

# Monoplex/multiplex linear-after-the-exponential-PCR assays combined with PrimeSafe and Dilute-‘N’-Go sequencing

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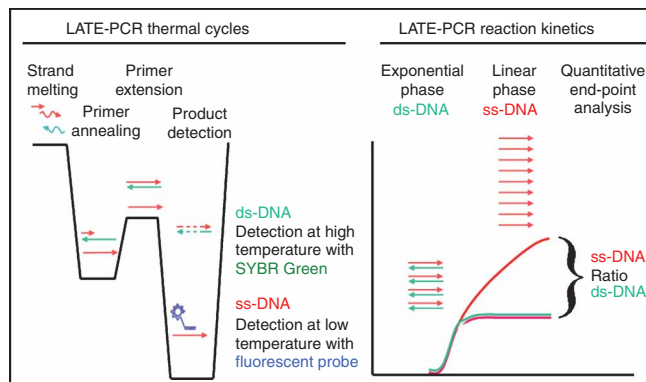
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This protocol describes the design and execution of monoplex and multiplex linear-after-the-exponential (LATE)-PCR assays using a novel reagent, PrimeSafe, that suppresses all forms of mispriming. LATE-PCR is an advanced form of asymmetric amplification that uses a limiting primer and an excess primer for efficient exponential amplification of double-stranded DNA, followed by linear amplification of one strand. Each single-stranded amplicon can be quantitatively detected in real time or at end point. By separating primer annealing from product detection, LATE-PCR enables product analysis at low temperatures. Alternatively, each single strand can be sequenced by a convenient Dilute-‘N’-Go procedure. Amplified samples are diluted with individual sequencing primers without the use of columns or spins. We have amplified and then sequenced 15 different single-stranded products generated in a single multiplexed LATE-PCR comprised of 15 pairs of unrelated primers. Dilute-‘N’-Go dideoxy sequencing is more convenient, faster and less expensive than sequencing double-stranded amplicons generated via conventional symmetric PCR. The preparation of LATE-PCR products for Dilute-‘N’-Go sequencing takes only 30 seconds.

## INTRODUCTION

For the past 20 years, symmetric PCR followed by dideoxy sequencing of the resulting double-stranded products has been the gold standard method for amplification and analysis of genomic DNA fragments, one fragment at a time<sup>1</sup>. But now in the era of genomics, faster, cheaper and easier processing of ever more samples, particularly small samples, is urgently needed. Asymmetric PCR, originally conceived and used by Gyllensten and Erlich and others, and its variant thermal asymmetric PCR were developed as means of preparing single-stranded DNA for DNA sequencing of the accumulated single strand<sup>2–5</sup>. These strategies, however, failed to achieve widespread use because asymmetric amplification as originally described tended to be inefficient and idiosyncratic owing to errors in primer design that we have described recently<sup>6–8</sup>. As demonstrated here, we have now developed LATE-PCR, an advanced form of asymmetric PCR that exponentially generates double-stranded amplicons and then predictably switches to linear amplification of single-stranded DNA<sup>6</sup>. These advances were made possible by improvement in primer design, separation of the steps of primer annealing and product detection within the thermal cycle, and modification of probes for detection of single-stranded products at lower temperatures. **Figure 1** provides an overview of the LATE-PCR thermal cycle and kinetics, illustrating the switch from double-stranded to

single-stranded amplicons and the detection of these products. In addition, each of the single-stranded excess primer strands generated during LATE-PCR can be sequenced conveniently by spiking an aliquot of the end product with a few picomoles of the limiting primer used up during amplification, as this primer can act as the sequencing primer during dideoxy cycle sequencing. The Dilute-‘N’-Go protocol we use for cycle sequencing does not require any sample clean-up such as that required following conventional symmetric PCR amplification. Because LATE-PCR and its allied technologies are platform technologies, their possible uses are at least as broad as those of conventional PCR. We are currently using both monoplex and multiplex LATE-PCR assays to detect loss of heterozygosity in precancerous cells as well as to detect and analyze different bacterial pathogens and strains of pathogenic DNA viruses. In addition, we have optimized the conditions required for both monoplex and multiplex reverse transcriptase LATE-PCR detection and analysis of gene transcripts; however, reverse transcriptase LATE-PCR is outside the scope of this protocol and will be described elsewhere.



**Figure 1** | Use of SYBR Green dye and a low- $T_m$  Cy5-labeled ResonSense probe permits detection of both double-stranded and single-stranded DNA products in LATE-PCR assays. Left side: Hybridization of the low- $T_m$  Cy5-labeled ResonSense probe to the accumulating single-stranded DNA is accomplished by introduction of a separate low-temperature detection step after each extension step or at end point of the LATE-PCR. Right side: The ratio of SYBR Green to ResonSense Cy5 signals provides an empirical means of establishing the amount of single-stranded product in any individual LATE-PCR sample (see text for details). ss-DNA, single-stranded DNA; ds-DNA, double-stranded DNA.



The relative ease with which we are now able to design and use LATE-PCR monoplex and multiplex reactions depends on avoidance and suppression of mispriming. Mispriming is an inherent limitation of all forms of PCR<sup>9</sup> and takes many guises, including primer-dimer formation, mispriming of unintended targets at low temperature before the start of the reaction and similar mispriming events during the early phase of amplification when target genomes are denatured and primers are in vast excess. In addition, the 3' ends of single-stranded amplicons generated in asymmetric reactions can prime on other single-stranded molecules accumulating in the reaction, particularly when the temperature is lowered. Several forms of hot start polymerases are commercially available to reduce primer-dimer formation and mispriming at low temperature before the start of the reaction, but few of these products suppress mispriming that occurs after the first heating step that destroys the adduct or modification and activates the enzyme. At least one commercial preparation of *Taq* DNA polymerase (Eppendorf Hotmaster *Taq* DNA Polymerase) includes an added proprietary ligand that acts throughout amplification to reduce mispriming by inhibiting the enzyme. Unfortunately, this reagent is not flexible and tends to decrease amplification efficiency. As illustrated here, a new class of molecules known as PrimeSafe, available from Smiths Detection by emailing [biodetection@smithsdetection.com](mailto:biodetection@smithsdetection.com), can be added to both symmetric and asymmetric reactions to suppress all forms of mispriming throughout amplification. One of the clear benefits of PrimeSafe is that it can be adjusted in terms of precise composition and concentration to minimally impact the efficiency of each monoplex or multiplex reaction. Monoplex and multiplex LATE-PCRs can readily be constructed without the use of PrimeSafe, but PrimeSafe significantly decreases the need to perfect primers to avoid possible interactions.

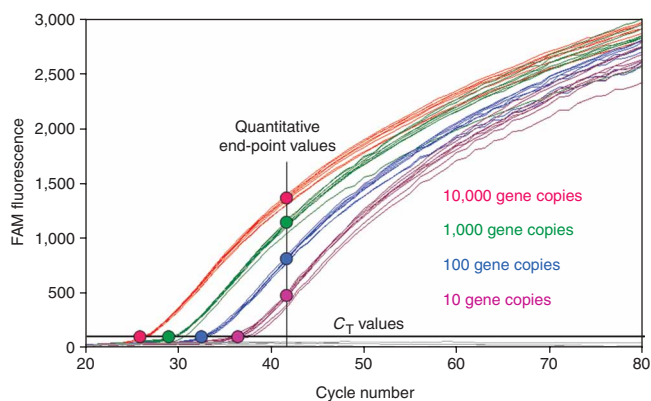
### Experimental design

**Primer design for LATE-PCR.** LATE-PCR assays are asymmetric and therefore use an excess primer (X) and a limiting primer (L) having concentration ratios ranging from at least 5:1 to as much as 100:1 (see ref. 6). Typically, we employ primers at a 10:1 or 20:1 ratio, and all of the primer pairs described here, including those used in the multiplex reactions, were used at 1,000 nM of each excess primer and 50 nM of each limiting primer. The key to designing a good pair of LATE-PCR primers lies in the fact that their melting temperatures ( $T_m$ ) obey the rule  $T_m^L - T_m^X \geq 0$ , where each  $T_m$  is calculated using the nearest-neighbor formula

adjusted for monovalent salt concentration, magnesium concentration and, most critically, the actual concentration of each primer present at the start of the reaction<sup>6</sup>. We routinely use Visual OMP (DNA Software) to calculate  $T_m^L$  and  $T_m^X$ , although other less expensive software is also available on the web, for instance IDT's OligoAnalyzer (<http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/>) and the Pasteur Institute's Melting program<sup>10</sup> (<http://bioweb.pasteur.fr/seqanal/interfaces/melting.html>). We also typically design the limiting primer to have a starting concentration-adjusted  $T_m^L$  that is about 5 °C higher than the starting concentration-adjusted  $T_m^X$  of the excess primer. This discrepancy makes it possible to set the annealing temperature of the thermal cycle 0–5° below the  $T_m^X$  of the excess primer to enable high stringency for that primer and high amplification efficiency for the limiting primer<sup>7</sup>.

**Real-time and end-point quantitative analysis.** LATE-PCR allows both real-time and end-point quantification of nucleic acids. LATE-PCR assays generate double-stranded amplicons exponentially and then abruptly switch to linear amplification of the excess primer strand. This switch occurs when the limiting primer runs out and no more limiting primer strand is made. Thus the rate of excess strand accumulation depends on the amount of limiting primer strand accumulated. In our experience, 50 nM of limiting primer guarantees that the accumulation of the excess primer strand is detectable with a fluorescently labeled probe very early during the linear amplification phase during real-time PCR analysis<sup>6–8</sup>. **Figure 2** shows a typical set of replicate reactions spanning four tenfold dilutions of the starting genome. The accumulating single strands are detected with a low-temperature molecular beacon probe<sup>6,11</sup>. Alternatively, the DNA binding dye SYBR Green can be used to follow just the exponential phase of double-stranded DNA amplification (not shown). **Figure 2** also shows that the amount of single-stranded amplicon detected during the first 10–15 linear cycles can be quantified and used like a  $C_T$  value to establish the number of gene copies present in the starting sample. This is a unique feature of LATE-PCR and is very useful because, unlike symmetric PCR<sup>12</sup>, it allows quantitative end-point analysis in the absence of real-time analysis. In the case of end-point analysis, the reaction is run without a low temperature detection step during each thermal cycle. When the desired number of cycles is reached, the temperature is lowered after the last round of extension to allow the low-temperature probe to bind to the accumulated single-stranded amplicons<sup>13</sup>.

**PrimeSafe suppression of mispriming in symmetric PCR and LATE-PCR.** Primer design software such as Visual OMP can also be

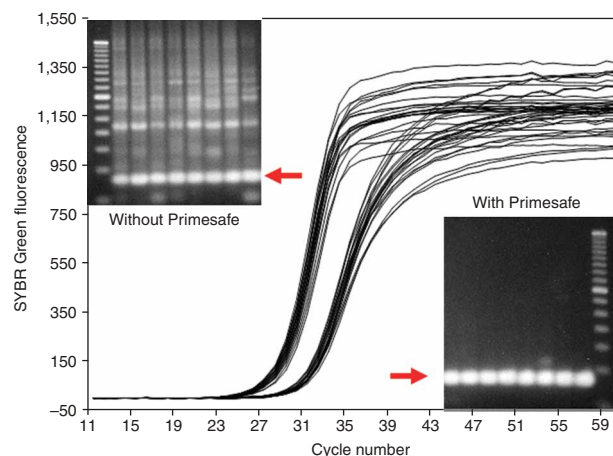


**Figure 2** | Detection of LATE-PCR amplification products in samples containing different initial concentrations of DNA using a low- $T_m$  molecular beacon, labeled with FAM (6-carboxylfluorescein). Optimized LATE-PCR amplification of the  $\Delta F508$  region of the CFTR gene was carried out using 10,000 (red), 1,000 (green), 100 (blue) and 10 (purple) copies of human genomic DNA. Curves show molecular beacon fluorescence increase in eight replicate samples at each starting template concentration. The molecular beacon only detects the accumulating single-stranded amplicons. SYBR Green staining of the corresponding double-stranded DNA for each LATE-PCR typically reaches its threshold cycle ( $C_T$  value) six cycles earlier than the corresponding molecular beacon signal. The amount of single-stranded amplicon detected during the first 10–15 linear cycles can be quantified and used like a  $C_T$  value to establish the number of gene copies present in the starting sample. Notice that all LATE-PCR amplifications eventually produce roughly the same amount of single-stranded amplicon, regardless of the amount of starting substrate.



**Figure 3** | Effect of adding 0.4× PrimeSafe-045 to a symmetric PCR on product specificity (determined by agarose gel electrophoresis) and the SYBR Green kinetic plot under conditions that promote substantial mispriming (use of native *Taq* polymerase and incubation at room temperature for 30 min before the start of PCR). Red arrow points to the expected specific product. PrimeSafe reduced detectable mispriming and thereby increased the yield of the intended double-stranded amplicon. Suppression of mispriming was also accompanied by a shift to the right in the kinetic plot of SYBR Green fluorescence of total double-stranded DNA accumulation because the correct amplicon is smaller than the misprimed products.

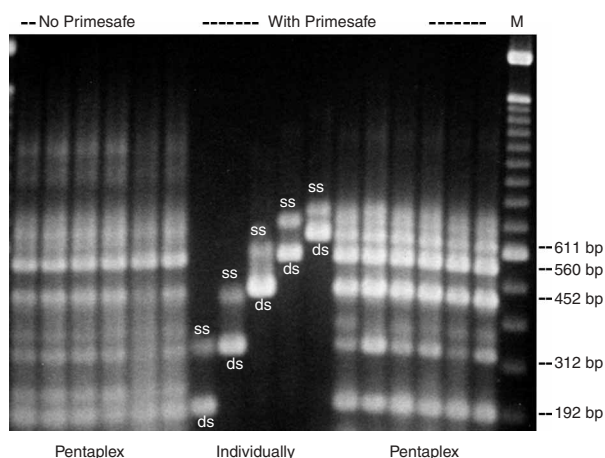
used to scan *in silico* for possible primer-dimer formation. Primers with gross incompatibilities (i.e., primers that have more than a three-nucleotide overlap at the 3' end or primers with 3' end interactions with  $-\Delta G$  smaller than  $-5$ ) are generally not used. But in reality, it is impractical to eliminate all potential primer-dimers, and, in most cases, it is unnecessary because PrimeSafe suppresses primer-dimer formation as well as other forms of mispriming. PrimeSafe is available from Smiths Detection by emailing [biodetection@smithsdetection.com](mailto:biodetection@smithsdetection.com) and comes in several different formulations, each as 100× stocks. Generically, they act as temperature-dependent inhibitors of *Taq* polymerase and can be used singly or in mixtures that are optimized empirically. For instance, we found that 0.4× PrimeSafe-045 worked well to suppress primer oligomerization in a symmetric PCR that had an annealing temperature of 55 °C (see Fig. 3). Suppression of mispriming was accompanied by a change in the SYBR Green fluorescence of total double-stranded DNA accumulation because the correct amplicon is smaller than the misprimed products. Experience has taught us that too high a concentration of any one of the available types of PrimeSafe can delay or even inhibit amplification, but this problem is easily remedied by using a lower concentration or changing the type of PrimeSafe. To date, the most effective universal PrimeSafe formulation is 0.3× PrimeSafe-001. Monoplex and multiplex LATE-PCRs can readily be constructed without the use of PrimeSafe, but PrimeSafe significantly decreases time spent, both *in silico* and at the bench, to optimize primer design, magnesium concentrations, enzyme concentrations and all other factors needed to avoid possible interactions of multiple sets of primers<sup>14</sup>. Higher order LATE-PCR multiplex reactions containing entirely unrelated pairs of primers can also be readily constructed



without the addition of PrimeSafe, if a relaxed degree of product specificity is acceptable. For instance, for the reaction shown in Figure 4, the pairs of primers were chosen because they generated a well-spaced ladder of products on an agarose gel and no attempt was made to minimize interactions between any of the five pairs. Addition of PrimeSafe ‘cleaned up’ but did not fundamentally change the pattern of the amplification products.

**Designing monoplex and multiplex reactions.** The choice of whether to design a monoplex or a multiplex LATE-PCR assay is entirely up to the investigator, but, in either case, it is made easier by two factors. First, addition of PrimeSafe to multiplex reactions suppresses unintended interactions of primers for different amplicons (see Fig. 4). Thus, the problem of nonspecific product formation in multiplex reactions can be viewed as a form of mispriming<sup>15–16</sup>. Second, as explained above, LATE-PCR primer pairs are chosen on the basis of a logical rule  $T_m^L - T_m^X \geq 0$ . This same rule can be applied to single pairs of primers or multiple pairs of primers in the same reaction (see Fig. 5).

In practice, we have found it convenient to construct duplex LATE-PCR assays in which one pair of primers amplifies an internal positive control amplicon whose synthetic template is relatively short (< 100 nucleotides) and is added to the reaction mixture at a low copy number (10–100 copies). Two separate probes (see below) visualize the unknown and internal control targets separately. A further potential assay design uses three primers, two different limiting primers and an excess primer, which is used by both the internal control amplicon and the sought-for target. Because the two amplicons compete for the same primer, amplification of the internal control goes down as the number of sought-for targets goes



**Figure 4** | Example of a LATE-PCR pentaplex amplification. A pentaplex assay was constructed using five pairs of LATE-PCR primers that generate double-stranded amplicons ranging in size from 192 to 611 bp, but were not otherwise optimized *in silico* for compatibility. One set of replicate reactions was prepared without PrimeSafe and a second set containing 0.1× PrimeSafe-022 plus 0.025× PrimeSafe-060. Each amplicon was also amplified individually, and all reactions were initiated with 6 ng (~1,000 genomes) of human DNA and were carried out for 45 total cycles. The electrophoretic results clearly show that the pentaplex reactions containing PrimeSafe gave cleaner mixtures of the expected double-stranded and single-stranded LATE-PCR amplicons with less nonspecific product. ds, double-stranded DNA product; ss, single-stranded DNA products; M, 100 bp ladder.

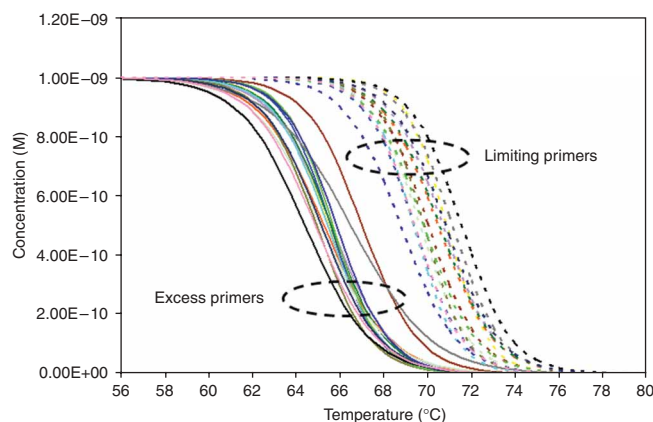
up. And in an even more compact assay design, we have used a single pair of LATE-PCR primers to amplify both the sought-for target (if present) and the low-copy number internal control. In this case, the internal control target contains a sequence complementary to one probe, whereas a different probe is used to distinguish the sought-for target. In this case, amplification of the low-copy number internal control (10–50 molecules) is readily suppressed when the number of sought-for targets is present in even modest excess.

**LATE-PCR as an engine for single-strand synthesis.** Figure 2 also reveals that all LATE-PCR amplifications eventually produce roughly the same amount of single-stranded amplicon, regardless of the amount of starting substrate. This is because the accumulating single strand eventually competes with the excess primer for hybridization to the limiting primer strand. Thus, the extent of single-stranded amplification in LATE-PCR depends on the relative melting temperatures of the excess primer,  $T_m^X$ , and the excess primer strand,  $T_m^A$ , both of which compete for hybridization to the limiting primer strand. Sustained production of single-stranded amplification is achieved when  $T_m^A - T_m^X \leq 18^\circ\text{C}$ . We routinely use Visual OMP software to calculate  $T_m^A$  via the %GC formula<sup>9</sup>, although reasonable  $T_m$  comparisons can be obtained using other formulae (see ref. 7 for details).

Experience has taught us that high levels of any single-stranded DNA amplicon up to at least 650 nucleotides (we have not tried longer amplicons) can be obtained using LATE-PCR in 40 linear cycles, although far fewer cycles are typically needed. Using a large number of linear cycles does not increase the risk of accumulating variants owing to PCR errors, because the limiting primer strands that serve as the templates for linear amplification stop accumulating when double-strand synthesis stops. It is possible to calculate the amount of excess primer strand DNA generated in a LATE-PCR using the equation  $(\text{nM limiting primer}) + (\text{nM limiting primer}/2) \times (\text{the number of linear cycles})$ , which takes into account the concentration of the limiting primer and the efficiency of the linear phase of the reaction<sup>2</sup>. Regardless of the number of starting genomes, virtually all 25  $\mu\text{l}$  LATE-PCR assays initiated with 50 nM limiting primer and 1,000 nM excess primer produce approximately 500 fmol per  $\mu\text{l}$  single-stranded DNA over the first 20 linear cycles, after which point the efficiency of linear amplification slows down.

**Product detection in monoplex and multiplex reactions.** Detection of specific single-stranded products can be accomplished in both monoplex and multiplex reactions by inclusion of fluorescently labeled probes that can be designed in several different ways, as described below. All such probes hybridize to their target strand at temperatures below the  $T_m$  of the limiting primer; thus, none of these probes are cleaved by the 5' exonuclease of *Taq* polymerase during primer extension. Indeed, Taqman probes are not advantageous in LATE-PCR, and low-temperature molecular beacons are used. These probes to the excess primer strand have a loop of 15–20

**Figure 5** | *In silico* melt curves from Visual OMP (DNA software) for 15 pairs of primers used for a 15-plex LATE-PCR assay. All 15 limiting primers (at 50 nM each) were designed with  $T_m$  values around 71 °C and all 15 excess primers (at 1,000 nM each) were designed with  $T_m$  values around 65 °C. Each pair of primers produced its own amplicon when tested



separately using 6 ng of human DNA and all 15 amplicons were similar in length (81–190 bp). The 15 different reactions had widely different amplification efficiencies, as judged by the spread of their  $C_T$  values over seven cycles (27–34), but no changes in primer concentrations were made for construction of the multiplex assay. The multiplex reaction constructed from these primers was supplemented with  $0.5 \times$  PrimerSafe-001 plus  $0.025 \times$  PrimeSafe-060. The resulting mixture of single-stranded and double-stranded products could not be easily resolved by electrophoresis, but amplification of total double-stranded DNA was followed in real time using SYBR Green and had a  $C_T$  value of 24 (results not shown). One end-point (55 cycles) 15-plex reaction was aliquoted into 15 fractions, each of which was diluted and spiked with a different limiting primer, before being sent out for Dilute-*N*-Go sequencing, as described in Figure 8. Comparison of the sequences from the 15 monoplex reactions and 15 aliquots from the multiplex reaction verified the presence of all 15 excess primer strands.

nucleotides with probe–target  $T_m$  5–20 °C below the  $T_m$  of the limiting primer<sup>6,11</sup>. The stem of such molecular beacons is typically 5 bp long and has a  $T_m$  5 °C above the  $T_m$  of the loop<sup>17</sup>. Hybridization of the low-temperature molecular beacon to the accumulating single-stranded DNA is accomplished by introduction of a separate low-temperature detection step after each extension step or at end point of the LATE-PCR<sup>6</sup>. Because their loops are relatively short, low-temperature molecular beacons are even more sequence specific than conventional molecular beacons. Low-temperature molecular beacons, in contrast to conventional molecular beacons, can be used at 300–600 nM, to saturate the target strand and generate the highest possible signal without the risk of inhibiting primer extension. The number of targets that can be visualized in such reactions depends on the number of fluorescent colors a particular instrument can detect. LATE-PCR amplification should not be monitored with hybridization probes labeled with the same fluorophores that will be used for cycle sequencing. F. Kramer (personal communication) has shown that two and three colors can also be used to label the same molecular beacon. This approach, known as color doublet or color triplet coding, increases the number of possible targets that can be visualized in a single LATE-PCR.

LATE-PCR single-stranded amplicons can also be detected using linear fluorescent probes such as ResonSense probes<sup>18</sup>, as described in the following protocol for monoplex LATE-PCR. These probes use a dye, such as SYBR Green, to excite the fluorophore of the probe via fluorescence energy transfer. Because SYBR Green binds only to double-stranded DNA, unbound probe molecules are not excited, but probes hybridized to their target strands are excited. Cy5 is the fluorophore of choice with these probes. But in LATE-PCR, the SYBR Green also binds to the total double-stranded DNA generated during the exponential phase of the reaction. The amount of double-stranded DNA stops accumulating in LATE-PCR whereas the amount of single-stranded DNA continues to accumulate. Thus, as shown in Figure 1, ResonSense probes can be

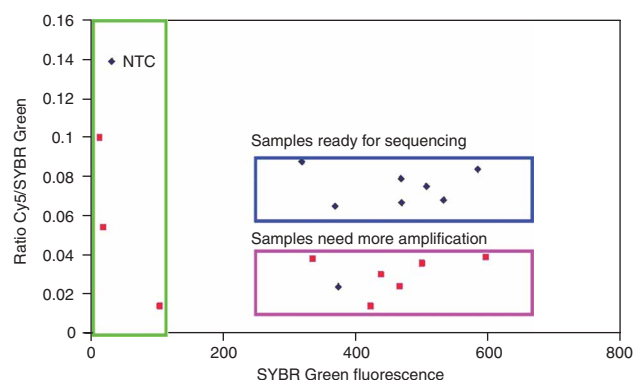
used with LATE-PCR to simultaneously measure the accumulated double-stranded DNA at an upper temperature, for instance 72 °C, as well as the accumulating single-stranded target at a lower temperature at which the probe is bound and excited<sup>19</sup>. The ratio of the two signals can be used to establish the amount of single-stranded product in any individual sample. For the reactions shown in **Figure 6**, the ratio of 0.06–0.1 was found to correspond to sufficient single-stranded amplicon to proceed to Dilute-*N*'-Go dideoxy sequencing (see below). The numerical range of this ratio, however, has to be established empirically for each amplicon because amplicons of different length bind different amounts of SYBR Green.

ResonSense probes used to estimate single-stranded DNA yield cannot, however, be used in multiplex reactions because such monitoring requires probes with different fluorophores for each amplicon and ResonSense probes are labeled only with Cy5. In the multiplexed assays described here, individual products are not quantified before Dilute-*N*'-Go sequencing, as LATE-PCR generates an excess of single-stranded DNA, which allows a wide degree of latitude for subsequent sequencing, and visualizing the products via gel electrophoresis can be used to verify the presence of abundant single-stranded DNA. The types and the best combinations of probes to use for multiplex LATE-PCR would depend on the number of targets to visualize, the number of colors that can be detected by the available equipment and the temperature range used for detection.

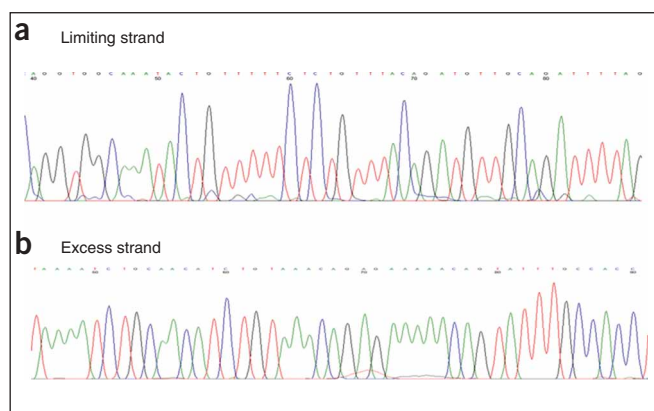
**Dilute-*N*'-Go dideoxy sequencing.** Using LATE-PCR, we have revisited the possibility of directly sequencing single-stranded DNA generated asymmetrically. Indeed, we have already demonstrated that 30 cycles of linear amplification generates enough single-stranded DNA to permit direct Pyrosequencing following enzymatic

**Figure 6** | An example of how to determine the number of linear LATE-PCR cycles required to generate sufficient single-stranded DNA for Dilute-*N*'-Go dideoxy sequencing.

Mitochondrial DNA was prepared from 5 mm human hair samples and used as a template for LATE-PCR amplification of the hypervariable region 1 (HV1) in the presence of SYBR Green dye and an HV1 Cy5-labeled ResonSense probe. The ratio of Cy5 to SYBR Green fluorescent signals was determined for each replicate sample after 45 cycles (pink squares) and after 65 cycles (black diamonds) and plotted relative to total SYBR Green fluorescence. The boxes in the figure indicate the range of Cy5 to SYBR Green ratios versus SYBR Green fluorescence values expected for the no-template controls (NTC, green box), samples with inadequate amounts of single-stranded DNA products in need of more linear amplification (pink box) and samples with sufficient amounts of single-stranded DNA for Dilute-*N*'-Go sequencing (blue box). The dimensions of these boxes have to be established empirically for each amplicon because amplicons of different length bind different amounts of SYBR Green.



depletion of unincorporated dNTPs when 100 nM limiting primer and 1 μM excess primers are used<sup>13</sup>. Dideoxy sequencing is far more sensitive than Pyrosequencing because cycle sequencing provides secondary amplification. Thus, we now report that it is possible to combine LATE-PCR with a convenient method we call the Dilute-*N*'-Go method of dideoxy sequencing (see **Fig. 7**). Typically, a 5 μl sample from a 25 μl LATE-PCR that has completed approximately 20 linear cycles is diluted 1:20 in distilled water. A 2 μl aliquot is then diluted to 25 μl in distilled water containing 10 pmol of the same limiting primer used in the LATE-PCR. The added limiting primer now serves as the sequencing primer using the abundant excess primer strand as template. These samples are then sent to a local commercial sequencing company that uses 5 μl of sample containing ~10 fmol of single-stranded DNA to synthesize dideoxy partials in a 25 μl reaction. LATE-PCR Dilute-*N*'-Go sequencing can also be used to sequence the excess primer strand. In this case, the reaction products are simply diluted in distilled water without the inclusion of an additional primer. The excess primer already present in the reaction serves as the sequencing primer in the cycle-sequencing reaction, and the limiting primer strand (1–10 fmol per 25 μl cocktail) serves as the template. The Dilute-*N*'-Go method works for dideoxy sequencing of both strands because the limiting primer is depleted and the excess primer strand becomes significantly more abundant than the limiting primer strand during the linear phase of a LATE-PCR. Neither of these conditions exist in a standard symmetric PCR. Symmetric PCR typically necessitates use of either a separate step for clean-up of the reaction products or careful titration of both primers and dNTPs to assure that these components of the reaction are depleted over the course of amplification<sup>20–25</sup>. Dilute-*N*'-Go sequencing can be used to analyze each of the excess primer strands generated in a multiplex reaction (see **Fig. 8**). Indeed, Dilute-*N*'-Go sequencing of the individual limiting primer strands from multiplex reactions provides a convenient method of verifying the presence of each of the excess primer strands when they are too similar in size to be resolved by agarose gel electrophoresis or too numerous to be probed individually (see **Fig. 5** for details).

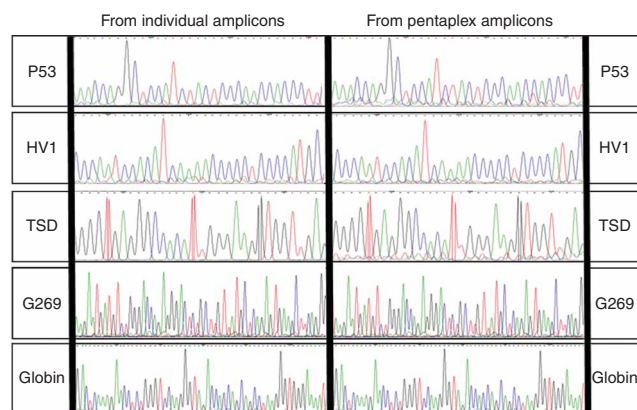


**Figure 7** | Example of LATE-PCR Dilute-*N*'-Go cycle sequencing. A region from the human p53 gene in human chromosome 17 was amplified using LATE-PCR to generate sequence data from both the limiting primer strand (**a**) and the excess primer strand (**b**) DNA amplification products. There was no need for PCR product quantification or purification before dideoxy sequencing.

**Negative and positive test reactions.** As in the case of any PCR for low-copy-number templates, it is critical to include no template controls in all such experiments. These must come up negative to convincingly demonstrate that reagents are clean of errant templates contaminating the laboratory. To minimize the risk of contamination, our laboratory is organized into different work spaces for preparation of the reaction mixture, addition of the target DNA and amplification. Tubes are opened only for sequencing and/or electrophoresis on another floor of the building. SYBR Green labeling of no template controls is also very useful for identifying primer-dimers that usually have melting temperatures many degrees lower than bona fide amplicons.

At an early stage in the development of both monoplex and multiplex LATE-PCR assays, we find it instructive to carry out dilution experiments for each amplicon in the reaction. Accordingly, a synthetic single-stranded target for each pair of primers is purchased (Operon, Sigma). For reasons of cost, the length of such synthetic targets may have to be shorter than the actual sought-for target sequence. The manufacturer's concentration for each target is used as a starting point to create a diluted series that stretches in tenfold steps downward from  $10^7$  copies to  $10^{-1}$  copies, using five replicate reactions at each step. Accumulation of the double-stranded products from each amplification is followed in real time using SYBR Green. The single-stranded products of each reaction can also be followed in real time if a target-specific probe has already been constructed. Analysis of the SYBR Green plots will reveal the following: (i) if the anticipated concentration of the synthetic amplicon is close to the value given by the manufacturer, every replicate in the series down to a 10 molecules per reaction

**Figure 8 | Dilute-'N'-Go dideoxy sequencing of multiplexed amplification products shown in Figure 4.** One replicate of the plus-PrimeSafe pentaplex reaction shown in Figure 4 was divided into five fractions, each of which was spiked with one of the five limiting primers used for amplification. As a reference, monoplex reactions for each of the same amplicons were sequenced in parallel. In each case, each limiting primer served as the cycle-sequencing primer for its corresponding excess primer strand. Comparison of the resulting sequences demonstrates that each of the Dilute-'N'-Go sequences from the pentaplex reaction was identical to the corresponding monoplex reactions. The labels in the figure correspond to the individual human gene target sequences used in these assays (P53, TP53 gene; HV1, mitochondrial DNA hypervariable region 1; TSD, HexA gene implicated in Tay-Sachs disease; G269, allelic variant of the HexA gene; Globin, beta globin gene).



will give a signal, and melting analysis of that signal will reveal the presence of a single double-stranded product; (ii) if the amplification is efficient, the mean  $\Delta C_T$  value for each tenfold step will be between 3.2 and 3.5 cycles. If condition (i) or (ii) is not achieved, it is likely that changes will have to be made in the design of the primers, the annealing temperature or the concentration or formulation of PrimeSafe. (iii) at each tenfold dilution through 100 molecules per tube all five replicates should have  $C_T$  values that are within one cycle of each other; at the dilution of 10 molecules per tube there should be variation among the replicates, as it is unlikely that every replicate will contain precisely 10 molecules, and, at the dilution of 1 molecule per tube, only 1–3 of the replicates will generate a signal at all. If these features are not observed, it is likely that the starting concentration of the target molecule provided by the manufacturer is not accurate.

**MATERIALS REAGENTS**

- 10× PCR buffer: 200 mM Tris-HCl (pH 8.4) and 500 mM KCl (Invitrogen, cat. no. 10966-018)
- 50 mM MgCl<sub>2</sub> (Invitrogen, cat. no. 10966-018)
- 100 mM dNTP set (dATP, dCTP, dGTP, dTTP) (Promega, cat. no. U1330); individual dNTPs are combined and diluted with 10 mM Tris-HCl pH 8.3 to obtain a mix of each dNTP at 10 mM
- 10 mM Trizma preset crystals, pH 8.3 (Sigma, cat. no. T-5128)
- 100× PrimeSafe (Smiths Detection, email: biodetection@smithsdetection.com)
- ▲ **CRITICAL** PrimeSafe comes in various types. PrimeSafe type and concentration must be adjusted empirically in individual reactions to achieve maximum amplification specificity without sacrificing amplification efficiency. To date, the most effective universal PrimeSafe formulation is 0.3× PrimeSafe-001.

- Recombinant Taq polymerase: 5 U  $\mu\text{l}^{-1}$  (Invitrogen, cat. no. 10342-053)
- SYBR Green I nucleic acid gel stain, 10,000× concentrate in DMSO (Invitrogen, cat. no. S-7567); dilute to 10× stock solution with distilled water
- Human genomic DNA (Sigma-Aldrich, cat. no. D-6537) ▲ **CRITICAL** We recommend that genomic DNA from cellular sources be prepared as described by Pierce *et al.*<sup>26</sup>
- 100  $\mu\text{M}$  ResonSense probe (see Experimental design)
- 10  $\mu\text{M}$  limiting oligonucleotide primer (0.05  $\mu\text{M}$ ; see Experimental design)
- 100  $\mu\text{M}$  excess oligonucleotide primer (1  $\mu\text{M}$ ; see Experimental design)
- Oligonucleotides were desalted and probes were HPLC-purified

**EQUIPMENT**

- ABI PRISM 7700 Sequence Detector (Applied Biosystems)
- Optical PCR tubes (Applied Biosystems, cat. no. N801-0933)
- Optical PCR caps (Applied Biosystems, cat. no. 4323032)

**PROCEDURE**

**LATE-PCR amplification of template DNA**

1| LATE-PCR amplification of template DNA for Dilute-'N'-Go dideoxy sequencing can be achieved using either monoplex LATE-PCR (option A) or multiplex LATE-PCR (option B).



**(A) Monoplex LATE-PCR amplification of template DNA for Dilute-‘N’-Go dideoxy sequencing ● TIMING 2–2.5 h**

(i) Prepare a PCR for each sample to be amplified, as follows:

Reagent	Volume (μl)
10× PCR buffer	2.5
50 mM MgCl <sub>2</sub>	1.5
10 mM dNTP mix	1
10× PrimeSafe-001	0.75
5 U μl <sup>-1</sup> recombinant <i>Taq</i>	0.25
100 μM excess primer	0.25
10 μM limiting primer	0.125
10× SYBR Green	0.6
6 ng μl <sup>-1</sup> genomic DNA	1
100 μM ResonSense probe	0.25
H <sub>2</sub> O	Adjusted to 25 μl final

**▲ CRITICAL STEP** Add PrimeSafe and *Taq* polymerase before adding the primers to the PCR master mix.

(ii) Prepare a negative control reaction as follows:

Reagent	Volume (μl)
10× PCR buffer	2.5
50 mM MgCl <sub>2</sub>	1.5
10 mM dNTP mix	1
10× PrimeSafe-001	0.75
5 U μl <sup>-1</sup> recombinant <i>Taq</i>	0.25
100 μM excess primer	0.25
10 μM limiting primer	0.125
10× SYBR Green	0.6
100 μM ResonSense probe	0.25
H <sub>2</sub> O	Adjusted to 25 μl final

(iii) Perform real-time PCR with appropriate cycling conditions, as follows:

	Cycle	Temperature (°C)	Duration (s)
55 cycles	Denaturation	95	10
	Annealing	64	20
	Extension and SYBR Green detection	72	20
	Cy5 detection	45	20

**▲ CRITICAL STEP** Optimization of this reaction requires (a) adjusting the PrimeSafe concentration from 0.3× to 1× to achieve maximum product specificity and (b) adjusting the annealing temperature in the range ±2–3 °C relative to the *T<sub>m</sub>* of the excess primer.

(iv) Determine the ratio of Cy5 to SYBR Green fluorescent signals to provide an empirical value with which to establish the amount of single-stranded product in any individual sample; a ratio of 0.06–0.1 indicates sufficient single-stranded DNA product to proceed to Dilute-‘N’-Go dideoxy sequencing.

**▲ CRITICAL STEP** The detection of double-stranded and single-stranded DNA products using ResonSense probes is also useful for determination of the appropriate dilution of the products when sequencing the limiting primer strand (see Step 3A(i)). However, this is optional because the amount of excess primer strand (which is the substrate for sequencing the limiting primer strand) can alternatively be calculated on the basis of the limiting primer concentration, the number of linear amplification cycles and the efficiency of the linear phase of the reaction (see Experimental design).

**(B) Multiplexed LATE-PCR amplification for Dilute-‘N’-Go dideoxy sequencing ● TIMING 2–2.5 h**

(i) First, ensure that each individual primer set works efficiently and generates a specific product in a monoplex reaction (see Step 1A) under the conditions that will be used for multiplexing before combining them into a multiplex reaction (see Experimental design).



## PROTOCOL

(ii) For multiplexed LATE-PCR amplification, prepare a PCR for each sample to be amplified, as follows:

Reagent	Volume ( $\mu\text{l}$ )
10 $\times$ PCR buffer	2.5
50 mM MgCl <sub>2</sub>	1.5
10 mM dNTP mix	1
10 $\times$ PrimeSafe-022	0.25
10 $\times$ PrimeSafe-060	0.0625
5 U $\mu\text{l}^{-1}$ Recombinant <i>Taq</i>	0.3
100 $\mu\text{M}$ excess primer (5–15 different primers)	0.25
10 $\mu\text{M}$ limiting primer (5–15 different primers)	0.125
10 $\times$ SYBR Green	0.6
6 ng $\mu\text{l}^{-1}$ genomic DNA	1
H <sub>2</sub> O	Adjusted to 25 $\mu\text{l}$ final

**▲ CRITICAL STEP** Add PrimeSafe and *Taq* polymerase before adding the primers to the PCR master mix.

(iii) Perform real-time PCR with appropriate cycling conditions, as follows:

Cycle	Temperature ( $^{\circ}\text{C}$ )	Duration (s)
55 cycles	Denaturation	95
	Annealing	64
	Extension and SYBR Green detection	72
		10
		20
		60

(iv) Monitor SYBR Green fluorescence to verify overall amplification success.

**▲ CRITICAL STEP** The ratio of SYBR Green to Cy5 signals used in monoplex reactions with ResonSense probes to estimate single-stranded DNA yield cannot be used in multiplex reactions because such monitoring requires probes with different fluorophores for each amplicon and ResonSense probes are labeled with only Cy5 (see Experimental design for further discussion of product detection).

(v) Alternatively, if the amplification products can be resolved by size, confirm specificity of multiplexed amplification by gel electrophoresis (see details in ref. 7).

**▲ CRITICAL STEP** As LATE-PCR generates both double-stranded and single-stranded DNA products, gel electrophoresis may reveal one or two bands for each product depending on whether the band corresponding to single-stranded DNA products is resolved from the double-stranded DNA band (see Fig. 4).

### Preparation of PCR amplification products for Dilute-N'-Go sequencing

2| Take 1  $\mu\text{l}$  of monoplex or multiplex PCR products (from Step 1A(ii) or 1B(iii)) and add 19  $\mu\text{l}$  of distilled H<sub>2</sub>O.

3| Set up monoplex PCR products for cycle sequencing using option A and multiplex PCR products for sequencing using option B.

#### (A) Setting up of monoplex PCR products for cycle sequencing ● TIMING 30 s

(i) To sequence the limiting primer strand, set up samples for cycle sequencing as follows:

Reagent	Volume ( $\mu\text{l}$ )
Diluted PCR products (Step 2)	2
10 $\mu\text{M}$ limiting primer or desired sequencing primer	1
H <sub>2</sub> O	Adjust to 25 $\mu\text{l}$ final

**▲ CRITICAL STEP** If the exact yield of single-stranded DNA products is not known (if ResonSense probes are not used), it might be necessary to adjust the volume of PCR products within a 2- to 4-fold range to obtain a satisfactory sequencing trace.

(ii) To sequence the excess primer strand from monoplex reactions, set up samples for cycle sequencing as follows:

Reagent	Volume ( $\mu\text{l}$ )
Diluted PCR products (Step 2)	2
H <sub>2</sub> O	Adjust to 25 $\mu\text{l}$ final

**▲ CRITICAL STEP** Do not add any sequencing primers because the existing excess primer serves as a sequencing primer.

**■ PAUSE POINT** Diluted PCR products can be stored indefinitely at  $-20^{\circ}\text{C}$ .

**(B) Setting up of multiplex PCR products for cycle sequencing of individual products ● TIMING 30 s**

(i) Set up samples for cycle sequencing as follows:

Reagent	Volume (μl)
Diluted PCR products (Step 2)	2
10 μM limiting primer	1
H <sub>2</sub> O	Adjust to 25 μl final

▲ **CRITICAL STEP** Thus far, we have only attempted to sequence the limiting primer strand in multiplexed LATE-PCR amplification because of the presence of multiple excess primers in the reaction.

▲ **CRITICAL STEP** As the exact yield of single-stranded DNA products is not known, it might be necessary to adjust the volume of PCR products within a 2- to 4-fold range to obtain a satisfactory sequencing trace.

■ **PAUSE POINT** Diluted PCR products can be stored indefinitely at -20 °C.

4| Supply 12 μl of the diluted PCR products (from Step 3A(i), 3A(ii) or 3B(i)) to a commercial sequencing facility. Specify that 5 μl of this volume be used for cycle sequencing in a 25 μl reaction.

● **TIMING**

Step 1: 2–2.5 h in ABI 7700 Sequence Detection System

Steps 2–4: 30 s

? **TROUBLESHOOTING**

Troubleshooting advice can be found in **Table 1**.

**TABLE 1** | Troubleshooting table.

Problems	Possible reasons	Solutions
<b>Monoplex LATE-PCR</b>		
Decrease or absent SYBR Green signals	No amplification	Check amplification conditions and LATE-PCR primer design
	Excess PrimeSafe concentration	Adjust PrimeSafe concentration
Good SYBR Green signals but reduced or absent ResonSense Cy5 signals	Limited number of linear cycles	Increase the number of cycles
	Nonspecific PCR products titrating SYBR Green	Perform melting curve analysis to confirm product specificity, adjust PrimeSafe concentration and/or PCR annealing step
	Secondary structure in probe or target region	To avoid probe secondary structure, redesign ResonSense probe  To avoid secondary structures at probe target sequence, increase probe concentration, lengthen detection time and/or raise detection temperature
Inadequate Cy5 to SYBR Green signal ratio	Not enough linear cycles	Increase the number of cycles
	Nonspecific PCR products	Perform melting curve analysis to confirm product specificity, adjust PrimeSafe concentration and PCR annealing step
Background on limiting-strand sequence traces	Inadequate dilution of PCR products	Test increased range of PCR product dilutions
	Poor linear amplification efficiency	Increase the number of cycles
Sequence trace corresponds to the wrong strand	Inadequate dilution of PCR products	Test decreased range of PCR product dilutions
	Incorrect strand targeted by primer	Check strands targeted by the limiting and excess primer



TABLE 1 | Troubleshooting table (continued).

Problems	Possible reasons	Solutions
<b>Multiplex LATE-PCR</b>		
Too much material in sequence trace	Excess single-stranded DNA products used for cycle sequencing	Increase the range of PCR product dilutions
Only a limited number of amplification products generate sequences	Nonspecific PCR products	Adjust PrimeSafe concentration

ANTICIPATED RESULTS

In a monoplex LATE-PCR, the  $C_T$  values obtained for ten gene copies of starting material with a hybridization probe are approximately 38 for the reactions, as shown in **Figure 2**, and each tenfold increase in copy number results in a 3.3–4 cycle decrease in the  $C_T$  value. Larger  $\Delta C_T$  values indicate that the exponential phase of the reaction is not efficient. The DNA-binding dye SYBR Green can be used to follow just the exponential phase of double-stranded DNA amplification, typically yielding a  $C_T$  value around 30–32 for ten gene copies of starting material (not shown). Multiplexed LATE-PCR assays will simultaneously amplify 15 and probably more unrelated amplicons in a single tube reaction. The presence of each amplification product in the reaction can be confirmed by dideoxy sequencing of the limiting primer strand following a simple dilution of total amplification products once LATE-PCR is completed. The use of PrimeSafe facilitates multiplexing by suppressing all forms of mispriming errors (see **Figs. 3,4**). **Figure 7a,b** shows sequences for the limiting primer strand and the excess primer strand generated using these LATE-PCR Dilute-*N'*-Go cycle-sequencing protocols.

**COMPETING INTERESTS STATEMENT** The authors declare competing financial interests (see the HTML version of this article for details).

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