Probe-based qPCR: Probe Compatibility and Multiplexing with Luna® Universal Probe qPCR Master Mix

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INTRODUCTION

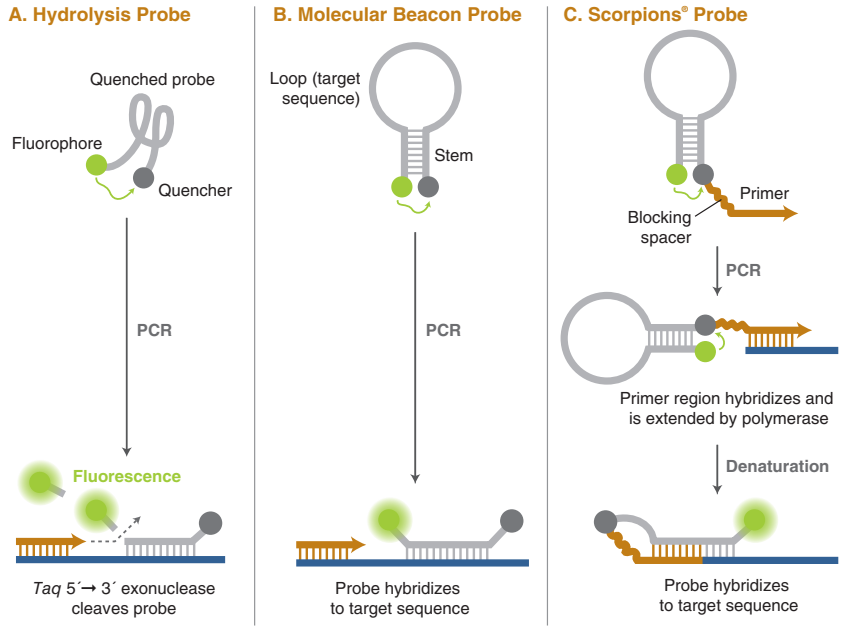
Quantitative PCR (qPCR) is a commonly used molecular biology technique that enables the precise determination of the amount of DNA or RNA in a sample of interest. By measuring the amount of DNA present at each cycle of a PCR, the concentration in an unknown sample can be calculated by comparison to a set of known standard samples. This real-time measurement of the PCR product is achieved using fluorescent reporters, broadly grouped into two classes:

1) intercalating dyes (e.g., SYBR® Green I) that significantly increase their fluorescence intensity when bound to double-stranded DNA (dsDNA)
2) dual-labeled (fluorophore and quencher) oligonucleotide probes that fluoresce via various mechanisms after annealing to a specific sequence in the target DNA

Although there are several mechanisms by which probes can be used (discussed in detail below), the major distinction between the two classes centers on specificity: SYBR and intercalating dyes detect all dsDNA produced during PCR, whereas probes are specific to a particular region of a target sequence. Accordingly, any nonspecific synthesis that occurs in a reaction is detected by intercalating dyes but not by probes, as generation of probe fluorescence signal is dependent upon specific annealing. Denaturation or "melt" curves at the end of the PCR cycling protocol permit detection of nonspecific products using dye-based methods, but the ability to verify the nonspecific product does not prevent it from occurring and interfering with quantitative analysis. Some probe types can be analyzed using denaturation curves (e.g., hybridization probes), but other probes, including hydrolysis (i.e. TaqMan™) probes may require gel-based or other analysis methods to confirm reaction specificity. Additionally, probe-based methods enable the specific detection and quantitation of multiple targets in a single reaction, commonly referred to as "multiplexing." The ability to multiplex reactions allows for internal controls or standards to validate reactions and for the measurement of numerous targets simultaneously in one reaction. Many qPCR fluorimeters are designed to measure fluorescence from a spectrum of different fluorophores simultaneously, and probes can be designed for multiple targets and synthesized with different fluorophore-quencher pairs that can be monitored independently. Assay performance should be validated with each amplicon independently and in combination, and a qPCR master mix formulated to maintain high performance in multiplex reactions is essential to performance.

FIGURE 1:
Schematic depiction of various qPCR probe chemistries

A) Hydrolysis (TaqMan) Probe: Initial quenched state results from coiling of ssDNA, but during amplification stages probes anneal to target sequence. As *Taq* DNA Polymerase extends from the forward primer, it encounters the 5´ end of the annealed probe, and cleaves the fluorophore moiety from the probe through 5´→3´ flap endonuclease activity, increasing fluorescence signal with each cycle. B) Molecular Beacon: Probes contain a stem-loop structure which anneals, bringing quencher and fluorophore in close proximity and quenching fluorescence. During amplification, copies of target sequence are amplified, allowing annealing of probe via the target complement, separating the stem-loop and producing fluorescence. C) Scorpions® Probe: Similar to the beacon, a stem-loop structure quenches probe fluorescence. A 3´ extension of the stem serves as a primer in the amplification reaction, and after extension from the 3´ primer end probe target region anneals downstream and generates fluorescent signal.



COMMON PROBE TYPES

Hydrolysis Probes

Perhaps the most commonly used probe is the hydrolysis probe (i.e., TaqMan probe). A typical hydrolysis probe consists of a short (10–30 base) oligonucleotide comprising a sequence in between the primers of a particular target amplicon, synthesized with modified 5´ and 3´ ends to have a fluorophore and corresponding dark quencher (e.g. Black Hole Quencher®, LGC Biosearch; Iowa Black®, IDT; or DABCYL). Some more recent probe designs include an additional internal quencher (e.g. Zen® quenchers). In solution, these single-stranded oligos are entropically collapsed and the fluorophore and quencher are kept in close proximity, resulting

in a quenching of the fluorescent signal. As amplification proceeds and the target sequence is produced in increasing number, the sequence-specific probes anneal to the complementary strand. As *Taq* DNA Polymerase extends the primer upstream to the probe, it will encounter the 5′ end of the probe. *Taq* DNA Polymerase intrinsically contains a 5′→3′ exonuclease (or flap endonuclease) domain, and upon encountering the labeled 5′ end degrades the probe through nuclease hydrolysis (Figure 1A). As the 5′ end is cleaved, the quencher and fluorophore moieties are permanently separated, producing fluorescence that accumulates in proportion to the increasing number of copies in the reaction.

Molecular Beacon

Beacon probes are superficially similar to hydrolysis probes, with a short oligonucleotide sequence labeled with a fluorophore and quencher on the 5′ and 3′ ends respectively. However, the sequence of the beacon is arranged such that flanking each side of the central 15–25 bases that anneal to the target sequence are short, complementary non-target stretches that form a stem-loop structure in solution (typical stem is ~7 bp). This stable structure brings the labeled 3′ and 5′ ends of the beacon close together, resulting in fluorescence quenching. When the target sequence is present, the beacon will hybridize to the target, separating the stem ends and producing fluorescence (Figure 1B). Optimization of the beacon and primer concentrations, and the extension temperature is critical to beacon assay performance.

Scorpions® Primers/Probes

Scorpions also utilize a stem-loop beacon structure, but this beacon is covalently linked via a blocking spacer group to the 5′ end of one of the primers. The Scorpions probe is thus a bifunctional molecule, performing roles of both primer and probe in a single oligonucleotide. In the first round of amplification, the primer region functions as any primer, binding to target sequence with subsequent extension by the DNA polymerase. After denaturation in the subsequent cycle, the target sequence present upstream in the beacon region binds intramolecularly downstream of the primer, spatially separating the quencher and fluorophore to enable detection. The arrangement of the beacon and its ability to bind downstream gives the method its name (see Figure 1C). Though more difficult to synthesize, as the probe contains a blocking spacer group and internal quencher, only two oligonucleotides are necessary for the reaction and less optimization may be required.

PROBE CONSIDERATIONS AND RESOURCES

qPCR assays using any probe type require use of efficient, well-designed oligonucleotide primers and probes. For standard qPCR assays, Luna qPCR mixes perform well with primers that have a T_m of approximately 60°C, which is typical of most primer sets used for qPCR. However, the nature of the different probe types imposes other considerations and limitations. Fortunately, numerous resources are available to facilitate successful probe qPCR. When designing a new assay, use of qPCR design software is strongly recommended, and many programs will choose an optimal probe location in the amplicon. Many targets have been used in qPCR assays by others, and databases such as RTPrimerDB (www.rtprimerdb.org) collects primers and probes for thousands of targets from different organisms, including several probe types and assays for different applications (e.g., CNV, gene expression, etc.).

Fluorophore choice is also important for any probe qPCR. The most common fluorophore is 6-carboxyfluorescein (FAM), as it is spectrally compatible with the SYBR detection channel of standard qPCR instruments. Paired with an appropriate quencher, FAM is a robust and reliable fluorophore for probe qPCR. For instruments with multiple fluorescence channels, appropriate fluorophores should be chosen to pair with the desired channels. For example, Cy5 can be used in Channel 4 of a Bio-Rad CFX Touch™ instrument and Channel 5 of an Applied Biosystems™ 7500 instrument. ROX is a bright fluorophore readily available for conjugation to oligonucleotides, but is used as a reference dye for normalization in some instruments (e.g. ABI® StepOne™, 7500). The Luna mixes contain a universal ROX reference dye, which requires normalization to be turned off if a ROX-labeled probe is used in a qPCR assay (see Figure 2, page 3).

OPTIMIZATION OF PROBE qPCR

Each probe type and application is unique, therefore different conditions and considerations should be used for assay optimization. Although the Luna qPCR products have been optimized for the most commonly used type of probe (hydrolysis/TaqMan), they are also compatible with other probe chemistries. Here we present examples of multiplexing with the Luna products and the simple optimizations that were used to achieve best performance with alternative probe designs.

Hydrolysis Probes and Multiplexing

As with most commercial qPCR mixes, the Luna products recommend the use of 0.4 µM each forward and reverse primer, with 0.2 µM hydrolysis probe. These conditions along with an extension step of 30s at 60°C generally supports efficient qPCR results. An example assay using a FAM-labeled probe to detect GAPDH from cDNA produced using Jurkat total RNA is shown in Figure 2. Figure 3 (page 4) shows the same GAPDH detection reaction as part of a multiplex assay, where standard conditions (without optimization) were used to simultaneously detect 3 targets using FAM (GAPDH), HEX (L32g), and Cy5 (SMG1) fluorophores. Importantly, as can be seen by comparing C_q and efficiency of the reactions, performance is maintained when all three amplicons are included in the multiplex reaction. These criteria should be evaluated by running single- and multiplex reactions for any amplicon chosen for use in a multiplex assay.

If copy numbers vary significantly for the different targets in a multiplex reaction, it can prove helpful to adjust primer concentrations to achieve a more uniform amplification. In some cases, with differential copy number or amplicon performance, PCR efficiencies or sensitivity can vary accordingly with the different targets. However, primer concentrations of 100–900 nM and probe concentrations of 100–500 nM can be used to optimize multiplex reactions with disparate copy number targets if one or more of the amplicons shows suboptimal performance. Lower primer/probe concentrations can be used for very high copy targets along with higher concentrations for the low copy targets to provide a more balanced reaction. Pairing lower fluorescence dyes with high abundance targets and bright dyes with lower abundance targets may also be useful.

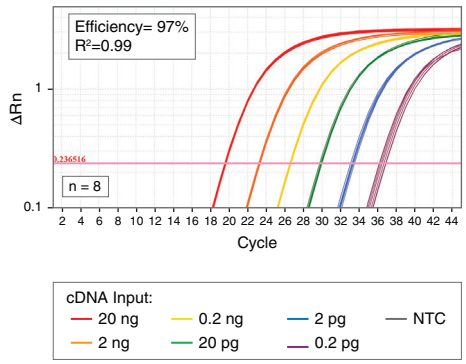
To enable compatibility with instruments that recommend ROX normalization, the Luna qPCR products contain a universal ROX reference dye for use with both high ROX and low ROX instruments. Despite the presence of ROX in the mix, a ROX-labeled probe may still be used. However, the settings of the qPCR instrument should be considered, and for instruments where ROX normalization is used (e.g. ABI instruments) the normalization setting should be set to "None" for a ROX probe. For ROX-independent instruments (e.g. Bio-Rad CFX) simply select the ROX fluorophore where applicable. An example of ROX (SRY) probe performance is shown in Figure 2, with the ROX data obtained on a CFX-96 Touch in a 3-plex reaction containing HEX and Cy5 amplicons.

FIGURE 2:

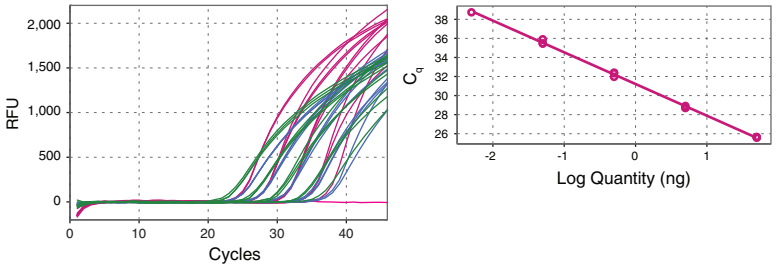
Performance of Luna Universal Probe qPCR Master Mix with hydrolysis probes

A) Amplification curves produced using a GAPDH target and probe. Six dilutions of cDNA produced from Jurkat total RNA were each run using 8 replicates of each dilution. B) Luna Probe Mix is compatible with detection of a ROX-labeled probe, with no interference of the universal passive reference dye. SRY target was detected using ROX probe in the presence of GAPDH (FAM) and L32g (HEX) targets. C) Three different probes and amplicons were amplified separately (left) or together in a 3-plex reaction (right). Performance of all targets was maintained when combined in a single reaction.

A. Hydrolysis Probe: GAPDH cDNA

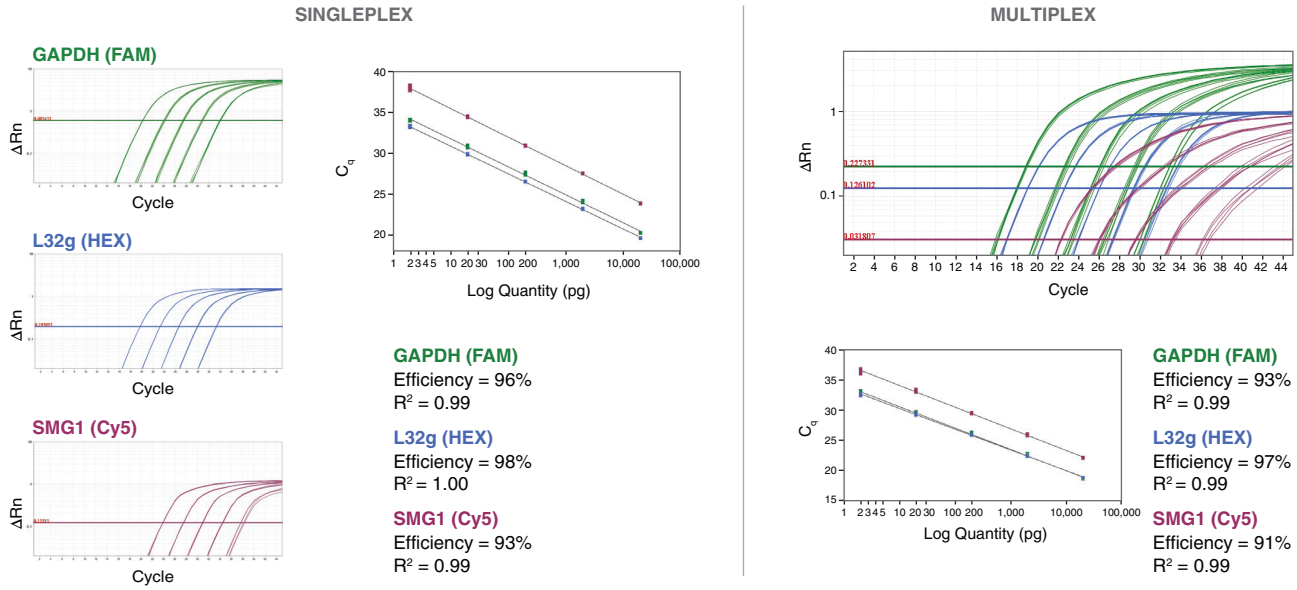


B. ROX Probe



SRY (ROX)
Efficiency=100%
R^2=0.99

C. Hydrolysis Probe: Multiplexing



GAPDH (FAM)
Efficiency = 96%
R^2 = 0.99

L32g (HEX)
Efficiency = 98%
R^2 = 1.00

SMG1 (Cy5)
Efficiency = 93%
R^2 = 0.99

GAPDH (FAM)
Efficiency = 93%
R^2 = 0.99

L32g (HEX)
Efficiency = 97%
R^2 = 0.99

SMG1 (Cy5)
Efficiency = 91%
R^2 = 0.99

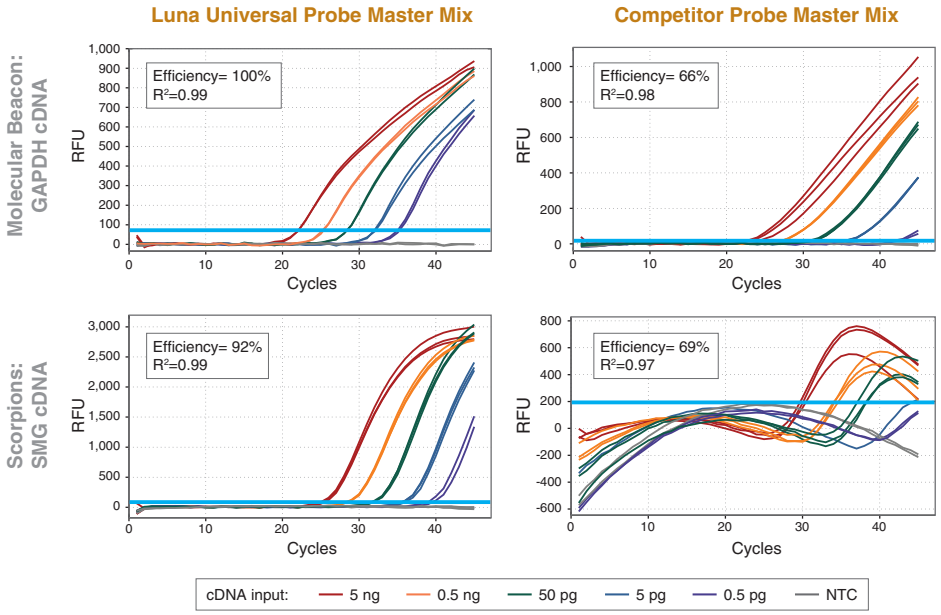
Other Probe Types

For reactions involving less common probe types, adjustments to the standard conditions may be necessary to achieve optimal performance. For example, we evaluated Luna and a multiplex-compatible competitor probe mix using a molecular beacon assay selected from RTPrimerDB targeting GAPDH transcripts from human cDNA/RNA. As an initial test, standard probe conditions were used, but unsatisfactory detection and amplification were observed for both mixes. Luna achieved a PCR efficiency of 73%, with low fluorescence values and inconsistency at low input, while the competitor mix failed to provide any interpretable data (data not shown). To optimize beacon assays, both primer/probe concentration and annealing temperature can be adjusted to improve performance. As hybridization stability is key to the detection of a molecular beacon, a gradient between 50–60°C was conducted, followed by a melting/denaturation curve covering 40–95°C to determine a temperature that produced optimal fluorescent signal during amplification. Results from reactions at 56°C showed improved performance, as did increasing the primer and probe concentration to increase fluorescence and amplification efficiency. Best results for this target were obtained using 900 nM forward and reverse primer with 600 nM beacon, and an example of these conditions at 56°C is shown in Figure 3. Using these straightforward optimizations, Luna was able to achieve 100% efficiency, whereas the competitor mix achieved only 66% efficiency and failed to amplify the low input cDNA samples.

Similarly, a Scorpions assay with FAM-labeled primer-probe targeting SMG in human cDNA/RNA was tested using Luna and a competitor probe mix. The probe was designed to contain a gene-specific 3´ region, a C18 blocking spacer, and a stem-loop structure with internal dark quencher and 5´ FAM (Biosearch). In a Scorpions assay, only two oligonucleotides are present, and various concentrations were tested to achieve optimal performance. As with the Molecular Beacon assay, standard conditions were tried initially. Luna achieved 90% efficiency although with low RFU values, while the competitor mix failed to provide signal above background. To improve performance, concentrations from 200–900 nM primer/probe were evaluated, and produced efficient amplification, although fluorescence and curve shape differed due to varying concentrations of the primer/probe. Best results for this target were seen using 600–900 nM each reverse primer and forward Scorpions primer. Again Luna could be easily optimized to achieve high efficiency (Figure 3, 92%) but the same attempts to optimize the competitor mix were only able to achieve 60% efficiency and with poor curve shape.

FIGURE 3:
Performance of Luna Probe Master Mix with Molecular Beacon and Scorpion Probes

To obtain highest efficiencies and performance, reaction parameters were adjusted from standard conditions. 900 nM primers and 600 nM beacon with a 30 second extension at 56 °C enabled 100% efficiency and detection of 5 pg target using Luna, while only providing slight improvement to the competitor mix (right). Luna achieved 92% efficiency and sensitive detection using 600 nM Scorpions probe and reverse primer, while the competitor mix failed with low input and produced poor curve shape.



CONCLUSION

Probe-based qPCR is a powerful diagnostic and quantitative tool, and a variety of approaches and chemistries exist. As demonstrated here, the Luna Universal Probe qPCR Master Mix can be successfully used with a variety of probe types, including hydrolysis and hybridization probes. Probe assays are inherently compatible with multiplexing, and the Luna Probe mix supports detection of multiple targets by simply including primers and probes for the different targets using spectrally distinct fluorophores. Standard conditions (400 nM primer, 200 nM probe, 30s 60°C extension step) support efficient qPCR for most targets, but some multiplexing may involve using targets with significantly different copy numbers or assays with unequal performance, and assay parameters can be adjusted accordingly (e.g., reducing concentration of the high copy target primers when multiplexed). Hydrolysis probes generally follow the standard guidelines, but when hybridization probes are used, optimal concentration of each target should be determined empirically, with consideration given to the effect of annealing temperature for a particular probe. Particular amplicons may require deviation from these conditions for optimal performance, and use of up to 900 nM primer and 500 nM probe is recommended to achieve highest detection and efficiency. In summary, Luna Universal Probe qPCR Master Mix provides a robust solution for probe-based qPCR using multiple targets and a variety of probe chemistries.

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