Chapter 3
Application of Molecular Beacons in Real-Time PCR

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Abstract Real-time PCR or quantitative PCR (QPCR) is a powerful technique that allows measurement of PCR product while the amplification reaction proceeds. It incorporates the fluorescent element into conventional PCR as the calculation standard to provide a quantitative result. In this sense, fluorescent chemistry is the key component in QPCR. Till now, two types of fluorescent chemistries have been adopted in the QPCR systems: one is nonspecific probe and the other is specific. As a brilliant invention by Kramer et al. in 1996, molecular beacon is naturally suited as the reporting element in real-time PCR and has been adapted for many molecular biology applications. In this chapter, we briefly introduce the working principle of QPCR and overview different fluorescent chemistries, and then we focus on the applications of molecular beacons-like gene expression study, single-nucleotide polymorphisms and mutation detection, and pathogenic detection.

3.1 Introduction

Soon after MBs were first introduced, they found important application in real-time PCR [1, 2], which requires prompt signal production with high specificity. In this chapter, some basic principles of real-time PCR are discussed, and the applications of MBs in single-nucleotide polymorphisms (SNPs) genotyping and pathogenic detection are summarized.

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3.2 Basic Principles of Real-Time PCR

The emergence of polymerase chain reaction (PCR) has enabled the amplification of one or many copies of DNA isolated from cell, tissue, or blood samples, subsequently sparking a revolution in biology. Nonetheless, standard PCR is only designed for positive recognition of the amplicons. Post-PCR analysis should be performed in order to characterize both the size and sequence of the product. A DNA gel electrophoresis to measure the size and the quantity of the amplicon is simple and inexpensive. However, it is not easy to discriminate among different amplicons having similar size by gel. Also, gel is not accurate enough to assess the starting target by visualizing the final PCR product with nucleic acid intercalating dyes, although, to some extent, more initial target leads to more PCR product. This can be explained by studying the kinetics of PCR [3].

A conventional PCR usually consists of three phases: exponential, nonexponential, and plateau. At the beginning, all the reagents are sufficient to guarantee good PCR efficiency, and amplification occurs in an exponential manner, with the initial DNA doubling after every cycle. As the cycles progress and reagents are consumed, the reaction starts to slow down. At this point, the PCR product is no longer doubled in every cycle, and nonexponential amplification dominates. Finally, after another several rounds of amplification, the PCR reaction no longer generates templates as a result of the lack of critical components in the reaction. This is commonly known as the plateau phase, or end point, of the PCR reaction [4]. In this sense, the final yield of PCR product is not primarily dependent on the target sequence in the sample.

Real-time PCR, which combines amplification and quantification of a target DNA molecule in a single assay, is now a routine and robust technique in molecular biology. By detecting the products generated every cycle in a "real-time mode" at the early exponential phase, real-time PCR can differentiate the three stages and also save time since it is not necessary to wait until the reaction terminates. Real-time PCR allows the PCR product to accumulate during every cycle to allow measurement through different fluorescent chemistries.

3.3 Fluorescent Measurements for Real-Time PCR

There are generally two types of measurements to acquire the fluorescent signal from the PCR product. The first type relies on DNA binding dyes, such as SYBR Green I, which binds nonspecifically to double-stranded DNA (dsDNA) and emits an enhanced fluorescence [5]. The other type is a probe-based approach. These probes are sequence specific, and most of them use fluorescence resonance energy transfer (FRET) as the reporting mechanism [6] and use the 5′-exonuclease activity of the DNA polymerase [7] to detect PCR amplification in real time.

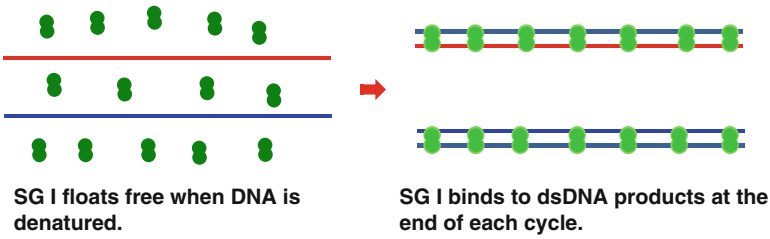

Fig. 3.1 A schematic of the working principle of SG I in real-time PCR

3.3.1 DNA Intercalating Dyes, SYBR Green I (SG I)

SG I shows a low fluorescence background when it is free in solution, while the fluorescence signal could increase up to 1,000-fold once it binds dsDNA (Fig. 3.1). This works universally for all dsDNA. Thus real-time PCR that utilizes SG I as reporter [5] is the most straightforward method by eliminating the complicated design of specific probes and reducing the cost of both time and money. As the PCR proceeds, more amplicons accumulate; accordingly, more SG I molecules are bound. The change in fluorescence can then be monitored using a thermocycler equipped with fluorescence detector. In other words, the fluorescence intensity is proportional to the amount of PCR products.

However, since SG I recognizes dsDNA in a nonspecific manner, nonspecific amplification products cannot be differentiated. Optimization of primers and template is therefore essential. First, the primers should be designed to generate amplicons with a suitable length (normally, 100–400 bp). Second, the concentration of primers should not be too high in order to achieve a high ratio of specific amplification versus primer-dimer signal. Third, the template should not include complicated secondary structures because this would contribute to the fluorescence signal. After real-time PCR is finished, a melting curve is usually recorded. If PCR generates a homogeneous sequence, only one transition point should be observed. Otherwise, nonspecific products or contaminants may exist. Most commonly, SG I is used for assays for which probe chemistry cannot be used or those assays which do not require high accuracy. SG I can also be used for optimization of primers prior to ordering the sequence-specific probe.

3.3.2 Probe-Based Chemistry

In probe-based chemistries, short oligonucleotides are used as an internal probe to hybridize with the region to be amplified. All these probes possess a quencher in close proximity to the reporter dye, where FRET occurs. In most cases, as the products form, the linkage between the quencher and dyes will be cut off, leading to a fluorescence enhancement. Compared to SG I, all probes are more specific since the hybridization only happens between probes and correct amplified products.

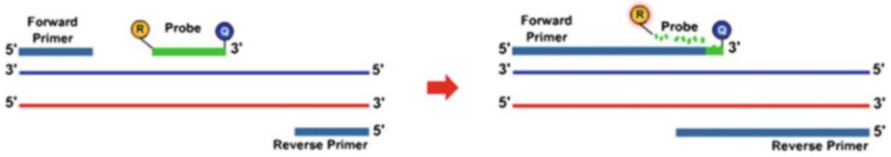

Fig. 3.2 Different states of TaqMan probe in real-time PCR: *left* – annealing; *right* – extension (Reprinted with permission from PREMIER Biosoft. Copyright ©1994–2013)

3.3.2.1 TaqMan Probe

TaqMan probe is a short single-stranded DNA (ssDNA) with a fluorophore at the 5′ end and a quencher at the 3′ end [8]. This DNA is complementary to the sequence within the template. Since the DNA is usually 20–30 bases in length, FRET is efficient when the probe is in free form. At this time, the fluorescence is quenched. It is only after the probe hybridizes to the template and is digested by Taq DNA polymerase (Taq polymerase is known to have 5′-exonuclease activity), as it extends the amplification primers, that the linkage between dye and quencher is cleaved, subsequently restoring fluorescence of the dye molecule (Fig. 3.2). Similar to SG I, the fluorescence increases in proportion to the amount of PCR products, since more probes will hybridize and will be cleaved. The advantages of the TaqMan probe include (1) analysis in real time without the need for post-PCR handling, thus reducing labor and cost; (2) specific hybridization-based detection, which eliminates nonspecific signal; and (3) labeling with different dyes with monitoring of different sequence amplifications in one tube. At the same time, however, TaqMan probes have some challenges, including (1) limited use in different assays and (2) limited design parameters. Normally, the probe should be designed close to the 5′ end to give a quick response. The length should be controlled in order to achieve sufficient FRET efficiency. In addition, guanine should not be placed next to the fluorescent dye since it is also an effective quencher.

3.3.2.2 FRET Hybridization Probes

The FRET probe system consists of two single-stranded fluorescent oligonucleotides such that probe 1 is labeled with a donor dye at the 3′ end, while probe 2 is labeled with an acceptor dye at the 5′ end [9, 10], typically having a 1–5 bases gap between them. During the annealing step, both probes will hybridize to the target, putting the donor in close proximity to the acceptor. The fluorescence signal of acceptor will then be detected, and the increase will be proportional to the products amplified (Fig. 3.3). Although this two-probe system gains specificity, it also increases the difficulty of hybridization.

Fig. 3.3 The working principle of FRET hybridization probes (Reprinted with permission from PREMIER Biosoft. Copyright ©1994–2013)

3.3.2.3 Molecular Beacons

The molecular beacons are the ideal hybridization-based probe for short oligonucleotide detection, and, thus, it is a suitable probe for real-time PCR [11]. In real-time PCR, MB hybridizes with template DNA at the annealing step and produces the fluorescent signal directly. Therefore, it does not need a polymerase with exonuclease activity, which is essential for the TaqMan probe. In the extension step, the polymerase will extend the sequence and displace the MB, returning it to the stem-loop conformation. In this case, the probe can be reused in the remaining cycles. MBs should be designed to hybridize 7–10 °C higher than primers, to ensure detection before primers are extended. Therefore, the stem should be just short enough to guarantee full hybridization, but not so short that can refold to the stem-loop structure after displacement of the molecular beacon by primer extension. Despite the difficulty in designing and optimizing a suitable MB, MB real-time PCR assays are simple, fast, sensitive, and accurate, allowing a high-throughput format and enabling the multiplexing detection in one tube using different labeling probes. MB-based PCR technique has been widely used in SNP analysis, real-time nucleic acid detection and quantitation, allele discrimination, and other clinical assays.

3.3.2.4 Scorpion Probe

The Scorpion probe is similar to molecular beacons in that it consists of a stem-loop structure when it is in free form. However, the Scorpion incorporates a primer into the sequence at the 3′ end, next to the quencher, via a blocker [12]. This blocker is a non-amplified monomer, which prevents the PCR from reading through the probe. In the extension stage, the polymerase binds the primer and synthesizes the complementary strand of target sequence just as it works in regular PCR. During the annealing step in next cycle, the loop will hybridize to the complementary strand within the same DNA. This separates the fluorophore and quencher, and an enhanced fluorescent signal is instantaneously observed (Fig. 3.4). Because the probe and primer are in the same molecule, the reaction kinetics is extremely fast. Also, intramolecular interaction is more favorable than intermolecular hybridization. This enables the Scorpion probe to provide a higher signal than other bimolecular systems, including either TaqMan or MB, but the design of the Scorpion is more difficult. Specifically, it reduces flexibility in probe

Fig. 3.4 The working principle of the Scorpion probe (Reprinted with permission from PREMIER Biosoft. Copyright ©1994–2013)

design where the loop should be engineered such that it is not too far from the complementary part to ensure high hybridization efficiency. Similarly, the stem should be long enough to stabilize the hairpin structure. The stem's Tm should be 5–10 °C higher than that of the primer-target hybrid.

3.4 MB Used in Real-Time PCR for SNPs and Mutation Detection Assays

The human genome consists of ten million single-nucleotide polymorphisms (SNPs). While most SNPs have no effect on health, some SNPs are believed to be related to the development of diseases [13]. Therefore, highly specific, simple, and accessible methods are needed for high-throughput SNPs detection (Fig. 3.5). MB-based assays provide a solution for screening SNPs in homogeneous assays [14]. Most of these assays require PCR to gain enough DNA targets, while MBs can specifically recognize these targets and present detectable signals in real time. Although other probes can also be used in real-time PCR, MBs have been demonstrated to be superior to them in certain aspects. For example, MBs have been proven to have better specificity than TaqMan probe in a detailed research report [15], and MBs have less complexity than Scorpion probe in design. Therefore, RT-PCR using MBs is perfect for SNPs analysis.

A key point for MBs in SNPs genotyping is to discriminate perfect match targets from single-base mismatch targets. The range of temperatures within which discrimination between the two targets is possible is wider for molecular beacons than it is for the corresponding linear probes. This is known as the window of discrimination, which is the basis for SNPs detection in homogeneous assays and is discussed at length in Chap. 4.

Early in 1998, 2 years after the MB was reported, Kramer et al. proposed the method of spectral genotyping human alleles using MB [11]. In their design, two MBs with different labeling were used: one specific for wild-type allele with green fluorophore and another for mutant allele labeled with red dye. The appearance of green, red, and both signals represented the homozygous wild type, homozygous mutant, and heterozygote, respectively.

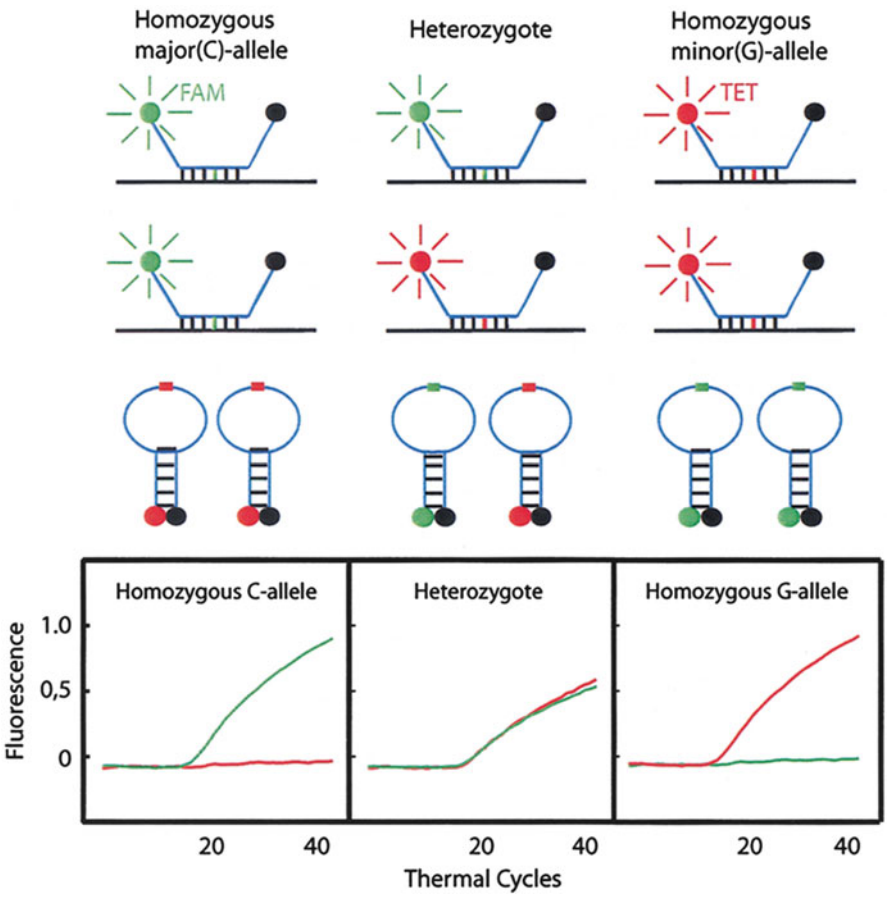

Fig. 3.5 Principle of spectral genotyping by PCR, exemplified by detection of a SNP in codon 325 of the estrogen receptor gene (Reprinted from Ref. [14]. Copyright 2001, with permission from Elsevier)

Later in 1998, in another report [16], they proved that MB-based sequence analysis could be adopted as an accurate assessment of DNA sequence. Five MBs, each complementary to a short fragment, were combined with 1–3 bases overlapped to span an 81 bp core region on the *rpoB* gene. This assay is simple and rapid. Most importantly, no contamination was observed since the tubes were not opened throughout the entire assay. Seventy-five clinical DNA isolates were correctly identified as drug susceptible or drug resistant. A broad range of point mutations, insertions, and deletions were detected successfully. Furthermore, in their paper in 1999 [17], up to four MBs, each with a different color, were used to explore four variants which differed from one another only by one base position. In four tubes, all the MBs were added with only one target variant. After PCR, only one fluorescence response was observed in each tube. This result indicates the extraordinary specificity of MB.

Since organic dyes often overlap each other in their emission spectra, 3–4 different dyes are the maximum that can be used at the same time. Another limitation comes from the instrument. Traditional thermal cyclers often have fixed excitation/emission filters. Under this condition, two dyes, which can be separate on a fluorometer, might not be able to be distinguished in PCR assays due to the lack of appropriate filters. Tyagi et al. proposed the construction of wavelength-shift MB [18], which emitted different fluorescent colors, but was excited with monochromatic light. This was realized by attaching a second fluorophore next to the fluorophore of a normal MB, which still contains a nonfluorescent quencher. In this design, one dye served as a harvester with strong absorption in the range of the light source. The other dye accepted the emission transferred from the harvester and emitted the desired color of fluorescence. This shift in emission spectrum is due to the fluorescence resonance energy transfer (FRET) from the harvester fluorophore to the emitter fluorophore. This only happened in opened probes that are bound to targets, and quencher has been separated from the fluorophores. By this method, the multiplex genetic analysis can be improved to be more simple and reliable.

3.5 MB Used in Real-Time PCR for Pathogenic Detection

Current techniques used to identify microbial pathogens usually rely on culturing and screening the samples to monitor the presence of pathogenic organisms, which are already well established. However, these suffer from a number of drawbacks. The assays are laborious, time-consuming, and expensive and require labile natural products [19]. More importantly, these routine tests do not directly characterize virulence factors. Efforts to overcome these problems in pathogenic detection have led to the development of DNA-based diagnosis. Today, culture-based methods for pathogen detection are rapidly being replaced by faster and more specific real-time PCR assays that discriminate different microorganisms based on a signal from specific nucleic acid sequences. Real-time PCR pathogen detection assays amplify target nucleic acid sequences from select microbes present in samples collected from complex biological environments. Specific amplification of target sequences is achieved by custom-designed primers and probes.

As pioneers in the study of molecular beacons, Kramer and colleagues described a multiplex MB assay to determine four pathogenic retroviruses [20]. Since then, scientists have applied this real-time PCR assay to detect all types of pathogenic organisms (Table 3.1).

Unlike assays that detect specific human DNA sequences present in samples, real-time PCR pathogenic detection assays must target genetic material from multiple microbial species in a single sample. This requires an assay which is capable of discriminating among the sequences from species of interest and other sequences from even the nearest evolutionary neighbors of the target species. Therefore, the detection of specific microorganisms requires the selection of an

Table 3.1 The utilization of MBs to detect different pathogens

Pathogens	Species	References
Bacteria and Fungus	*Aspergillus fumigatus*	[21]
	Bordetella pertussis	[22]
	Candida dubliniensis	[23]
	Chlamydophila felis	[24]
	Chlamydophila pneumoniae	[25]
	Clostridium	[26, 27]
	Escherichia coli	[28–31]
	Legionella pneumophila	[32]
	Mycobacterium tuberculosis	[33–44]
	Nitrifying bacteria	[45]
	Paracoccidioides brasiliensis	[46]
	Salmonella	[47–53]
	Scedosporiosis	[54]
	Staphylococcus	[55–58]
	Streptococcus	[59]
	Vibrio cholerae	[60]
Viruses	T-cell leukemia virus	[61]
	Adenovirus	[62, 63]
	Bluetongue virus	[64]
	Coronavirus	[65]
	Cytomegalovirus	[66]
	HAV	[67, 68]
	HBV	[69–74]
	HCV	[75–77]
	HPV	[78]
	Human immunodeficiency virus	[75, 79–83]
	Iridovirus	[84]
	Phage	[85]
	Plant virus	[86]
	Retrovirus	[20]
	Swine virus	[87]
	Syncytial virus	[88]
	West Nile virus	[89]

optimum target sequence to amplify. However, since many microbial sequences are unknown or have not yet been deciphered, the selection will be complicated and has no principle to rely on yet.

3.6 Conclusions

Real-time PCR represents one of the most important techniques in modern molecular biology, and it has become a routine and robust laboratory assay for gene expression analysis. In this chapter, different fluorescent chemistries applied in

real-time PCR were summarized, reflecting the prominent role of MBs in this area. Based on the capacity to distinguish perfect match target from false targets, MBs are naturally suited for SNPs and mutation detection. Similarly, MBs can be used to detect all kinds of pathogens, which may only differ by a few bases in the gene sequences. With the increasing use of real-time PCR in gene transcription studies, disease-related diagnostics, and food safety assessment, MBs will continue to play an irreplaceable role in this field and aid the development of real-time PCR.

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