วารสาร
เทคนิคการแพทย์และกายภาพบำบัด
JOURNAL OF MEDICAL TECHNOLOGY AND PHYSICAL THERAPY

บทความปริทัศน์
REVIEW ARTICLE

Linear After The Exponential (LATE) Polymerase Chain Reaction: Potential for clinical diagnostics

วันวิสาข์ บุญเลิศ [1, 2]*, Nicole L. Gentile[1], Anna M. Dillier[1], Richard F. Louie[1], Greg V. Williams[3], Gerald J. Kost[1]

บทคัดย่อ

Linear-after-the-exponential polymerase chain reaction (LATE-PCR) คือ เทคนิคใหม่ทางพีซีอาร์แบบไม่สมมาตร ซึ่งให้ผลผลิตเป็นดีเอ็นเอสายเดี่ยวภายใต้สภาวะที่จำเพาะ โดยการใช้ไพร์เมอร์ที่มีความเข้มข้นไม่เท่ากันเรียกว่า ลิมิตติงไพร์เมอร์ และเอคเซสไพร์เมอร์ เมื่อลิมิตติงไพร์เมอร์ถูกใช้หมดไปแล้ว เอคเซสไพร์เมอร์จะทำหน้าที่ต่อในการสร้างดีเอ็นเอสายเดี่ยว LATE-PCR มีศักยภาพในการแยกอัลลีลได้สูงขึ้น เนื่องจากอุณหภูมิที่โพรบสามารถจับกับดีเอ็นเอเป้าหมายจำเพาะและเป้าหมายที่มีลำดับเบสเปลี่ยนไปมีความแตกต่างกัน LATE-PCR แสดงให้เห็นถึงการมีความสามารถเป็นมัลติเพล็กซ์สูง เนื่องจากสามารถประยุกต์ใช้โพรบที่ติดฉลากด้วยสีเพียงตัวเดียวในการตรวจติดตามดีเอ็นเอเป้าหมายจำเพาะและเป้าหมายที่มีลำดับเบสเปลี่ยนไปได้หรือใช้โพรบที่จำเพาะกับหลายเป้าหมาย ณ อุณหภูมิที่แตกต่างกัน LATE-PCR ยังสามารถนำมาใช้ในการวินิจฉัยโรคทางพันธุกรรมด้วยการใช้เซลล์เดียว การทำจีโนไทป์ การตรวจหาลำดับเบสบนสายนิวคลีโอไทด์ และการตรวจหาซีโรไทป์ของไวรัสที่ก่อโรคในสัตว์ LATE-PCR มีความไว และความจำเพาะมากกว่าพีซีอาร์แบบดั้งเดิม มีความเร็วและความสามารถในการทำปฏิกิริยาแบบมัลติเพลกซ์ได้ ส่งผลให้ LATE-PCR มีศักยภาพสูงในการตรวจหากรดนิวคลิอิก และอาจนำไปใช้ประโยชน์ในการตรวจวินิจฉัยโรค ณ จุดดูแลผู้ป่วย

คำสำคัญ: ปฏิกิริยาพีซีอาร์แบบไม่สมมาตร, โมเลคคูลาเบคอนโพรบชนิดอุณหภูมิต่ำ, การจำแนกอัลลีล, พีซีอาร์ชนิดมัลติเพล็กซ์สูง, การตรวจหาเชื้อก่อโรคแบบเร็ว, การตรวจทางห้องปฏิบัติการ ณ จุดดูแลผู้ป่วย

[1]Point-of-Care Testing Center for Teaching and Research (POCT·CTR), University of California at Davis-Lawrence Livermore National Laboratory Point-of-Care Technology Center, University of California Davis, Davis, California, USA
[2]ภาควิชาเทคนิคการแพทย์ คณะสหเวชศาสตร์ มหาวิทยาลัยนเรศวร จังหวัดพิษณุโลก
[3]Smiths Detection Diagnostics, Edgewood, Maryland, USA
* ผู้รับผิดชอบบทความ

วารสาร
เทคนิคการแพทย์และกายภาพบำบัด
JOURNAL OF MEDICAL TECHNOLOGY AND PHYSICAL THERAPY

บทความปริทัศน์
REVIEW ARTICLE

Linear After The Exponential (LATE) Polymerase Chain Reaction: Potential for clinical diagnostics

Wanvisa Boonlert[1, 2]*, Nicole L. Gentile[1], Anna M. Dillier[1], Richard F. Louie[1], Greg V. Williams[3], Gerald J. Kost[1]

Abstract

The linear-after-the-exponential polymerase chain reaction (LATE-PCR) is an advanced asymmetric PCR method that generates single-stranded DNA amplicons under unique amplification conditions that involve unequal concentrations of limiting and excess primers. When the limiting primer is depleted, the excess primer will continue to form single-stranded DNA (ssDNA) products, in contrast to conventional PCR. Additionally, LATE-PCR potentially has increased allele discrimination because of the wide temperature probing window between mis-matched and matched targets. Mismatch-tolerant and temperature-dependent probe use enables highly multiplexing amplification. The LATE-PCR assay provides detection capability for single cell diagnosis of congenital diseases, genotyping, sequencing, and serotypes of viruses causing disease in animal. LATE-PCR has demonstrated higher sensitivity and precision than conventional PCR. Rapid, multiplex LATE-PCR has the potential to improve performance of nucleic acid detection and may be a useful diagnostic tool at the point of care.

Keywords: Asymmetric PCR reaction, Low temperature molecular beacons, Allele discrimination, Highly multiplexed PCR, Rapid pathogen detection, Point of care testing

[1]Point-of-Care Testing Center for Teaching and Research (POCT·CTR), University of California at Davis-Lawrence Livermore National Laboratory Point-of-Care Technology Center,
University of California Davis, Davis, California, USA
[2]Department of Medical Technology, Faculty of Allied Health Sciences, Naresuan University, Phitsanulok, Thailand
[3]Smiths Detection Diagnostics, Edgewood, Maryland, USA
*Corresponding Author: (e-mail: wanvisaboon@yahoo.com, wanvisab@nu.ac.th)

Introduction

Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) is a nucleic acid recognition (NAR) technique used for amplifying DNA segments of interest for numerous purposes. [1] PCR allows rapid generation of large quantities of specific DNA sequences by using a thermal cycler, DNA templates, primers, DNA polymerase (e.g., *Taq* polymerase), deoxynucleoside triphosphates (dNTPs) and magnesium ions (Mg^{2+}). The thermal cycler controls the temperature of three major steps required for successful amplification by PCR: target denaturation, primer annealing, and extension. In each thermal cycle, the target region of a double-stranded DNA (dsDNA) template is duplicated to achieve two dsDNA, each comprised of an original strand and a newly synthesized strand. These two dsDNA molecules (i.e. four strands) are then available for amplification in the next cycle, yielding four dsDNA copies: as the cycles are repeated, the number of copies thereby increases exponentially. With advanced optical technology coupled to thermocycling, PCR-based amplification can be detected by using fluorescent probes or dyes to monitor the increase in copies, either throughout the course of thermocycling (real-time detection) or at the end of cycling (end-point detection), with data analysis largely automated on commercial platforms. Consequently, the analytical power of PCR is increasingly exploited for clinical diagnostics, genetic analysis, bioengineering, forensic analysis, and recombinant DNA technology.

Symmetric and asymmetric PCR

Symmetric, also termed traditional, conventional, or "standard" PCR (**Figure 1A**) uses equal molar concentrations of each primer to anneal with the DNA template and produce double stranded DNA (dsDNA) copies, whereas asymmetric PCR (**Figure 1B**) uses unequal concentrations of primer. The combination of a "limiting" primer (LP) and an "excess" primer (XP) yields a predominantly single-stranded amplicon that is designed to be complementary to a fluorescently labeled nucleic acid probe. After an initial period of exponential amplification in which the limiting primer is partially depleted, reaction kinetics shift to favor extension from the excess primer, yielding the single-strand DNA (ssDNA) product. Studies have shown that the amplicon produced by asymmetric PCR from various dilutions of a virus sample generate a fluorescent signal 6-57% brighter than those obtained from conventional symmetric PCR. [2]

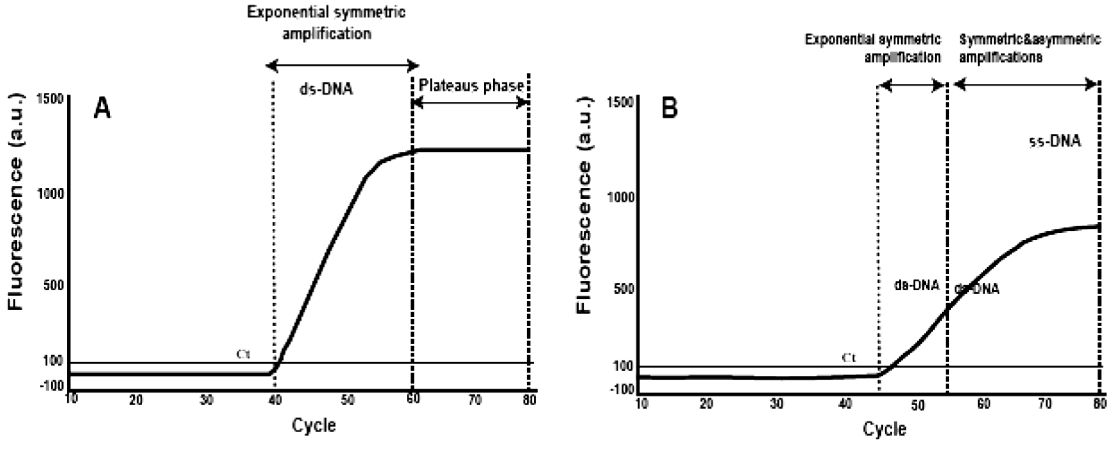

Figure 1 Symmetric and standard asymmetric PCR. A) Conventional symmetric PCR uses equal concentrations of primer to generate dsDNA amplicons exponentially. B) Standard asymmetric PCR generates only dsDNA amplicons exponentially prior to produce both dsDNA and ssDNA.

Conventional PCR has been used for DNA amplification for more than 20 years. The equal concentration and similar melting temperature (T_m) of a primer pair are standard design conditions for exponential amplification of a dsDNA target sequence. Such reactions eventually reach a plateau phase where little or no more product is formed, and in realtime monitoring the fluorescence signal approaches its maximum intensity (**Figure 1A**). Symmetric PCR exhibits highly variable kinetics with a large end-point range. Conversely, the XP in asymmetric PCR continues to generate ssDNA throughout thermocycling; LATE PCR assays are designed to switch from exponential amplification to linear (i.e. ssDNA product) shortly after the cycle threshold (C_T) (**Figure 1B**), thereby producing a delayed linear fluorescent signal over more cycles before it reaches plateau. However, asymmetric PCR produces low end-point signals [2], exhibits an overall efficiency of 60-70% in contrast with symmetric PCR, and has high variability. These characteristics make extensive optimization necessary to maximize synthesis of the specific amplicon product and minimize non-specific amplification; careful selection of appropriate hot-start enzymes is also important for asymmetric PCR performance. [2-4] Traditional asymmetric PCR requires improvements on reaction efficiency, which is inherently lower than that of symmetric PCR [3-4]. Optimization often requires LP and XP ratio adjustments and iterative primer re-designs, and these assays typically require more thermal cycles than a symmetric assay.

Linear-After-The-Exponential Polymerase Chain Reaction (LATE-PCR)

LATE-PCR is a novel form of asymmetric PCR that allows for high quality endpoint analysis. LATE-PCR was invented in the laboratory of Dr. Lawrence Wangh at Brandeis University (Waltham, MA, USA) in 2004 to alleviate limitations found with traditional asymmetric PCR. [5]

LATE-PCR chemistry favors production of single-stranded amplicon following a brief initial period of exponential amplification in which a LP, with a higher melting temperature than the XP, is consumed by the reaction. LATE-PCR uses unequal concentrations of primer in concert with conditions optimized to produce a ssDNA product efficiently (**Figure 2B**) whereas conventional symmetric PCR uses equal concentrations of primer to generate dsDNA product exponentially (**Figure 2A**). In LATE-PCR, detection of amplicon with a sequence-specific probe can be performed over a large temperature range (**Figure 2B**) in contrast to a probe optimally reactive only at high temperature as used in conventional PCR (**Figure 2A**). The single stranded amplicon obtained from LATE-PCR prevents the competition for probe binding normally seen with double-stranded PCR products. Fluorescence signal and cycle numbers of conventional symmetric PCR and LATE-PCR are shown in **Figure 2A** (below) and **Figure 2B** (below) respectively.

Effective primer and probe design for LATE-PCR, conventional symmetric, and traditional asymmetric PCR all rely on the oligonucleotide melting temperatures (T_m) which can be estimated using the formula:

$$T_m \text{ of primer or probe} = \%GC \text{ or } 2(A+T) + 4(G+C) \quad [6]$$

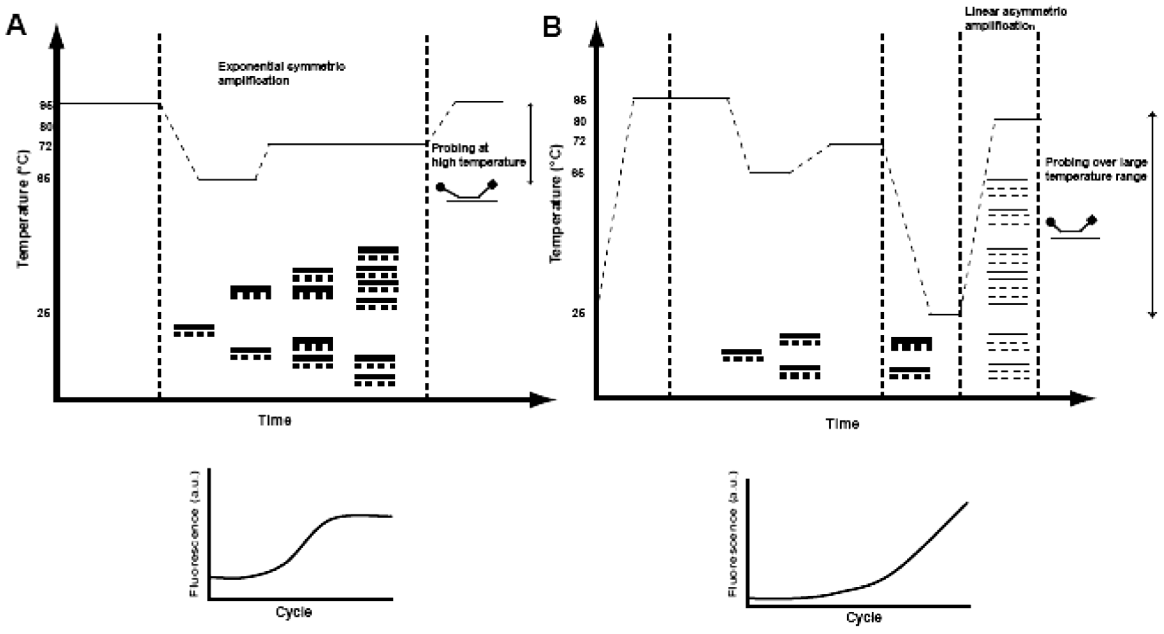

Figure 2 Conventional symmetric PCR and LATE-PCR. A) Conventional symmetric PCR uses equal concentrations of primer to generate dsDNA amplicons exponentially. B) LATE-PCR uses unequal concentrations of primer and other components that are optimized to produce ssDNA products efficiently. Probing of LATE-PCR is capable perform over a large temperature range (B) in contrast to a probing at only high temperature which found in conventional PCR (A). The amount of fluorescence signal when cycle numbers are increased for conventional symmetric PCR and LATE-PCR shows in 2A and 2B below.

By decreasing the concentration of the LP lower than the XP, the effective T_m of the LP (T_m LP) is shifted lower relatively to that of the XP (T_m XP). In practice, melting temperatures of primers and probes intended for LATE PCR are calculated by a more accurate method using the nearest-neighbor formula, defined as:

$$T_m = \frac{\Delta H}{\Delta S + R \ln (C/2)} + 12.5 \log[M] - 273.15 \quad (7\text{-}11)$$

Where ΔH and ΔS are thermodynamic parameters, R is the universal gas constant, and C is the initial concentration of the primer. The salt correction, [M], uses the total molar concentration of monovalent cations, sodium, and potassium in the PCR buffer. [12] T_m calculations can be made using the MELTING program available on the Internet site (http://bioweb.pasteur.fr/seqanal/interfaces/melting.html). [6]

Table 1 summarizes concentrations and melting temperatures of primers used in symmetric, traditional asymmetric, and LATE-PCR. T_mLP is higher than the T_mXP for LATE-PCR, while T_mLP is lower than T_mXP when using traditional asymmetric PCR. When temperature is decreased to allow primer annealing, the LP is favored to bind the template at a higher temperature than the XP, and enhances initial

production of dsDNA product. LATE-PCR generates ssDNA amplicons after a short period of exponential amplification; however, as the LP is consumed, reaction kinetics shift to favor the generation of ssDNA from the XP in a linear fashion. Traditional asymmetric reactions using a primer ratio of 1:100 were inefficient and exhibit delayed C_T values because (T_mLP- T_mXP) < 0. [5-6, 13] In LATE-PCR, altering the ratio of LP to XP (e.g., increased from 1:100 to between 1:10-1:40) and adjusting the relative primer melting temperatures (i.e., T_mLP- T_mXP ≥ 0), increased the fluorescent signal strength.

Table 1 Characteristics of primers used in symmetric, traditional asymmetric, and LATE-PCR.

Details	Symmetric PCR	Asymmetric PCR	LATE-PCR
Concentration (mM)	Equal C1 = C2	Unequal CLP < CXP	Unequal CLP < CXP
T_m (°C)	Equal T_m1- T_m 2 = 0	Unequal T_mLP- T_m XP < 0	Unequal T_mLP- T_mXP ≥ 0

Abbreviations: C_1, concentration of primer 1; C_2, concentration of primer 2; T_m, melting temperature ; T_m1, melting temperature of primer 1; T_m2, melting temperature of primer 2; T_mLP, melting temperature of limiting primer; T_mXP, melting temperature of excess primer

Another important consideration for LATE-PCR primer design is the T_m of the product (T_mA), which depends on the amplicon length and GC content. T_mXP must be 10 – 15 °C below T_mA in order for the XP to compete successfully with the accumulating ssDNA product for hybridization to the target strand and for continued generation of amplicon, thereby yielding the strongest detection signal.

Serveral types of oligonucleotide probes can be used in LATE-PCR. However, molecular beacons are prefered. 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 molecule into close proximity to a quenching moiety on the 3' end.

Characteristics of probes used in symmetric, asymmetric, and LATE-PCR are summarized in **Table 2**. Production of ssDNA amplicon enables use of lower T_m-molecular beacons with LATE-PCR relative to conventional PCR. However, because of the competition seen between dsDNA product and primer/probe binding to target, symmetric PCR requires primers and probes designed with higher T_m to ensure efficient binding during the anneal phase of the thermocycling reaction (**Figure 2A**). In contrast, the T_m of the probe for LATE-PCR should be lower than T_mLP to ensure that probe will not interfere with extension of the LP nascent strand. LATE-PCR demonstrates good amplification efficiency during the exponential phase and retains sensitivity even in the presence of high concentrations of probe. Low T_m-molecular beacons used in LATE-PCR have greater allele discrimination characteristics compared to the high-T_m probes commonly used in symmetric and asymmetric PCR, which provides a wider range of detection temperature for discrimination between specific sequence matched target and sequence mismatched target. [5-6, 13]

Table 2 Symmetric and traditional asymmetric, and LATE-PCR probings.

Details	Symmetric/ Traditional asymmetric PCR	LATE-PCR
T_m (°C)	High Tm $T_mXP - T_mLP > 0$	Low T_m $T_mXP - T_mLP \leq 5 \leq 10$
Saturates with increasing concentrations (mM)	Delayed CT value Low sensitivity	Increased amplification efficiency High sensitivity
Allele discrimination	Narrow temperature range for detection	Broad temperature range for detection

Abbreviations: T_m, melting temperature; T_mLP, melting temperature of limiting primer; T_mXP, melting temperature of excess primer.

Traditional probe design strategy is applicable to both symmetric PCR and LATE-PCR (**Figure 3A**). Mismatch-tolerant probing (**Figure 3B**) and temperature-dependent probing (**Figure 3C**) are novel probing methods of LATE-PCR that enable more efficient multiplexing capabilities. Multiple targets can be monitored in the same reaction by labeling different molecular beacon sequences with different fluorophores and/or different temperature ranges for probe binding. LATE-PCR makes it possible to use molecular beacons with shortened loop sequences to achieve greater allele discrimination and for mismatch-tolerant probes capable of binding to multiple targets (**Figure 3B**) or to use multiple probes having a common fluorescent tag but binding at different temperature (**Figure 3C**). These diverse probing strategies may prove useful for such applications as rapid pathogen detection of influenza viruses with divergent target sequence, or detection of polymicrobial bloodstream infections. Additional LATE-PCR applications are listed in **Table 3**. [14-19]

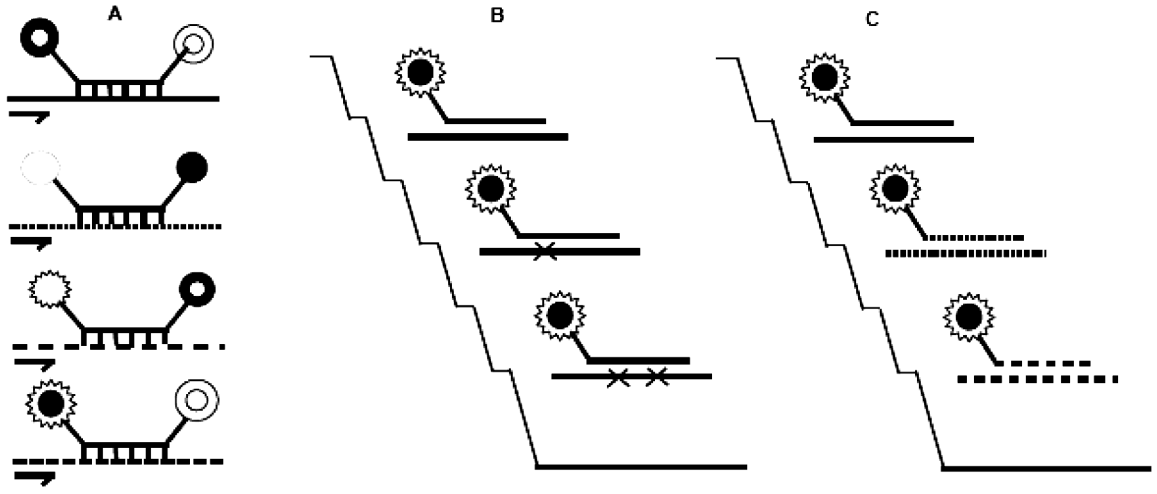

Figure 3 LATE-PCR permits highly multiplexed assay by probe applications. A) Traditional probing: Distinct primer/probe set for each target. B) Mismatch-tolerant probing: Single probe that binds to multiple sequences at different temperatures. C) Temperature-dependent probing: Multiple probes with a common fluorophor binding at different temperatures.

Table 3 LATE-PCR applications and advantages.

Publication	Application	Advantages
Pierce KE et al. 2003 [14]	Diagnosis of Cystic fibrosis by detecting of CFTR Δ*F508* alleles using a single lymphoblast	Improves allele discrimination and increases signal strength, thereby eliminating the need for mechanical or enzymatic separation and purification of single-stranded DNA
Sanchez JA et al. 2004 [5]	Diagnosis of Tay-Sachs disease by detect a defection on Human α-subunit of the β-N-acetylhexosaminidase (*HEXA*) gene from single human lymphoblast	Introduces a detection step distinct from the annealing step permitting the use of probes having greater allele discrimination capacity and improves signal-to-noise ratios
Sanchez JA et al. 2006 [15]	Genotyping the sequences near the human p53 tumor suppressor gene	Improves allele discrimination, eliminates the need for solid-phase sample preparation, and reduces the risk of laboratory contamination
Osborne A et al. 2009 [16]	Detecting mutations at specific loci within the HVI and HVII regions of the D-loop in mitochondria	Proves ready to use ssDNA from single target molecules for sequencing in parallel with using the Dilute-'N'-Go Sequencing
Salk JJ et al. 2006 [17]	Pyrosequencing at some regions of human chromosome for detection of the mutation at globin IVS 110 site to response to β-thalassemia	Provides ready-to-use ssDNA templates for pyrosequencing
Yang HY et al. 2009 [18]	Pyrosequencing to detect five SNPs related with human breast-cancers in the BRCA1 gene	Provides the simple ssDNA template for pyrosequencing
Pierce KE et al. 2009 [19]	Pathogen detection of foot and mouth disease virus (FMDV)	Decreases laboratory turnaround time, could be used as a point-of-care testing, applicable for a single mismatch-tolerant probe diagnosis of FMDV [seven discrete serotypes including O, A, Asia 1, C, SAT (South African Territories) 1, SAT 2 and SAT 3]
Boonlert W et al. 2011 [20]	Multi-pathogen detection in sepsis	Provides multiplex capability for multi-bacteria and fungi with low limit of detections (LoD)

Conclusions

LATE-PCR is a novel form of asymmetric PCR that utilizes novel primer and probe design and detection methods to achieve precision, sensitivity, and overall increased assay capability for allele discrimination compared to standard symmetric and traditional asymmetric PCR methods. LATE-PCR facilitates development of highly multiplexed assays and can be performed in real-time or with end-point analysis. Implementing molecular techniques, such as LATE-PCR, for diagnostic tests used in hospitals, emergency medicine, and disasters could improve standard of care in the future and ultimately may aid in early evidence-based treatment decisions of high value at the point of care.

Acknowledgements

The project described was supported by Award Number RC1EB010643 from the National Institute of Biomedical Imaging and Bioengineering. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of Biomedical Imaging and Bioengineering or the National Institutes of Health. We thank Dr. Lawrence J. Wangh and his colleagues at Brandeis University (Waltham, MA, USA) for expert assistance and colleagues at Smiths Detection Diagnosis (Edgewood, MD, USA) for their collaboration.

References

1. Clark DP. The polymerase chain reaction. In: Clark DP, ed. Molecular Biology. Elsevier Inc. 2005:634-57.
2. Poddar SK. Symmetric vs asymmetric PCR and molecular beacon probe in the detection of a target gene of adenovirus. Mol Cell Probes 2000; 14: 25–32.
3. McCabe PC. A guide to methods and applications. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, eds. PCR protocol. New York: Academic Press, 1999: 76–83.
4. Gyllensten UB, Allen M. Sequencing of in vitro amplified DNA. Methods Enzymol 1993; 218: 3–16.
5. Sanchez JA, Pierce KE, Rice JE, Wangh LJ. Linear-after-the-exponential (LATE)-PCR: An advanced method of asymmetric PCR and its uses in quantitative real-time analysis. Proc Natl Acad Sci USA 2004; 101: 1933-38.
6. Pierce KE, Wangh LJ. Linear-after-the-exponential polymerase chain reaction and allied technologies: Real-time detection strategies for rapid, reliable diagnosis from single cells. Methods in Molecular Medicine 2007; 132: 65-85.
7. Allawi HT, SantaLucia J. Thermodynamics and NMR of internal G.T mismatches in DNA. Biochemistry 1997; 36: 581–94.
8. SantaLucia J. A unified view of polymer, dumbbell, and oligonucleotide DNA nearest-neighbor thermodynamics. Proc Natl Acad Sci USA 1998; 95: 1460–65.
9. Owczarzy R, Vallone PM, Gallo FJ, Paner TM, Lane MJ, Benight AS. Predicting sequence-dependent melting stability of short duplex DNA oligomers. Biopolymers 1998; 44: 217–39.
10. Breslauer KJ. Methods for obtaining thermodynamic data on oligonucleotide transitions. In: Hinz H, ed. Thermodynamic data for biochemistry and biotechnology. New York: Springer-Verlag, 1986; 402–27.
11. Le Novère N. MELTING, computing the melting temperature of nucleic acid duplex. Bioinformatics 2001; 17: 1226-27.
12. SantaLucia J, Allawi HT, Seneviratne PA. Improved nearest neighbor parameters for predicting DNA duplex stability. Biochemistry 1996; 35: 3555–62.

13. Pierce KE, Sanchez JA, Rice JE, Wangh LJ. Linear-after-the-exponential (LATE)-PCR: Primer design criteria for high yield of specific single stranded DNA and improved real-time detection. Proc Natl Acad Sci USA 2005; 102: 8609-14.
14. Pierce KE, Rice JE, Sanchez JA, Wangh LJ. Detection of cystic fibrosis alleles from single cells using molecular beacons and a novel method of asymmetric real-time PCR. Mol Hum Reprod 2003; 9: 815-20.
15. Sanchez JA, Abramowitz JD, Salk JJ, Reis AH, Rice JE, Pierce KE, et al. Two-temperature LATE-PCR endpoint genotyping. BMC Biotechnology 2006; 6: 44.
16. Osborne A, Reis AH, Bach L, Wangh LJ. Single-molecule LATE-PCR analysis of human mitochondrial genomic sequence variations. PLoS ONE 2009; 4: e5636.
17. Salk JJ, Sanchez JA, Pierce KE, Rice JE, Soares KC, Wangh LJ. Direct amplification of single-stranded DNA for pyrosequencing using linear-after-the-exponential (LATE)–PCR. Anal Biochem 2006; 353: 124–32.
18. Yang HY, Xi T, Liang C, Chen ZY, Xu DB, Guo HZ. Preparation of single-stranded DNA for pyrosequencing by linear-after-the-exponential-polymerase chain reaction. Chinese J Anal Chem 2009; 37: 489-94.
19. Pierce KE, Mistry R, Reid SM, Bharya S, Dukes JP, Hartshorn C, et al. Design and optimization of a novel reverse transcription linear-after-the-exponential PCR for the detection of foot-and-mouth disease virus. J Appl Microb 2010; 109: 180–9.
20. Boonlert W, Gentile NL, Dillier A, Williams GV, Czajka JW, Reis AH, et al. Determining the limit of detection of a multiplex LATE-PCR assay for pathogen detection in critically ill patients. American Association for Clinical Chemistry Annual Meeting 2011: D-23 [Abstract].