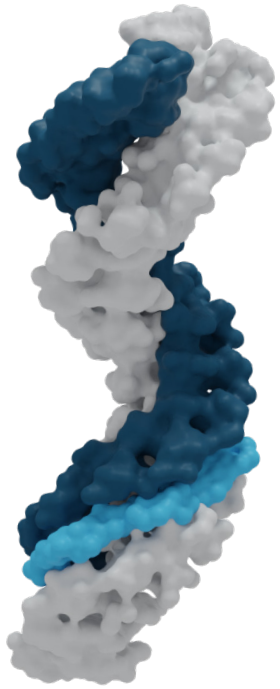
DSQ: The next evolution of real-time PCR

Summary

Since its inception in the 1990s, real-time PCR chemistry has seen countless advances in technology that offer new methodologies and applications. The inventions in fluorophore and quencher chemistry, their structural arrangements when linked to an oligonucleotide, and the many refinements of enzymes and reagents propelled real-time PCR into widespread adoption very shortly after its debut. Arguably one of the most impactful technological advances in real-time PCR was the invention, in 1995, of the minor groove binder (MGB)-oligonucleotide conjugate[1], or MGB probe, by ELITechGroup MDx, LLC (EG MDx). MGB probes, with the structure fluorophore-DNA oligo-quencher-MGB, became ubiquitous in the field. In 2018, EG MDx invented a hybrid structure to combine the functions of an MGB and a fluorescence quencher, simplifying labeled oligonucleotide chemistry, without compromising the DNA duplex stabilization and fluorescence quenching effected by the separate MGB and quencher molecules. Here we present the duplex stabilizing quencher[2], or DSQ, and describe the properties of the DSQ hydrolysis probe in reference to the gold standard MGB hydrolysis probes.



A DSQ probe (dark blue) hybridized to its target DNA template (white), showing the minor groove binding of the DSQ (blue), stabilizing the DNA duplex.

Minor Groove Binders (MGBs)

Molecules that bind to the minor groove of the double-stranded DNA helix, or MGBs, are found naturally in many microbes (e.g., *Streptomyces* species). MGBs encompass a diversity of molecular compositions with different DNA binding affinities and different natural functions. Driven by hydrophobic interactions, hydrogen bonding, and Van der Waals forces, MGBs form highly stable DNA complexes and facilitate enzymatic reactions for the host cell. The planar, crescent shape of many MGBs is an efficient configuration; the planar profile allows for a snug fit in the narrow, deep minor groove and the crescent shape approximately matches the curvature of a spiraling double helix of B-form DNA. The shape complementarity reduces the energy required for a conformational arrangement within the minor groove. An MGB bound to a double helix can prevent DNA processing and packaging functions (e.g., synthesis, transcription), making MGBs attractive models for therapeutics and molecular biology. MGBs were isolated as early as 1951, and described as such in the 1970s and 1980s[3]. The array of synthetic derivatives in development for in vivo use includes anti-cancer agents, antiparasitic agents, antifungals, antibiotics, and antivirals[4–7], and in vitro applications of MGB derivatives are numerous.

The invention of the MGB-oligonucleotide conjugate[1], or MGB probe, in 1995 by EG MDx was conceivably one of the most important contributions in real-time PCR. The increased stability of the DNA template-oligonucleotide duplex conferred by an MGB offered many new advantages. The MGB allows for the design of significantly shorter oligonucleotide probes (10-15 nucleotides) with the same melt temperature (T_m) ranges as traditional longer probes (20-25 nucleotides), increasing probe specificity[8,9], and bringing the fluorophore closer to its quencher, reducing background fluorescence and increasing sensitivity[8]. The increased specificity is especially useful for mismatch discrimination[8]. The MGB also allows for easier assay design. The shorter probe length reduces the potential for secondary structure or dimer formation among primers and probes. Additionally, the bound MGB stabilizes A-T

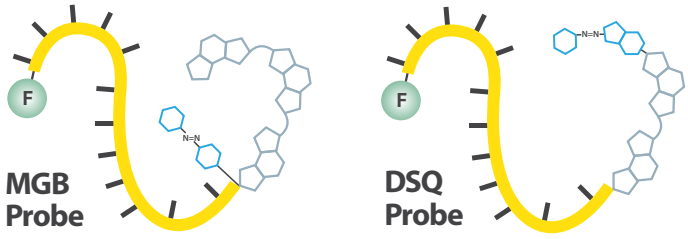


Figure 1. An MGB hydrolysis probe has a separate MGB and fluorescence quencher, while in a DSQ hydrolysis probe, the MGB and quencher are combined into one structure, simplifying probe chemistry. F, fluorophore.

nucleotide bonds more than G-C bonds, equalizing their binding affinity and thus reducing the influence of DNA sequence on the probe T_m[9]. This, coupled with the shorter length requirement, provided the opportunity for previously unfeasible assay designs, such as in AT-rich regions, in very short targets such as miRNAs, target regions rendered short due to polymorphisms, predicted secondary structures, or high substitution rate, or for high resolution detection of mismatches, splice variants, or different gene family members.

Fluorescence Quenchers

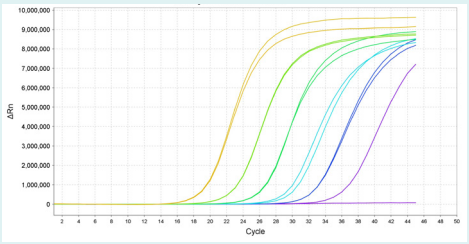
Fluorescence quenchers come in many varieties as well, and work via several mechanisms. The azo dyes, characterized by their internal -N=N- moiety, have long been an optimal quencher choice for probe-based real-time PCR. An azo dye quencher has a strong fluorescence resonance energy transfer (FRET) quenching property, whereby it absorbs the light emitted by a fluorophore within its proximity. FRET requires spectral overlap between fluorophore emission and quencher absorption spectra, necessitating optimal pairing of the two molecules. Azo dyes are also known to exert non-FRET quenching, so-called contact or collisional quenching, to varying degrees, which requires close proximity between fluorophore and quencher. EG MDx developed and patented in 2003 the first azo quencher, the Eclipse Dark Quencher® (EDQ)[10], with long-range and broad-spectrum FRET activity, now replicated in other popular, effective quenchers. The EDQ can be placed on the probe on the opposite end to a wide range of fluorophores and exert highly efficient FRET quenching, making this a convenient quencher choice. This molecular arrangement of the bis-terminally labeled probe is now widely used for hydrolysis probes, including the short MGB probes (<20 nucleotides) (Fig. 1) or the longer non-MGB probes (>20 nucleotides). Interestingly, the EDQ can also be used for hybridization probes with a 5′ MGB-fluorophore conjugate, where the MGB exerts highly effective contact quenching which, combined with the EDQ FRET quenching, effects very low background signal. In the 5′ MGB-fluorophore - 3′ EDQ orientation, i.e., Pleiades probes[11], hydrophobic interactions between the MGB and EDQ bring the fluorophore and EDQ into close proximity; upon probe-target hybridization, the quencher is spatially separated from the fluorophore while the MGB is bound within the minor groove, reducing both FRET and non-FRET quenching mechanisms and restoring fluorescence during the annealing stage of PCR[12].

Duplex Stabilizing Quencher (DSQ)

Now commercially available from EG MDx is the next iteration of real-time PCR probe, the DSQ probe, with a simplified architecture (Fig. 1) and equivalent or superior performance. The **duplex stabilizing quencher**[2], or DSQ, smoothly merges the DNA duplex stabilization of an MGB and the quenching power of an azo dye into one structure (Fig. 2). The single-structure creation was achieved by replacing one of the three subunits of the dihydropyrrole indole-carboxylate ($CDPI_3$) MGB (the

DSQ PROBE CHEMISTRY

The DSQ probe is constructed with a 5′ fluorophore and 3′ DSQ for hydrolysis chemistry. In hydrolysis probe-based real-time PCR, polymerase with 5′ exonuclease activity is necessary to generate a fluorescent signal for each primer and probe set. During each cycle of PCR, the primers and probe anneal to their target template, if present, and DNA is synthesized from the primers by the polymerase. The polymerase encounters the probe annealed to the template downstream of the primer, and the exonuclease activity of the polymerase hydrolyzes the probe, releasing the fluorophore from the proximity of the DSQ and allowing fluorescence emission. The PCR cycles result in exponential amplification of the target DNA and fluorescence levels.



A DSQ Alert™ amplification plot of a serial dilution of template DNA, illustrating high, clear fluorescence signal and maximum PCR efficiency.

most commonly used MGB in real-time PCR) with an azo dye derivative. The modified MGB ($CDPI_2$) and azo quencher preserve the planar, crescent shape of a full $CDPI_3$ MGB by using a rigid amide linker preventing internal folding and limiting rotation thus maintaining properties of both MGB and quencher in one entity[2].

The DSQ was designed for straightforward transition from MGB probe chemistry. The DSQ confers DNA duplex stabilization approximately equivalent to the traditional MGB-quencher combination. The same probe design algorithms are useful for both DSQ and MGB probes, and DSQ probe assays use typical thermal cycling conditions. Across several probes of varying oligo length, nucleotide sequence, and GC content, the use of the separate MGB and quencher versus the use of a DSQ resulted in approximately equal probe T_ms (Fig. 3); virtually any MGB hydrolysis probe can be switched to a DSQ probe with little to no impact on the optimal assay parameters and thermal cycling conditions. The DSQ was also designed to possess long-range and broad-spectrum fluorescence quenching abilities, similar to or greater than the EDQ, to retain the breadth of compatible fluorophores and the multiplexing capacity of an azo dye quencher. The inventors of the DSQ probe propose that the truncation of the MGB and the modifications to the azo dye in the DSQ maintain the template-probe stabilization and efficient universal quenching because i) the azo quencher behaves as the third subunit of a full MGB, supplementing the $CDPI_2$ structure with added DNA duplex support, and ii) a $CDPI_3$ MGB has natural fluorescence quenching abilities, not lost on the truncated $CDPI_2$ structure, that supplement the inherent fluorescence quenching abilities of the azo dye derivative. The DSQ effectively synergizes these properties into a simplified structure.

The DSQ probe has a number of advantages in assay performance. In addition to the numerous benefits of DNA duplex stabilization conferred by an MGB, the DSQ more efficiently quenches the fluorescence of unhybridized probes, reducing background signal and therefore the noise in an assay (Fig. 4). This reduced noise enhances the true signal-to-background fluorescence levels (Fig. 4) potentially improving PCR assay sensitivity. These features of the DSQ make switching to DSQ probes a simple and practical option for molecular laboratories.

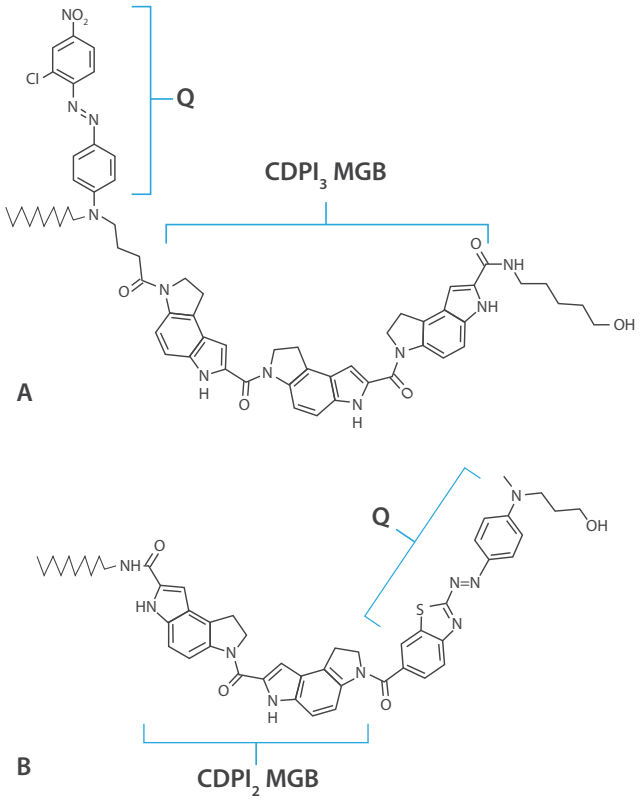


Figure 2. Molecular structure comparison between A. an MGB and separate azo quencher in a typical MGB hydrolysis probe, and B. a DSQ, in which one of the three subunits of the $CDPI_3$ MGB moiety is replaced with an optimized azo quencher molecule, joined by a rigid amide bond, with no ability for internal folding and limited rotation, so it acts as one entity. The wavy lines represent the linker and oligonucleotide portion of each probe. Q, quencher.

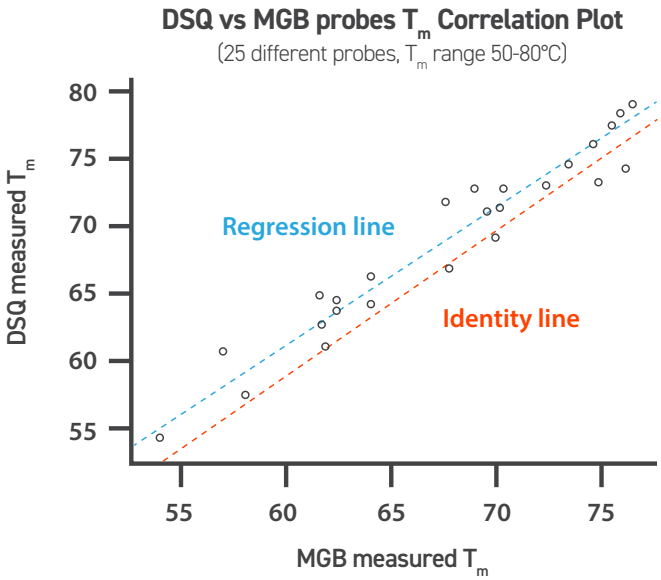


Figure 3. DSQ versus MGB-EDQ T_m correlation plot, showing the average slight increase in DNA duplex stability conferred by the DSQ, using 25 different probes over a T_m range of 50 to 80°C. The data illustrate that the same oligo sequence in an MGB probe and a DSQ probe results in similar probe T_m. DSQ probes are, on average, slightly more stable.

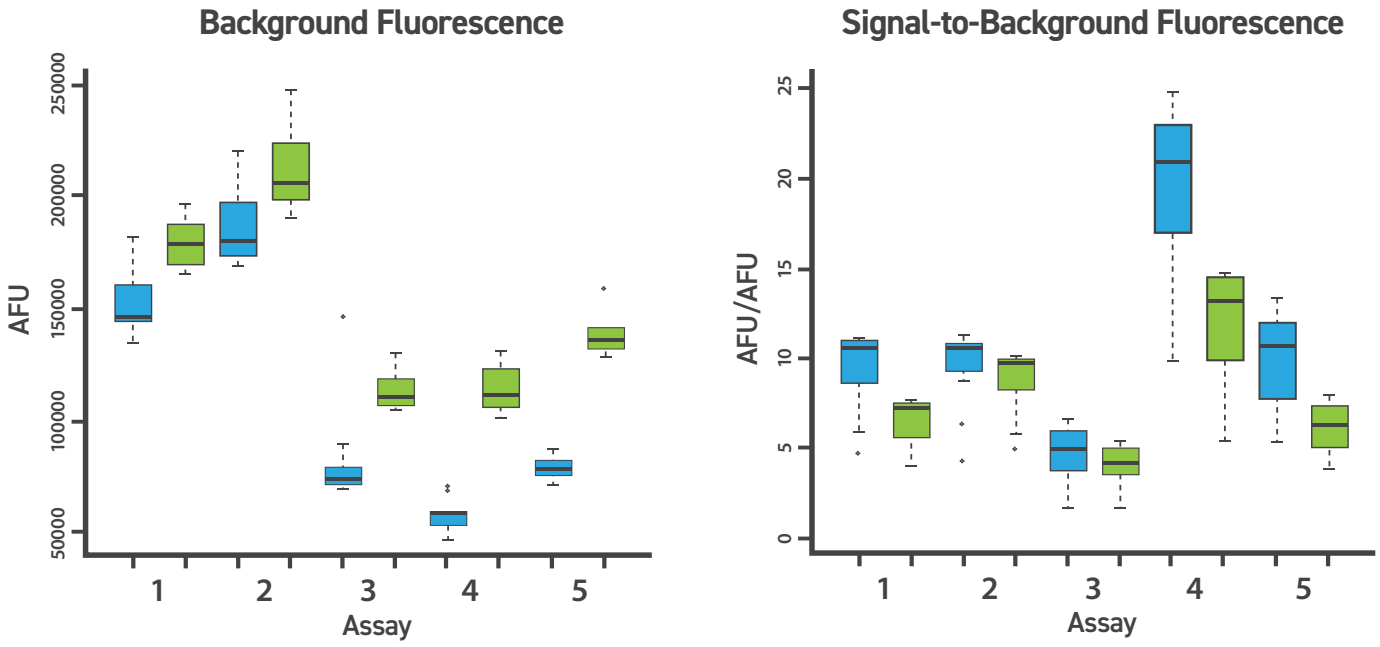


Figure 4. A comparison across five different identical pairs of assays, one of each pair using a DSQ probe (blue) and one an MGB-EDQ probe (green). The DSQ reduces background fluorescence of unhybridized probes by as mch as 50% compared to MGB-EDQ probes. This reduced noise translates to higher true signal-to-background fluorescence levels and better assay performance.

MGB probe technology has long been the gold standard for optimal probe architecture and improved sensitivity and accuracy of laboratory testing. Adding to the evolution of the innovation in real-time PCR is DSQ probe technology. DSQ reagents are now available as RUO reagents or ASRs, for laboratories running assays for research or creating LDTs.

References

1. *Kutyavin, I. V., Lukhtanov, E. A., Gamper, H., & Meyer Jr., R. B. Covalently linked oligonucleotide minor groove binder conjugates. U.S. Patent No. US5801155A. U.S. Patent and Trademark Office (1998).*
2. *Lukhtanov, E. A. & Scarr, N. Duplex stabilizing fluorescence quenchers for nucleic acid probes. U.S. Patent No. US10677728B2. U.S. Patent and Trademark Office (2020).*
3. *Nanjunda, R. & Wilson, W. D. Binding to the DNA minor groove by heterocyclic dications: from AT-specific monomers to GC recognition with dimers. Curr. Protoc. Nucleic Acid Chem. Chapter 8, Unit 8.8 (2012).*
4. *Barrett, M. P., Gemmell, C. G. & Suckling, C. J. Minor groove binders as anti-infective agents. Pharmacol. Ther. 139, 12–23 (2013).*
5. *Khalaf, A. I., Al-Kadhimi, A. A. H. & Ali, J. H. DNA Minor Groove Binders-Inspired by Nature. Acta Chim. Slov. 689–704 (2016).*
6. *Scott, F. J., Nichol, R. J. O., Khalaf, A. I., Giordani, F., Gillingwater, K., et al. An evaluation of Minor Groove Binders as anti-fungal and anti-mycobacterial therapeutics. Eur. J. Med. Chem. 136, 561–572 (2017).*
7. *Grande, F., Garofalo, A. & Neamati, N. Small molecules anti-HIV therapeutics targeting CXCR4. Curr. Pharm. Des. 14, 385–404 (2008).*
8. *Kutyavin, I. V., Afonina, I. A., Mills, A., Gorn, V. V., Lukhtanov, E. S., et al. 3'-minor groove binder-DNA probes increase sequence specificity at PCR extension temperatures. Nucleic Acids Res. 28, 655–661 (2000).*
9. *Lukhtanov, E. A., Kutyavin, I. V., Gamper, H. B. & Meyer, R. B. Oligodeoxyribonucleotides with conjugated dihydropyrroloindole oligopeptides: preparation and hybridization properties. Bioconjug. Chem. 6, 418–426 (1995).*
10. *Reed, M. W., Lukhtanov, E. A., Gall, A. A., Dempcy, R. O. & Vermeulen, N. M. J. Fluorescent quenching detection reagents and methods. U.S. Patent No. US6653473B2. U.S. Patent and Trademark Office (2003).*
11. *Lokhov, S. & Lukhtanov, E. A. Fluorescent probes containing 5'-minor groove binder, fluorophore and quenching moieties and methods of use thereof. U.S. Patent No. US7381818B2. U.S. Patent and Trademark Office (2008).*
12. *Lukhtanov, E. A., Lokhov, S. G., Gorn, V. V., Podyminogin, M. A. & Mahoney, W. Novel DNA probes with low background and high hybridization-triggered fluorescence. Nucleic Acids Res. 35, e30–e30 (2007).*

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