A probe for every occasion: A how-to guide for qPCR probe selection

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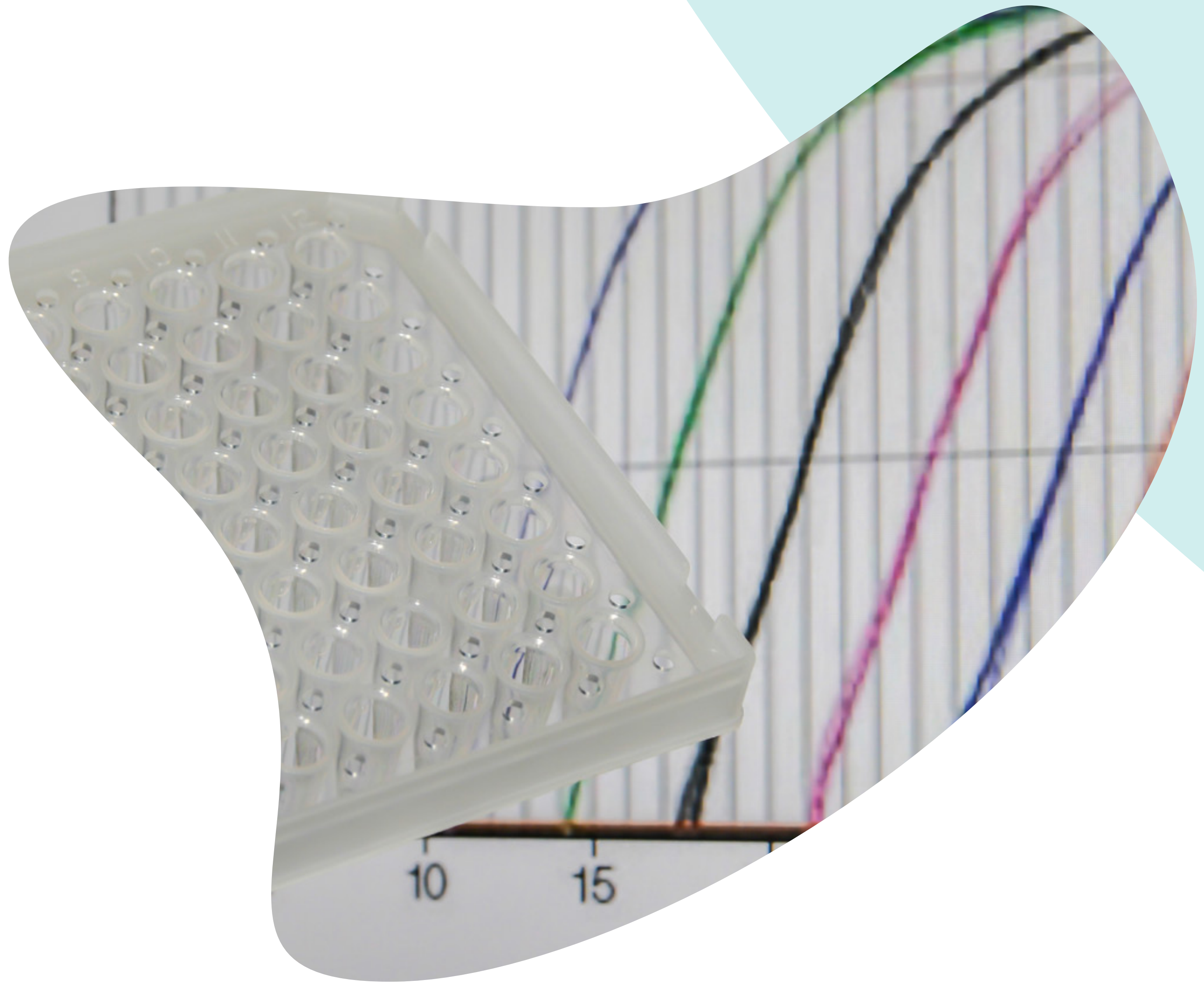
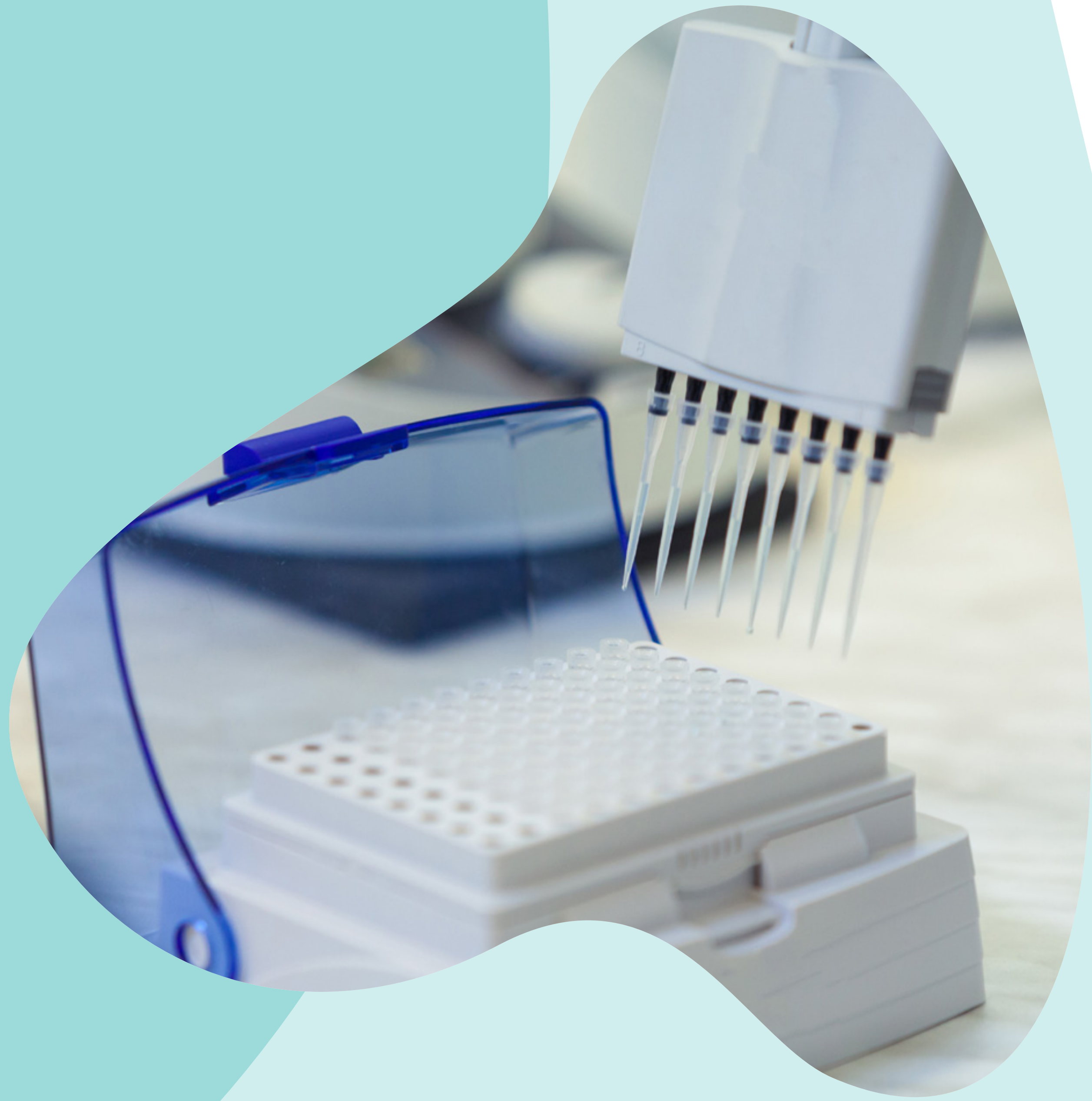




Starting with the sample

Quantitative real-time PCR (qPCR) is the most popular technique for quantifying nucleic acids. Simple to perform while offering excellent detection sensitivity, scientists employ qPCR in a diverse array of fields ranging from agricultural biology to molecular diagnostics.[1] Like conventional PCR, qPCR uses denaturation, annealing, and extension cycles to exponentially produce additional copies of nucleic acid sequences of interest. Unlike conventional PCR, qPCR attaches a signal-emitting agent—typically a dye or a probe—to these sequences of interest. Signal magnitude after each cycle is measured and compared against controls to quantitate DNA abundance.

Intercalated dyes were some of the earliest signal emitting agents used for qPCR. These small fluorescent molecules integrate into double-stranded DNA (dsDNA) during amplification, which dramatically increases their fluorescent light emission. However, this binding is non-specific: intercalated dyes will bind to any dsDNA, including primer dimers, contaminants, and other amplicons. In light of this, scientists developed fluorescent-labelled oligonucleotide probes that bind only to specific sequences. To prevent non-specific signal, many types of probes are also labelled with quenchers, which are only removed after probe-DNA binding *(see page 4 for more information on probe technology)*.

How samples influence primer and probe choices

There can be a tendency to only look downstream when thinking about probe selection and design. However, scientists need to factor in sample properties when making probe-related decisions. The first question is whether researchers are working with a DNA or RNA template, as RNA needs to be reverse transcribed into DNA prior to qPCR amplification. This process yields complementary DNA (cDNA), which differs from genomic DNA (gDNA) in that it does not contain non-coding sequences. Researchers must double check whether probe binding sites based on gDNA still exist within cDNA sequences. However, this can also prove advantageous, as a probe designed to bind a target sequence that exists only in cDNA (such as one that straddles two exons) can offer enhanced specificity and minimise the impact of DNA contamination.

Whether working with a DNA or RNA template, overall sample quality and integrity impacts probe binding affinity, which in turn affects design decisions. Nucleic acid degradation, for example, can result from prolonged sample storage, poor storage conditions, or the presence of nucleic acid cleavage enzymes, and can inhibit primer and probe binding due to target site fragmentation. This potentially creates a variety of amplicon sizes and alters probe binding efficacy, affecting the correlation of signal with gene expression or abundance. Sample contamination is another major challenge, as the presence of nucleic acid contaminants leads to non-specific primer and probe binding. Other contaminants create noise via background fluorescence emissions or unintended signal quenching. Alternatively, they may physically or chemically impede the function of probes, primers, reverse transcriptases, or polymerases.

Finally, sample logistics can affect probe design. Scientists use qPCR in many situations where sample scarcity is a factor, including forensic investigations and research involving ancient nucleic acids. In these cases, researchers may not have the luxury of extensive process optimisation or trial-and-error, and thus must opt for high specificity and sensitivity over cost or throughput concerns. The same also holds true for scientists working with genes of interest that are not endogenously abundant. Alternatively, qPCR may be employed in situations where turnaround and throughput are critical, such as clinical diagnostics and public health screening measures (e.g. wastewater surveillance). Here, probe design and selection may favour lower costs and faster reaction speeds.

A solid foundation

qPCR is used for a broad range of applications, and this versatility means that qPCR sample properties can vary dramatically. Researchers can overlook how the sample can influence qPCR probe effectiveness, resulting in sub-optimal data. Experimental design that starts with the sample is important for establishing a solid foundation for further troubleshooting and optimisation.

References

1. S.A. Bustin et al., "The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments," *Clin Chem*, 55(4):611-22, 2009.

The right probe for the right assay

Today, qPCR is the tool of choice for a wide range of applications,[1] including investigating gene expression changes in the context of health and disease, pathogen detection in clinical samples, and genotyping to make better informed breeding decisions for improved crop varieties, livestock, and aquaculture. Different applications come with different sensitivity, specificity, and throughput requirements. As such, probe selection and design depend heavily on the intended downstream application and overall research objectives.

Starting with research aims

Regardless of the downstream application, the most important aspect of probe design is ensuring that the probe is able to bind efficiently and specifically to the amplicon of interest. The probe should ideally bind in a location that is close to a primer binding site, but the binding sites should not overlap in order to avoid direct competition. Researchers should also judge probe length carefully. Longer probes allow for longer binding sites, potentially elevating specificity through enhanced binding-site uniqueness. However, lengthy probes are also more susceptible to dimerisation and hairpin formation, and excessively long probes can generate increased background signal because the quencher and fluorophore are too far apart. Longer binding sites may also facilitate binding despite a short sequence mismatch such as a single nucleotide polymorphism (SNP), making long probes less suited for SNP genotyping or allelic discrimination applications.

Additionally, researchers also have to consider their own experimental objectives. Scientists looking for the presence or absence of a gene (e.g. the detection of pathogens or genetically modified organisms) typically need less probe specificity compared to those seeking quantitative or comparative information on gene expression or dynamic modulation. Researchers can take several steps to obtain more sensitive and specific probes, such as opting for a dual-quencher model or selecting probes that incorporate duplex stabilising technology for enhanced probe-target binding.

Scientists have developed specialised probe configurations to streamline genotyping applications. For example, KASP™ assays use competitive allele-specific PCR with a fluorescence-based reporting system to detect nucleotide-level mutations such as indels or SNPs.[2] Using two allele-specific primers along with complementary fluorescent cassettes, KASP enables accurate bi-allelic discrimination of known SNPs and indels within a single experimental run.

Overcoming logistical obstacles

Probe-related decision making is also impacted by experimental logistics. If accuracy is essential (i.e. in clinical or forensic situations), then researchers should opt for more sensitive options such as shorter probes designed to maximise specificity or double-quencher probes that minimise background noise. Alternatively, if speed or throughput is the priority (e.g. diagnostic screening or genotyping for crop breeding), probes requiring shorter annealing and elogation cycle times can decrease the overall duration of the qPCR process.

Many researchers are turning to multiplexing—investigating multiple genes of interest within the same experimental run—as a way of adding depth to their studies, getting more from their samples, and improving experimental efficiency. Multiplexing can add a layer of complexity to probe selection and design. For example, researchers have to be careful that their probe emission frequencies do not spectrally overlap and that their instruments have enough distinct detection channels to distinguish each probe used in the experiment. Scientists also must take care that probes do not cross-react with other primers or probes in the reaction, that all probes have similar melting temperatures (ideally 5-10 °C above primer melting temperatures), and that quenchers do not unintentionally dampen signals from probes other than the one that they are paired with.

Informed decision making

While most probes will deliver some degree of result for most applications, selecting the best probe for a given application is instrumental to optimisation. Understanding how probe technology aligns with researcher aims and needs can provide increased data accuracy and reproducibility, streamlined logistics, and troubleshooting solutions.

References

1. H.D. VanGuilder et al., “Twenty-five years of quantitative PCR for gene expression analysis,” *Biotechniques*, 44(5):619-26, 2008.
2. C. He et al., “SNP genotyping: the KASP assay,” *Methods Mol Biol*, 1145:75-86, 2014.

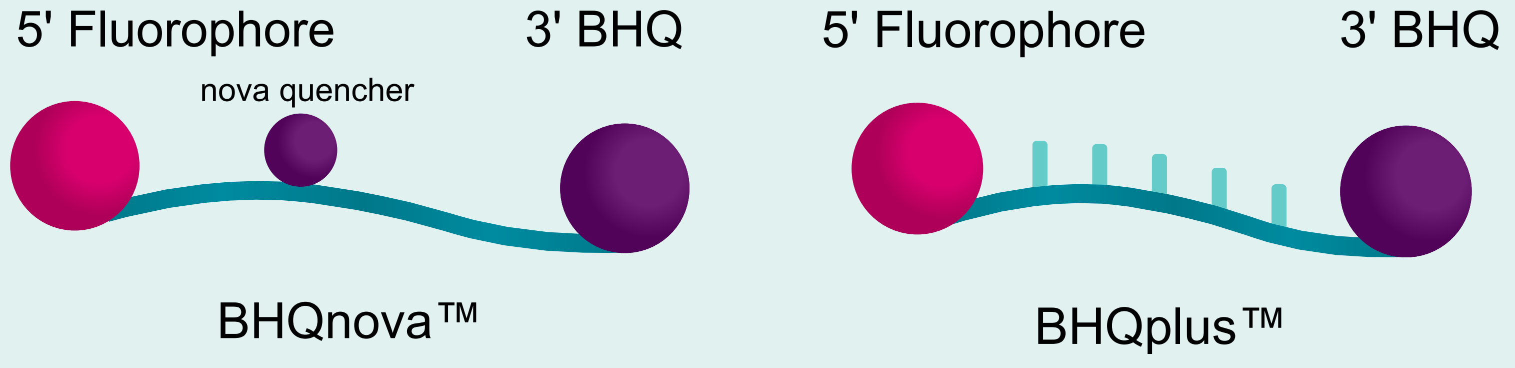
Understanding probe options

qPCR probes come in a variety of sizes and formats, each with their own qualities and advantages.

Hydrolysis Probes

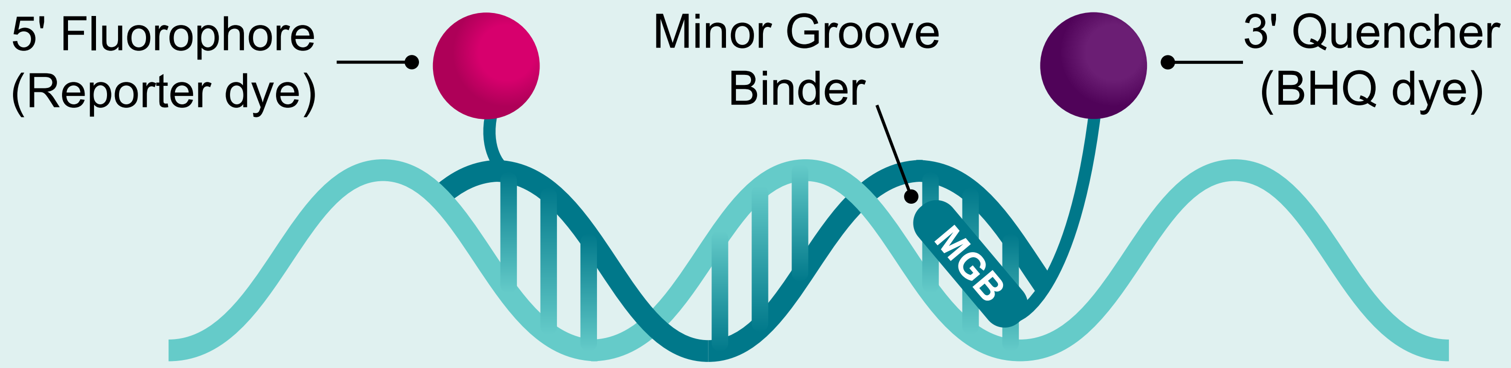
Hydrolysis probes are typically labelled with a fluorophore reporter at the 5′ end and a quencher [such as the Black Hole Quencher (BHQ™)] at the 3′ end. During amplification, the DNA polymerase exonuclease activity cleaves off the reporter, allowing for signal unquenching and detection.

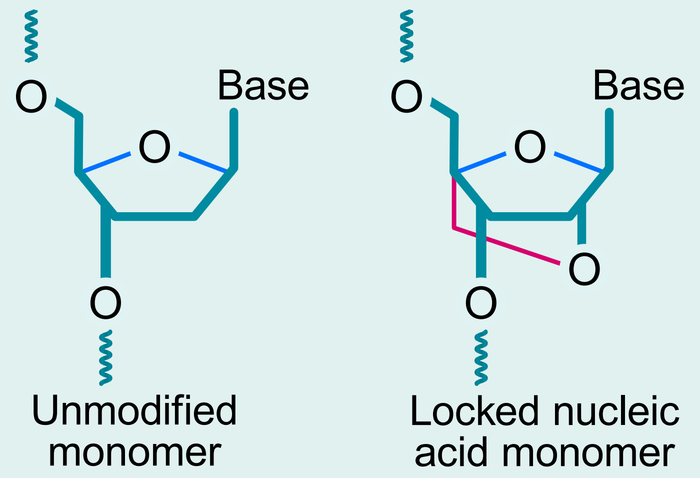
Hydrolysis probes can be amended for increased binding stability or signal specificity. For example, the BHQnova™ incorporates two quenchers to facilitate longer probe lengths without elevated background signal noise, while BHQplus™ probes use modified C and T nucleotides and are purposefully shorter in order to enhance specificity and improve mismatch discrimination.



Minor Groove Binders (MGBs)

MGBs selectively bind non-covalently to the minor groove, a shallow furrow in the DNA helix. Dual-labelled probes conjugated with MGB groups form extremely stable duplexes with single-stranded DNA targets, enabling shorter probe lengths and superior quenching.[1]



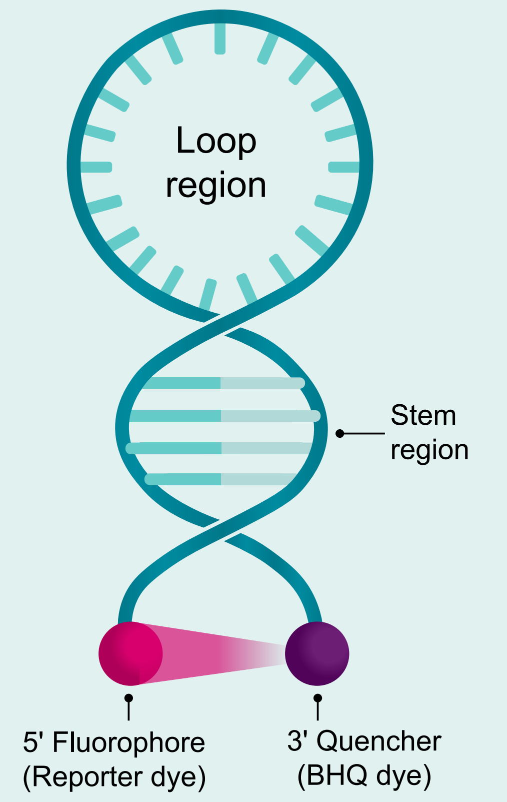


Locked Nucleic Acids (LNAs)

LNAs are modified RNA monomers that contain a methylene bridge bond linking the 2′ oxygen to the 4′ carbon in the RNA pentose ring that fixes the ring's conformation. When incorporated into probes, LNAs increase hybridisation melting temperatures, allowing for shorter probe lengths and enhanced sensitivity.

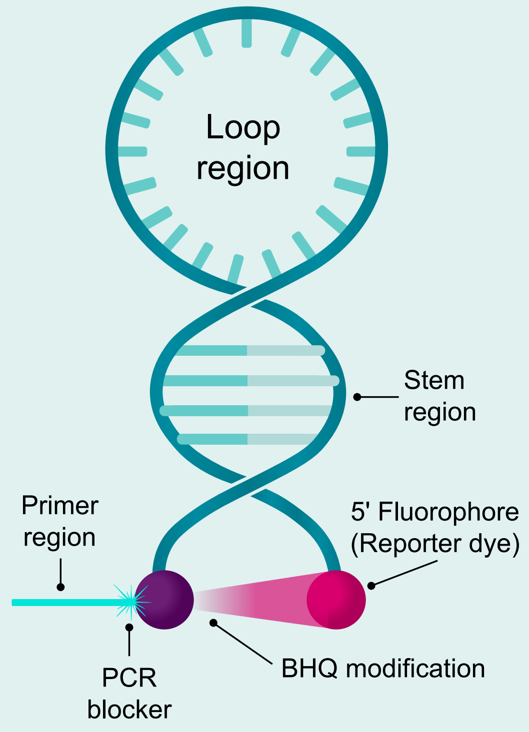
Molecular Beacons

Molecular beacons contain a target-recognition loop region flanked by two short complementary stem sequences containing a quencher and fluorophore respectively. The stem-loop structure brings the quencher and fluorophore into close proximity until target hybridisation, which opens the loop, separates the fluorophore and quencher, and enables fluorescence emission. Molecular beacons offer excellent stability and selectivity.[2]



Scorpions™ Primers

Scorpions™ primers also use a hairpin-loop conformation to quench fluorophore signal until hybridisation. This hairpin-loop structure is directly conjugated to the 5′ end of a PCR primer, with a blocker preventing extension in the wrong direction. Scorpions primers unfold and hybridise with newly synthesized target sequences during the second qPCR cycle and beyond, allowing for signal emission and detection.



FRET Probes

Fluorescence resonance energy transfer (FRET) probe systems consist of a pair of single-stranded fluorescent-tagged oligonucleotides. During qPCR annealing, these probes hybridise to their target regions, bringing the two probes—and their tags—into close proximity. Light excitation of the donor tag results in energy transfer via FRET to the acceptor tag, facilitating signal emission and detection.

References

1. I.V. Kutyavin et al., "3'-minor groove binder-DNA probes increase sequence specificity at PCR extension temperatures," *Nucleic Acids Res*, 28(2):655-61, 2000.
2. K. Wang et al., "Molecular engineering of DNA: molecular beacons," *Angew Chem Int Ed Engl*, 48(5):856-70, 2009.

Troubleshooting probe-based qPCR

While making an informed probe decision can ward off many potential problems, it is not the only factor affecting qPCR success or failure. Indeed, troubleshooting and maintenance is a part of all experiments, and qPCR is no exception. Troubleshooting qPCR involves understanding what can potentially cause experimental anomalies and discrepancies, the controls and metrics used to evaluate qPCR data integrity, how to recognise potential issues when they appear in the data, and ways to remedy issues and prevent them from occurring.

An ounce of prevention

Proper routine maintenance of qPCR instrumentation is important for data consistency and reliability and can identify potential problems before they affect experimental runs. Heating block function is critical for thermocycling, and variations can dramatically affect amplification. It is important to routinely test heating block function—both from a general standpoint and to detect potential discrepancies between different wells. Moreover, researchers should also examine the power supply, as surges or other fluctuations can affect heating block performance. Next, lamps and optical sensors should be routinely inspected and calibrated to ensure signal capture efficiency and consistency. This is especially important for researchers who employ multiplexing and compare data gathered across different detection channels. Software plays a large role in qPCR data acquisition, processing, collation, and analysis. Researchers should make sure that instrument firmware and analysis software suites are up to date and fully compatible with one another. Finally, the correct consumables are extremely important whether shipping/storing probes (using amber tubes can limit light exposure) or running the assay (using frosted or white tubes can prevent signal refraction).

Looking at one's tools

Most qPCR reagents are compatible with most qPCR instruments and applications. However, each instrument and application has their own optimal experimental conditions, and reagents play a large role in that optimisation. For example, $MgCl_2$ concentration in qPCR master mix impacts polymerase function, which in turn affects amplification and hydrolysis probe-cleavage efficiency. Similarly, primer and probe hybridisation efficiency is heavily affected by melting temperature (T_m) and concentration. Lower T_m values increase yields at the expense of specificity, while lower primer/probe concentrations increase specificity at the expense of yield.

While reagent and instrument manufacturers provide helpful starting points for optimisation by way of documentation and pre-assembled kits, kits have to be optimised for the specific experimental conditions and requirements of a given laboratory and its researchers. This, inevitably, involves some trial-and-error. Scientists often design or acquire reagents with a fixed value in mind. For example, most primers are designed to have a T_m around 60 °C, with most hydrolysis probes aiming for a T_m roughly 5-10 °C above that. Researchers then adjust other parameters, such as concentration, around that fixed value and look at how those adjustments affect amplification as reflected by C_q values.[1] Melt curves are also useful during this process to examine amplification specificity, detect primer dimers, and confirm the validity of desired C_q values.

Establishing control

qPCR experiments should also have myriad controls to assess different aspects of each assay. Negative controls screen for DNA contamination in a sample, whether genomic DNA, exogenous complementary DNA, or primer/probe dimerisation. The most general type of negative control is the no-template control, but researchers should also employ no-reverse transcription controls to look at contamination during RNA preparation and no amplification controls to look at background fluorescence and probe integrity.

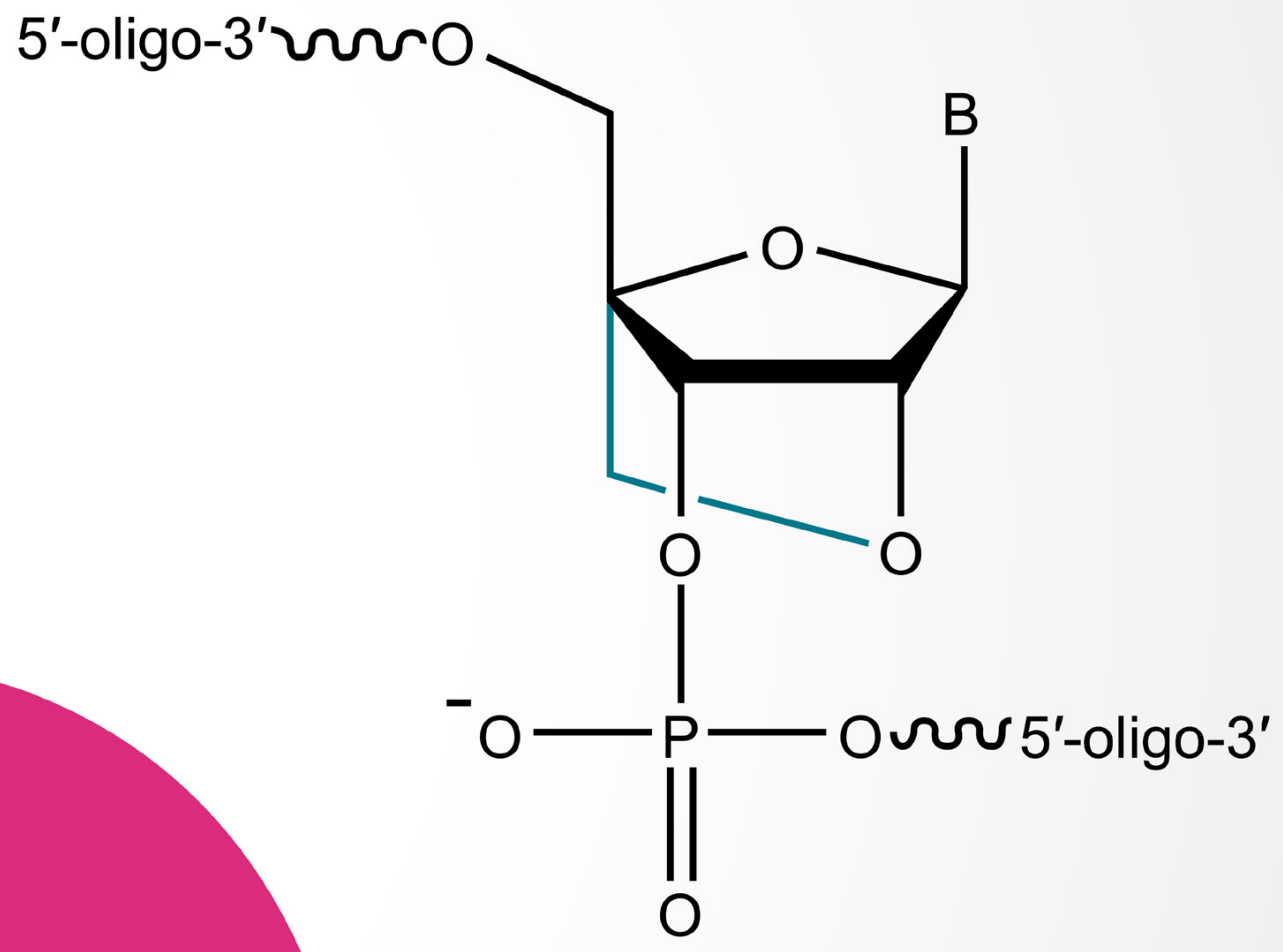
Positive controls give confidence to negative experimental results and are particularly important when measuring low copy numbers. Positive controls also help assess assay efficiency and detection thresholds and can signal the presence of reaction inhibitors. Control samples should reflect the test samples as much as possible: water-based controls are not ideal for blood-sourced samples, for example. It is important to prepare negative and positive controls alongside test samples using the same procedures, as well as run them at the same time.

Setting a standard for the future

qPCR involves many components and aspects, and scientists must be aware of the potential challenges that can arise in a variety of areas. In light of this, researchers have been working to standardise qPCR. The MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) guidelines,[2] first published in 2009, provides an extensive and thorough look at best practices, troubleshooting, process optimisation, ensuring accuracy and reproducibility, and data interpretation and transparency. Efforts such as these, along with technical support from reagent and instrument manufacturers, help guarantee that qPCR continuously becomes less cumbersome, more accessible, and just as reliable as time passes.

References

1. T. Nolan et al., “Good practice guide for the application of quantitative PCR (qPCR),” *LGC*, 2013.
2. S.A. Bustin et al., “The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments,” *Clin Chem*, 55(4):611-22, 2009.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