustrated by comparison to both TaqMan and Molecular Beacons reactions (Fig. 4). The molecular beacons signal starts low and grows through the segment, indicating that a substantial proportion of the molecules have adopted the quenched conformation but can "open up" as they collide with the single-stranded target. In addition, at the beginning of each cycle, the signal is less than it was at the end of the previous cycle. In

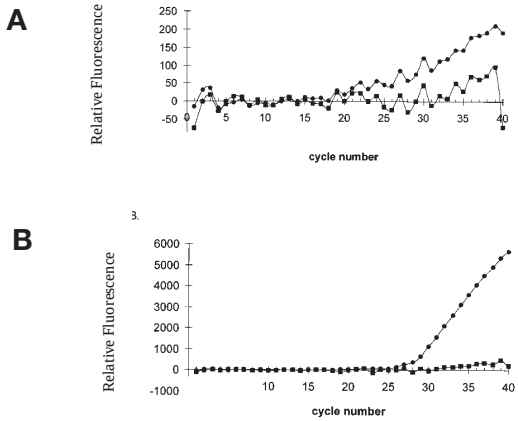

Figure 3. Comparison between bimolecular probing and the unimolecular probing in real-time PCR. Identical primer and probe sequences were used to amplify a region of the *BRCA2* gene in two formats: (A) probe free floating, or (B) probe attached to one of the amplimers. In each case, positives (●) contained C-variant genomic DNA while the negatives (■) contained only water.

© 1999 Nature America Inc. • http://biotech.nature.com

© 1999 Nature America Inc. • http://biotech.nature.com

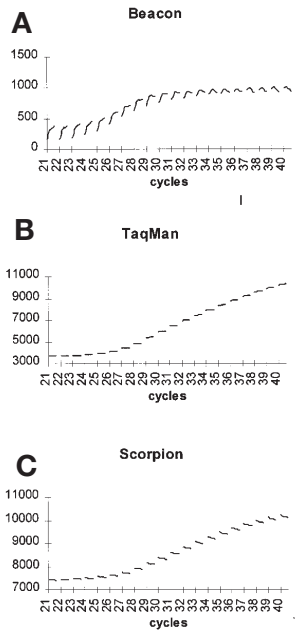

Figure 4. Fluorescence accumulation patterns for three fluorescence signaling methods. The accumulation of fluorescence throughout each 60°C anneal extend segment was monitored for three signal generation technologies: (A) Molecular Beacons (B) TaqMan, and (C) Scorpions.

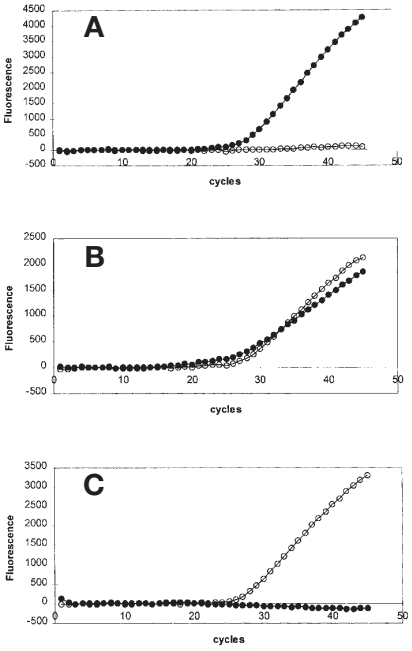

Figure 6. Single-tube genotyping. ARMS tests were combined and monitored simultaneously for both FAM (*G* allele, closed symbols) and TET (*T* allele, open symbols) fluorescence. Examples of each possible genotype are shown: (A) *G/G* homozygote (B) *G/T* heterozygote (C) *T/T* homozygote.

the later cycles of PCR, the growth in signal through a hold becomes more efficient, but is in competition against the reannealing of double-stranded amplicon, causing a decrease in signal. The displaced molecule can still revert to the thermodynamically stable "off" configuration.

In contrast, the TaqMan signal grows by increments and (unsurprisingly) never decreases. In fact the fluorescence accumulation, especially in the earlier cycles, is not visible during the 60°C hold.

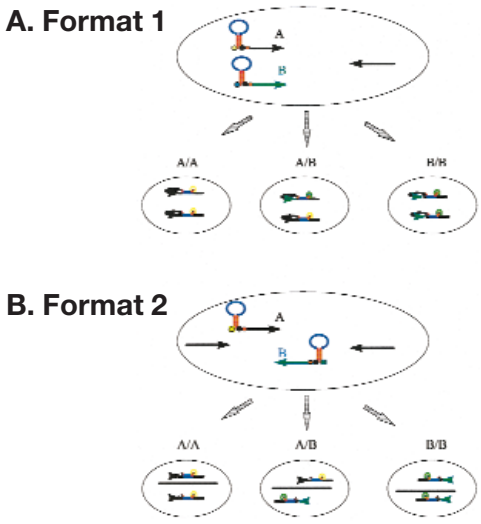

Figure 5. Two possible formats for combining two allelic reactions in a single tube. The figure shows the Scorpions and other primers and the possible outcomes of a genotyping experiment. Each specific ARMS primer carries a probe element labeled with a different fluorophore. In this way, the priming of a specific primer triggers signals of a certain wavelength. (A) In format 1, the two Scorpions prime in the same direction and share a single unlabeled "common" primer. For this reason, the reactions are expected to interact (compete) so that in heterozygotes, the signal for each fluorophore will be half its maximum in the homozygote. (B) In format 2, the Scorpions/ARMS primers are in opposite directions, overlapping only at the polymorphic site. Each amplification has a corresponding opposing primer and the reactions proceed independently of each other, although the two outer unlabeled primers amplify in all samples without directly generating signals.

This indicates that the extension and cleavage occur outside of that segment either before the hold ("on the way down" from the denature step) or after it ("on the way up" to the next denaturation). In the later cycles, as the PCR becomes less efficient, there is evidence of the fluorescence accumulating during the hold.

For Scorpions reactions, the fluorescence starts higher and tends to decline slightly through the segment. We believe that the amplicon quickly assumes the "on" position as it cools from the denaturation step (before the measurement begins) and becomes displaced to the "off" conformation, either by the oncoming Taq polymerase, or by the complementary second strand, during the 60°C step. Given that the fluorescence decline appears more extreme in the later cycles of PCR, this second mechanism of displacement seems more likely. Thus, fluorescence measurements would be better taken at as early a stage as possible.

Since the probes do not substantially cross-hybridize, we were able to multiplex ARMS reactions together (Fig. 6) to perform STG in two formats. If the probe element of one primer had been able to probe the product of the other allele-specific primer, there would have been mixed signals for both variants, and this would have prevented such clear-cut genotyping.

In spite of the additional sequence present on the 5′ ends of Scorpions primers, no decrease in priming specificity has been observed and PCRs appear to proceed with undiminished efficiency (data not shown).

The presence of the tail sequence on the primers does affect the way assays are designed, so that care must be taken to avoid the possibility of primers hybridizing to a probe element, thereby "unraveling" all the probe in an amplification-independent manner and causing significant, target-independent fluorescence increases. These issues are no greater, however, than for Molecular Beacons or other free-probe methods, unless the interaction is between the 3′ end of a Scorpion and its attached 5′ tail.

We have successfully designed more than 100 fully functional Scorpions primers–amplicons with only a single assay failure. Among the successes were assays for amplicons recalcitrant to Molecular Beacons probing (probably because of secondary structures within the amplicon). Thus unimolecular fluorescent probes may improve homogeneous diagnostic assays by increasing both assay design reliability and speed of signaling, thereby potentially shortening assay completion time.

© 1999 Nature America Inc. • http://biotech.nature.com

© 1999 Nature America Inc. • http://biotech.nature.com

Experimental protocol

Design and mechanism of Scorpions primers. Scorpions primers were produced by modifying the design of existing, working PCR primers (or ARMS primers) by the addition of a nonamplifiable monomer, typically hexethylene glycol (HEG) (Oswel DNA services, Southampton, UK), which prevents copying of the elements further in the 5′ direction[13], a methyl red monomer (Oswel) to act as a nonfluorogenic (dark) quencher of the fluorophore, a probe element flanked by self-complementary stems (hairpin), and a fluorophore. It is important to eliminate copying of the extension in order to prevent the quenched tail structure from "unravelling" and thus giving rise to sequence-independent signaling.

Hairpin design and amplicon probing was modeled using the DNA *mfold* program on the Michael Zuker website, using the thermodynamic parameters established by John Santalucia[19,20].

For allele-specific hybridization, the tails were designed such that a single mismatch between the probe element and the primer extension products was less thermodynamically favored than the stem-loop "off" configuration, while the fully matched extension product was more thermodynamically favored than the "off" position.

Primers and Scorpions primers. Primers were synthesized in house, and Scorpions primers were purchased from Oswel DNA Services. Sequences were designed using the Oligo 5.0 software (National Biosciences Inc., Plymouth, MN) to minimize primer dimer and other primer secondary structures and to ensure primers were matched for PCR.

Allele-specific amplification, (ARMS)[21], was used for allele discrimination, and additional mismatches were introduced near the 3′ terminus of the primers to enhance specificity. Where two different allele-specific primers were to be multiplexed together, the additional mismatches were placed in different positions to minimize the possibility of any "cross-priming" against products derived from the other primer. The introduction of such additional mismatches reduces the primer's efficiency such that product tends to appear somewhat later than might be expected (a delay of typically about five cycles).

BRCA2 reactions. B2098-*BRCA2* Scorpions primer (reverse direction): FAM-CGCACGATGTAGCACATCAGAAGCGTGCG-MR-HEG-TTGGAGATTTGTCACTTCCACTCTCAAA. The probe portion is specific for the C variant of the *BRCA2* polymorphism. R186-98: untailed equivalent of B2098: TTGGAGATTTGTCACTTCCACTCTCAAA. R187-98: opposing primer to R186-98 and B2098 (forward direction): GTGGAACCAAATGATACTGATCCATTAGATTC. Z3702: the probe segment of the Scorpion B2098 (also C specific): FAM-CGCACGATGTAGCACATCAGAAGCGTGCG-MR.

Il1a Scorpions and primer sequences. D0680: T-variant specific ARMS primer labeled with TET: TET-CCCATGCCGTATTTCACATTGCTCAGGAAGGCATGGG-MR-HEG GGTTTTAGAAATCAAGCCTAGGTCGT. D0681: G-variant specific ARMS primer labeled with FAM: FAM-CCCATGCCGTATTTCACATTGCTCAGGAAGGCATGGG-MR-HEG-GGTTTTAGAAATCAAGCCTAGGTTAG. R913-98: opposing (reverse) primer: CATTGGCTCGAATTAACTTTGATTGA.

K-*ras* reactions. MB001-98: FAM-CGCGGUGCCUUGACGAUACAGCUAAUUCAGAACGCG-MR. This probe hybridzes to exon 1 of the human K-*ras* gene; it is made from 2'-*O*-methyl-RNA, to ensure the none of the signal could be generated via cleavage by the 5¢-3¢ exonuclease of Taq polymerase. The sequence in boldface type overlaps that of the TaqMan probe (KR-TaqMan). KR-TaqMan: FAM-CAAGAGTGCCTTGACGATACAGCTA-TAMRA. C2101: FAM-CCCGCCATATTACTGGTGCAGGCGGG-MR-HEG-CTCATGAAAATGGTCAGGAAACCTTTATC, exon 1 reverse primer, tailed. R844-97: CTCATGAAAATGGTCAGAGAAACCTTTATC, exon 1 reverse primer, unlabeled. R015-98: TGACTGAATATAAACTTGTGGTAGTTGGCG, exon 1 forward primer.

Template DNA. For the *BRCA2* polymorphism and K-*ras* studies, cell lines with known genotypes were used as the source of DNA, which was prepared by proteinase K/phenol methods[22]. For the IL1A test, a panel of DNA samples prepared using an alkali-boiling method[22] from the blood of 100 informed consenting donors was tested by ARMS reactions to identify suitable homozygous and heterozygous samples.

PCR. Reactions (25 or 50 μl) comprised 1× ARMS buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.2 mM $MgCl_2$, 0.01% wt/vol gelatin), containing 500 nM primer or Scorpions primer, 1 unit of AmpliTaq Gold (Perkin-Elmer/Applied Biosystems, Warrington, UK) per 25 μl reaction mix. Human genomic DNA (5–50 ng) or water for no-template controls was added to each reaction. Where appropriate, a Molecular Beacon or TaqMan probe was included at 500 nM.

AmpliTaq Gold was activated by preincubation at 94°C for 20 min, before cycling for 40 cycles of 94°C, 45 s; 60°C, 45 s. Amplifications were performed in Perkin-Elmer 9600 thermocyclers or, for continuous fluorescence monitoring, in an ABI Prism 7700 machine. Unless otherwise stated, fluorescence measurements were "clipped data," that is, the mean of the last three fluorescent readings in each cycle (fluorescence measurements for each well are collected every 6–7 s).

Acknowledgments

We thank Dr. Neil Gibson for helpful discussions, Kay Callaghan and Kemal Haque for sharing of unpublished K-ras data, and Dr. Julian Sorrell for information concerning IL-1A polymorphisms. The methods described in this paper form part of worldwide patent applications. ARMS is the subject of European patent number 0332435 (ZENECA Ltd.) and corresponding US patent #5595890. The use of nonamplifiable tails and blocking monomers are the subjects of European patent 0416817 (ZENECA Ltd.) and corresponding US patent #5525494.

1. Ishiguro, T. *et al*. Homogeneous quantitative assay of hepatitis C virus RNA by polymerase chain reaction in the presence of a fluorescent intercalater. *Anal. Biochem*. **229**, 207–213 (1995).
2. Tseng, S.Y. *et al*. An homogeneous fluorescence polymerase chain reaction assay to identify *Salmonella*. *Anal. Biochem.* **245**, 207–212 (1997).
3. Wittwer, C.T. *et al*. The Lightcycler™ a microvolume multisample fluorimeter with rapid temperature control. *Biotechniques* **22**, 176–181 (1997).
4. Wittwer, C.T., Herrmann, M.G., Moss, A.A. & Rasmussen, R.P. Continuous fluorescence monitoring of rapid cycle DNA amplification. *Biotechniques* **22**, 130. (1997).
5. Nazarenko, I.A., Bhatnagar, S.K. & Hohman, R.J. A closed tube format for amplification and detection of DNA based on energy transfer. *Nucleic Acids Res.* **25**, 2516–2521 (1997).
6. Brownie, J. *et al*. The elimination of primer-dimer accumulation in PCR. *Nucleic Acids Res.* **25**, 3235–3241 (1997).
7. Gibson, N. J. *et al*. A homogeneous method for genotyping with fluorescence polarization. *Clin. Chem.* **43**, 1336–1341 (1997).
8. Holland, P.M., Abramson, R.D., Watson, R. & Gelfand, D.H. Detection of specific polymerase chain reaction product by utilizing the 5'–3' exonuclease activity of *Thermus aquaticus* DNA polymerase. *Proc. Natl. Acad. Sci. USA* **88**, 7276–7280 (1991).
9. Gelmini, S. *et al*. Quantitative polymerase chain reaction-based homogeneous assay with fluorogenic probes to measure C-Erbb-2 oncogene amplification. *Clin. Chem.* **43**, 752–758 (1997).
10. Livak, K.J., Marmaro, J. & Todd, J.A. Towards fully automated genome-wide polymorphism screening. *Nat. Genet.* **9**, 341–342 (1995).
11. Tyagi, S. & Kramer, F.R. Molecular beacons—probes that fluoresce upon hybridization. *Nat. Biotechnol.* **14**, 303–308 (1996).
12. Tyagi, S., Bratu, D.P. & Kramer, F.R. Multicolor molecular beacons for allele discrimination. *Nat. Biotechnol.* **16**, 49–53 (1998).
13. Newton, C.R. *et al*. The production of PCR products with 5′ single-stranded tails using primers that incorporate novel phosphoramidite intermediates. *Nucleic Acids Res*. **21**, 1155–1162 (1993).
14. Ibrahim, M.S., Esposito, J.J., Jahrling, P.B. & Lofts, R.S. The potential of 5′ nuclease PCR for detecting a single-base polymorphism in orthopoxvirus. *Mol. Cell. Probes* **11**, 143–147 (1997).
15. Ibrahim, M.S. *et al*. Real-time microchip PCR for detecting single-base differences in viral and human DNA. *Anal. Chem.* **70**, 2013–2017 (1998).
16. Couch, F. J. *et al*. BRCA2 germline mutations in male breast cancer cases and breast cancer families. *Nat. Genet.* **13**, 123–125 (1996).
17. Ferrie, R.M., Ellison, G., Callaghan, K. & Fox, J.C. UK Patent Application, number 9815224.2 (1998).
18. van den Velden, P.A. & Reitsma, P.H. Amino acid dimorphism in IL1A is detectable by PCR amplification; *Hum. Mol. Genet.* **2**, 1753 (1993).
19. Zuker, M. *mfold* Program website. http://www.ibc.wustl.edu/~zuker.
20. Santalucia, J. A unified view of polymer, dumbbell, & oligonucleotide DNA nearest-neighbor thermodynamics. *Proc. Natl. Acad. Sci. USA* **95**, 1460–1465 (1998).
21. Newton, C.R. *et al*. Analysis of any point mutation in DNA. The amplification refractory mutation system (ARMS). *Nucleic Acids Res*. **17**, 2503–2516 (1989).
22. Ferrie, R.M. *et al*. Development, multiplexing, and application of ARMS tests for common mutations in the CFTR gene. *Am. J. Hum. Genet.* **51**, 251–262 (1992).

© 1999 Nature America Inc. • http://biotech.nature.com