

METHODS IN MOLECULAR MEDICINE™

Single Cell Diagnostics

Methods and Protocols

Edited by

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Preface

Until recently, the world of diagnostics revolved around large chunks of tissue, whole blood samples, cell suspensions, or extracted DNA in microgram quantities. Given such a large starting template, problems of contamination and assay failure are rare but do still occur. When working with extremely small templates such as single cells or small groups of cells and their metabolites, these diagnostic challenges are simply magnified. Single cell diagnostics is a fast moving area even though the field has focused primarily on the analysis of embryonic blastomeres for the preimplantation diagnosis of single gene and chromosomal disorders—a relatively tiny area of diagnostics in terms of test volume. In just over a decade, there has been a diagnostic shift in the field from relatively simple singleplex PCR assays analyzed with ethidium based gel electrophoresis through sensitive fluorescent PCR involving multiplexing to expression analysis of multiple genes. Finally, the seemingly ubiquitous microarray technology has been applied to single cells for the identification of chromosome abnormalities and DNA alterations. *Single Cell Diagnostics: Methods and Protocols* is intended for clinical and research scientists as well as those providing care for couples seeking treatment for infertility or preimplantation genetic diagnosis. The aim is for all readers to extend their knowledge and expertise in analysis of single cells (whether or not that is their specific need). The majority of readers may not require assays of such exquisite sensitivity, but it has been my experience that many excellent research and diagnostic laboratories have learned useful tips from those of us struggling to salvage accurate diagnostic information from a single cell without contamination. *Single Cell Diagnostics: Methods and Protocols* starts with laser-assisted cell collection, noninvasive assessment of single cells and moves through the techniques of standard fluorescence *in situ* hybridization and polymerase chain reaction (PCR). As the reader moves through the book, the scope and complexity of each technique gradually increases as real-time quantitative PCR, isothermal whole genome amplification, comparative genomic hybridization, real-time gene expression analysis and the production of RNA and cDNA libraries are covered. The book closes with the application of customized microarrays to the study of single cells.

The future may see (1) a further shift away from preimplantation genetic diagnosis and more toward more routine diagnostic analysis in diseases such as cancer (in situations where very little tissue might be available for analysis)

and (2) direct proteomic analysis and indirect analysis (via the secretome) from single cells.

Alan Thornhill

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Linear-After-The-Exponential Polymerase Chain Reaction and Allied Technologies

Real-Time Detection Strategies for Rapid, Reliable Diagnosis from Single Cells

Kenneth E. Pierce and Lawrence J. Wangh

Summary

Accurate detection of gene sequences in single cells is the ultimate challenge to polymerase chain reaction (PCR) sensitivity. Unfortunately, commonly used conventional and real-time PCR techniques are often too unreliable at that level to provide the accuracy needed for clinical diagnosis. Here we provide details of linear-after-the-exponential-PCR (LATE-PCR), a method similar to asymmetric PCR in the use of primers at different concentrations, but with novel design criteria to ensure high efficiency and specificity. Compared with conventional PCR, LATE-PCR increases the signal strength and allele discrimination capability of oligonucleotide probes such as molecular beacons and reduces variability among replicate samples. The analysis of real-time kinetics of LATE-PCR signals provides a means for improving the accuracy of single cell genetic diagnosis.

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Key Words: Asymmetric PCR; cell lysis; fluorescent probes; molecular beacons; proteinase K; real-time PCR.

1. Introduction

The polymerase chain reaction (PCR) provides a method for identifying alleles of specific genes, or the mRNA transcribed from those genes. Through the 1980s and most of the 1990s, the products of PCR amplification were characterized using postamplification methods such as restriction enzyme treatment followed by electrophoresis through agarose or polyacrylamide gels. These and other postamplification detection strategies are time-consuming and increase the risk of contaminating subsequent assays. This is particularly problematic in the case of single cell samples, because a single product molecule inadvertently introduced into a sample tube can generate a false positive result and lead to a misdiagnosis.

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Real-time PCR using fluorescent probes (**1–3**) allows the kinetics of the amplification process to be observed and analyzed. Moreover, the fact that real-time PCR is carried out in closed tubes greatly reduces the risk of laboratory contamination, saves time, and is amenable to automation. Real-time assays using TaqMan™ probes have become popular for many applications, primarily owing to the “assays on demand” program from Applied BioSystems for primer and probe design. However, the TaqMan assay requires digestion of the probe by the exonuclease activity of Taq polymerase, a process that requires probes with a relatively high melting temperature (T_m). This, in turn, makes it more difficult to distinguish allelic variants and can also reduce amplification efficiency. Molecular beacons and several other types of commercially available probes have greater allele-discriminating capacities than TaqMan probes but have design constraints of their own.

Regardless of which type of probe is used to monitor a symmetric real-time amplification, hybridization of the probe to its target must compete with the reannealing of the complementary amplicon strands. By the end of the reaction, amplicon strand reannealing predominates and the probe detects only a fraction of the total number of amplicons produced (**Fig. 1A**). To circumvent this problem, we investigated the possible use of asymmetric PCR. Asymmetric PCR uses unequal concentrations of primers first to amplify both DNA strands exponentially, then shifts to linear amplification of one strand on depletion of the limiting primer. The DNA strand that is produced by the extension of the excess primer during the linear phase is freely accessible for hybridization to the probe (**Fig. 1B**). However, traditional asymmetric PCR that makes use of primers designed for symmetric amplifications (**4**) is inefficient, highly variable, and tends to generate high levels of nonspecific product. Those undesirable characteristics can be overcome if primers are designed for use at unequal concentrations. The resulting amplification strategy, termed Linear-After-The-Exponential PCR (LATE-PCR) is efficient and specific (**5,6**). **Figure 2** shows a comparison of symmetric PCR and LATE-PCR for the detection of the $\Delta F508$ allele of the cystic fibrosis gene (CFTR) in single cells.

LATE-PCR also makes it possible to use lower temperature detection, because the probe does not need to compete with hybridization and extension of the limiting primer during the early, exponential phase of the reaction. Hybridization of probe and target is unimpeded once the limiting primer is depleted and can be done either by lowering the annealing temperature at that point, or by introducing a low-temperature detection step between the extension and melting steps. Probes with lower melting temperatures are easier to design, more allele discriminating, and have lower background fluorescence. Moreover, because the probe dissociates from its target strand well below the extension temperature of the reaction, sufficient probe can be added to the reaction to

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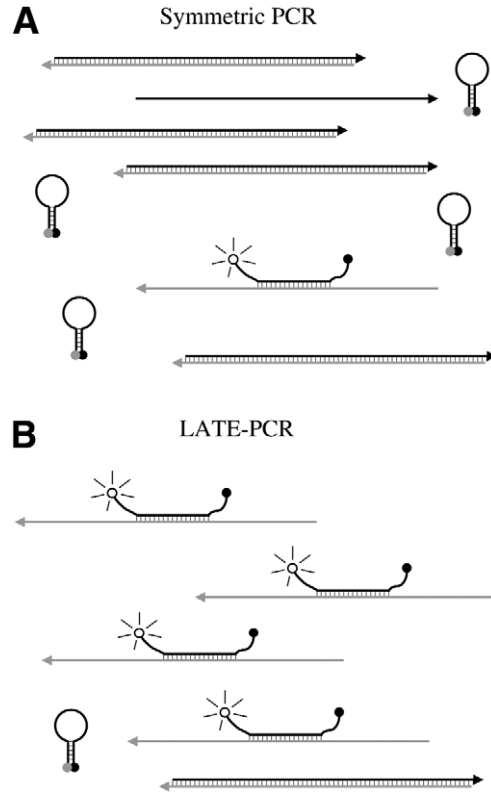


Fig. 1. Schematic comparison of symmetric PCR and LATE-PCR for amplicon detection using molecular beacons. Near the completion of symmetric PCR (A), the complementary strands of the amplicon (black and gray lines with arrows representing the 3' ends) reach high concentrations and reanneal. Molecular beacon molecules unable to hybridize with those targets remain in the nonfluorescent, hairpin configuration. LATE-PCR (B) generates an excess of the amplicon strand that is the target of the molecular beacon. Molecular beacons readily hybridize to those strands and emit fluorescence, generating a much greater total fluorescent signal from the LATE-PCR sample.

measure all product strands without inhibiting the amplification reaction (5). We have used these features of LATE-PCR for constructing single cell assays for several alleles of cystic fibrosis ([7]; unpublished data), Tay-Sachs disease (5), and B-thalassemia and p53 (unpublished data). Here we provide practical information for the design and use of LATE-PCR assays.

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2. Materials

1. Cells with desired genotypes for positive controls (Coriell Cell Repositories).
2. Microscope of choice for cell analysis and transfer.

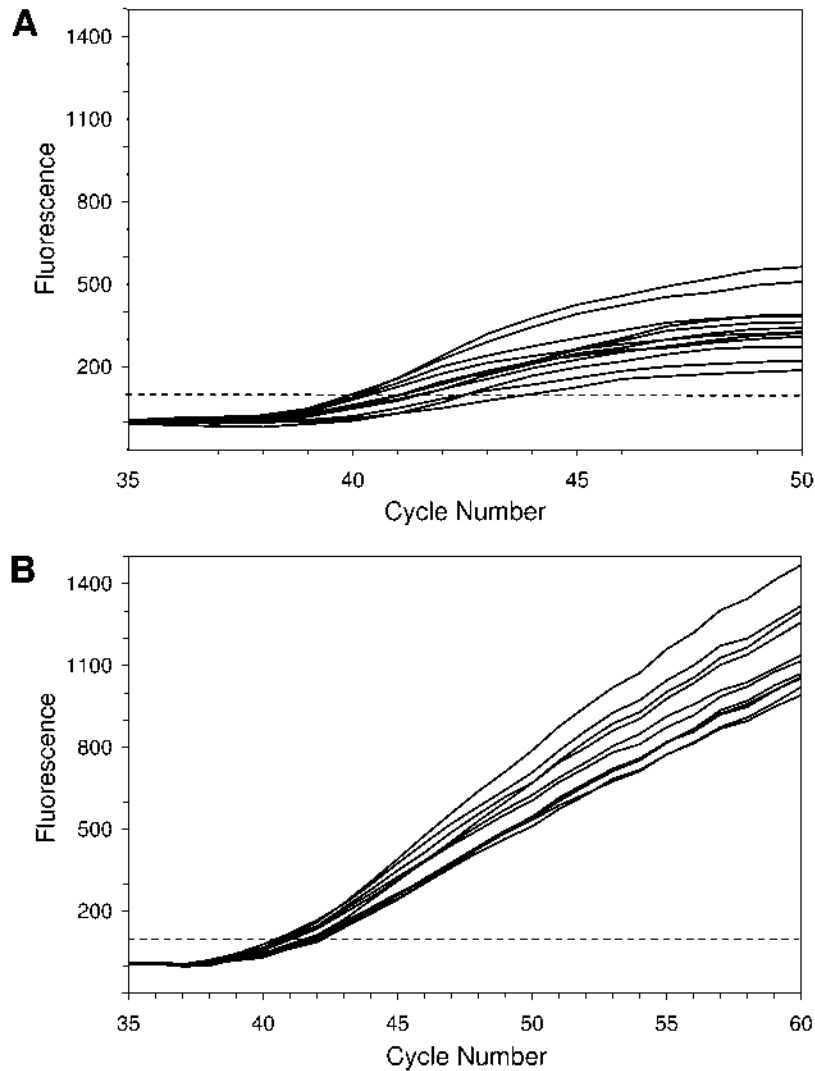


Fig. 2. Real-time PCR results for detection of $\Delta F508$ allele in single, heterozygous lymphoblasts using molecular beacons. (A) Symmetric PCR replicate samples exhibit wide ranges of C_T values (the point at which fluorescence reaches the dashed threshold line) and low final fluorescence. (B) LATE-PCR replicate samples have relatively low variation in C_T values and much higher final fluorescence.

3. PCR enclosure hoods (e.g., Labconco Purifier™).
4. Low-attachment culture dishes (e.g., Corning 6-well; cat. no. 3471).
5. Narrow-bore cell transfer pipets and micromanipulators.
6. Mechanical pipettors and aerosol-resistant pipet tips.

7. Calcium-free, magnesium-free phosphate-buffered saline (PBS) (Sigma).
8. Nonacetylated bovine serum albumen (BSA) or polyvinylpyrrolidone (PVP) (optional) (Sigma).
9. Lysis solution: 100 µg/mL of proteinase K (Roche); 5 µM sodium dodecyl sulfate (SDS) (Sigma); 10 mM Tris-HCl, pH 8.3 (TRIZMA® Pre-Set Crystals, Sigma).
10. PCR primer design software.
11. Thermal cycler with fluorescence detection capability (e.g., ABI PRISM® 7000 or 7700, Bio-Rad iCycler, Stratagene Mx3000P™ or Mx4000®, or Cepheid SmartCycler®).
12. Optical sample tubes appropriate to the thermal cycler.
13. Racks for placing sample tubes on ice (e.g., ABI MicroAmp® Bases).
14. Standard thermal cycler or heating blocks (with heated cover) for lysis reaction (optional).
15. PCR reagents:
 - a. Taq polymerase with hot-start capacity (either with anti-Taq antibodies such as Platinum Taq [Invitrogen], or with modified enzymes such as AmpliTaq Gold [ABI]).
 - b. Buffers containing Tris and KCl (usually supplied with commercial Taq polymerases).
 - c. MgCl₂ stock solution at 25 or 50 mM.
 - d. Custom oligonucleotide primers and probes.
 - e. Deoxynucleotide triphosphates (dNTPs), PCR grade (Promega).
 - f. Water, molecular biology grade.
 - g. SYBR Green I (Molecular Probes) (optional).

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3. Methods

Obtaining reproducible results from samples of single cells requires (1) sample preparation that avoids inhibitors of PCR and removes chromosomal proteins from the DNA; (2) the use of primers and probes that maximize amplification efficiency, specificity, and signal strength; and (3) analysis of real-time signal kinetics from tested samples and from controls with known genotypes.

3.1. Preparation and Lysing of the Cell

The choice of methods for isolating single cells will vary considerably depending on the cell type and available equipment. For instance, cells in suspension can be isolated individually by hand-controlled micromanipulation or by fluorescence-activated cell sorting. Alternatively, fixed or embedded cells can be isolated using laser capture microscopy, although the required equipment is expensive and not widely available. This chapter provides only general information on this topic with the intention of pointing out potential pitfalls that can affect cell lysis, genomic DNA preparation, and subsequent PCR.

3.1.1. Isolation and Washing of the Cell

When cell isolation is carried out manually, cells should first be diluted to a density that facilitates picking up individual cells using either a handheld pipet or a pipet in a micromanipulator. Adherent cells should be dissociated by repeated pipetting, preferably in a calcium-free, magnesium-free medium or PBS. Petri dishes or microtiter plates with low-adhesion surfaces can reduce the chances of cell damage or loss. Solution additives such as BSA or PVP can also be used for this purpose, but any additive should be carefully evaluated for its effect on cell lysis and amplification (*see Note 1*).

Several components present in culture medium or used in cell isolation techniques can inhibit PCR and must be removed by transferring the cell through PBS or culture medium that lacks the inhibitors (*see Note 2*). One or two rinses may be sufficient if the transferred volume can be kept to a minimum (e.g., overall volume dilution of 1:100 or greater per step). Transfers should be practiced before attempting to manipulate valuable, limited-source cells. First aspirate a small amount of the wash solution into the transfer pipet, and then aspirate the cell into the tip of the pipet. Carefully expel the contents of the pipet into the wash while examining under the microscope. As soon as the cell exits the pipet, remove the pipet from the wash dish, expel the remaining solution into a separate container, and rinse the pipet in unused wash solution. Repeat this procedure as necessary to reduce the concentration of potential PCR inhibitors. All washes should be brief.

3.1.2. Preparation of Lysis Solution and Transferring of the Cell

Using real-time detection of multicopy genes, we demonstrated that a properly buffered solution containing proteinase K and SDS provides the greatest number of targets for amplification (**8**). This lysis reagent can be prepared in advance and stored for up to at least 1 yr at -20°C in a constant-temperature freezer (i.e., not frost free). Other tested lysis methods resulted in more variable recovery and/or delayed detection, presumably owing to either DNA damage or incomplete removal of chromosomal proteins from the DNA. A delay in PCR signals can also indicate inefficient amplification owing to the presence of PCR inhibitors.

Shortly before preparing the cells, an aliquot of the lysis reagent is thawed on ice and 10 μL is pipetted to each PCR sample tube (*see Note 3*). It is extremely important to use procedures that minimize the likelihood of contamination when preparing or working with lysis solution and PCR reagents (*see Note 4*). Sample tubes should be kept on ice until the cells have been transferred, because proteinase K is self-digesting at rates that are temperature dependent. Any unused solution should be discarded, because repeated freezing and thawing may reduce enzyme activity.

The final cell transfer is done directly into lysis solution in a PCR tube (*see Note 5*), keeping the volume of the transferred wash solution to a minimum. Depending on the type of sample tube, it may not be possible to observe the cell during this transfer. Careful observation of the fluid height in a fine-bore pipet is usually sufficient to ensure the transfer of the cell and avoid adding an excessive volume of wash solution. The sample tube should be centrifuged briefly (a few seconds) to ensure that all liquid is at the bottom of the tube and returned to ice until the completion of all cell transfers.

3.1.3. Lysis Incubation

Lysis incubation should be carried out in a temperature-controlled block or thermal cycler separate from that used for amplification (*see Note 6*). Incubate samples at 50°C for 30 min, then 95°C for 15 min. It may be possible to shorten the 50°C incubation depending on the cell type. The high-temperature incubation is required to inactivate proteinase K completely. A heated cover must be in place over the samples to prevent condensation. If condensation is present on the cap or sides of the tubes following this incubation, subsequent amplification efficiency may be reduced. Reaction tubes can be frozen at -20°C (constant-temperature freezer) or placed on ice for immediate use.

3.2. LATE-PCR

There are three main criteria for LATE-PCR design. First, the concentration-adjusted melting temperature of the limiting primer (T_m^L) at the start of the reaction must be at least as high as that of the excess primer. This is achieved by making the limiting primer either longer or higher in percentage of guanine and cytosine (G + C) relative to the excess primer. Second, the concentration-adjusted temperature of the excess primer (T_m^X) must be reasonably close to the melting temperature of the double-stranded amplicon (T_m^A) in order for that primer to compete successfully with the accumulating single-stranded product for hybridization to the target strand. Third, if real-time detection is utilized, the concentration of the limiting primer should be chosen such that the limiting primer is depleted approximately when the probe signal reaches the detection threshold, i.e., at the C_T value of the reaction.

3.2.1. Designing of Limiting and Excess Primers

Primers originally designed for symmetric PCR can be modified to fit LATE-PCR criteria (usually by lengthening the primer chosen as limiting), or primers can be newly selected according to those criteria. In either case, primer design software should be used to evaluate internal stability characteristics and 3' dimer formation in the same manner as would be done for symmetric PCR

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primers. Computer software can also be helpful in selecting primers. Preferred primer software provides input for primer concentration and should calculate T_m according to nearest-neighbor methods using accurate thermodynamic values (**9–11**). Do not rely on primer T_m calculations based on the earlier estimates of nearest-neighbor thermodynamic values by Breslauer (**12**).

When selecting new primers for LATE-PCR, it is useful to scan the sequences neighboring the site to be probed (e.g., mutation or polymorphism) for a region with relatively high GC content. The initial choice of a limiting primer can be made from that region. It generally does not matter which DNA strand is chosen for the sequence of the limiting primer as long as the hybridization probe is later chosen from a sequence on the same strand. An initial evaluation of the region to be amplified also can provide an estimate of T_m^A , which will be needed to determine the required T_m^X value.

The concentration of limiting primers should be about 50 nM (1.25 pmol/25- μ L reaction) when used in combination with molecular beacons labeled with FAM or TET. At that concentration, a limiting primer length of approx 24–32 nucleotides is needed to achieve T_m^L in the vicinity of 65°C (*see Note 7*). Excess primer concentration is usually 1 or 2 μ M. Optimal amplification efficiency and specificity are achieved with T_m^X about 5° below T_m^L when the primer concentration ratio is in the 20:1 to 40:1 range (**6**). Primer T_m calculations are made using the nearest-neighbor formula (**13**):

$$T_m = \frac{\Delta H}{\Delta S + R \ln(C/2)} + 12.5 \log[M] - 273.15$$

The thermodynamic values ΔH and ΔS are calculated according to Allawi and SantaLucia (**9**). R is the universal gas constant and C is the initial concentration of the primer. The salt correction is that of SantaLucia et al. (**14**) using $[M]$ as the total molar concentration of monovalent cations, sodium, and potassium in the PCR buffer. The T_m calculations can be made using the MELTING program available on the Internet site <http://bioweb.pasteur.fr/seqanal/interfaces/melting.html>.

Another consideration in designing primers for LATE-PCR is T_m^A . That value depends primarily on amplicon length and GC content. Short amplicons (about 100 nt) are preferred for gene expression analysis or diagnosis of a specific genetic allele. When multiple alleles are tested or sequencing information is desired, longer regions can be successfully amplified. We have been able to amplify a 650-nt segment of the p53 gene using LATE-PCR criteria and using samples directly for sequencing (unpublished data).

Even for short amplicons, T_m^A does not vary significantly with concentration, because the helix growth steps dominate the helix initiation step, producing a pseudo-first-order equilibrium for which no concentration effect is

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observed (12). Therefore, good estimates of T_m^A are obtained using a “%GC” formula (15):

$$T_m^A = 81.5 + 16.6 \log \frac{[M]}{1 + 0.7[M]} + 0.41(\%G + \%C) - 500 / \text{length}$$

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The formulas do not include a factor for magnesium concentration, which can raise the actual T_m several degrees, but still provide valuable comparisons for designing amplification reactions.

As already mentioned, T_m^X must be reasonably close to T_m^A in order for that primer to compete successfully with the accumulating single-stranded product for hybridization to the target strand. We have observed the strongest real-time detection signals when $T_m^A - T_m^X$ is about 10–15°C, as calculated using these formulas (6). Signal strength was lower as $T_m^A - T_m^X$ increased and was unacceptably low when it exceeded 20°C. Therefore, primers must have a higher T_m for amplicons that are long or GC rich.

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3.2.2. Probe Design

We describe the design of molecular beacons for LATE-PCR, although it should be recognized that many other types of oligonucleotide probes can be used with this amplification technique (see **Note 8**). Molecular beacons are fluorescently labeled oligonucleotides that assume a stem-loop structure in the absence of homologous target, bringing a fluorophore on the 5' end of the molecule into close proximity of a quenching moiety (e.g., DABCYL) on the 3' end (**Fig. 1**) (1). The molecular beacon is able to hybridize with a DNA strand (such as a PCR product) with sequence homologous to its loop. In that configuration, the fluorophore emits its fluorescent signal when illuminated at particular wavelengths. Thus, increasing PCR product in the presence of the homologous molecular beacon generates corresponding increases in fluorescent signal (**Fig. 2**). Multiple targets can be monitored in the same reaction by labeling different molecular beacon sequences with different fluorophores. LATE-PCR makes it possible to use molecular beacons with shortened loop sequences and greater allele discrimination.

The sequence of the molecular beacon loop (or any other probe that fluoresces on hybridization) must be chosen from the same DNA strand as the limiting primer. If the probe is used for distinguishing a single-nucleotide polymorphism (SNP), that site should be in the center third of the loop. The T_m of the beacon loop sequence to its target (T_m^P) should be at least 5° and preferably about 10° below T_m^L . This contrasts with the situation in symmetric PCR, in which T_m^P must be greater than T_m^L . The lower T_m^P value ensures that the probe will not interfere with extension of the limiting primer. Thus, amplification efficiency during the exponential phase of LATE-PCR remains high even in the presence of high concentrations of probe.

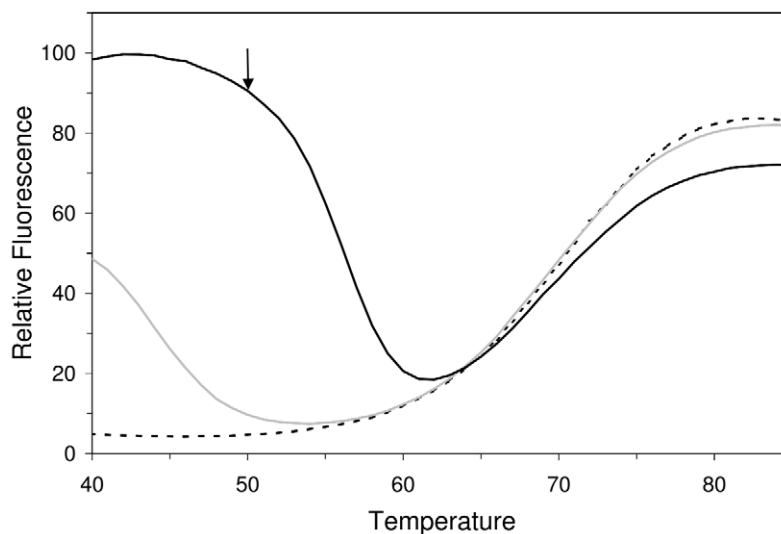


Fig. 3. Melting analysis of $\Delta F508$ molecular beacon in absence of target (broken line), with mismatched normal allele target (gray line), and with $\Delta F508$ target (black line). The measured T_m of this molecular beacon with the complementary $\Delta F508$ target is about 56°C . These results show that detection during PCR at 50°C fluorescence will provide close to maximum fluorescence with that target (arrow), but only background fluorescence with the mismatched target.

Good estimates of T_m^P can be obtained using the same nearest-neighbor formula used for determining T_m^L and T_m^X , even though variations in the molecular beacon stem do affect the empirically determined T_m of the beacon-target hybrid. Stem designs are similar to conventional design, typically 5 to 6 bp, predominantly G and C. The stem T_m is estimated using the intramolecular hybridization program mfold (16), available on-line at <http://www.bioinfo.rpi.edu/applications/mfold/>. mfold is also useful in identifying sequences that can form stable nonhairpin structures. Unlike conventional designs for molecular beacons in which T_m^P and stem T_m are both typically $7\text{--}10^\circ\text{C}$ above the annealing temperature, we prefer to increase the stem T_m $5\text{--}10^\circ\text{C}$ above T_m^P in order to ensure lower background fluorescence at the annealing temperature.

Molecular beacons should be tested using synthetic target oligonucleotides prior to use in LATE-PCR. The complementary oligonucleotide target should include at least 3 nt beyond each end of the molecular beacon loop, using the sequence of the target gene, so that possible interaction between the stem and target is included in the empirically determined T_m (see **Note 9**). A melting analysis of molecular beacon in the absence of target, with complementary target, and with mismatched target (in the case of SNP analysis) is carried out to determine the best temperature for allele-specific detection (**Fig. 3**). Molecular

beacon is used at a concentration that will be present during LATE-PCR, typically $1\ \mu\text{M}$, and the concentration of targets should be about $0.5\ \mu\text{M}$, the estimated final concentration of single-stranded product following LATE-PCR. Sodium, potassium, and magnesium concentrations should be the same as those used for amplification. Additional details on the design, synthesis, and testing of molecular beacon are available in the scientific literature (17) and on the Internet site <http://www.molecular-beacons.org>.

3.2.3. Components of LATE-PCR

With the exception of the primer and probe concentrations, other components used in LATE-PCR samples are similar to those used in symmetric reactions for single cells. The use of a “hot-start” method to prevent mispriming prior to the first denaturation step is required. Several commercially available Taq polymerases are modified so that they become active only after the initial high-temperature incubation. We prefer to use Taq polymerase with antibodies, because the required denaturation step is usually shorter. Taq polymerases from different commercial sources are supplied with buffers containing sodium Tris (or other buffer) and KCl. Begin testing using the recommended buffer solution, keeping in mind that varying the concentration of the monovalent cations will affect primer and probe T_m .

The dNTPs (specifically dATP, dCTP, dGTP, and dTTP) should be PCR grade and included at about $0.2\ \text{mM}$ each. Higher concentrations may be needed for multiplex reactions, and lower concentrations are useful if the single-stranded product will be used directly for sequencing. Remember that dNTPs chelate magnesium ions and thereby affect the free magnesium concentration in the sample. Therefore, changes to dNTP concentration may affect reaction efficiency and specificity. We generally use a magnesium concentration of $3\ \text{mM}$. That concentration works well with most Taq polymerase enzymes and molecular beacon probes.

3.2.4. LATE-PCR Cycling Parameters

3.2.4.1. INITIAL CYCLING STEPS AND DURATION

An initial denaturation step of 95°C for 2 min is followed by 25–35 initial cycles with steps for primer annealing, primer extension, and product denaturation. Fluorescence detection is not needed during these cycles. The annealing step should be no more than 10–15 s. Longer incubations promote nonspecific amplification. The extension step is usually carried out at 72°C , at which Taq polymerase has maximal activity. If amplicon size is only 100–200 nt, 15 s is more than sufficient to complete primer extension. A denaturation step of 5 s at 95°C should separate the DNA strands of most amplicons, enabling hybridization with primers during the subsequent annealing step.

Testing replicate samples containing low, equal concentrations of genomic DNA (e.g., 10 or 100 genome equivalents) at three or four different annealing temperatures is usually sufficient to identify optimal conditions (*see Note 10*). That optimum is usually close to the calculated T_m^X value in samples containing 3 mM magnesium, 20 mM sodium, and 50 mM potassium. Amplification efficiency at different annealing temperatures is evaluated by comparing mean C_T values of replicate samples. Lower C_T values (earlier detection) indicate higher amplification efficiency. Reaction specificity can be evaluated by analyzing products using gel electrophoresis. Alternatively, the DNA-binding dye SYBR Green I can be substituted for the hybridization probe, and product melting analysis can be done following amplification (*18*) (*see Notes 11 and 12*).

One of the difficulties of traditional asymmetric PCR is low amplification efficiency, which for real-time reactions causes delays in detection and the inability to obtain quantitative information. Another problem is the high level of nonspecific amplification, which can reduce the yield of specific product and the resulting signals from hybridization probes. By designing primers for which T_m^L is higher than T_m^X , LATE-PCR makes it possible to use annealing temperatures that are low enough to ensure high amplification efficiency by the limiting primer, yet high enough to minimize mispriming by the excess primer.

3.2.4.2. FLUORESCENCE DETECTION DURING LINEAR AMPLIFICATION

Molecular beacon signals for single-copy targets usually reach detection threshold around cycle 40–45, depending on the detection equipment and the specific molecular beacon. Detection threshold will be reached about four cycles earlier for each 10-fold increase in the initial target concentration. Fluorescence detection should be included during cycling starting about 10 cycles before reaching threshold, and those initial values are then used to determine a fluorescence baseline for subsequent readings (*see below*).

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Detection can be carried out either during the annealing step of a standard thermal cycle or, preferably, during a step added after extension, since most of the amplicon strand detected by the probe remains single stranded during the linear phase of LATE-PCR. The temperature at which detection is done is chosen based on the tests with the probe and synthetic targets (*see Subheading 3.2.2. and Fig. 3*). That temperature should be low enough to provide strong signal from the complementary targets, but high enough to avoid signal from mismatched targets. Dropping the temperature a few degrees below the optimal annealing temperature at this point of the reaction usually does not present a problem in terms of nonspecific amplification. However, large drops in temperature should be avoided, because mispriming by amplicon strands may produce a phenomenon we refer to as “product evolution” (*see Note 12*).

The C_T value of the reaction should be reached at or slightly before the limiting primer is depleted. Under these circumstances, the observed C_T value will

reflect the number of copies of the target sequence present in the sample at the start of the reaction, as in the case of symmetric real-time PCR. It may be necessary to test higher concentrations of the limiting primer (e.g., 100 or 200 nM) if the C_T values are higher than anticipated or the subsequent rate of increase in fluorescence is low. Conversely, a nonlinear increase in fluorescence may reflect limiting primer concentrations that are too high. Remember that altering the concentration of the primers will change their T_m .

3.2.5. Preparation and Running of Diagnostic Assays on Lysed Cells

Large volumes of solutions containing all PCR reagents except Taq polymerase can be prepared and stored frozen in aliquots sufficient for concurrently tested samples, including positive and negative controls. Using the same mixture provides the highest reproducibility between assays run on different days. Taq polymerase should be added to the thawed aliquot just before use. The reagent solution should be thoroughly mixed before being added to individual samples containing lysed cells. A final volume of 25 μ L is used for most applications. Samples should be kept on ice to ensure minimal polymerase activity during preparation. Even in the presence of antibodies, some mispriming may occur if samples are kept at room temperature for long periods prior to PCR.

The thermal cycler is programmed with the optimal cycling parameters with the detection step included about 10 cycles before the anticipated C_T values. In most cases with single-copy genes, this will mean that the detection step will be added after the first 30 cycles. We typically run a total of 60 cycles. Specific requirements for selecting sample wells, programs, and detection wavelengths will vary with different thermal cyclers.

3.3. Analysis of Real-Time Signals

To display and analyze real-time signals properly, a fluorescence baseline is set using readings in the cycles before amplicon detection. Using a baseline normalizes background variations and gradual increases in fluorescence unrelated to the amplicon. The baseline readings can include any or all of the cycles before an increase in fluorescence. At least five cycles are usually necessary. Baselines are determined separately for each fluorophore used. Thermal cycler manufacturers typically suggest a threshold of 5 or 10 SDs above baseline detection values. We have found that choosing a threshold with a specific fluorescent value often provides better reproducibility between assays run at different times using the same PCR reagent mixture.

One of the advantages of real-time PCR is the ability to identify samples with atypical signal kinetics. Assay accuracy can be increased by excluding such samples from diagnosis (**19,20**). LATE-PCR increases final fluorescence intensity and reduces sample-to-sample variation, thereby improving the kinetic analysis.

To establish limits for diagnosis, cells with known genotypes should be tested at the same time or at least using the same PCR mixture as unknown samples. Each genotype should be represented by about 10 or more samples. Mean values for C_T , increase in fluorescence (slope), and final fluorescence are determined for samples with the same genotype. Individual sample values are evaluated using the extreme studentized deviate method to identify outliers (21). Any positive control sample that does not yield the expected signals, or yields a value that is a statistical outlier, is not used for establishing diagnostic limits. We typically set those limits at 3 SDs from the means.

Figure 4 illustrates this method for final fluorescence values obtained from human lymphoblasts homozygous or heterozygous for the $\Delta F508$ mutation in the cystic fibrosis gene (CFTR), or homozygous normal at that locus. An initial assay was run to establish diagnostic limits, including the limits for final fluorescence indicated by the dashed lines for homozygous normal cells (box 1), homozygous mutant cells (box 2), or heterozygous cells (box 3). Because LATE-PCR yields a narrower range of final fluorescence values compared to other real-time methods, the size of these boxes is relatively small and gives a useful first step for data analysis. Individual data points shown include all results from a second assay using the same PCR reagent mixture, simulating “unknown” sample testing. The large majority of data points fall within the boxes established for the corresponding genotypes. A few samples did not give the expected results. Two samples with values outside the boxes might have been misdiagnosed but are excluded based solely on quantitative analysis of final fluorescence. First, one heterozygous cell yielded an extremely low fluorescence value for the $\Delta F508$ allele (open diamond near the upper right corner of box 1). Such a preferential amplification result would have almost certainly been misdiagnosed as homozygous normal using conventional PCR and electrophoresis, but the molecular beacon provides the sensitivity to detect the mutant allele. Second, a single homozygous mutant sample generated a low-level signal for the normal allele, presumably owing to contamination (solid square to the left of box 3). That signal, however, was outside the limits for final fluorescence, and well outside the limits for C_T value (latter not shown in **Fig. 4**) and, therefore, would not be misdiagnosed as heterozygous.

Evaluating C_T values and rates of increase in fluorescence (slope) provides additional means for reducing misdiagnosis. **Figure 5** plots those values for the samples that yielded signals only from the normal allele, i.e., those indicated by the data points in box 1 of **Fig. 4**, including two heterozygous cell samples that failed to generate a $\Delta F508$ allele signal. The diagnostic limits for C_T values and rates of increase in fluorescence were established using data from the prior assay, as described in the preceding paragraph. One of the heterozygous cell samples generated a C_T value above those limits for diagnosis as homozygous

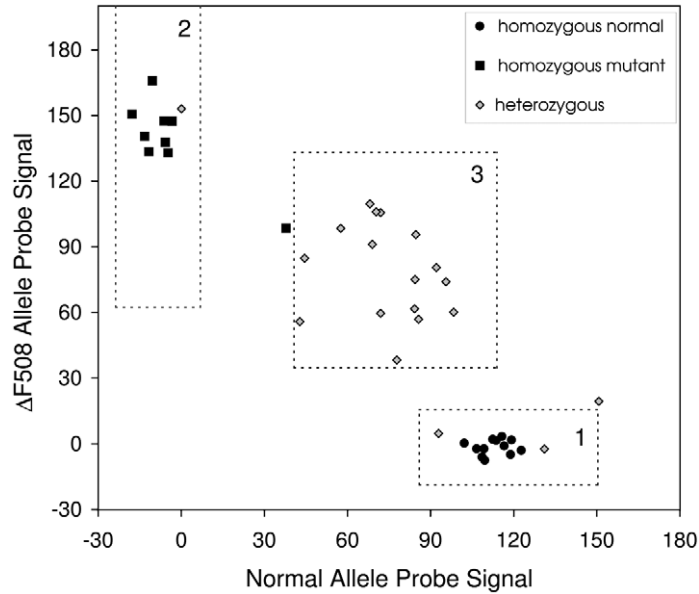


Fig. 4. Scatter plot of LATE-PCR final fluorescence values in replicate samples of individual lymphoblasts homozygous normal for CFTR (solid circles), homozygous for $\Delta F508$ mutation (solid squares), or heterozygous for the $\Delta F508$ mutation (shaded triangles). The boxes labeled 1, 2, and 3 indicate diagnostic limits for those genotypes that were established by previously tested samples. The accuracy of the assay is improved by excluding from diagnosis all samples outside these limits. See the text for details.

normal. The other heterozygous cell sample gave values within the limits, although the slope was higher than that from any of the homozygous cells. In the setting of preimplantation genetic diagnosis (PGD) in which both parents carry the same mutant allele, failure to identify allele dropout (ADO) of the $\Delta F508$ allele has no phenotypic consequence but becomes extremely important as the assay is extended to multiple mutation sites within a gene (*see Sub-heading 3.4*). Analysis of the real-time signals using the 3-SD limits reduces misdiagnosis of ADO by about half for both symmetric PCR and LATE-PCR ([7,20]; unpublished data).

3.4. Assays for Detecting Compound Heterozygotes

Genotyping multiple mutation or SNP sites within a gene requires either a single amplicon that encompasses both sites or coamplification of the two regions using separate pairs of limiting and excess primers. If possible, the single amplicon approach is preferred, because it is simpler to design and optimize and provides a means to detect ADO in PGD cases. (Absence of the normal

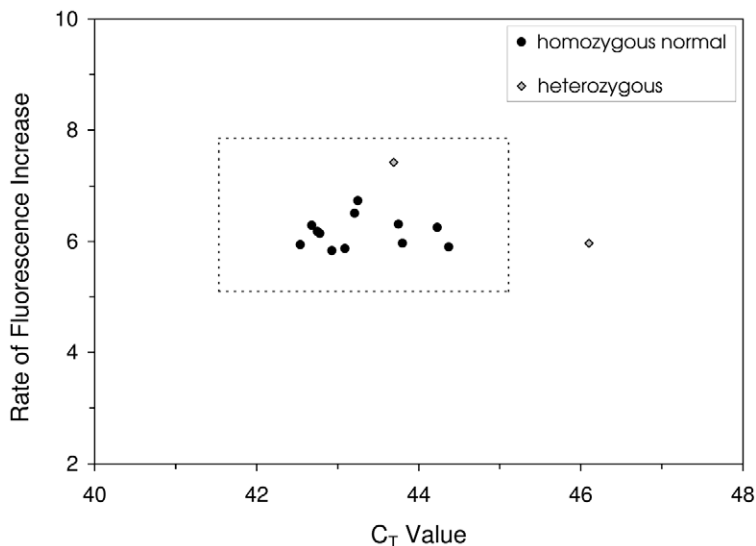


Fig. 5. Scatter plot of LATE-PCR C_T values and rates of increase in fluorescence for samples in box 1 of **Fig. 4** (i.e., those generating only normal allele signal). Assay accuracy can be increased using the diagnostic limits indicated by the broken lines. A similar analysis can be done for samples generating 1 only $\Delta F508$ allele signals (box 2 of **Fig. 4**) and for samples generating both signals (box 3 of **Fig. 4**). See the text for additional details.

allele signal at either site is an indication of ADO.) In contrast to symmetric real-time assays in which signal strength declines rapidly as amplicon size increases, LATE-PCR can generate strong signals with amplicons several hundred nucleotides long. Although we have not tested the limits in this area, strong signals have been obtained for a 650-nt amplicon. Limits may depend more on T_m^A values, for reasons discussed in **Subheading 3.2.**, and thus be longer when the %GC of the amplicon is low and relatively short when %GC is high.

In cases in which the distance between the mutation sites is too great for single amplicon design, the individual sites can be coamplified using LATE-PCR design criteria for each. Primers need to be evaluated for possible 3' dimer formation, as would be the case for any multiplex PCR, with particular attention paid to possible interaction between the two excess primers. Optimizing PCR reagent concentrations and the annealing temperature enables coamplification of both targets to similar levels. Moderate increases in the concentrations of one pair of primers can be used to equalize amplification efficiencies. We have successfully coamplified CFTR exon 10 and exon 11 sequences using LATE-PCR (unpublished data).

ADO has greater consequences when multiplex amplification is used in PGD to identify compound heterozygotes, because a failure to amplify either of the

mutant alleles can lead to misdiagnosis and transfer of an affected embryo. Several PGD centers have implemented tests that include amplification of polymorphic sites found near the tested gene (22). This approach, however, only detects loss of the entire region of the gene (e.g., owing to aneuploidy) or poor accessibility of the DNA in that entire region owing to inadequate cell lysis. Those situations are also detected by the absence of one of the normal allele sequences when testing two sites within the gene. When the target gene copies are both present, amplification from different sites within or neighboring the gene are independent events and, therefore, coamplification of neighboring polymorphic sites has limited value. By contrast, the analysis of real-time kinetics provides a means of identifying samples that exhibit atypical amplification including ADO. The strong signals and reliability of LATE-PCR offer the best opportunity to increase the diagnostic accuracy of single cell PCR.

4. Notes

1. PVP at 0.01 mg/mL in the final cell wash step in a cystic fibrosis assay does not delay detection (19). Preliminary tests did show that detection was delayed slightly when higher concentrations of that solution were added to the lysis solution. Much larger and more variable detection delay was found with polyvinyl alcohol, possibly owing to interference with fluorescence detection.
2. We have observed that calcium can inhibit amplification. Serum and other culture additives such as hemoglobin, immunoglobulin, and heparin also interfere with amplification (23,24). Adding BSA (nonacetylated, nuclease free) can improve PCR efficiency in the presence of some of these inhibitors (24).
3. The volume of the lysis solution can be adjusted for the specific application and final PCR volume. Volumes below 10 μ L can be used if the volume of transferred PBS is less than about 10% of that volume. Higher volumes are limited only by the volume of subsequently added PCR reagents and the final PCR volume.
4. A cell inadvertently introduced at any step can provide a DNA template for amplification. Contamination control measures should include dedicated pipettors for preparation of solution, aerosol-resistant pipet tips, lab coats, disposable caps, masks, extended-cuff gloves, and containment hoods, all in rooms separate from the PCR amplification area. Although ultraviolet treatment offers some protection from contaminating DNA, its effectiveness is limited. Treating surfaces with 10% bleach (1% sodium hypochlorite) is more effective for eliminating contaminating cells and DNA. The work area should be a "DNA-free zone" that resembles a "sterile field" in an operating room. Only clean gloves should touch items within that zone (e.g., sample tubes, pipettors, pipet tip boxes, reagent vials), and gloves that come in contact with any area outside the zone should be changed immediately. These procedures should be used when handling sample tubes at any step prior to PCR.
5. We have found that proteinase K-based lysis is not as effective in PCR tubes with glasslike properties, such as those for the Cepheid Smart Cycler, presumably owing to the adhesive properties of those tubes. Doing the lysis step in a standard sample

- tube, then adding PCR reagents and transferring to the required PCR tube is an alternative, but losing material during that transfer increases the possibility of ADO. Preliminary results indicate that alkaline lysis *without* dithiothreitol (DTT) can produce acceptable results in the Smart Cycler tubes. DTT was present in the initial protocol for reducing protamines in sperm (25) but is unnecessary for most cell types, and residual DTT reduces PCR efficiency (8).
6. Contamination of a thermal cycler block with PCR product is nearly impossible to avoid, even when great care is taken not to open tubes following PCR. If the same thermal cycle is used in a subsequent assay for the lysis incubation, it is possible to introduce those product molecules into samples when the tubes are opened to add PCR reagents. If the same block must be used for both lysis and PCR, it must be decontaminated between each assay. The block should be flooded with 10% bleach and then rinsed thoroughly with water.
 7. If an adequate limiting primer cannot be designed using the actual DNA sequence of the target gene, T_m^L can be increased by substituting one or two guanine bases for adenine near the 5' end of the primer. Hybridization of that primer with the initial target will have low-affinity G-T pairing, but not destabilizing mismatches, and subsequent hybridization with complementary amplicon strands will provide high amplification efficiency during the exponential phase of LATE-PCR. Another option is the addition of cytosine or guanidine to the 5' end of the primer, irrespective of the target sequence. Because the annealing temperature during the initial cycles cannot be lowered without risking mispriming by the excess primer, these options have obvious limits, particularly with a low initial target number, and, therefore, the T_m of the limiting primer with the initial target sequence should not be more than 5°C below T_m^X .
 8. We have recently developed allele-specific assays using double-stranded displacement probes (26,27). These probes are easy to design and are relatively inexpensive, because each oligonucleotide is modified with a single fluorophore or quencher, not both. Extensive purification is not necessary, greatly increasing the manufacturing yield relative to dual-labeled probes such as molecular beacons. In general, the LATE-PCR benefits of increased signal strength and allele specificity can be accrued using any probe that signals on hybridization. Although TaqMan probes could be designed to work with LATE-PCR amplification, the need for hydrolysis requires that those probes have high melting temperatures and hybridize with the extension products of the limiting primer, rather than the accumulating single-stranded product. Therefore, benefits with TaqMan probes are limited.
 9. When designing the molecular beacon stem, it is worthwhile to check for complementarities with nucleotides in the target sequence. It is usually possible to modify the stem slightly to minimize hybridization between the stem and target. Alternatively, those hybridizations can be allowed but should be taken into account when predicting T_m^P .
 10. Alternatively, annealing temperature can be held constant and magnesium concentration is varied to identify optimal annealing conditions. We have observed that increasing the magnesium concentration from 3.0 to 3.5 mM has a similar effect to

lowering the annealing temperature 2°. Large changes in magnesium concentration, however, may affect Taq activity and change hybridization characteristics of molecular beacons and other probes. In addition, note that these tests can be done on genomic DNA rather than single cells, since the limiting primer becomes depleted once it makes sufficient product to reach the detection threshold, regardless of the initial target concentration. Using 600 pg of DNA (equivalent to about 100 genomes) will lower the C_T value about eight cycles compared to single cells but does not change the subsequent linear signal kinetics (6).

11. SYBR Green I binds to double-stranded DNA regardless of nucleotide sequence. Fluorescence therefore plateaus after the limiting primer is exhausted. Following PCR cycling, fluorescence is monitored as temperature is gradually increased. As PCR products denature, a large drop in fluorescence is observed. Multiple drops in fluorescence, usually evaluated as “melting peaks” on plots of temperature vs the rate of decrease in fluorescence, indicate the presence of nonspecific product. Specific reactions should have a single melting peak about 3–6° above the calculated T_m^A value, depending on the magnesium concentration.
12. If LATE-PCR is continued for many linear cycles, a second rise in SYBR Green fluorescence may be observed. This corresponds to a phenomenon that we call “product evolution,” which involves the single strands priming on one another with a resulting increase in product size and melting temperature. Product evolution usually can be avoided by limiting the number of linear cycles and minimizing the drop in temperature needed for probe detection. In rare cases, it may be necessary to modify the 5' end of the limiting primer, thereby changing the 3' end of the amplicon single strands, in order to avoid this type of mispriming.

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