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基于实时 PCR 的多靶检测技术研究

Study on Real-Time PCR-Based Approaches for Multiple Targets Detection

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摘要

作为重要的均相检测技术，实时 PCR 因具有快速、简便、灵敏、特异、可定量等优点，已被广泛应用于分子生物学研究和医学研究等领域。然而，受目前实时 PCR 仪可检测的荧光通道数目的限制，实时 PCR 存在检测容量小的问题，难以在单次反应中检测多个靶序列。针对这个问题，本论文对实时 PCR 多靶检测技术展开了研究，研究工作主要包括对多色组合探针编码技术的研究及探针熔解曲线分析技术的研究。

第一部分，提出了多色组合探针编码技术，并考察了该技术分别用于多靶识别和多突变检测的可行性。在该部分第一章，以多种食源性致病菌为检测对象，利用改良分子信标作为检测探针，将多色组合探针编码技术用于八种食源性致病菌的识别，所建立的检测体系可以准确识别八种食源性致病菌的任意一种，具有快速、简便、通量高、特异性好等优点。第二章，详细考察了荧光双链置换探针受 *Taq* DNA 聚合酶的 5'→3'核酸外切酶活性的影响及其规律，为多色组合探针编码技术用于多种突变的检测打下基础。第三章，以 β-珠蛋白基因多种突变为检测对象，通过结合 HAND 系统和荧光双链置换探针技术，将多色组合探针编码技术用于多种突变的检测，在单个反应管中实现对 5 种中国常见 β-地中海贫血突变的检测和 21 种基因型的分型。

第二部分，发展了新的探针熔解曲线分析技术。多色荧光检测和 T_m 多重检测是实现实时 PCR 多靶检测的有力工具。然而，目前的荧光探针具有难以同时结合多色荧光检测和 T_m 多重检测的缺陷。因此，在该部分第一章，首先系统考察了目前常用的荧光探针用于熔解曲线分析的可行性，比较各探针用于熔解曲线分析的优缺点，并发现改良分子信标探针是特别适合用于熔解曲线分析的荧光探针。第二章，以 β-珠蛋白基因多种突变为检测对象，考察了改良分子信标探针熔解曲线法用于检测多种突变的能力，并在此基础上，根据中国常见 β-地中海贫血突变发生频率，建立了两管多重多色突变检测体系，实现了对中国常见 15 种 β-地中海贫血突变的检测和基因分型。

关键词：实时 PCR；多色组合探针编码技术；熔解曲线分析；β-地中海贫血

ABSTRACT

Real-time PCR has become a powerful tool for quantitative and qualitative nucleic acids analysis, and has been broadly applied in the scientific, medical, and diagnostic communities. However, the number of targets that can be detected in a single real-time PCR is limited to four or five due to the limited number of channels in a real-time PCR apparatus. To address this issue, this dissertation focuses on the development of new real-time PCR strategies for multiple targets detection. It consists of two parts. The first part is the development of a novel multiplexing strategy called multicolor combinational probe coding technology, and the second part is the development of the probe melting curve analysis.

In the first part, a new strategy that significantly increases the number of targets identifiable in a single real-time PCR was developed. The strategy, termed multicolor combinational probe coding (MCPC) technology, uses fluorophore combinations in addition to single fluorophores to label probes. The combination rule allows n types of fluorophores to label $N= C_n^1+C_n^2 +\dots +C_n^n= 2^n-1$ different probes in a combinational manner. Thus, up to 15 probes can be labeled using 4 different fluorophores, and 15 targets can be detected on a 4-color real-time PCR machine. The feasibility of MCPC technology was tested by identifying multiple targets or detecting multiple mutations.

In chapter one of this part, the feasibility of MCPC technology was tested by identifying 8 foodborne pathogens in a single real-time PCR. With MCPC, 8 pairs of species-specific tagged primers, 1 pair of universal primers, and 8 single-labeled or mix-labeled molecular beacon probes were included in a single reaction tube. As demonstrated by both proof-of-principle experiments and blind test of 118 samples, all 8 foodbrone pathogens targeted were accurately identified and distinguished from other pathogens using MCPC technology.

In chapter two, the effect of 5' to 3' nuclease activity of *Taq* DNA polymerase on displacing probes was studied in order for MCPC technology to detect multiple mutations. Using both kinetic studies and matrix-assisted laser desorption ionization

time-of-flight mass spectrometry, we comprehensively examined the 5'-nuclease activity of *Taq* DNA polymerase on fluorogenic displacing probes of varied structures. We observed that displacing probes with unstable 5'-terminal could be hydrolyzed, and the major cleavage was the removal of the 5'-terminal fluorophore-labeled nucleotide. These observations can serve as guidance for better design of displacing probes with reduced or eliminated background for real-time PCR detection.

In chapter three, the concept of MCPC technology was extended to multiplex mutations detection in a single gene, and this was exampled by detecting the five common Chinese β-thalassemia mutations and an internal control in a single real-time PCR. By coupling MCPC technology and Homo-Tag Assisted Non-Dimer System (HANDS), 3 pairs of specific tagged primers, 1 universal primer, and 6 single-labeled or mix-labeled displacing probes were included in a single reaction tube. Totally 21 genotypes including wild-type, heterozygous, homozygous and compound heterozygous were covered by this assay. The specificity of the assay was 100% when tested against 129 normal, 98 carriers, and 12 patients. Accurate results could be acquired with genomic DNA ranging from 100 ng to 10 pg per reaction. The assay provides a rapid, accurate, and sensitive means for detecting multiple β-thalassemia mutations.

In the second part, a new probe melting curve analysis strategy was developed. While multiplex PCR by both probe color and melting temperature (T_m) could greatly expand the power of real-time PCR analysis for multiple targets detection, the existing fluorogenic probes for this aim are inherently limited in combining color multiplexing and T_m multiplexing. Thus, in the first chapter, a variety of fluorogenic probes were evaluated regarding their potentials in melting curve analysis. The result showed that nearly all probes studied could be used for melting curve analysis. Particularly interesting finding was that the self-quenching probes and shared-stem molecular beacons, which were never used for melting curve analysis before, were found to be comparable with the FRET probe. Shared-stem molecular beacons were advantageous in their high signal to noise ratio, narrow melting peak, and more important, in their flexibility of combining color multiplexing and T_m multiplexing. In

the second chapter, a new probe melting analysis strategy was established by taking advantage of the shared-stem molecular beacons. The significance of this strategy was that several mutations could be detected by a single probe, thus more mutations could be detected by several differently labeled probes in one reaction. For a proof-of-principle study, more than 15 mutations of β-globin gene were detected through the new probe melting curve strategy. The specificity of the multiplex assays was 100% for the detection of 118 clinical samples. Accurate results could be acquired with genomic DNA ranging from 50 ng to 5 pg per reaction. We concluded that the new probe melting curve analysis strategy provided a rapid, accurate, sensitive, low-cost, and high-throughput means for detecting multiple mutations.

Keywords: Real-time PCR; Multicolor Combinational Probe Coding Technology; Melting curve analysis; β-thalassemia

前　言

§1 实时 PCR 概述

所谓实时聚合酶链式反应（Real-time PCR，简称实时 PCR），就是利用荧光探针结合在线分析技术，实时分析 PCR 扩增动力学过程，实现起始模板的定性、定量分析。简单地说，实时 PCR 就是 PCR 的在线分析。自从 1992 年 Higuchi 等报道实时 PCR 以来[1]，因其具有简便、快速、灵敏、特异、重现性好、无需 PCR 后处理、易于自动化等优点，实时 PCR 在基因定量分析、定性检测、基因分型等方面得到越来越广泛的应用[2-8]。

实时 PCR 可以利用不同形式实现对扩增产物的荧光检测[9]。根据使用的荧光探针类型，实时 PCR 可以分为荧光嵌入染料型和荧光标记探针型两种。荧光嵌入染料型实时 PCR 是利用双链 DNA 嵌合染料（如溴化乙锭[10]、YO-PRO-1[11]、SYBR Green I[12]、SYBR Gold[13]、BEBO[14]、BOXTO[15]、LCGreen[16]、SYTO9[17]）来指示扩增产物的增加。荧光标记探针型实时 PCR 则是利用与靶序列特异杂交的探针（详述如下）或特殊设计的引物（如 Amplifluor™引物[18]、LUX™引物[19]、蝎子引物[19]、荧光双链引物[20]）来指示产物的存在。荧光标记探针型可以采用不同荧光基团标记探针或者引物，因此可用“多色”分析检测多个靶序列。荧光嵌入染料型则因为对所有双链产物都发出相同光谱类型的荧光，无法直接区分不同产物，但是可以通过熔解曲线根据产物熔点差异加以区分。总之，实时 PCR 经过十多年的发展，在探针化学上已取得长足进步并日臻成熟。

在荧光标记探针中，靶序列特异杂交探针最为重要，这类探针通过与靶序列杂交指示扩增产物的有无或多少，特异性更高。常见的靶序列杂交荧光探针包括 TaqMan 探针[21, 22]、分子信标探针(Molecular Beacons) [23]、荧光共振能量转移探针[24]（FRET 探针）、荧光双链置换探针[25]等，这些探针几乎无一例外地都采用荧光共振能量转移原理或者基态荧光淬灭原理，指示杂交前后荧光信号的变化。

TaqMan 探针又称水解探针，是目前实时 PCR 应用最广泛的荧光探针，该探针为单链寡核苷酸探针，在探针两端分别标记荧光基团和淬灭基团。探针完整时，因荧光基团和淬灭基团的距离很近而使荧光基团发出的荧光被淬灭；PCR 延伸阶段，探针与靶序列杂交，具有 5'到 3'外切酶活性的 *Taq* DNA 聚合酶可以将探

针降解成小片段，使荧光基团和淬灭基团分离，荧光基团发出荧光。随着 PCR 扩增产物的增加，释放出来的荧光基团就不断的积累，故 TaqMan 探针检测的是积累的荧光信号。

分子信标探针是一种发夹型探针，其环状部分与靶序列互补，位于茎干部分的 5'端和 3'分别标记荧光基团和淬灭基团。因此，未与靶序列杂交时，分子信标探针呈发夹结构，荧光基团和淬灭基团互相靠近，荧光几乎完全淬灭；PCR 扩增的退火阶段，探针与靶杂交，发夹展开，荧光基团与淬灭基团分开，荧光得以恢复，其荧光信号的强度也与 PCR 扩增的产物量相对应。

FRET 探针又称杂交探针，由两条与模板互补、且相邻的特异探针组成，其中一条为锚定探针（标记供体荧光基团），另外一条为检测探针（标记受体荧光基团）。当没有靶序列存在时，两条探针处于游离状态，受体荧光基团不能被激发，因此不能检测到荧光信号；当有互补的靶序列存在时，两探针同时结合在互补的模板上，供体荧光基团和受体荧光基团相互靠近，激发供体荧光基团产生的荧光能量被荧光受体基团吸收而产生特定波长的荧光信号，其荧光信号强度与被扩增的模板量成正比。

荧光双链置换探针具有双链结构的探针，由两条不同长度的互补的寡核苷酸链组成。在双链的对齐的一端，长链 5'末端标记荧光基团，短链 3'末端标记淬灭基团，因此当探针形成双链结构时，荧光基团和淬灭基团互相靠近，荧光几乎完全淬灭。在 PCR 退火阶段，长链将优先与靶序列杂交而恢复荧光，未杂交的长链则与短链退火而不引起背景荧光，其荧光信号的强度与 PCR 扩增的产物量成正比。

在新的探针技术不断涌现的同时，实时 PCR 的仪器技术同样取得了突飞猛进的发展，从早期的单激发光源，到现在的多激发光源、多通道检测、再到最近的大容量并行检测仪器，使得成千上万的实时 PCR 反应得以同步进行，使得实时 PCR 进入数字 PCR 时代[26-28]。可以说，作为在线分析的实时 PCR 在越来越多的领域取代那些离线的检测系统，在基因定量和突变分析上已成为越来越受欢迎的新一代基因分析工具。

和近年来出现的另一类基因分析工具——二代测序技术[29-33]相比，实时PCR的突出优点在于均相在线检测，由此引发的不仅是操作的简便性，本质上则是提