

## I See the Light! And I See It Again and Again!

Mikael Kubista\*

In 1983, Kary Mullis at Cetus conceptualized the most important of biotechnological reactions—the polymerase chain reaction (PCR) (1). The idea, for which Mullis was awarded the 1993 Nobel Prize in chemistry, was as simple as it was brilliant. Given the natural ability of polymerases to copy nucleic acids in the presence of short complementary oligonucleotides it should be possible to perform the reaction in cycles to produce large numbers of copies of virtually any DNA sequence. In the beginning, fresh polymerase had to be added at each cycle, because it was irreversibly denatured by the heating. In 1985, Susanne Stoffel and David Gelfand successfully isolated DNA polymerase from *Thermus aquaticus* (i.e., *Taq*), and Randy Saiki showed that it could be used to automate the process (2). Heating to 95 °C separated the strands of the nucleic acid template, a temperature between 50 °C and 60 °C allowed primer oligonucleotides to hybridize with template, and raising the temperature to 72 °C activated the heat-stable *Taq* polymerase to extend the primers to generate a copy of the template molecule. With the PCR, virtually any DNA molecule could be amplified to large amounts from a single copy. The technique rapidly became important in biological research, for species identification, in forensic applications, and in qualitative testing for infectious disease. When Robert Watson, who was working with Russell Higuchi, accidentally ran a PCR in the presence of ethidium bromide, which was intended for post-PCR gel staining, and found that the reaction was not totally inhibited, they realized it should be possible to monitor the amplification in real time via the dye's fluorescence (3). The capability to monitor the number of amplification cycles needed to produce the amount of product that generates a certain threshold fluorescence signal allowed the PCR to become quantitative (4, 5).

The intercalating dye ethidium bromide, which had been used to demonstrate the concept of real-time PCR, was soon replaced with asymmetric cyanine dyes that were less inhibitory to the PCR (6). In particular, SYBR Green I from Life Technologies has become popular. Dyes are sequence-nonspecific reporters. They do

not distinguish between PCR products during the kinetic phase, and they bind to any aberrant products formed by interacting primers only (so-called primer-dimer products). These features make them less suitable for multiplex analyses, and false-positive signals can appear in regular singleplex assays. Multiple targets in a single tube can be quantified with sequence-specific reporters. The hydrolysis probe, also known as TaqMan® (Applied Biosystems), was the first probe to be invented (7, 8). It is an oligonucleotide labeled with a fluorophore and a quencher that are kept in proximity in the intact probe so that the fluorescence is quenched. Upon primer extension by *Taq* polymerase, the enzyme's nuclease activity degrades the probe (9), the labels are released, and the freed fluorophore fluoresces (10). Other probe designs include the molecular beacons (11), adjacent probes (12, 13), LightUp™ probes (Invitrogen) (14), and NuPCR (Illumina) (15). These probes are also labeled oligonucleotides, but they are designed to fluoresce upon hybridization to the target. A third category of reporters are labeled/modified primers that generate fluorescence when they are incorporated into amplicons. These reporters include Scorpions® primers (16), Amplifluor® primers (17), QZyme™ (18), Snake™ (19), and the Lion™ Probes (20). Labeled primers are highly suited for multiplexing, but those that lack an internal hybridization sequence have a compromised specificity and may report the formation of aberrant products.

Although primers are regular oligonucleotides that are easy to synthesize and can be purified by precipitation or by using a cartridge, probes are complex modified oligonucleotides that require HPLC or electrophoretic purification. This characteristic makes dye-based reporter assays substantially less costly and therefore preferred for singleplex quantitative PCR. Probes are used for multiplexing or in singleplex when false-positive signals cannot be tolerated. In an attempt to obtain probe specificity at a reduced cost, Exiqon invented the Universal ProbeLibrary (UPL), which Roche has commercialized. The UPL is based on hydrolysis probes containing locked nucleic acid residues, which confer stability and for which 8 or 9 nucleotides are sufficient for probe hybridization. This short length makes the probes degenerate, and the 165 probes in the UPL target most of the transcripts in the genome (>99% coverage of the human transcriptome). This feature is an excellent solution for taking

TATAA Biocenter, Goteborg, Sweden.

\* Address correspondence to the author at: TATAA Biocenter, Odinsgatan 28, Goteborg 411 03, Sweden. E-mail mikael.kubista@tataa.com.

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advantage of probe specificity at low assay cost. In this issue of *Clinical Chemistry*, Faltin et al. describe a novel approach for obtaining the same result, but with a single fluorogenic probe only. A mediator probe, which is a low-cost oligonucleotide, is designed to recognize the template. The probe is partially degraded by the polymerase's exonuclease activity in a template-specific reaction, and the product triggers the cleavage of a universal fluorogenic probe. The new system is as sensitive as state-of-the-art hydrolysis probe assays but has the advantage that a single universal probe can be used for all singleplex assays. Aberrant primer-dimer products do not generate a signal, and multiple universal probes can be used in a single reaction to achieve multiplexing. It will be interesting to learn how difficult it is to design mediator probes for new targets, how much sensitivity is compromised in multiplex systems, and how robust the system is with respect to inhibitors and interfering substances that may be present in extracts from complex matrices.

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