Clinica Chimica Acta xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/clinchim

Invited critical review

Real-time PCR detection chemistry

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ARTICLE INFO

Article history:
Received 18 March 2014
Received in revised form 9 October 2014
Accepted 11 October 2014
Available online xxxx

Keywords:
Real-time PCR
DNA detection chemistries
DNA binding dye
Fluorescent primer-probe
Fluorescent probe
Nucleic acid analogues

ABSTRACT

Real-time PCR is the method of choice in many laboratories for diagnostic and food applications. This technology merges the polymerase chain reaction chemistry with the use of fluorescent reporter molecules in order to monitor the production of amplification products during each cycle of the PCR reaction. Thus, the combination of excellent sensitivity and specificity, reproducible data, low contamination risk and reduced hand-on time, which make it a post-PCR analysis unnecessary, has made real-time PCR technology an appealing alternative to conventional PCR. The present paper attempts to provide a rigorous overview of fluorescent-based methods for nucleic acid analysis in real-time PCR described in the literature so far. Herein, different real-time PCR chemistries have been classified into two main groups; the first group comprises double-stranded DNA intercalating molecules, such as SYBR Green I and EvaGreen, whereas the second includes fluorophore-labeled oligonucleotides. The latter, in turn, has been divided into three subgroups according to the type of fluorescent molecules used in the PCR reaction: (i) primer-probes (Scorpions, Amplifluor®, LUX™, Cyclicons, Angler®); (ii) probes; hydrolysis (TaqMan, MGB-TaqMan, Snake assay) and hybridization (Hybprobe or FRET, Molecular Beacons, HyBeacon™, MGB-Pleiades, MGB-Eclipse, ResonSense®, Yin-Yang or displacing); and (iii) analogues of nucleic acids (PNA, LNA®, ZNA™, non-natural bases: Plexor™ primer, Tiny-Molecular Beacon). In addition, structures, mechanisms of action, advantages and applications of such real-time PCR probes and analogues are depicted in this review.

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http://dx.doi.org/10.1016/j.cca.2014.10.017
0009-8981/© 2014 Published by Elsevier B.V.

1. Introduction

Higuchi et al. [1,2] pioneered the analysis of Polymerase Chain Reaction (PCR) kinetics by constructing a system that detected amplification products as they accumulated. This "real-time" system included intercalating ethidium bromide in each amplification reaction, an adapted thermal cycler to irradiate the samples with ultraviolet light and detection of the resulting fluorescence with a computer-controlled cooled CCD camera. The increase in fluorescence was due to the intercalation of ethidium bromide into the increasing amounts of double-stranded DNA (dsDNA) produced during each amplification cycle. By plotting this fluorescence increment versus cycle number, the system produced a graph that provided a more complete picture of the PCR process than analyzing the accumulation of products by electrophoresis after the reaction.

Very quickly, this technology matured into a competitive market, becoming commercially widespread and scientifically influential. This is evidenced by the large number of companies offering real-time PCR instrumentation as well as the rapid growth rate of scientific publications pertaining to quantitative real-time PCR (qPCR). Such instrumentation was first made available by Applied Biosystems in 1996 [3]. At present, Applied Biosystems and other companies such as BioGene, Bioneer, Bio-Rad, Cepheid, Corbett Research, Idaho Technology, MJ Research, Roche Applied Science, and Stratagene all offer devices for qPCR [4–6].

The deployment of this interesting methodology is growing exponentially in many molecular biology and clinical laboratories and, hence, it is replacing conventional PCR. The main advantage of qPCR over the traditional PCR assays is that the starting DNA concentration is determined with accuracy and high sensitivity. Thus, the obtained results can be either qualitative (showing the presence or absence of the DNA sequence of interest) or quantitative. In contrast, conventional PCR is, at best, semiquantitative. Moreover, the amplification reactions are run and data are analyzed in a closed-tube system, eliminating the need for post-amplification manipulation and therefore reducing opportunities for contamination [7–9]. Real-time PCR technology has proven its versatility and usefulness in different research areas including biomedicine, microbiology, veterinary science, agriculture, pharmacology, biotechnology and toxicology. It also offers interesting new applications, such as for the quantification and genotyping of pathogens, gene expression, methylated DNA and microRNA analysis, validation of microarray data, allelic discrimination and genotyping (detection of mutations, analysis of SNPs and microsatellites, identification of chromosomal alterations), validation of drug therapy efficacy, forensic studies and quantification of genetically modified organisms (GMOs).

Basically, the qPCR instrument consists of a thermal cycler with an integrated excitation light source (a lamp, a laser or LED: light emitting diode), a fluorescence detection system or fluorimeter and software that displays the recorded fluorescence data as a DNA amplification curve, it being necessary to add a dsDNA intercalating dye or fluorophore-labeled probe to the reaction mixture.

In the last 15 years, a large number of methods for DNA detection in qPCR have been described. This review offers a useful classification as well as a detailed description of such detection methods. They have been classified into two principal groups based on the fluorescent agent used and the specificity of the PCR detection. The first group uses dsDNA intercalating agents such as SYBRGreen I and EvaGreen, leading to the detection of both specific and non-specific amplification products. On the other hand, the other group employs fluorophores attached to oligonucleotides and only detects specific PCR products. It has been further divided into three subgroups according to the type of fluorescent molecules added to the reaction: (i) probes acting as primers, called primer-probes; (ii) hydrolysis probes emitting fluorescent light upon degradation during the extension phase, and hybridization probes that give a fluorescent signal when binding to the DNA target during the amplification reaction; and (iii) analogues of nucleic acids. In addition to their structures and mechanisms of action, advantages and applications of each DNA detection method are described in this review.

2. PCR chemistries for the detection and quantitation of nucleic acids

There are two main proceedings of DNA analysis in qPCR: methods enabling both specific and non-specific detection of amplified products using dsDNA binding dyes, and those that only detect specific PCR products via employing fluorophore-linked oligonucleotides (primer-probes or probes). Table 1 summarizes the structures, mechanisms of action and advantages of the different fluorescent molecules used in qPCR.

2.1. DNA binding dyes

There is a wide variety of commercially available fluorescent DNA dyes, including ethidium bromide [1], YO-PRO-1 [10,11], SYBR® Green I [12], SYBR® Gold [13], SYTO [14,15], BEBO and BOXTO [16], and EvaGreen [17]. The use of DNA binding dyes allows the detection of specific products, nonspecific products and primer-dimers produced during the qPCR reaction.

2.1.1. Structure

The most commonly used is SYBR® Green I [18], an asymmetrical cyanine dye (2-[N-(3-dimethylaminopropyl)-N-propylamino]-4-[2,3-dihydro-3-methyl-(benzo-1,3-thiazol-2-yl)-methylidene]-1-phenylquinolinium) with two positive charges under standard PCR reaction conditions contributing to its high dsDNA binding affinity [18–21]. The resulting DNA-dye complex absorbs blue light (λ_{max} = 497 nm) and emits green light (λ_{max} = 520 nm). More recently, several authors have described that EvaGreen and certain SYTO dyes (−9,−13 and −82) are more stable and sensitive than SYBR Green I for DNA quantification by qPCR [14,15,17,22,23].

2.1.2. Mechanism of action

When such a dye binds to the minor groove of dsDNA, its fluorescence is increased and can be measured in the extension phase of each cycle of qPCR [19]. Given that nonspecific products and primer-dimers can be formed during the PCR process [24], a melting curve analysis is highly recommended to check the specificity of the amplified fragments. This analysis consists of applying heat to the sample (from 50 °C to 95 °C) and monitoring the fluorescence emission during the process. The temperature of DNA denaturation is shown as a sharp drop in the fluorescence signal due to dissociation of the dye. Nonspecific products and primer-dimers are denatured at lower temperatures than the specific products [12]. In fact, PCR products of different length and/or nucleotide content show distinct peaks when the derivative of fluorescence is plotted with respect to temperature (−dT/dF), due to the fact that they are denatured at different temperatures.

2.1.3. Advantages

The costs of employing DNA binding dyes in qPCR are much lower than those of methods requiring fluorescent probes. However, a melting curve analysis is necessary after the completion of each qPCR assay for selective detection of amplicons of multiplex PCRs. The most commonly used is SYBR® Green I but, despite its popularity, it presents some limitations, including limited dye stability and dye-dependent PCR inhibition [14,22]. EvaGreen is a third generation dsDNA binding dye that offers several advantages such as being less inhibitory to PCR than SYBR® Green I, and it can be used under saturating conditions to generate greater fluorescent signals. EvaGreen is also well suited for HRM [high resolution melt] analysis [22].

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2.1.4. Applications

These dyes can be used to detect either single or two or more different DNA sequences in a single PCR reaction (multiplex assays). SYBR® Green I is mainly employed for pathogen detection [25], gene expression [26], mutation detection, SNP detection and GMO (genetically modified organismos) detection [27]. Furthermore, EvaGreen is being used for pathogen detection [28,29], gene expression [26], mutation detection [30], genotyping [31], SNP detection [32,33] and GMO detection [34].

2.2. Fluorophore-labeled oligonucleotides

Fluorophores are small fluorescent molecules that are attached to oligonucleotides in order to function as probes in qPCR technology. These fluorescent oligonucleotides are classified as either: (i) primer-probes, (ii) probes or (iii) analogues of nucleic acids.

There are two types of fluorophores: donor or reporter and acceptor or quencher. When a donor fluorophore absorbs energy from light, it rises to an excited state. The process of returning to the ground state is driven by the emission of energy as fluorescence. This emitted light from the donor has a lower energy and frequency and a longer wavelength than the absorbed light and can be transferred to an acceptor fluorophore. If both fluorophores are within a specific distance, usually 10 to 100 Å [35–37], the transfer of excited-state energy from a reporter to a quencher is denoted as Fluorescence Resonance Energy Transfer (FRET) [38,39]. There are two different FRET mechanisms, based on how the energy transferred to the acceptor fluorophore is dissipated; (i) FRET-quenching [40] in which the electronic energy of the quencher (a non-fluorescent molecule) is dissipated as heat, and (ii) FRET in which the transferred energy is emitted as fluorescence because the acceptor molecule is fluorescent.

At present, there are a wide variety of donors and acceptors with different excitation and emission spectra that can be used in qPCR [5].

2.2.1. Primer-probes

Primer-probes are oligonucleotides that combine a primer and probe in a single molecule. They can be classified into three groups: Harpins, Cyclicons and Angler® primer-probes. Fluorescence emitted from primer-probes is detected and measured during the denaturation or extension phase of the qPCR, depending on the type of primer-probe used. The use of these primer-probes can lead to amplification of unspecific products or dimer-primers during the PCR reaction; therefore, melting curve analysis to determine the efficiency of the reaction is recommended.

2.2.1.1. Hairpin primer-probes. Hairpin primer-probes are single-stranded (ss) oligonucleotides that contain: (i) a hairpin secondary structure, in which the loop of the structure specifically binds to the target DNA [7]; (ii) a short tail sequence of 6 nucleotides (CG) at the 5′-end of the probe complementary to the 3′-end region; (iii) one or two fluorophores attached at the ends [41]; and (iv) in some cases, the probe also contains a primer linked to the hairpin structure. Hairpin primer-probes include Scorpions, Amplifluor® and LUX™.

2.2.1.1.1. Scorpion primer-probes

2.2.1.1.1.1. Structure. Described in 1999 by Whitcombe et al. [42], the hairpin structure has a reporter at the 5′-end and an internal quencher at the 3′-end. The 3′-end of the hairpin is attached to the 5′-end of the primer by a HEG (hexathylene glycol) blocker, which prevents primer extension by the polymerase [42] (Fig. 1A).

2.2.1.1.1.2. Mechanism of action. In solution, the reporter and quencher are in close proximity and energy transfer via FRET-quenching is produced. After binding of the primer-probe to the target DNA, the polymerase copies the sequence of nucleotides from the 3′-end of the primer. In the next denaturation step, the specific sequence of the probe binds to the complementary region within the same strand of newly amplified DNA. This hybridization opens the hairpin structure and, as a result, the reporter is separated from the quencher leading to a fluorescent signal proportional to the amount of amplified PCR product [42].

2.2.1.1.1.3. Advantages. The primer-probe combines the binding and detection mechanisms in the same molecule, making it an inexpensive system. Oligonucleotides with hairpin structures prevent the formation of primer-dimers and non-specific PCR amplification products [43] because the intramolecular binding of such structures is kinetically favorable and highly effective. The use of stems offers additional benefits, such as minimal background signals as the unincorporated primer-probes are switched off [42]. Furthermore, in this system enzymatic breakdown of the primer-probe is not necessary and the fluorescent signals are stronger than those produced when other probes are used [44,45].

2.2.1.1.1.4. Applications. Scorpion primer-probes can be used in single and multiplex formats for pathogen detection [46], viral/bacterial load quantitation, genotyping, SNP allelic discrimination [47,48] and mutation detection [49,50]. It is important to note that the addition of a nucleic acid analogue (LNA) to a Scorpion primer-probe containing reaction is recommended in order to obtain greater accuracy in SNP detection and allele discrimination, given that the thermal stability and hybridization specificity of such probes are increased.

2.2.1.1.2. Amplifluor™ primer-probes

2.2.1.1.2.1. Structure. Described by Nazarenko et al. [41]. This system was later reported as the Sunrise system [51] and commercialized under the name Amplifluor™ by Oncor/Intergen (Gaithersburg, MD; USA) [52]. The reporter is located at the 5′-end and the internal quencher is linked at the 3′-end of the hairpin. The 3′-end acts as a PCR primer [41] (Fig. 1B).

2.2.1.1.2.2. Mechanism of action. It is similar to that described for Scorpion primer-probes. When the primer-probe is not bound, the hairpin structure is intact and the reporter transfers energy to the quencher via FRET-quenching. DNA amplification occurs after binding of the primer-probe to the target sequence. In the next step of denaturation, reporter and quencher are separated and, as a result, the emitted fluorescence of the donor is measured by the fluorimeter [41,53].

2.2.1.1.2.3. Advantages. These probes display the same advantages as those described previously for Scorpion primer-probes.

2.2.1.1.2.4. Applications. Amplifluor™ primer-probes can be used in single and multiplex formats for pathogen detection, viral/bacterial load quantitation [54], genotyping, allelic discrimination, mutation detection, SNP detection [55] and GMO detection [27].

2.2.1.1.3. LUX™ primer-probes

2.2.1.1.3.1. Structure. LUX™ (Light-Upon-eXtension) primer-probes were first described by Nazarenko et al. [43]. The 3′-end acts as a primer and contains a single reporter located in the guanosine rich region of the primary sequence [56]. Unlike Scorpion and Amplifluor primer-probes, they do not require the presence of an internal quencher [43] (Fig. 1C).

2.2.1.1.3.2. Mechanism of action. The hairpin structure confers the ability to decrease the fluorescence signal when the primer-probe is free and increases the signal exponentially when it binds to its target sequence. The maximum fluorescence emission is generated after the incorporation of LUX™ primer-probes into dsDNA [43] (Fig. 1C). Fluorescence is measured during the extension phase.

2.2.1.1.3.3. Advantages. The advantages of this system are similar to those methods that rely on Scorpions and Amplifluor primer-probes. The employment of these primer-probes offers high sensitivity and specificity despite their containing only a single fluorescent molecule [57].

2.2.1.1.3.4. Applications. They can be used in single and multiplex formats for pathogen detection [58,59], viral/bacterial load quantitation [57], genotyping, allelic discrimination, mutation detection, SNP detection [43] and gene expression analysis [56] and GMO detection [27].

2.2.1.2. Cyclicon primer-probes

2.2.1.2.1. Structure. Described by Kandimalla and Agrawal in 2000 [60], cyclicons contain a long primer-probe (complementary to the

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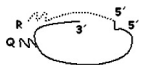
Table 1
Detection systems for DNA amplified in qPCR.

	Structure	Mechanism of action	Advantages
Detection of specific and non-specific PCR products			
Ethidium bromide [1], **YO-PRO-1** [10,11], **SYBR® Green I** [12], **SYBR® Gold** [13], **SYTO** [14,15], **BEBO and BOXTO** [16], **and EvaGreen** [17]	**Intercalating dye**	Its binding to the minor groove of the amplified DNA sequences leads to fluorescence emission. Extension phase[a]	The costs of its employ are much lower than that of probes, but melting curve analysis is necessary to check the specificity of amplified fragments.
Detection of specific PCR products			
Primer-probes			
Hairpin primer-probes			
Scorpions [42]	**5′RΩ3′Q_HEG_Primer**	After probe binding to target, DNA polymerase copies the target sequence. In the next denaturation step, the specific sequence of the probe binds to the complementary region within the same strand of newly amplified DNA, leading to fluorescence emission. Denaturation phase[a]	This technology combines the primer and the probe in the same molecule, making it a cheap system. Its use prevents the formation of primer-dimers and non-specific PCR products. In solution, minimal fluorescence background is registered as primer-probes are switched off in solution.
Amplifluor® or Sunrise [41]	**5′RΩ 3′Q _Primer**		
LUX™ [43]	**5′ΩR3′**	Its incorporation into dsDNA leads to fluorescence emission. Extension phase[a]	This system allows melting curve analysis to be performed. Its use offers high sensitivity and specificity without using a fluorescent acceptor molecule.
Cyclicons [60]	 ••• Modifier oligo – Primer-probe	When it binds to the complementary sequence, the cyclic structure is opened up and the fluorophores are separated far enough to disrupt FRET-quenching, resulting in fluorescence emission. Extension phase[a]	This system allows short oligonucleotides to be employed, reducing the costs of qPCR assays.
Angler® [62]	**5′Cy5_HEG_3′Rvprimer** Acceptor moiety (Cy5)	SYBR® Gold DNA intercalating dye is employed as the donor fluorescent moiety. When the probe binds to its target sequence, the SYBR®Gold intercalates in the newly amplified DNA and emits fluorescence, behaving as a donor moiety in a FRET pair with the acceptor moiety of the probe. Denaturation phase[a]	This system allows melting curve analysis to be performed. Its use in qPCR assays leads to the detection of both non-specific (SYBR®Gold) and specific (Angler® primer-probe) amplification products.
Probes			
Hydrolysis probes			
TaqMan [44]	**5′R—Q3′**	In solution, the fluorescent signal is quenched due to the fact that the two fluorophores of the probe are in close proximity. In the extension phase, the bound hydrolysis probe is degraded by the 5′-3′-exonuclease activity of DNA polymerase, generating fluorescence from the reporter. Extension phase[a]	Easy design and synthesis of the probe.
TaqMan-MGB [88]	**5′R—Q3′-MGB**		Easy design and synthesis of the probe. The presence of MGB increases the DNA specificity of the probe, allowing the use of short oligonucleotides
Snake assay [72]	**Forward Snake primer** 5′ Flap sequence———3′ **+ Hydrolysis probe**	In this system, target amplification and the detection of fluorescence are two processes separated in time and space. The hydrolysis of the probe leads to fluorescence emission. The Snake assay needs an asymmetric PCR format (reverse > forward primer), since one of the strands is preferentially amplified (sense amplicon). Extension phase[a]	Specialized software is required for designing the 5′-flap sequences of the Snake primers. This system favors the use of short probes, which reduces fluorescence background. The cost-effectiveness ratio of this type of assay is lower than that of TaqMan systems.

Hybridization probes				
Hyprobe o FRET [75,77]	**5′—R3′ + 5′Q—3′-Ph**	During hybridization, the binding of the probe to the target sequence brings the fluorophores into close proximity, producing energy transfer by FRET. Annealing phase[a]	This system allows melting curve analysis to be performed. The designing and synthesis of the probes, as well as, the optimization of PCR reaction is quick and easy.	
Molecular Beacon probes [45]	**5′RΩQ3′**	During the annealing phase, the Beacon probe unfolds and binds to the target DNA sequence, leading to fluorescence emission. Annealing phase[a]	The system allows performing a melting curve analysis. The binding specificity of these probes is higher than that of hairpin probes. Their use allows discriminating between sequences which differ in a single nucleotide.	Q3 Q4
HyBeacon™ [84]	**5′—FlUb————3′Ph**	The amount of fluorescence emitted from hybridized HyBeacons is considerably greater than that of ss-probes, permitting the detection of target sequences. Extension phase[a]	This system allows performing a melting curve analysis.	Q5
MGB Probes				
MGB-Pleiades [89] *MGB-Eclipse* [90]	**MGB-5′R—Q3′** **MGB-5′Q—R3′**	The probe is straightened out when binding to the target, leading to fluorescence emission. Annealing phase[a]	This system allows melting curve analysis to be performed. The MGB-probe forms a highly stable duplex, increasing the DNA specificity of the probe. The presence of a non-fluorescent quencher (NFQ) greatly reduces background fluorescence.	
ResonSense® [62]	**5′Cy5.5———3′Ph** Acceptor moiety (Cy5.5)	SYBR® Gold DNA intercalating dye is employed as the donor fluorescent moiety. During the PCR reaction, the binding of the probe to the target and the simultaneous intercalation of SYBR®Gold results in energy transfer by FRET. Annealing phase[a]	This system allows melting curve analysis to be performed. Its use in qPCR assay is cost effective.	
Yin-Yang [107]	**5′R——————————3′ Ph** **3′Q————5′**	During the annealing phase, the shorter strand is displaced by the target, leading to fluorescence emission. Annealing phase[a]	This system allows melting curve analysis to be performed. Its binding to the target is highly specific and the design is much easier than that of dual-dye labeled probes.	
Analogues of nucleic acids				
PNAs [113]	**PNA + either intercalating dye, primer-probes or probes**	The mechanism of action of primer-probes or probes in which PNA/LNA have been introduced is identical to that of the conventional oligonucleotides.	PNA containing probes are more resistant to nucleases and proteases and can interact with DNA at lower salt concentrations than standard probes/primer-probes.	
LNAs [142]	**LNA are inserted in primer-probes or probes**		LNA containing probes are resistant to degradation by nucleases. LNA molecules increase the DNA specificity of the probe.	
ZNAs [119,120]	**ZNA™ are inserted in primer-probes or probes**	ZNAs are cationic moieties and are able to increase the affinity of primers or probes for their targets by decreasing the electrostatic repulsion between the two nucleic acids. Hybridization occurs when the ZNA™ oligonucleotide meets its complementary sequence.	ZNA containing oligonucleotides exhibit an exceptionally high affinity for their target DNA sequences.	
Plexor primers [121]	Only one primer labeled **5′R-idCTP————————3′** Add into reaction mix: **Q-idGTP**	Fluorescence emission is produced when they are in solution. The incorporation of Iso-dG into DNA brings the quencher and reporter into close proximity, producing quenching of the initial fluorescent signal. Extension phase[a]	This system allows melting curve analysis to be performed. These primers are easy to design.	
Tiny-Molecular Beacon probes [167]	Molecular Beacon probe synthesized from **2′-O-methyl RNA/LNA chimeric** nucleic acids	They display the same mode of action as Molecular Beacon probes. Annealing phase[a]	These probes are very resistant to nucleases, remain stable within a cellular environment and have a high affinity and specificity for RNA sequences.	

ds: double stranded; **5′**: 5′end; **Ω:** Hairpin probe; **R:** reporter; **3′:** 3′-end; **Q:** quencher; **HEG:** HEG (hexathylene glycol) blocker PCR; **LUX:** Light Upon Extension; **FRET**: Fluorescence Resonance Energy Transfer; **Cy5:** Acceptor moiety (Cy5); **MGB:** Minor Groove Binders; **Tm:** melting temperature; **FlUb:** fluorophore-labeled uracil base; **Ph:** phosphate group; **Cy5.5:** Acceptor moiety (Cy5.5); **PNAs:** Peptide Nucleic Acids; **ss:** single stranded; **NAs:** Nucleic Acids; **LNAs:** Locked Nucleic Acids; **ZNAs:** Zip Nucleic Acids; **idCTP:** non-natural nucleotide; **idGTP:** non-natural nucleotide; **MB:** Molecular Beacon.

[a] qPCR phase in which the fluorescence is measured.

target DNA sequence) and a short modified oligo attached through 5′–5′ ends, which binds to six-eight nucleotides at the 3′-end of the primer-probe forming a cyclic structure with two 3′-ends [61] (Fig. 1D). Cyclicons have a reporter at the free 3′-end of the modified oligo and a quencher placed on a thymine base at the 5′-position in the primer-probe sequence [60].

2.2.1.2.2. Mechanism of action. In the absence of the target sequence, reporter and quencher molecules are in close proximity and energy transfer occurs via FRET-quenching (Fig. 1D). The binding of Cyclicon probes to DNA opens up the cyclic structure and leads to extension of the 3′-end primer-probe by DNA polymerase without any interference from the quencher. The 3′-end of the modified oligo is not extendible since it does not bind to the target DNA and because its 3′-end is blocked by a reporter. The separation between donor and acceptor molecules results in emission of fluorescence, which is measured during the extension phase [60].

2.2.1.2.3. Advantages. The integrated primer-probe structure of cyclicons is an important benefit for DNA detection in qPCR systems. It allows the use of shorter oligonucleotides, reducing the costs of the assay, simplifies the reaction set up and avoids unnecessary carry-over contaminations (Kandimalla and Agrawal, 2000) [60]. On the other hand, the linkage between the long primer-probe and the short oligo can also be through their 3′–3′-ends. In this case, Cyclicons would function as probes similar to TaqMan probes [44] and Molecular Beacons [45] (see Section 2.2.2 Probes). Interestingly, it has been reported that Cyclicons with a 5′–5′-attached structure give less fluorescence background in reactions with polymerases devoid of nuclease activity [60].

2.2.1.2.4. Applications. They can be used in single and multiplex qPCR for pathogen detection, viral/bacterial load quantitation, genotyping, allelic discrimination, mutation detection and SNP detection. Cyclicons can also be directly fixed to solid supports on chips for high-throughput screening in solid-phase PCR [60].

2.2.1.3. Angler® primer-probes

2.2.1.3.1. Structure. Described in 2002 by Lee et al. [62]. The probe component is a DNA sequence identical to that of the target, which is bound to a reverse primer through a hex-ethylene glycol (HEG) linker [63]. It has an acceptor fluorescent moiety at its 5′-end. SYBR® Gold

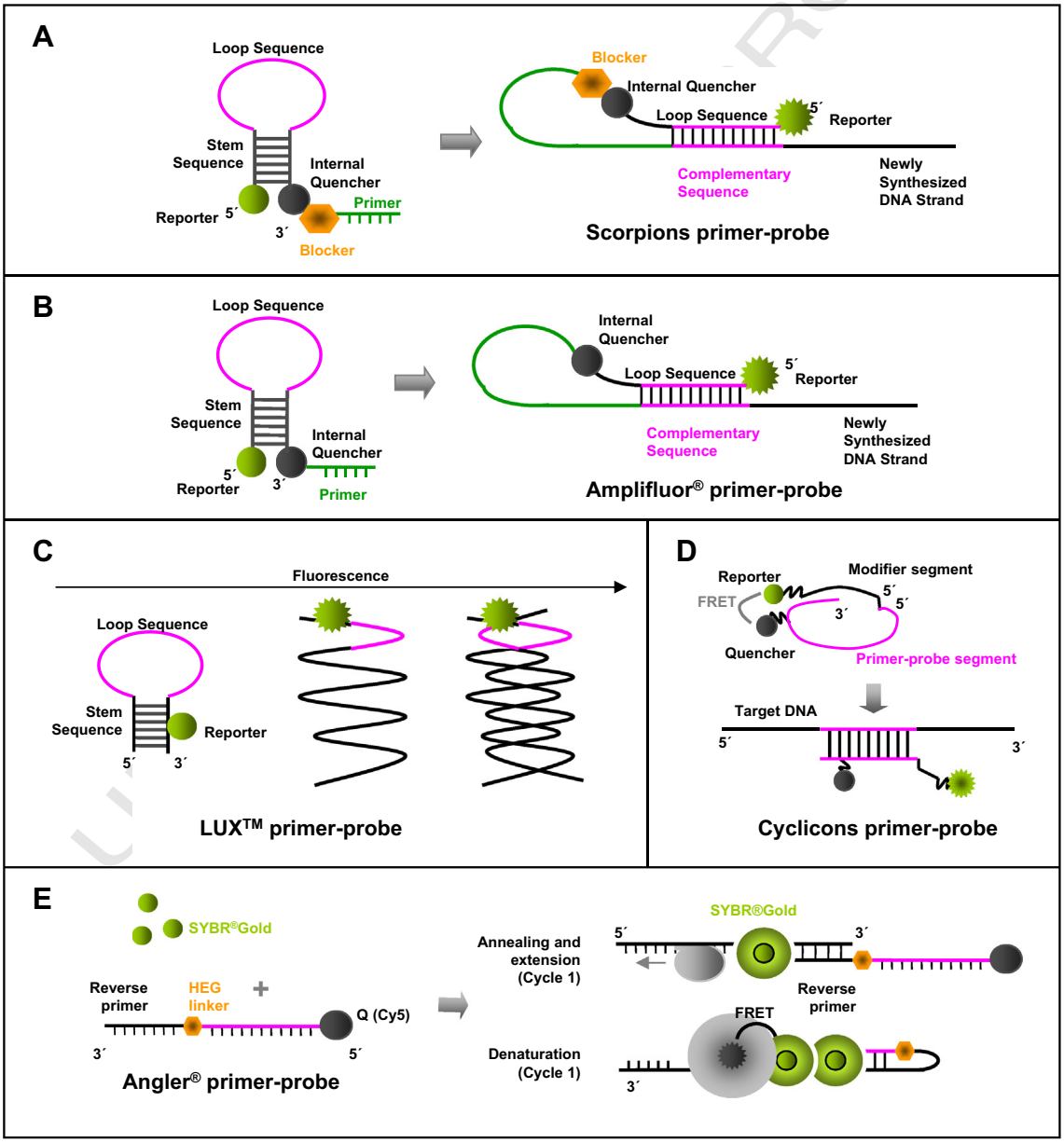


Fig. 1. Structure and mechanism of action of primer-probes. (A) Scorpion; (B) Amplifluor®; (C) LUX™ and (D) Cyclicon; (E) Angler®.

DNA intercalating dye is employed in the assay as the donor fluorescent moiety [62] (Fig. 1E).

2.2.1.3.2. Mechanism of action. In solution, the primer-probe does not emit fluorescence since there is no donor fluorescent moiety close enough for FRET. When the Angler® primer-probe binds to its target DNA during the annealing step, DNA polymerase starts the extension of the 3′-end reverse primer. Subsequently, during the denaturation phase, the specific sequence of the probe binds to the complementary region of newly amplified DNA, producing a dsDNA fragment in which SYBR®Gold dye can be intercalated to generate fluorescence [39,64,65]. Hence, the emitted fluorescence is measured during the denaturation step in each cycle.

2.2.1.3.3. Advantages. The combination of a dsDNA intercalating agent and a primer-probe in qPCR allows non-specific (SYBR® Gold) and specific (Angler® primer-probe) amplified products to be distinguished without performing melting curves. In the PCR instrument, the 520 channel of the optical detector is used to detect the fluorescent signal from non-specific intercalation of the SYBR® Gold dye while the 705 nm channel recognizes the signal generated by the specific binding of the Angler® primer-probe. This faster system offers better cost effectiveness than other methods [62].

2.2.1.3.4. Applications. They can be used in single or multiplex formats for rapid detection of DNA, in studies of gene expression, allelic discrimination, genotyping, SNP detection, identification and quantitation of infectious organisms, and screening of environmental and biological samples.

2.2.2. Probes

The probes are oligonucleotides with an attached-donor and/or -acceptor fluorophore. There are two types: hydrolysis and hybridization probes.

2.2.2.1. Hydrolysis probes. Their mechanism of action relies on the 5′–3′ exonuclease activity of Taq polymerase, which degrades the bound probe during amplification. This also prevents performing a melting curve analysis. In this system, the fluorescence is measured at the end of the extension phase and is proportional to the amount of amplified specific product [66].

2.2.2.1.1. TaqMan probes

2.2.2.1.1.1. Structure. Described in 1991 by Holland et al. [44]. These probes are oligonucleotides containing a donor fluorescent moiety at the 5′-end and an acceptor fluorescent moiety at the 3′-end that quenches the fluorescence emitted from the donor molecule due to their close proximity [67]. The hydrolysis probe is designed to bind to a specific region of the target DNA [44] (Fig. 2A).

2.2.2.1.1.2. Mechanism of action. In solution, the fluorescent signal from the donor fluorophore is suppressed by the acceptor fluorophore, although a residual fluorescence can be detected [68]. During the extension phase, the bound hydrolysis probe is degraded by the 5′–3′-exonuclease activity of DNA polymerase, generating fluorescence from the donor [67,69]. This process is repeated in each cycle without interfering with the exponential synthesis of the PCR products [67].

2.2.2.1.1.3. Advantages. The design and synthesis of TaqMan probes are easy but if they are not well designed, primer-dimers might be formed during qPCR assay.

2.2.2.1.1.4. Applications. They can be used in single and multiplex formats for virus detection [70], viral/bacterial load quantitation, gene expression, microarray validation, allelic discrimination, mutation detection [71], SNP detection and GMO detection [27].

2.2.2.1.2. MGB-TaqMan probes. Minor Groove Binding-TaqMan probes are described in: MGB-conjugated DNA probes (see Section 2.2.2.2.4).

2.2.2.1.3. Snake assays

2.2.2.1.3.1. Structure. This assay, described in 2010 by Kutyavin et al. [72], combines Snake primers and TaqMan probes in order to amplify DNA regions with a high percentage of secondary structures [72] (Fig. 2B).

2.2.2.1.3.2. Mechanism of action. Given that snake assays employ hydrolysis probes, the fluorescence signals are generated by the 5′-nuclease activity of DNA polymerase over the probe [73]. In this system, target DNA amplification and detection of fluorescence are two processes separated in time and space [72]. Fig. 2B illustrates the mechanism of Snake systems [72]. A forward primer containing a 5′-flap sequence binds to the target DNA sequence site located downstream from the primer binding site. Extension of this primer results in the synthesis of an antisense strand, which provides a double stranded amplicon (stage A). After strand separation (95 °C), a reverse primer hybridizes to the antisense strand and DNA polymerase extends the complex (stage B), resulting in another double stranded amplicon. Since the 5′-flap of the forward primer functions as a template for DNA synthesis, a complementary sequence appears at the 3′-end of the sense amplicon strand (stage C, linear form). After another round of strand separation, the sense amplicon (synthesized in stage B) folds into a secondary structure in which the 3′-terminal nucleotide remains mismatched (stage C, folded form). The hydrolysis probe binds to the sense strand of the amplicon creating an optimal cleavage structure for 5′-nuclease (stage D). Then, this structure is subsequently cleaved in stage E, releasing a detectable fluorescent signal, stage E [72].

The Snake assay needs an asymmetric PCR format (reverse > forward primer) because one DNA strand is preferentially amplified (sense amplicon) [72,73].

Fig. 2B also shows an alternative pathway which could be taken by the sense amplicon during the PCR assay [72]. Briefly, there is a small fraction of linear form (C) amplicon that might be accessible to a forward 5′-flap primer (stage F). In this context, the strand DNA replication would be accomplished through a passive hybridization (pathway C → F). In addition, the active hybridization of the forward Snake primer in stage G might be followed by a strand displacement in stage H, which substantially accelerates the replication process [73].

2.2.2.1.3.3. Advantages. The Snake assay favors the use of short probes with reduced fluorescence background [72]. Thus, the cost-effectiveness ratio of such assays is less than that of TaqMan systems. However, specialized software is required for the primer design since the length and base composition of the 5′-flap sequences in Snake primers determine the stability of the secondary structures in the folded PCR amplicons [73].

2.2.2.1.3.4. Applications. The assays can be used in single and multiplex formats for pathogen detection, viral/bacterial load quantitation, gene expression, microarray validation, allelic discrimination, mutation detection and SNP detection [72].

2.2.2.2. Hybridization probes. The fluorescence emitted by binding hybridization probes can be measured either during the annealing or the extension phase. The use of these probes allows amplified fragments to be analyzed by performing melting curves, this being the main advantage over hydrolysis probes. The amount of fluorescent signal detected is directly proportional to the amount of the target amplified during the qPCR reaction [66].

2.2.2.2.1. Hybprobes or FRET probes

2.2.2.2.1.1. Structure. Hybprobes, also known as FRET probes, were first described in 1985 by Heller and Morrison [74]. This system consists of a pair of oligonucleotides binding to adjacent target DNA sequences [75]. The first probe carries a reporter fluorophore at its 3′-end and the second probe contains a quencher at its 5′-end and a phosphate group attached to its 3′-end to prevent DNA amplification [39,75] (Fig. 3A).

2.2.2.2.1.2. Mechanism of action. The sequences of the probes are designed to hybridize to the target DNA sequences in a head-to-tail orientation so that the two fluorophores are in close proximity [76,77]. During the annealing phase, in which the probes are adjacently bound, the quencher emits fluorescence due to the fact that it has been previously excited by the energy released from the reporter [78] (Fig. 3A).

2.2.2.2.1.3. Advantages. It has been reported that the design and synthesis of these probes as well as the optimization of the PCR reaction conditions

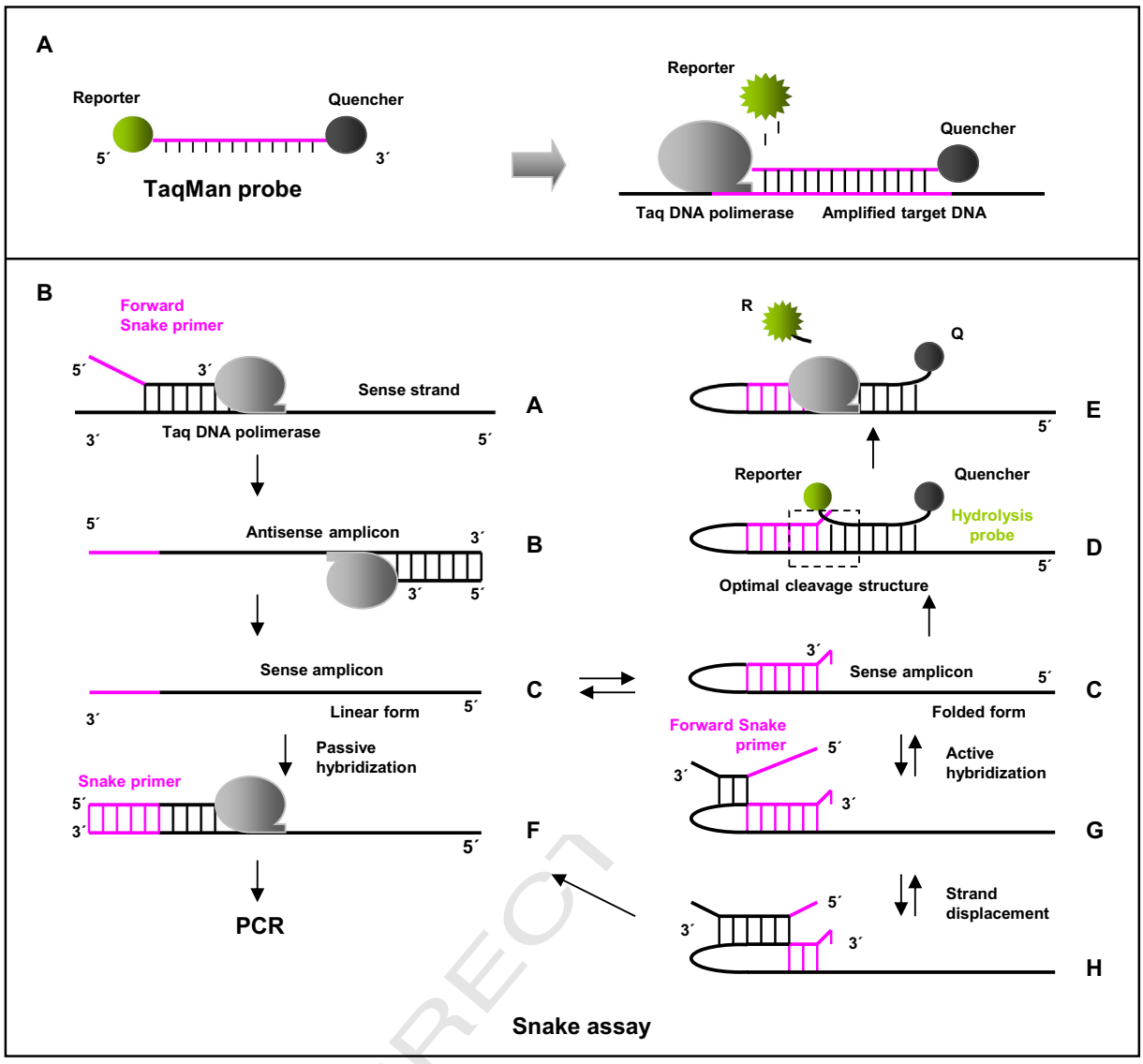


Fig. 2. Structure and mechanism of action of hydrolysis probes. TaqMan probe (A) and scheme of Snake system (B).

is quick and easy (http://www.fluoresentric.com/documents/HybProbe.pdf).

2.2.2.2.1.4. Applications. They can be used in the multiplex format for pathogen detection [79,80], viral/bacterial load quantitation, microarray validation, genotyping [81], allelic discrimination, mutation detection and SNP detection.

2.2.2.2.2. Hairpin probes: Molecular Beacon Probes

2.2.2.2.2.1. Structure. Molecular beacons were first described by Tyagi and Kramer [45]. They are single stranded hairpin shaped oligonucleotide probes divided into four parts: (i) a loop, a fragment of 18–30 bp complementary to the target DNA sequence; (ii) a stem, which is formed by two complementary sequences of 5–7 bp located at each end of the probe; (iii) a fluorescent reporter attached to the 5′-end and (iv) a non-fluorescent quencher attached to the 3′-end, which absorbs the emitted fluorescence from the reporter when the Molecular Beacon probe is in closed form [82] (Fig. 3B).

2.2.2.2.2.2. Mechanism of action. During the annealing phase, this probe unfolds and binds to the target, emitting fluorescence since the reporter is not quenched any longer. This fluorescent signal is proportional to the amount of amplified PCR product. If the Molecular Beacon probe and target DNA sequences are not perfectly complementary, there will be no emission of fluorescence because the hairpin structure prevails over the hybridization [82].

2.2.2.2.2.3. Advantages. The binding specificity of Molecular Beacon probes is higher than that of fluorescent oligonucleotides because they are able to form a hairpin stem. Hence, the use of such probes allows discrimination between target DNA sequences which differ in a single nucleotide [82]. However, employing Molecular Beacon probes requires a thermodynamic study to ensure that the binding energy of the loop-target is more stable than that of hairpin formation.

2.2.2.2.2.4. Applications. They can be used in single and multiplex formats for pathogen detection, viral/bacterial load quantitation, genotyping, allelic discrimination, mutation detection [83], SNP detection, mRNA analysis in living cells and GMO detection [27].

2.2.2.2.3. Hybridization Beacon probes or HyBeacon™ probes

2.2.2.2.3.1. Structure. HyBeacon™ probes, described by French et al. [84], consist of ss-oligonucleotide sequences containing fluorophore moieties attached to internal nucleotides, and a 3′-end blocker (3′-phosphate or octanediol), which prevents their PCR extension [84] (Fig. 3C).

2.2.2.2.3.2. Mechanism of action. The amount of fluorescence emitted from hybridized HyBeacons when they bind to their target is considerably greater than the emission of ss-probes in solution [84]. The fluorescence is measured during the extension phase.

2.2.2.2.3.3. Advantages. This system allows melting curve analysis to be carried out to address the specificity of the amplified product and the efficiency of the reaction. Other benefits displayed by the HyBeacon

technology derive from their simple mode of action, ease of design and relatively inexpensive synthesis [84].

2.2.2.2.3.4. Applications. They can be used in single and multiplex analysis sequence for detection, DNA quantification, genotyping [85], SNP detection [86] and allelic discrimination [87].

2.2.2.2.4. MGB-conjugated DNA probes. In the last years, several types of probes including TaqMan [88], Pleiades [89] and Eclipse [90] have been attached through their 3′ or 5′ ends to Minor groove binding (MGB) ligands in order to improve target DNA-binding specificity and sensitivity.

2.2.2.2.4.1. Structure. MGB ligands are small molecule tripeptides, including dihydrocyclopyrroloindole tripeptide (DIP) or 1, 2-dihydro-(3H)-pyrrolo [3.2-e] indole-7-carboxylate (CDPI) that form a non-covalent union with the minor groove of dsDNA [91–94]. This type of ligand selectively binds to AT-rich sequences, favoring the inclusion of aromatic rings by van der Waals and electrostatic interactions. This interaction produces very minimal distortion in the phosphodiester backbone but greatly stabilizes the DNA structure [92,95]. Some features of these MGB-probes are listed in Table 2.

2.2.2.2.4.2. Mechanism of action. As shown in Fig. 4, FRET-quenching occurs when the random coiling form of the probe brings the non-fluorescent quencher and the fluorophore reporter together. The probe is straightened out when it binds to its target, causing an increase in the fluorescent signal [90].

2.2.2.2.4.3. Advantages. The highly stable interaction between the MGB-probe and the target increases the T_m of the probe [96] and prevents the amplification of non-specific products [88]. Moreover, the use of a non-fluorescent or dark quencher (NFQ) in the MGB-probe greatly reduces the background fluorescence. This method enables the use of shorter probes capable of detecting short conserved genomic sequences. In addition, post-amplification melt-curve analysis can be performed when MGB-Pleiades and MGB-Eclipse probes are used.

2.2.2.2.4.4. Applications. They can be used in single and multiplex formats for pathogen detection [97,98], viral/bacterial load quantitation [99,100], gene expression, microarray validation, allelic discrimination, mutation detection [101], SNP detection [72,102], GMO detection [27] and forensic analysis [103].

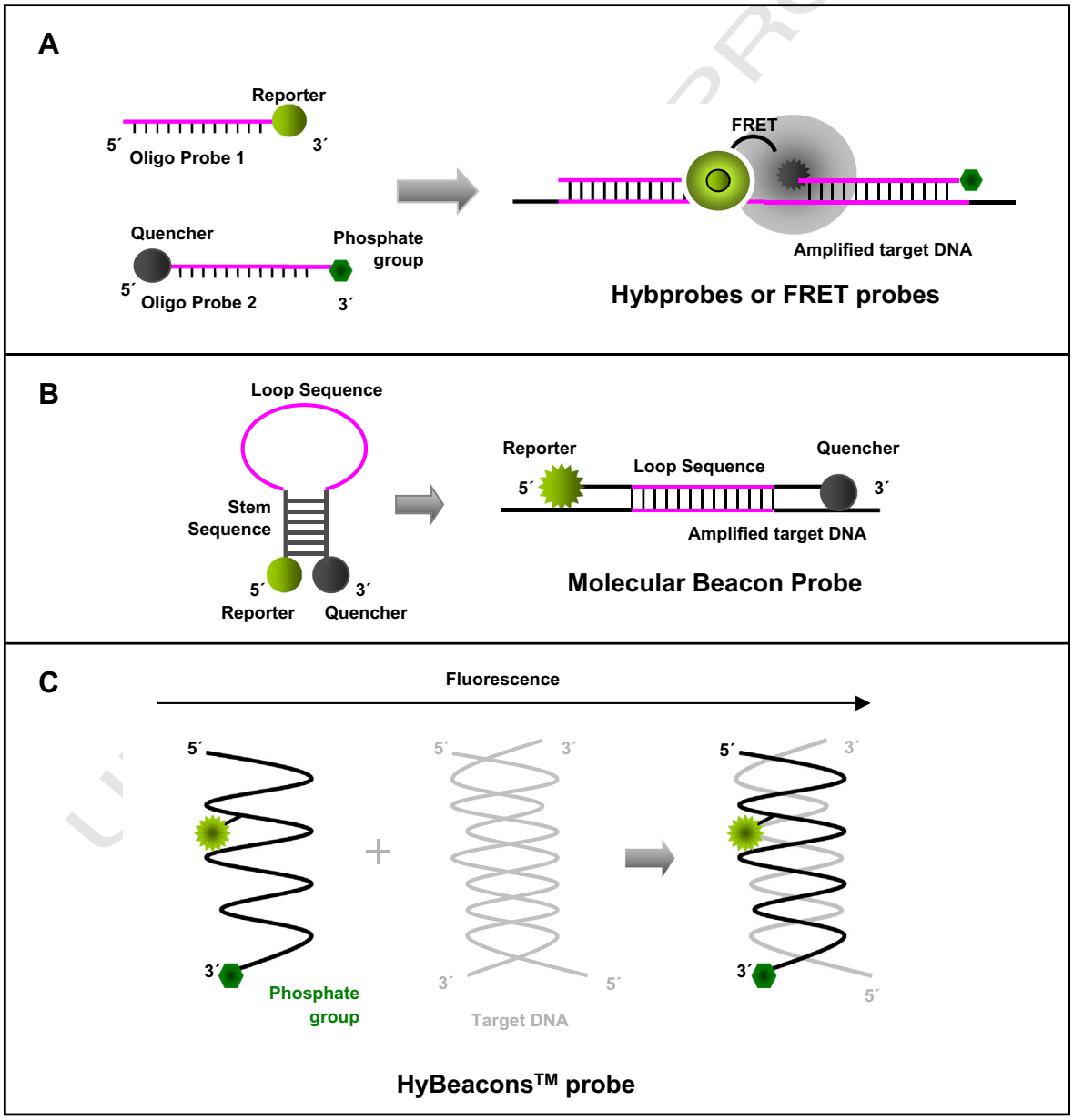


Fig. 3. Chemistry of hybridization probes. (A) Hybprobes/FRET probe; (B) Molecular Beacon Probe and (C) HyBeacons™ probe.

Table 2
MGB-probes.

MGB-probes	Reporter	NFQ[a]	MGB	Probe type	Log fluorescence
MGB-TaqMan	5′ end	3′ end	3′ end	Hydrolysis	Extension phase
MGB-Pleiades	5′ end	3′ end	5′ end	Hybridization	Annealing phase
MGB-Eclipse	3′ end	5′ end	5′ end	Hybridization	Annealing phase

MGB: Minor Groove Binding.
[a] NFQ: non-fluorescent quencher.

2.2.2.2.5. ResonSense® probes

2.2.2.2.5.1. Structure. ResonSense® probes and Angler® primer-probes have similar features. These probes, described by Lee et al. in 2002 [62], have a Cy5.5 fluorescent-Fluor at the 5′-end as an acceptor fluorescent moiety and a phosphate group at the 3′-end to prevent DNA polymeration. The real-time PCR reaction also contains the binding dye SYBR®Gold as fluorescence donor, which intercalates into the DNA duplex formed by the probe and its target [62,104] (Fig. 5A).

2.2.2.2.5.2. Mechanism of action. In solution, fluorescence is not emitted from the probe due to the absence of a fluorescent donor close enough to the acceptor. During the annealing phase, energy transfer by FRET is produced as a result of simultaneous binding of the probe to the target and intercalation of the DNA dye into the probe-target duplex [39,64,65]. The fluorescence signal is proportional to the concentration of target DNA sequences.

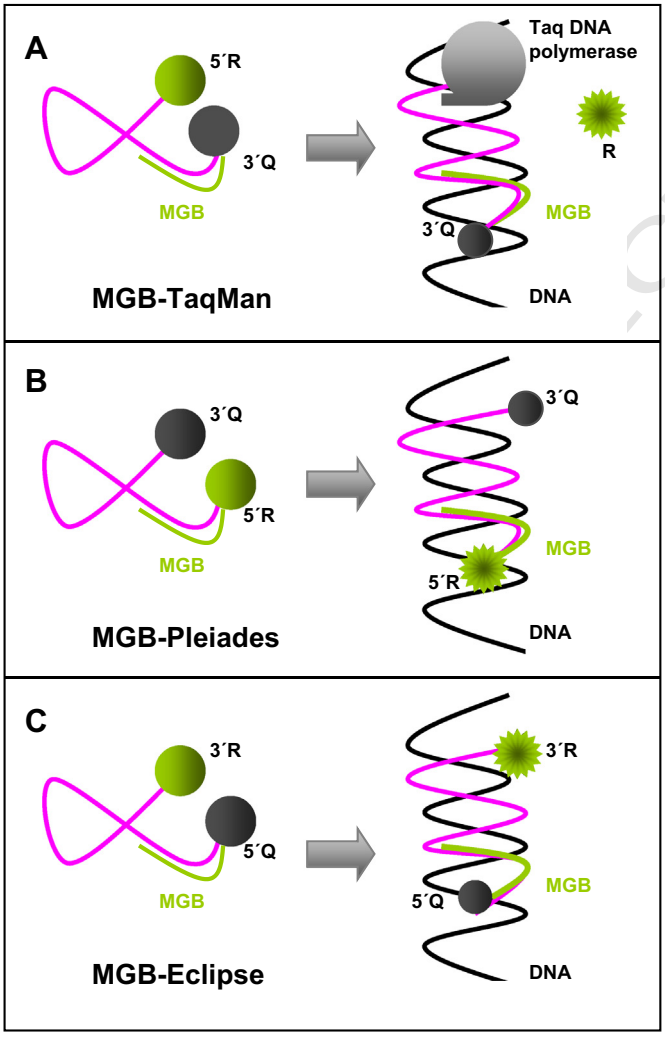


Fig. 4. Structure and mechanism of action of hybridization probes: MGB-probes. (A) MGB-TaqMan hydrolysis probe; (B) MGB-Pleiades hybridization probe and (C) MGB-Eclipse hybridization probe.

2.2.2.2.5.3. Advantages. The use of a DNA binding dye and a probe in the same reaction allows the signal coming from both non-specific and specific amplified products to be monitored [46]. In addition, the synthesis of this type of probe, which contains a unique fluor, significantly reduces the assay costs [62].

2.2.2.2.5.4. Applications. They can be use in single and multiplex formats for rapid detection, gene expression, allelic discrimination, genotyping [105], SNP detection, mutation detection [106], identification and quantitation of infectious organisms (bacteria and viruses) and for analysis of environmental and biological samples.

2.2.2.2.6. Yin-Yang probes or 'displacing probes'

2.2.2.2.6.1. Structure. These double-stranded probes are composed of two complementary oligonucleotides of different lengths. The 5′-end of the longer positive strand is labeled with a fluorophore reporter and blocked with a phosphate group at its 3′-end, whereas the 3′-end of the shorter negative strand contains a fluorophore quencher [107] (Fig. 5B).

2.2.2.2.6.2. Mechanism of action. In solution, the shorter negative oligonucleotide, which acts as a competitor, forms a stable DNA duplex with the longer probe. This interaction prevents the fluorescent emission due to the fact that the reporter and quencher remain in close proximity. During the annealing phase, the shorter strand is displaced by the target leading to the emission of fluorescence. These so-called 'displacing probes' were first reported in 2002 by Li et al. [107]. In such a system, an ideal competitor must be competitive enough to prevent non-specific hybridizations but not too much so, in order to favor the formation of perfectly matched probe-target duplexes. The authors proposed that a single-stranded oligonucleotide with the same nucleotide sequence but shorter than that of the target would be a suitable competitor [107].

2.2.2.2.6.3. Advantages. The binding of Yin-Yang probes to the target is highly specific and their design is much easier than that of dual-dye-labeled probes. In addition, their synthesis is cost effective because it only involves a single-dye modification [107].

2.2.2.2.6.4. Applications. They can be used in single and multiplex formats for a wide-range of applications including pathogen detection or viral/bacterial load quantitation as well as mutations detection [108], analysis and genotyping SNPs [109], in which discrimination single nucleotide substitutes are required. Furthermore, the use of these probes has been proposed for tracing mRNAs in living cells or for the construction of biosensors and biochip detection devices [107].

2.2.3. Nucleic acid analogues

Nucleic acid analogues are compounds that are analogous (structurally similar) to naturally occurring RNA and DNA. An analogue may have alterations in its phosphate backbone, pentose sugar (either ribose or deoxyribose) or nucleobases [110]. Normally, the analogues incorporate all of the advantages of native DNA but are more stable in biological fluids and have increased affinity for complementary nucleic acid targets [111].

A variety of nucleic acid analogues have been described in the last years (Fig. 6):

- 2′-O-methyl oligodeoxyribonucleotides or 2′-O-methyl RNA [112],
- Peptide Nucleic Acids (PNAs) [113],
- 2′-Fluoro N3-P5′-phosphoramidites [114],
- 1,5-anhydrohexitol nucleotides (HNAs) [115,116],
- Phosphorodiamidate Morpholino Oligomer (PMO) [117],
- Locked Nucleic Acids (LNAs) [118],
- Zip nucleic acids (ZNAs) [119,120],
- Non-natural bases: isoguanine (iG) and 5′-methylisocytosine (iC) [121].

Some of these analogues, including PNAs, LNAs, ZNAs and non-natural bases (iG and iC) are currently used for different real-time PCR applications.

Please cite this article as: Navarro E, et al, Real-time PCR detection chemistry, Clin Chim Acta (2014), http://dx.doi.org/10.1016/j.cca.2014.10.017

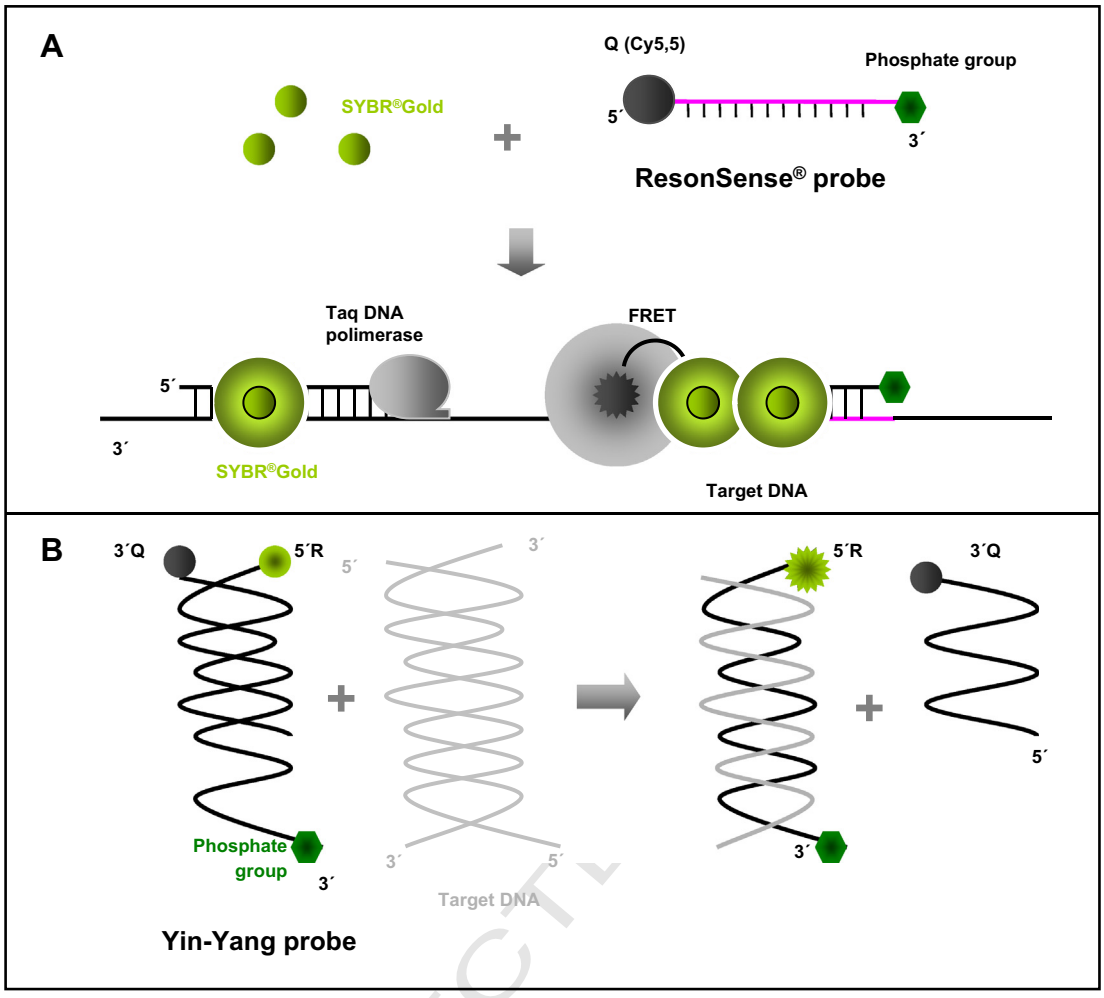


Fig. 5. Structure and mechanism of action of hybridization probes. (A) ResonSense® probe and (B) Yin-Yang probe.

2.2.3.1. PNAs

2.2.3.1.1. Structure. Peptide nucleic acids were first described by Nielsen et al. [113]. They are achiral and electrically neutral DNA analogues in which the sugar-phosphate backbone has been replaced by a peptide of N-(2-aminoethyl)-glycine units linked to the nitrogenous bases by metilencarbonilo [113,122]. PNA hybridizes to complementary oligonucleotides obeying the Watson–Crick hydrogen-bonding rules [123] (Fig. 6A).

PNAs are able to interact with either dsDNA or RNA with higher affinity and greater specificity than conventional oligonucleotides. This is due to its electrically neutral character, which prevents the phenomenon of repulsion between chains [113,124]. This binding takes place by strand displacement rather than by triple helix formation [125]. This nucleic acid analogue is attached to a molecule of thiazole orange or a fluorophore for qPCR reactions [126].

2.2.3.1.2. Mechanism of action. The mechanism of primer-probes or probes in which PNA molecules have been introduced is identical to the method of action of conventional probes. Noteworthy, the binding of PNAs to double-stranded DNA does not interfere with their properties as probes.

2.2.3.1.3. Advantages. PNA containing probes are more resistant to nucleases and proteases and can interact with DNA at lower salt concentration than standard probes/ primer-probes [123,127,128].

2.2.3.1.4. Applications. Their high affinity for DNA allows such probes to interact easily with target dsDNA sequences by strand invasion [129–131]. It is highly recommended to employ these nucleic acid analogues in order to induce DNA recombination or block PCR amplification of specific genes [132]. Uniquely, allelic discrimination of single nucleotide polymorphisms can be accomplished by using PNA-molecular beacons [133]. Furthermore, they can also be used in mutation detection [134,135], pathogen mutation [136] and for discriminating between DNA and cDNA sequences in prokaryotes [137].

2.2.3.2. LNA®

2.2.3.2.1. Structure. Locked Nucleic Acids, first described by Wengel and co-workers in 1998 [118,138,139], are DNA or RNA sequences in A conformation that contain one or more modified nucleotides [139]. Specifically, they have a methylene bridge between atoms 2′-O and 4′-C in the ribose ring to form a bicyclic ring [139] (Fig. 6B).

2.2.3.2.2. Mechanism of action. LNA containing primer-probes or probes exhibit the same mode of action as that of conventional primer-probes or probes.

2.2.3.2.3. Advantages. Like the PNA system, LNA probes are resistant to degradation by nucleases [140]. LNA® nucleotides are often used in combination with non-modified DNA/RNA nucleotides to increase the thermal stability of the probe [141,142], resulting in a high specificity for their target sequences [143,144]. Table 3 shows an example of the increment in T_m values based on the number of LNA® nucleotides introduced into the oligonucleotide.

2.2.3.2.3.1. Applications. LNA® nucleotides can be introduced into most primer-probes and probes described in this review [145]. For instance, the use of LNA-Molecular Beacon and LNA TaqMan probes has been reported for SNP detection of *Mycobacterium tuberculosis* [146,147], GMO detection [27], determination of the presence of *Helicobacter pylori*

[148], allele specific mutational analysis of *KRAS* and *BRAF* [149] as well as quantifying hepatitis B virus DNA in serum [150].

2.2.3.3. ZNA™

2.2.3.3.1. Structure. Zip nucleic acids, developed by the Polyplus-transfection company, are a novel type of synthetic modified oligonucleotide [119,120]. The introduction of ZNA™ molecules into oligonucleotides increases their affinity for the target by decreasing the electrostatic repulsion between the two nucleic acids [151,152]. This is achieved by conjugating cationic moieties (Z units), such as derivatives of spermine, to an oligonucleotide (Fig. 6D).

A number of Z units can be placed at the 5′ or 3′-ends or in the middle of primer-probes and probes [153]. The melting temperature of ZNA-containing oligonucleotides is linearly dependent on the number of cationic units grafted on this structure, providing a convenient means to fine tune hybridization temperatures [151]. The T_m and global charge of the ZNA™ are easily predictable using a simple mathematical relation [119,153].

2.2.3.3.2. Mechanism of action. During the annealing phase, ZNA™ oligonucleotides are attracted towards the nucleic acid strands due to their polycationic nature, starting their scanning of DNA sequences.

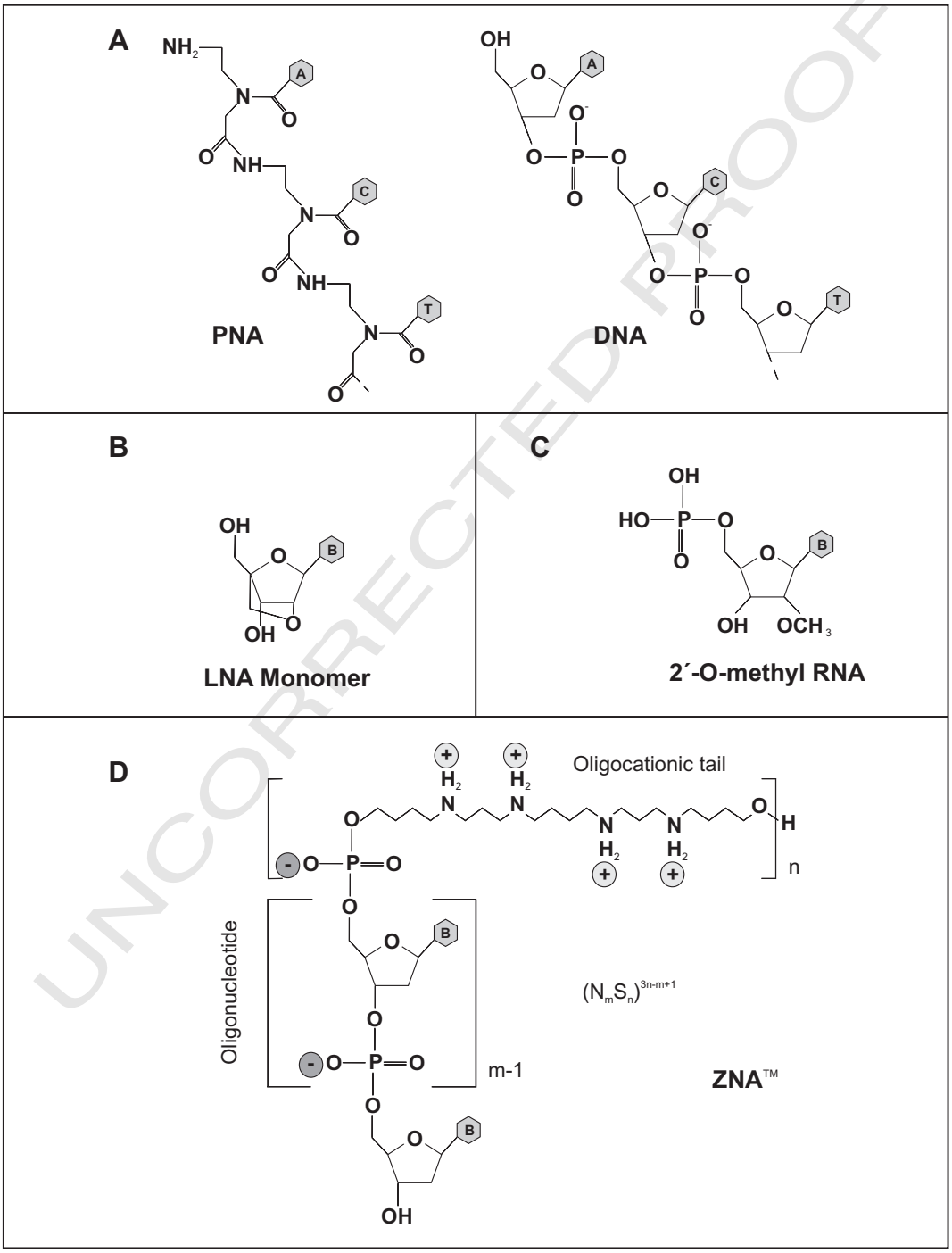


Fig. 6. Chemical structures of nucleic acid analogues. (A) PNA probe; (B) LNA® Monomer; (C) 2′-O-methyl RNA and (D) ZNA™. The global charge of ZNA™ is calculated by applying the following equation: $(N_mS_n)^{3n-m+1}$, where N: number of nucleotides and S: number of spermine cationic units.

Please cite this article as: Navarro E, et al, Real-time PCR detection chemistry, Clin Chim Acta (2014), http://dx.doi.org/10.1016/j.cca.2014.10.017

Table 3
Increase in T_m with oligonucleotides carrying LNA® monomers.

Probe sequence (5′–3′)	LNA® base	$T_{melting}$	$\Delta T_{melting}$
dG dG dT dA dA dT dG dT dC	–	29 °C	–
dG +G dT dA +A dT +G dT dC	3	44 °C	15 °C
+G +G +T +A +A +T +G +T +C	9	64 °C	35 °C

+ symbol denotes the LNA® base.

Next, hybridization takes place by zipping up when the ZNA™ oligonucleotide meet its complementary sequence [119] (Fig. 7A).

2.2.3.3.3. Advantages. ZNA™ oligonucleotides display an exceptionally high affinity for their targets, mainly due to the presence of the Z units, which enhance the interaction with the DNA target.

2.2.3.3.4. Applications. ZNA™ represent a potent new tool for numerous nucleic-acid-based applications, including: real-time PCR, capture probes, Northern/Southern Blotting, microarrays and in situ hybridization. In PCR assays, these oligonucleotides can be used in single and multiplex formats for pathogen detection [154], gene expression, microarray validation, allelic discrimination, mutation/SNP detection, and viral/bacterial load quantitation [155]. In particular, LNA-based methods have been described to efficiently detect Hepatitis B virus resistance to drugs in patients [156]. Interestingly, it has been reported that the use of ZNA™ primers improves the synthesis of cDNA from total RNA, making them the best choice for the quantification of low-abundant transcripts [151].

2.2.3.4. Non-natural bases: Plexor™ primers. The development of organic chemistry has made it possible to enlarge the number of standard nucleotides beyond those known in nature [121,157,158]. Two modified bases, isoguanine (Iso-dG) and 5′-methylisocytosine (Iso-dC), which generate novel base pairings, have been successfully designed to allow protein recognition and site-specific enzymatic incorporation [158–161].

2.2.3.4.1. Structure. Plexor™ primers, described by Sherrill et al. in 2004 [121], take advantage of the highly specific interaction between two modified nucleotides: Iso-dG and Iso-dC. In Plexor™ reactions, one PCR primer contains an Iso-dC residue and a fluorescent reporter label at the 5′-end, whereas the second one is an unlabeled oligonucleotide that carries standard nucleotides. In this system, Iso-dG nucleotides, covalently coupled to a quencher, are added into the qPCR reaction [121] (Fig. 7B).

2.2.3.4.2. Mechanism of action. During the amplification phase, the incorporation of Iso-dG nucleotides brings the quencher and reporter into close proximity, producing the quenching of the fluorescent signal released from the labeled primer [121]. In this system, the decrease in initial fluorescence is proportional to the starting amount of target (Fig. 7B).

2.2.3.4.3. Advantages. Plexor-primer based-technology takes advantage of the highly specific interaction between Iso-dG and Iso-dC. These two modified nucleotides are not recognized either by nucleases nor proteases.

2.2.3.4.4. Applications. Plexor™ primers can be used in single and multiplex formats for pathogen detection [162], viral/bacterial load quantitation, gene expression, genotyping, SNP detection [163] and GMO detection [27].

2.2.3.5. Tiny-Molecular Beacon probes

2.2.3.5.1. Structure. Molecular Beacon probes, described by Bratu et al. in 2011 [164], are redesigned as small hairpins and synthesized from 2′-O-methyl RNA/LNA chimeric nucleic acid analogues [164,165].

2.2.3.5.2. Mechanism of action. They display the same mode of action as that of Molecular Beacon probes (see Section 2.2.2.2.2).

2.2.3.5.3. Advantages. These probes have been reported to be very resistant to nucleases and stable within a cellular environment. Furthermore, they have high affinity and specificity for RNA sequences, due to the incorporation of LNA into RNA oligonucleotides in the probe structure [165].

2.2.3.5.4. Applications. The user-friendly synthesis protocol of these probes as well as their ability to couple to a variety of fluorophores make Tiny-Molecular Beacons the optimal technology to detect less abundant, highly structured mRNAs and small RNAs such as microRNAs, small nucleolar RNAs and nuclear RNAs. Recently, this technology has also been used to visualize native mRNAs in living cells [164,165].

3. Primer and probe design

The design of primers and probes is a very important requirement for most applications of qPCR [166]. The choice of specificity, length, GC content, 3′ end stability, sequence complexity, melting temperature, and location in the target sequence of the primers determines amplicon length, melting temperature and amplification efficiency [166,111]. The choice of chemistry and probe design are a very personal matter and there are, as always, numerous options that need to be considered prior to sitting down and designing the probes [111]. We should consider, (i) if we want to quantity DNA, profile mRNA or perform allelic discrimination assays; (ii) which chemistry is most appropriate for our experiment; (iii) if we wish to detect DNA, RNA or both; (iv) if it is necessary to distinguish between closely related sequences, e.g., to detect and quantify a determined pathogen that belongs to a family with several species; (v) which fluorescent reporter/ quencher combinations should be used; (vi) if our probe should contain DNA analogues, MGB factors or any other modifications; and finally (vii) if the assay is multiplex [111].

Nowadays, numerous in silico tools have been developed to guide the design of qPCR assays and analyze any resulting quantitative data [167]. Many tools are freely available online, while others are bundled with qPCR instruments or available from various software houses [167]. Some in silico tools are Primer3 [168,169], FastPCR software [170,171], Java web tools [172], PerlPrimer [173], IDTSciTools [174], UniPrime [175], and Primer-BLAST [176]; in addition, it is important to analyze the secondary structure of primers using an additional software program like mFold (http://www.idtdna.com/Scitools/Applications/mFold/). MPprimer is a program for multiplex PCR primer design [177]. This program employs the program Primer3 [168] for the primer design and the program MFEprimer for assessing primer specificity [178]. Recently, several authors have presented detailed descriptions, step by step, of a qPCR assay design [167,179]. The MIQE guidelines also provide clear guidance on the steps that are important for assay design [180,181].

Several research companies offer useful guidelines on their websites for designing primers and probes:

- Qiagen's website: “Critical factors for successful real-time PCR” (http://www.qiagen.com/resources/resourcedetail?id=f7efb4f4-fbcf-4b25-9315-c4702414e8d6&lang=en).
- Cepheid's website http://www.cepheid.com/us/component/phocadownload/…/2-support?…). “Designing Real-Time Assays” (SmartNote 6.1), “Optimizing and Analysing Real-Time Assays” (SmartNote 6.2), “Dye-Quencher Considerations” (SmartNote 6.3) and “Guidelines for the “Advance to Next Stage”” (SmartNote 6.5) on the SmartCycler® II System.
- Applied Biosystems' website: “Getting started guide: Designing primers and probes for quantification assays and allelic discrimination” (http://www3.appliedbiosystems.com/cms/groups/mcb_support/documents/generaldocuments/cms_041902.pdf), Designing MGB-TaqMan® probes design for allelic discrimination (http://www3.appliedbiosystems.com/cms/groups/mcb_support/documents/general documents/cms_042997.pdf) or Designing MGB-TaqMan® probe and primers sets for gene expression (http://www6.appliedbiosystems.com/support/tutorials/pdf/taqman_mgb_primersprobes_for_gene_expression.pdf).

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- Roche Applied Science's website: "Designing Primers and Probes" (https://www.roche-applied-science.com/wcsstore/RASCatalogAssetStore/Articles/Fast_and_Convenient_Primer_Probe_Design_for_Multiplex_Assays_with_the_LightCycler_Probe_Design_Software%202.0.pdf)
- Invitrogen has developed the software called LUX™ Designer (http://tools.lifetechnologies.com/content/sfs/manuals/luxprimers_man.pdf) for designing LUX™ primer-probes.

It is noteworthy that, for DNA analysis of higher organisms, primers and probes should be designed to avoid complex regions contained in their genomes, such as repeat elements (LINEs, SINEs, alu), pseudogenes, and large duplications.

4. Real-time PCR instruments

qPCR instruments basically consist of a thermal cycler with an integrated excitation light source, a fluorescence detection system and software, which performs the quantitative analysis of the detected fluorescence during the assay. These instruments are able to simultaneously detect different wavelengths [4].

There are three basic devices: lamps, light emitting diodes (LED) and lasers, which emit excitation energy. The lamps are instruments of the emission spectrum, while LEDs and lasers are more restricted. These devices containing lamps (usually tungsten halogen or quartz tungsten halogen) include filters to limit excess excitation. Some examples are the ABIPrism 7000 from Applied Biosystems, the MX4000 and

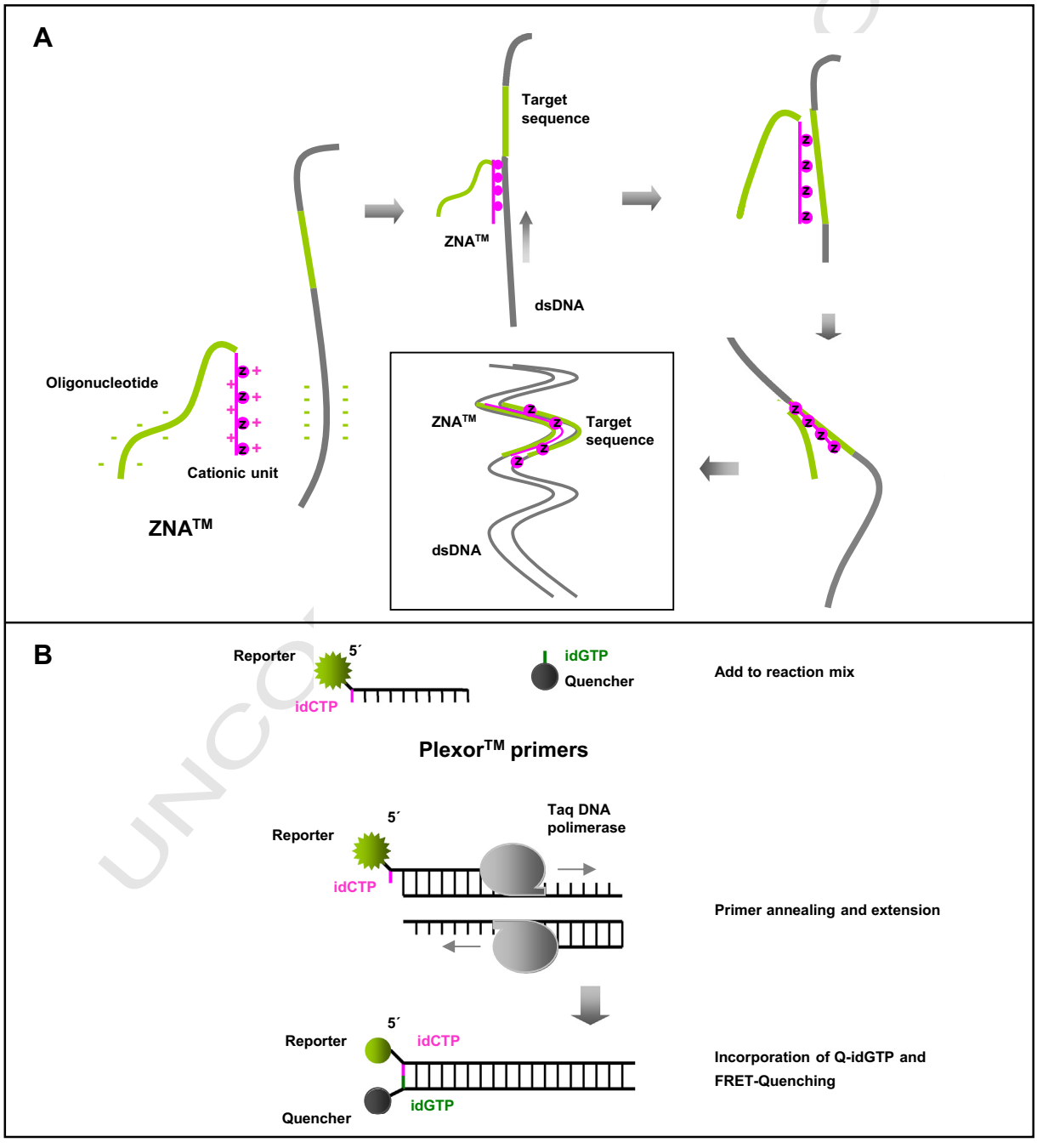

Fig. 7. Mode of action of nucleic acid analogues: ZNA™ (A) and Plexor™ probe (B).

Mx3000P from Stratagene, and the iCycler iQ Bio-Rad. The LED system is represented by the Roche LightCycler, the Cepheid SmartCycler, the Corbett Rotor-Gene and DNA Engine Opticon 2 from MJ Research. The ABI Prism 7900HT is the only device using a laser that excites in the range of 350–750 nm (Valasek and Repa, 2005) [4].

The number of channels available for reading fluorescence is also important in qPCR experiments, given that it allows researchers to identify different targets in the same reaction (Multiplex PCR) and the presence of PCR inhibitors (Costa J, 2004) [8].

To record data, the energy emitted at discrete wavelengths by fluorophores is monitored in detectors, including chambers loaded with coupled devices, photomultiplier tubes or other photodetectors. Generally, filters or channels are used to detect short wavelength ranges.

On the one hand, a common and unaccounted for source of error in qPCR data is the PCR instrument itself. PCR instruments are subjected to vast and sudden changes in temperature (cycles of expansion and contraction), leading to material fatigue. Thus, the device must be in perfect operating condition to guarantee temperature homogeneity for every well position [182–184]. So, there is a dispute about whether the PCR efficiency is constant [185], which has led to the development of different methods to analyze amplification curves. For example, Ruijter et al. have evaluated the bias, resolution and precision as well as implications of qPCR curve analysis methods for reliable biomarker discovery [185].

The instrumentation of qPCR is not complete without hardware and software for data analysis. The software simplifies analysis of the data and presents the results in graphs. In particular, amplification curves allow one to quantify the starting DNA, whereas dissociation curves show the purity of the final DNA product.

The first qPCR thermocycler, the ABI 7700, was produced and marketed by Applied Biosystems in 1996 [3]. At present, large companies (Applied Biosystems, Roche, Stratagene, Cepheid, Corbett, Eppendorf and BioRad) are offering different models of qPCR platforms. Logan and Edwards have accurately described the device features of numerous brands of PCR apparatus, including company, model, laser/lamp, detector, thermocycling, filters/detection channels, format (96-well plates, 0.2 ml tubes, 8-strips tubes among others), time (40 cycles), reaction volume, fluorescence chemistry, supports multiplexing, passive reference, dimensions (H × W × D), weight and also software for primer and probes design [6,186].

5. MIQE guidelines

Currently, a lack of consensus exists on how best to perform and interpret qPCR experiments. The problem is exacerbated by the lack of information that characterizes most reports of studies that have used this technology, with many publications not providing sufficient experimental detail to permit the reader to critically evaluate the quality of the results presented or to repeat the experiments [180], which makes it very difficult to compare results between several studies.

To promote consistency between laboratories, increase experimental transparency, and ensure the integrity of the scientific literature, guidelines for the Minimum Information required for the publication of qPCR Experiments (MIQE) were formulated by Bustin et al. in 2009 [180,181]. MIQE is a set of guidelines that describe the minimum information necessary for evaluating qPCR experiments [187], which are separated into nine major components (Experimental design, Sample, Nucleic acid extraction, Reverse transcription, qPCR target information, qPCR oligonucleotides, qPCR protocol, qPCR validation and Data analysis) that contain detailed information on pre- and post-assay parameters as well as comprehensive documentation of the experimental protocol [180].

By providing all relevant experimental conditions and assay characteristics, reviewers can assess the validity of the protocols used; in addition, it can enable other investigators to reproduce the results [180]. MIQE details should be published either in abbreviated form or as an online supplement [180,188].

Today, the rationale underlying the MIQE guidelines has become widely accepted [180] and used [189–193], with more than 2200 citations by March 2014 and editorials in Nature and related publications acknowledging the enormity of the problem [187]. However, it will be some time before the many contradictions apparent in every area of the life sciences are corrected [187].

6. Concluding remarks

The aim of the current review is to bring together, classify and display available information and knowledge published on detection chemistries for qPCR in the last years, approaching PCR-based DNA analysis in a comprehensive way. Although several authors have also described this information previously [194–198], this review offers a useful classification as well as a detailed description of all such detection methods. They have been classified into two groups based on the fluorescent agent used and the specificity of PCR detection: dsDNA intercalating agents and fluorophores attached to oligonucleotides. The latter have been further divided into three subgroups according to the type of fluorescent molecules added to the reaction: primer-probes, probes and analogues of nucleic acids. In addition to their structure and mechanism of action, advantages and applications of each DNA detection method are described in this review.

Several novel methods for DNA detection in real-time PCR have recently been described, but the tendency in this field has been to introduce new molecules such as MGB ligands or to combine distinct PCR systems in order to improve target DNA-binding specificity and sensitivity. As shown in the paper, the incorporation of MGB ligands increases the melting curve of the primer/probe in order to enhance its specificity of interaction with the target DNA sequence. This makes MGB probes quite attractive for use in SNP detection and allelic discrimination. Interestingly, combinations of dsDNA intercalating agents (SYBR® Gold) with fluorescent primer-probes (Angler®) or fluorescent probes (ResonSense®) in the same real-time PCR reaction are rapidly becoming popular within studies to detect non-specific and specific amplified products. On the other hand, nucleic acid analogues (PNA, LNA, ZNAs) exhibit very high affinity and excellent DNA and RNA binding specificity. Furthermore, primers and probes containing modified nucleotides display novel attractive features, such as resistance to the action of nucleases or proteases and to changes in pH or ionic strength.

When we design a qPCR assay, it must take into account the MIQE guidelines [180] for correct design, implementation and publication of our study. Therefore, this study will provide sufficient experimental detail to permit the reader to critically evaluate the quality of the results presented or to repeat such experiments [180]. Since 2009, when Bustin described the MIQE guidelines, many authors have applied these MIQE guidelines [180] in their qPCR assays [189–193]. In addition, new articles have been published on how to improve the design, qPCR protocol, qPCR validation and data analysis of qPCR assays. For example, Tuomi et al. observed a bias in the threshold cycle (Ct) or quantification cycle (Cq) with hydrolysis probes that can be corrected with the estimated PCR efficiency value [199], and Ruijter et al. evaluated this bias in different chemistries (DNA-binding dyes, hybridization probes, hydrolysis probes, LUX primers, hairpin primers and the QZyme system) and have described how it requires a correction of the observed Cq [200]. Although it will be some time before the many contradictions apparent in every area of the life sciences are corrected [187], we must be aware that adherence to the MIQE guidelines by the scientific community is vital, because basic studies may be reversed in subsequent clinical studies. Also, it is useless to simply publish studies if they cannot be compared due to not having followed the MIQE guidelines.

The experience of our group on the use of real-time PCR is focused on molecular diagnosis of human brucellosis. We developed a Taqman probe-PCR method to detect and quantify *Brucella melitensis* DNA in

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the blood and serum of patients with acute brucellosis [201]. Despite this PCR assay being highly reproducible, sensitive and specific in acute patients, it failed to detect and quantify *B. melitensis* DNA in chronic patients [202]. This failure is likely due to lower DNA concentrations in blood and sera from chronic patients. At present time, we are testing other probes (TaqMan-MGB, probes with PNAs, LNA®, ZNA™ and Plexor primers™) in order to increase the detection efficiency of *Brucella* DNA in such patients, which will extremely be useful for setting up a new qPCR-based diagnostic tool for chronic brucellosis.

Thus, given our prior knowledge of such technology and that reported by other authors [27,72], we highly recommend prior evaluation, including determinations of sensitivity, cost-effectiveness and simplicity of probe-design, of different PCR chemistries and conditions to determine the most appropriate qPCR assay for a particular scientific/clinical application, as well as to follow the MIQE guidelines [180,181] for each qPCR assay.

Acknowledgments

The English grammar/syntax of the manuscript have been revised by Proof-Reading-Service.com.

This review was supported by the Spanish Ministry of Health (Fondo de Investigación Sanitaria grant G03/204), Government of Castilla La-Mancha (Convocatoria de Ayudas a la Investigación en Biomedicina y Ciencias de la Salud), grants PI2007-27, PI2010-005 and G2010/C004, and Investigadores Expertos' Fellowship MOV-2010-IE003 to E.N.

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