

# Detection of cystic fibrosis alleles from single cells using molecular beacons and a novel method of asymmetric real-time PCR

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We present a method for rapid and accurate identification of the normal and  $\Delta F508$  alleles of the cystic fibrosis (CF) gene in single human cells that utilizes LATE (linear after the exponential)-PCR, a newly invented form of asymmetric PCR. Detection of the single-stranded amplicon is carried out in real time, using allele-specific molecular beacons. The LATE-PCR method permits controlled abrupt transition from exponential to linear amplification and thereby enhances the fluorescent signals and reduces variability between replicate samples relative to those obtained using typical real-time PCR. Of 239 single lymphoblasts generating amplification signals, 227 (95%) exhibited signals that met objective quantitative criteria required for diagnosis. Among these samples, 222 were genotyped correctly, for an assay accuracy of 98%. The small number of diagnostic errors was due to allele drop-out among heterozygous lymphoblasts, 4/119 (3.4%), and contamination among homozygous  $\Delta F508$  lymphoblasts, 1/57 (1.8%). LATE-PCR offers a new strategy for preimplantation genetic diagnosis and other fields in which accurate quantitative detection of single copy genes is important.

*Key words:* cystic fibrosis  $\Delta F508$ /fluorescent oligonucleotide probes/linear DNA amplification/preimplantation genetic diagnosis/quantitative PCR

## Introduction

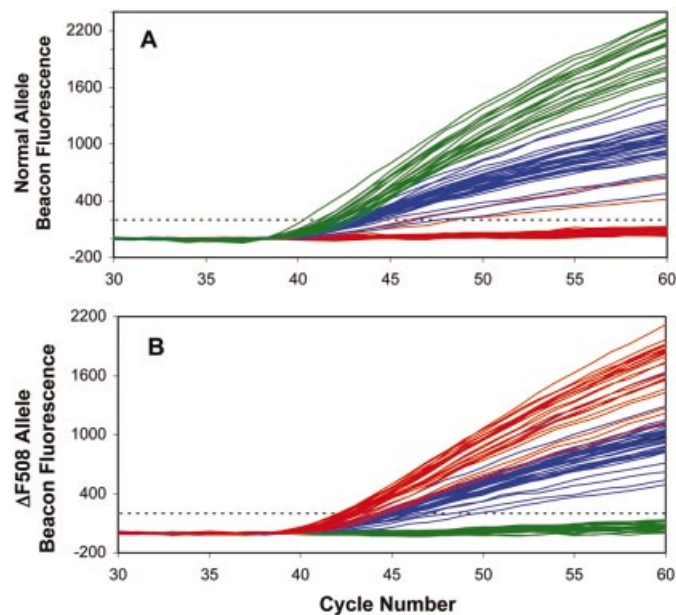
Cystic fibrosis (CF) is the most common inherited disease among Caucasian populations with an incidence of ~1 in 2500 births (Welsh *et al.*, 1995). Couples in which both individuals carry a mutant copy of the CF gene have a one in four chance of having an affected child. The conditions caused by these mutations range from mild to life-threatening. Preimplantation genetic diagnosis (PGD) offers such couples an opportunity to determine the genetic composition of their embryos before starting a pregnancy and thereby avoid either having an affected child or terminating a pregnancy of an affected fetus. However, PGD is technically difficult because each assay is carried out on a single cell recovered from a cleavage stage embryo.

A 3 base pair (bp) deletion, designated  $\Delta F508$ , accounts for nearly 70% of CF cases and causes severe manifestations of the disease. It results in the absence of phenylalanine at position 508 of the cystic fibrosis transmembrane conductance regulator (CFTR) protein and this error prevents normal processing and translocation of the polypeptide chain to apical membranes of epithelial cells (Cheng *et al.*, 1990). The first tests for  $\Delta F508$  in single cells used nested PCR to amplify the requisite sequence followed by verification of the final product either by restriction enzyme digestion (Coutelle *et al.*, 1989), hybridization to allele-specific oligonucleotides (Wu *et al.*, 1993), or by heteroduplex formation (Liu *et al.*, 1993; Avner *et al.*, 1994). The first clinical reports of PGD for CF also utilized heteroduplex analysis of the PCR products (Handyside *et al.*, 1992; Verlinsky *et al.*, 1992; Ao *et al.*, 1996). More recent PCR assays have used fluorescently labelled primers to increase sensitivity and reduce the rate of allele drop-out (ADO), a failure to amplify one allele from a heterozygous cell (Findlay *et al.*, 1995; Verlinsky and Kuliev, 2000; Goossens *et al.*,

2000). When this approach is employed, the fluorescently labelled products are separated and identified by electrophoresis after PCR amplification is finished.

In this report, we describe the use of real-time PCR with molecular beacons to identify the normal and  $\Delta F508$  alleles of cystic fibrosis in single human cells. Molecular beacons are fluorescently tagged single-stranded oligonucleotides that only fluoresce when bound to a complementary sequence. Several molecular beacons, each with a differently coloured fluorophore, can be used simultaneously to monitor accumulation of PCR products that differ by as little as a single base (Tyagi and Kramer, 1996; Kostrikis *et al.*, 1998) and thereby offer exquisite allele-discrimination capabilities. Molecular beacon assays are carried out in closed tubes that permit fluorescence detection during product amplification, thereby shortening the duration of the assay and minimizing the risk of laboratory contamination.

In order to increase the intensity of the molecular beacon signals and reduce sample variation, we developed a unique PCR method in which amplification of a double-stranded DNA molecule abruptly shifts to linear amplification of the single strand to which the molecular beacons bind. We call this new method LATE-PCR, an acronym for 'linear after the exponential'. As with all asymmetric PCR, LATE-PCR uses primers at different concentrations, but unlike typical asymmetric PCR, amplification is efficient due to improved primer design. LATE-PCR with molecular beacons also has similarities to symmetric real-time PCR assays, such as those developed for detection of the human Y chromosome (Pierce *et al.*, 2000) and detection of Tay-Sachs disease (Rice *et al.*, 2002), in that both generate signals that reflect the number of target molecules present in



**Figure 1.** Examples of specific molecular beacon fluorescence increase during real-time PCR in samples containing single lymphoblasts homozygous normal for CF (green), heterozygous  $\Delta F508$  (blue), or homozygous  $\Delta F508$  (red). (A) Fluorescent signal from the molecular beacon detecting the normal allele. (B) Fluorescent signal from the molecular beacon detecting the  $\Delta F508$  allele. Dashed lines indicate the threshold of 200 units ( $\sim 10$  SD above baseline readings) used for determining  $C_T$  values.

the initial sample. However, LATE-PCR consistently generates strong signals because the absence of product strand reannealing permits unhindered hybridization of the molecular beacon to its target strand and continued accumulation of that strand beyond the cycle at which symmetric reactions typically plateau (Sanchez *et al.*, 2003).

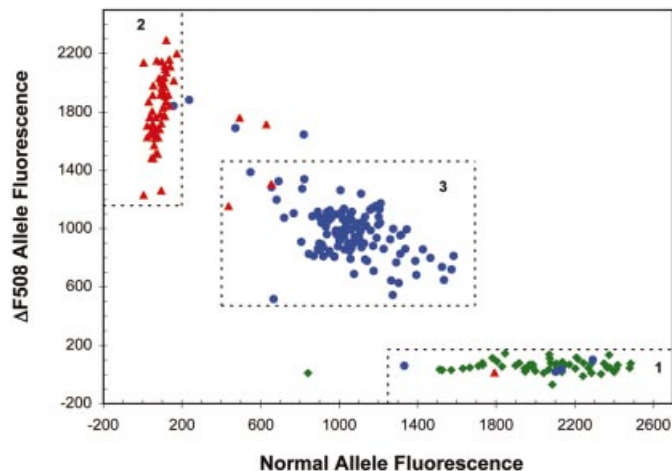
## Materials and methods

### Lymphoblasts

Human lymphoblast cell lines homozygous or heterozygous for CF  $\Delta F508$ , or homozygous for the normal CF gene sequence, were obtained from Coriell Cell Repositories (USA). Lymphoblasts were prepared and manipulated as described elsewhere (Pierce *et al.*, 2000). Single lymphoblasts were transferred directly to MicroAmp optical PCR tubes (PE Applied Biosystems, USA) containing 10  $\mu$ l QuantiLyse (Hamilton Thorne Biosciences, USA), an optimized lysis solution containing proteinase K (Pierce *et al.*, 2002). Samples were incubated at 50°C for 30 min, then 95°C for 10 min in an ABI 7700 (PE Applied Biosystems).

### Molecular beacons and primers

Molecular beacons were designed according to previously described methods (Tyagi and Kramer, 1996; Pierce *et al.*, 2000) and were purchased from Biosearch Technologies (Novato, USA). The molecular beacon sequence for the normal allele was 5'-FAM-CGCGCTTATCATCTTTGGTGTTCCTATAGCGCG-Dabcyl-3' and for the  $\Delta F508$  allele was 5'-TET-CGCGCTAAAATATCATTGGTGTTCCTAAGCGCG-Dabcyl-3'. FAM and TET are fluorescent molecules and Dabcyl quenches that fluorescence when the beacon is in a hairpin configuration in the absence of its target sequence. Primers were designed to amplify an 84 bp region that includes the  $\Delta F508$  location. The upper primer sequence was 5'-CCTGGATTATGCCTGG-CACCAT-3' and the lower primer sequence was 5'-CCTTGATG-ACGCTTCTGTATCTA-3'. The melting temperatures of these primers were estimated using a formula based on nearest-neighbour thermodynamics (Allawi and SantaLucia, 1997) and a salt concentration adjustment (SantaLucia *et al.*,



**Figure 2.** Final fluorescence values from normal and  $\Delta F508$  allele-specific molecular beacons following amplification from single lymphoblasts homozygous normal for the CF gene (green diamonds), heterozygous  $\Delta F508$  (blue circles), or homozygous  $\Delta F508$  (red triangles). Dashed lines indicate 3 SD from the means of samples generating only normal signal (box 1), only  $\Delta F508$  signal (box 2), or both signals (box 3).

1996). A program that computes these values is available at <http://bioweb.pasteur.fr/seqanal/interfaces/melting.html>. The  $T_m$  of the upper primer at the limiting concentration of 50 nmol/l is 59°C and the  $T_m$  of the lower primer at a concentration of 1  $\mu$ mol/l is 58°C, each calculated at a total monovalent cation concentration of 70 mmol/l. Specific amplification of the 84 bp product from single lymphoblasts was confirmed using SYBR Green detection during amplification and product denaturation, and by electrophoresis through agarose gels.

### PCR conditions

Fifteen microlitres of concentrated PCR reagent mixture was added to each tube containing a lysed cell (or no cell control) to yield a final sample volume of 25  $\mu$ l with final concentrations of 1 $\times$ PCR buffer (Invitrogen, USA), 3.75 mmol/l MgCl<sub>2</sub>, 0.25 mmol/l dATP, 0.25 mmol/l dCTP, 0.25 mmol/l dGTP, 0.75 mmol/l dUTP, 50 nmol/l upper primer, 1  $\mu$ mol/l lower primer, 1.2  $\mu$ mol/l of each molecular beacon, and 1.5 IU Platinum Taq DNA polymerase (Invitrogen). Amplification and fluorescence detection were carried out in an ABI 7700. Thermal cycling consisted of an initial 5 min denaturation at 95°C followed by four cycles of 95°C for 10 s, 55°C for 2 min, and 72°C for 30 s, followed by 21 cycles of 95°C for 10 s, 55°C for 30 s, and 72°C for 30 s, followed by 35 cycles of 95°C for 10 s, 52°C for 30 s, and 72°C for 30 s, with fluorescence acquisition during the 52°C step. The total duration of the PCR is 3 h. Contamination control procedures for reagent preparation and sample handling were described previously (Pierce *et al.*, 2000).

## Results

Previous reports from our laboratory have emphasized that real-time PCR is particularly useful for PGD because kinetic analysis allows optimization of PCR conditions otherwise not possible (Pierce *et al.*, 2000; Rice *et al.*, 2002). Efficient amplification is indicated by early detection signals with minimal variance among replicates. The present analysis of cystic fibrosis began with an evaluation of several pairs of primers at equimolar concentration and several molecular beacons under a variety of concentrations and buffer conditions. None of these proved entirely satisfactory because signal strength was low. In order to overcome this problem we explored the use of asymmetric PCR in which primers are used at unequal concentrations in order to generate a single-stranded product that is more accessible to a molecular beacon.

We realized that reducing the concentration of an oligonucleotide reduces its melting temperature ( $T_m$ ), a fact that is not taken into account in traditional asymmetric PCR. We discovered that the primer used at limiting concentrations must have a concentration-adjusted  $T_m$  equal to, or above, that of the primer used in excess in order to achieve efficient exponential amplification of both DNA strands and then abruptly switch to linear amplification when the limiting primer is exhausted. Moreover, by adjusting the concentration of the limiting primer, it is possible to cause this switch to occur coincident with the cycle at which the fluorescent signals reach the detection threshold, i.e. the  $C_T$  value. Accumulation of the single-stranded product can be monitored by including one or more molecular beacons that target this strand without competition from the complementary strand. This protocol, designated LATE-PCR, is an improvement over traditional approaches to asymmetric PCR. LATE-PCR is particularly useful for analysis of single target molecules because it generates brighter molecular beacon signals, reduces sample variance, and allows amplification to continue for many more cycles without plateau (Sanchez *et al.*, 2003).

All reactions described here contained two molecular beacons. One, labelled with the fluorophore TET, was complementary to 22 nucleotides at the site of the  $\Delta F508$  3 bp deletion; the other, labelled with the fluorophore FAM, was complementary to 22 nucleotides in the same region of the normal allele. The specificity of each molecular beacon for single target sequences was confirmed by PCR analysis of single lymphoblasts homozygous for either the normal allele, or the  $\Delta F508$  allele. As anticipated, homozygous normal lymphoblasts generated fluorescent signals from only the molecular beacon specific to the normal allele, while fluorescence from the molecular beacon to the  $\Delta F508$  allele always remained below threshold (green lines in Figure 1A, B). Conversely, lymphoblasts homozygous for  $\Delta F508$  generated fluorescent signal from the molecular beacon specific to the  $\Delta F508$  allele, while fluorescence from the normal allele beacon remained below threshold in the vast majority of samples (red lines in Figure 1A, B). Figure 1 also illustrates that the increasing signal observed with LATE-PCR is linear rather than sigmoidal.

When lymphoblasts heterozygous for the  $\Delta F508$  and normal alleles of cystic fibrosis were assayed by LATE-PCR, all but a few samples generated both signals (blue lines in Figure 1A, B). Comparison of the three sets of signals in Figure 1 reveals that the  $C_T$  values of most reactions from heterozygous cells (blue lines) are slightly higher than the  $C_T$  values of reactions from cells homozygous for either allele (green and red lines). In addition, the mean slope of the lines from heterozygous cell reactions is approximately one-half of the mean slope of the lines from homozygous cells. These observations are consistent with the fact that heterozygous cells contain only a single copy of each allele whereas homozygous cells contain two copies of one of the alleles. The mean strength of the signals from heterozygous cells is more than double that obtained using symmetric PCR and the SD is vastly reduced (Pierce *et al.*, 2001).

The above results illustrate the quantitative dimensions that real-time LATE-PCR technology adds to PCR analysis. The kinetics of signal detection provide additional information that is useful for sample analysis. Since clinical samples with unknown genotypes can only be evaluated based on the signals generated (normal only, mutant only, or both), each lymphoblast sample was analysed on that basis, regardless of its known genotype. Although most reactions within each of these groups had similar kinetics, a few samples generated  $C_T$  and/or final fluorescence values far from the mean values for that set. Such variants may reflect problems with target availability, PCR amplification, or detection of products. In the context of PGD, such deviants could increase the chances of a misdiagnosis. Therefore, each set of reactions was evaluated in terms of both  $C_T$  values and final

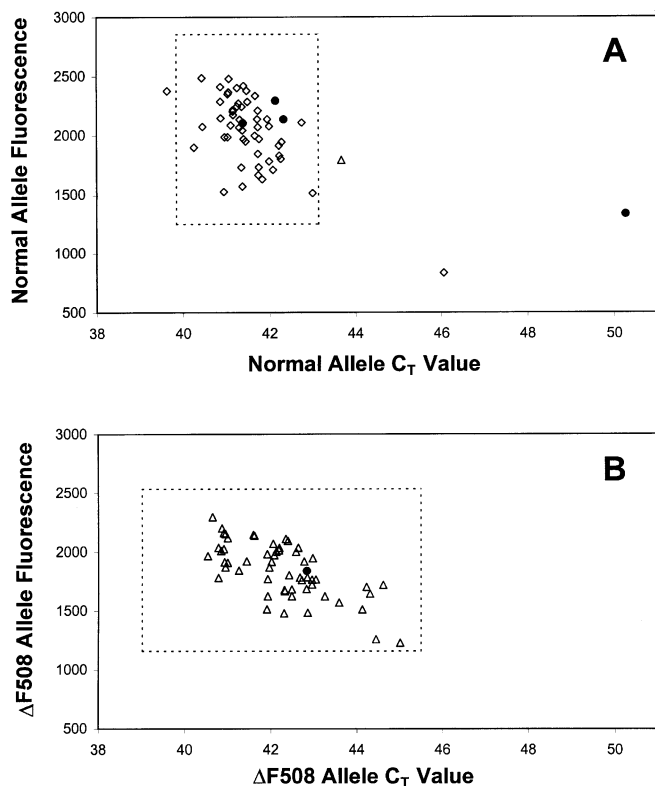
fluorescence (fluorescence intensity after 60 PCR cycles). The resulting analysis established objective quantitative criteria with which to judge whether a particular reaction should or should not be considered diagnosable. Samples having either a  $C_T$  value or a final fluorescence value  $>3$  SD from the mean values for the corresponding set were categorized as atypical and were not used to evaluate the accuracy of the LATE-PCR assay (see below).

Figure 2 displays the final fluorescence values of all lymphoblast samples, as well as the boundaries 3 SD away from each mean value. The set of reactions generating only normal allele signals (box 1) includes all homozygous normal lymphoblasts (green diamonds) plus four of the 125 heterozygous lymphoblasts (blue circles). The latter are examples of ADO, as they did not generate the expected  $\Delta F508$  signal. In addition, one lymphoblast homozygous for  $\Delta F508$  (red triangles) unexpectedly generated only a normal allele signal, presumably the result of contamination combined with failure to either transfer the lymphoblast to the PCR tube, or to amplify the  $\Delta F508$  allele within that cell. The  $C_T$  values and final fluorescence for these normal allele signals are presented in Figure 3A. The contaminated sample, one of the heterozygous samples, and two homozygous normal samples exhibited  $C_T$  values  $>3$  SD from the mean  $C_T$  value and were eliminated from further evaluation based on this objective criterion.

The final fluorescence values for all samples exhibiting only the  $\Delta F508$  allele signal are presented in box 2 of Figure 2. These data include the great majority of the homozygous  $\Delta F508$  lymphoblasts (red triangles), as well as one heterozygous lymphoblast (blue circle) that failed to generate a signal for the normal allele, a case of ADO. All final fluorescence and  $C_T$  values for all points in this data set are within 3 SD of the corresponding means (Figure 3B).

Also included in Figure 2 are the final fluorescence values for all reactions generating both signals. These data include 120 heterozygous lymphoblasts (blue circles), of which 117 had values within the threshold boundaries (box 3). Three samples displayed preferential amplification of the  $\Delta F508$  allele and therefore fell above the upper boundary of the box. Four homozygous  $\Delta F508$  lymphoblasts also generated both signals, presumably due to contamination. However, for these samples the final fluorescence values for the normal allele were among the lowest observed and for  $\Delta F508$  were of unusually high intensity in two of the four samples, placing them above the upper boundary of box 3. All samples that fell within box 3 were evaluated further by examining  $C_T$  values for both signals (Figure 4). Two heterozygous lymphoblasts (filled circles) and one of the two remaining homozygous  $\Delta F508$  lymphoblasts (open triangles) had a  $C_T$  value  $>3$  SD above the mean, as indicated by the symbols outside the box. Thus, only one of the four samples contaminated with normal DNA could not be excluded as atypical based on either final fluorescence or  $C_T$  values. Of the 120 heterozygous samples, 115 yielded both signals with final fluorescence and  $C_T$  values within 3 SD of the means.

These results are summarized in Table I. Of the total 256 lymphoblasts tested, 239 exhibited at least one signal above threshold, yielding an assay utility of 93%. The utility ranged from 83 to 98% depending on the cell line used, suggesting that the PCR assay *per se* was not responsible for the majority of the amplification failures. Of the 239 samples that generated at least one fluorescent signal, 227 (95%) had  $C_T$  and final fluorescence values within 3 SD of the appropriate mean values. We use the term diagnostic efficiency to refer to the fraction of samples generating signals within these limits and recommend that only such samples should be used for clinical diagnostic purposes (Pierce *et al.*, 2000). Had the 227 diagnosable samples presented clinically, four heterozygotes would have been misdiagnosed as homozygotes due to ADO, and one  $\Delta F508$

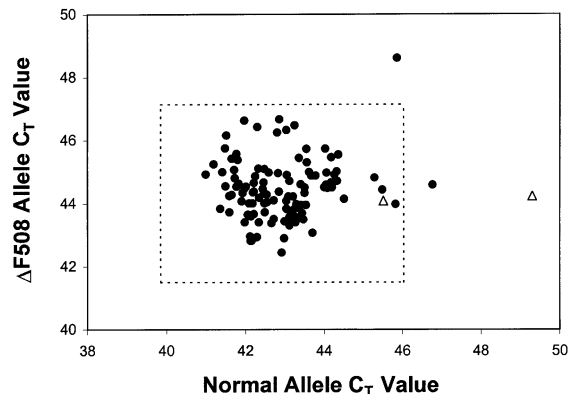


**Figure 3.** Scatter plots of  $C_T$  values and final fluorescence from single lymphoblast samples generating (A) only normal allele, or (B) only  $\Delta F508$  allele signal. Lymphoblasts include those homozygous normal for the CF gene (open diamonds), heterozygous  $\Delta F508$  (filled circles), or homozygous  $\Delta F508$  (open triangles). Dashed lines indicate 3 SD from the means.

homozygote would have been misdiagnosed as heterozygous due to contamination. Thus, the LATE-PCR assay described here has a diagnostic accuracy of 98% (222/227).

## Discussion

Molecular beacons are effective tools for identifying individual alleles in real-time closed-tube reactions (Kostrikis *et al.*, 1998). The results presented here demonstrate that the normal and  $\Delta F508$  alleles of the cystic fibrosis gene in single cells can be distinguished rapidly and accurately using this technology in combination with LATE-PCR. Improved accuracy is achieved, in part, by utilizing the quantitative features of real-time detection. Samples that do not exhibit amplification kinetics within a defined range can be objectively excluded from diagnosis. We previously used such criteria to develop an assay that was >99% accurate for detection of the multi-copy TSPY gene on the human Y chromosome in lymphocytes, as well as in blastomeres from embryos having <30% fragmentation (Pierce *et al.*, 2000). We have now extended the same analytic approach to alleles of a single copy gene in single cells. Quantitative analysis of the data allowed us to objectively eliminate samples with atypical signals and thereby improve the accuracy of the assay from 96 to 98%. Although a few samples that gave the expected fluorescent signals were also eliminated from diagnostic evaluation, we believe that the increase in accuracy is worth the 5% reduction in diagnosable samples, even if a small percentage of normal embryos is excluded from transfer when this assay is applied to PGD. Similar improvement in accuracy was obtained when this strategy was used for the most common mutant allele of the Tay-Sachs gene (Rice *et al.*, 2002).



**Figure 4.**  $C_T$  values for normal and  $\Delta F508$  allele signals from single lymphoblast samples that are within the 3 SD limits for final fluorescence of both signals (i.e. are within box 3 in Figure 2). This data set includes 117 heterozygous  $\Delta F508$  lymphoblasts (filled circles) and two homozygous  $\Delta F508$  lymphoblasts (open triangles). Dashed lines indicate 3 SD from the means.

The ADO rate observed among the 119 heterozygous lymphoblasts with diagnosable signals was 3.4% (4/119). This rate is lower than rates observed using conventional PCR and similar to the best rates observed using fluorescent primers (Lissens and Sermon, 1997; Goossens *et al.*, 2000; Verlinsky and Kuliev, 2000). One reason for low ADO is likely to be the use of QuantiLyse, a proteinase K-based lysis solution that has been optimized using real-time PCR to ensure maximum availability of DNA targets for amplification (Pierce *et al.*, 2002). Cell lysis in water as well as some versions of solutions containing proteinase K that have been used for PGD were clearly inferior for releasing DNA for amplification. Alkaline lysis solutions containing dithiothreitol (DTT) also gave inferior results, possibly due to the effect of residual DTT on PCR efficiency. Another possible reason for our low ADO rate rests in optimization of PCR conditions using real-time technology (Pierce *et al.*, 2000), since the choice of thermal cycling profile and amplicon size are among factors that influence ADO rates (Piyamongkol *et al.*, 2003). In addition, the kinetics of real-time amplifications can be used to evaluate the validity of the signals generated. For instance, one of the heterozygous  $\Delta F508$  lymphoblasts exhibited a single, late signal that could be distinguished from that of known homozygotes. In the case of Tay-Sachs heterozygotes, one of two examples of ADO could be similarly identified based on atypical kinetics (Rice *et al.*, 2002). Conventional PCR strategies only analyse final PCR products without visualizing the kinetics of the reaction and therefore inherently have higher risk of misdiagnosis.

Another advantage of real-time PCR is the reduced risk of laboratory contamination with amplicon molecules, because the tubes are not opened following amplification. Consistent with this decreased risk of amplicon contamination, no signals were observed in any of 60 no-cell control reactions run after a large number of amplification reactions had already been carried out in our laboratory during the development of the CF assay.

Contamination of assays with cellular DNA is another possible serious problem in PGD. Five of 64 reactions prepared with homozygous  $\Delta F508$  lymphoblasts showed normal allele signals. However, by analysing the kinetics of amplification in these reactions, all but one was found to have at least one atypical signal, illustrating yet another advantage of the real-time PCR assay. Thus, the contamination rate in the data set presented here was 1.8% of the homozygous  $\Delta F508$  cells that had diagnosable signals, or 0.4% of all

**Table I.** Evaluation of real-time PCR for diagnosis of cystic fibrosis (CF) genotype

Genotype	Total	Any signal	Utility <sup>a</sup> (%)	Diagnosable signals	Efficiency <sup>b</sup> (%)	Diagnosis			Accuracy <sup>c</sup> (%)
						+/+	+/-	-/-	
Homozygous CF normal	64	53	83	51	96	51	0	0	100
Heterozygous $\Delta F508$	128	125	98	119	95	3	115	1	97
Homozygous $\Delta F508$	64	61	95	57	93	0	1	56	98
All lymphoblasts	256	239	93	227	95				98
No-cell control	60	0							

<sup>a</sup>Percentage of samples that generate any signal.

<sup>b</sup>Percentage of samples with signals that generate only diagnosable signals, i.e. within 3 SD of means for  $C_T$  and final fluorescence.

<sup>c</sup>Percentage of diagnosable samples that exhibit the expected signals.

diagnosable samples. We anticipate lower contamination rates in the future as we focus on improving protocols for handling large numbers of single cell samples.

This study describes the first application of a generalizable design for real-time amplifications, which we have named LATE-PCR. The method results in linear accumulation of single-stranded DNA, and when used in conjunction with molecular beacons, increased signal intensity and reduced sample variation. These features are particularly useful for real-time PCR initiated with single cells. Standard real-time PCR requires ~40 cycles to reach levels of detection using molecular beacons, but typical exponential amplification begins to slow down and plateau within a small number of additional cycles (Rice *et al.*, 2002). LATE-PCR circumvents this amplification plateau, enabling continued increase in signal for many additional cycles while minimizing the increased sample-to-sample variation typically observed at the end of PCR. LATE-PCR is related to asymmetric PCR (Gyllensten and Erlich, 1988) in the use of primers at different concentrations. However, traditional asymmetric PCR is most often done using primers designed for symmetric PCR. Reducing the concentration of one of those primers reduces amplification efficiency, a generally accepted phenomenon that can be verified using real-time detection (unpublished data). LATE-PCR design includes the use of a limiting primer that has a concentration-adjusted  $T_m$  at least as high as that of the excess primer. This enables efficient exponential amplification until the limiting primer is depleted and the reaction makes an abrupt transition from exponential to linear amplification. By adjusting the concentration of the limiting primer, this transition can be timed to coincide with the threshold cycle at which the target strand is first detected. Additional LATE-PCR design features are published elsewhere (Sanchez *et al.*, 2003).

The new technologies described here promise higher accuracy, reduced handling, and reduced total assay time for clinical PGD. These improvements can be achieved using the most recent generation of lower cost, real-time PCR equipment (unpublished data), suggesting that PGD could soon be a service available at more IVF clinics. In order to make this goal a reality, assays for additional alleles of CF, Tay-Sachs and other genetic diseases will have to be developed. We are currently developing such assays using the LATE-PCR strategy because of its improved sensitivity and low ADO rates.

## Acknowledgements

The authors thank Dr Fred Kramer and Dr Sanjay Tyagi for assistance with molecular beacon technology. This research was supported in part by a grant from Hamilton Thorne Biosciences, Inc.

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Submitted on June 11, 2003; resubmitted on August 5, 2003;  
accepted on August 11, 2003