Roche Applied Science

Technical Note

No. LC 18/2004

Assay Formats for Use in Real-Time PCR

Purpose of this Note

The LightCycler Instrument uses several detection channels to monitor the amplification of PCR products in real-time and online. For maximum flexibility, the LightCycler System supports several fluorescent analysis formats and can use a broad range of probes and dyes:

- SYBR Green I (dye to detect all double-stranded [ds] DNA)
- SimpleProbe probes (single, sequence-specific, single-labeled probe)
- Hybridization probes (pair of sequence-specific, single-labeled probes)
- Hydrolysis probes (sequence-specific, double-labeled probes; commonly called "TaqMan" probes)

The flexibility of the LightCycler System allows a variety of sophisticated analyses. For example:

- During PCR, the instrument uses discrete fluorescence measurements (once per cycle) to estimate starting copy number.
- After PCR, the instrument continuously monitors fluorescence, while slowly heating the sample, to provide qualitative information about the product.
- By analyzing the melting of short duplexes, such as those formed with hybridization probes, the LightCycler Instrument can identify even single base alterations in the amplicon.

The LightCycler 2.0 Instrument has expanded possibilities and can simultaneously analyze signals from multiple dyes, *e.g.* for multiplexing experiments.

The LightTyper Instrument was specifically designed for SNP-detection and genotyping using HybProbe (LightCycler Red 640) and SimpleProbe probes.

This Technical Note will describe four different LightCycler assay formats and show applications for each.

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1. Principles of Fluorimetric Online Detection

Introduction

All real-time PCR systems detect a fluorescent dye, then correlate this fluorescence signal with the amount of PCR product in a reaction. There are several methods for detecting and evaluating fluorimetric PCR reactions. The most commonly used fluorescence formats fall into two classes:

1. Sequence-Independent Detection Assays

Rely on fluorophores that bind to all double-stranded DNA molecules regardless of sequence; for example:

- SYBR Green I
- Ethidium bromide (not used in LightCycler PCR due to low sensitivity and specificity)

2. Sequence-Specific Probe Binding Assays

Rely on fluorophores coupled to sequence-specific oligonucleotide hybridization probes that only detect certain PCR products; for example:

- Single labeled probes (SimpleProbe format)
- Hybridization probes (HybProbe format)
- Hydrolysis probes ("TaqMan" format)

Note: Other assay formats may also be adapted for real-time PCR or used in the LightCycler Instrument. For example, adaptable probe formats include Bi-Probes (iFRET-Probes), Molecular Beacons and Scorpions™. These will not be described in this Technical Note.

2. Sequence-Independent Detection with SYBR Green I

Monitoring PCR with the SYBR Green I Dye

One simple and inexpensive approach to real-time PCR is based on SYBR Green I, a fluorescent dye that binds to dsDNA. SYBR Green I barely fluoresces when it is free in solution, but its fluorescence emission is greatly enhanced when it binds to DNA (due to conformational changes in the dye). Thus, the increase in SYBR Green I signal (measured at 530 nm) correlates with the amount of product amplified during PCR.

This assay format can easily be applied to well-established PCR assays, because it does not require additional fluorescence-labeled oligonucleotides. However, since this format detects both specific and non-specific PCR products, the assay format must be carefully optimized and the product must be identified after the PCR run.

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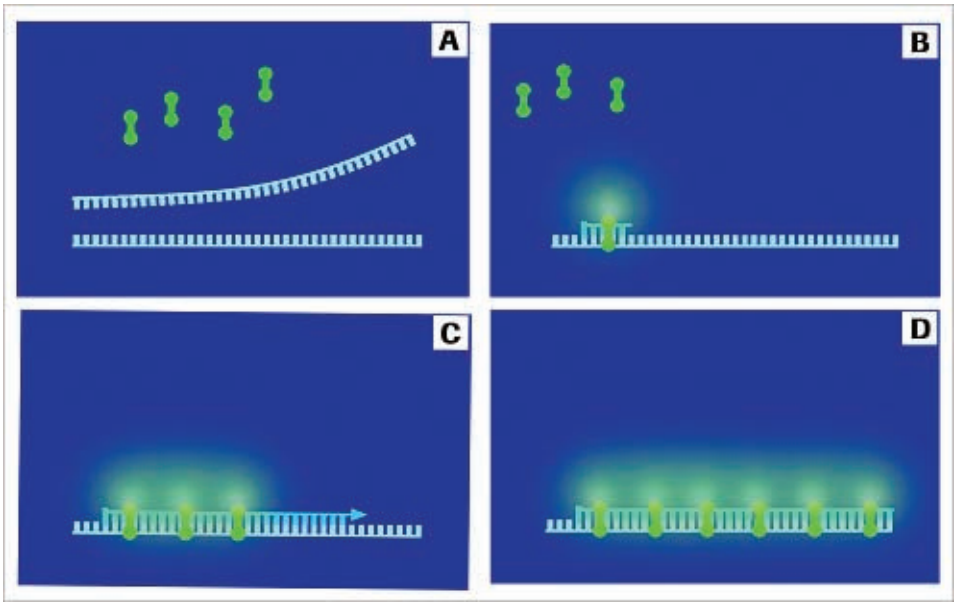
2. Sequence-Independent Detection with SYBR Green I continued

Note: For more information, see Technical Note LC 01/update 2002 "Optimization of Reactions to Reduce Formation of Primer-Dimers" and LC 9/2000 "Optimization Strategy."

Performing PCR with SYBR Green I

When SYBR Green I binds to dsDNA, its fluorescence emission increases over 100-fold. During the various stages of PCR, the intensity of the fluorescence signal will vary, depending on the amount of dsDNA that is present. After denaturation, all DNA becomes single-stranded (Figure 1A). At this stage of the reaction, SYBR Green I dye will not bind and the fluorescence intensity is low. During annealing, the PCR primers hybridize to the target sequence, creating small regions of dsDNA that SYBR Green I dye can bind, thereby leading to increased fluorescence (Figure 1B).



△ **Figure 1A-D**: PCR in the presence of SYBR Green I

In the elongation phase of PCR, PCR primers are extended and more SYBR Green I dye can bind (Figure 1C). At the end of the elongation phase, the entire DNA is double-stranded and a maximum amount of dye is bound (Figure 1D).

For optimal results, the fluorescence signal (at 530 nm) needs to be acquired at the end of each elongation phase. Increasing amounts of PCR product can then be monitored from cycle to cycle.

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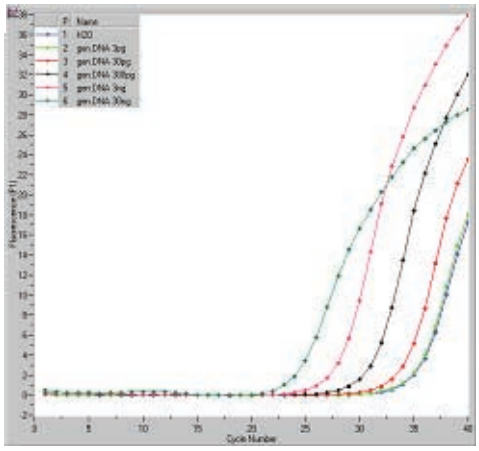
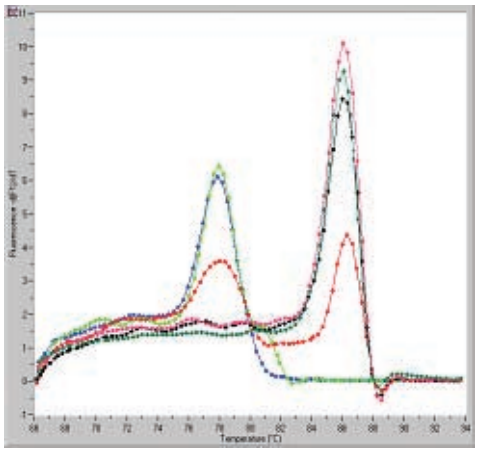
2. Sequence-Independent Detection with SYBR Green I continued

SYBR Green I - Application Example

2A



2B



△ **Figure 2A:** Using SYBR Green I to detect amplification of β-actin from different starting amounts of human genomic DNA. Note that a signal is also detected in the sample that contains no specific target sequence (see sample 1, "H_2O" = no template control); this signal is generated by primer-dimers.
△ **Figure 2B:** A melting curve analysis, the only way to distinguish product from primer-dimers, must always be included in each SYBR Green I program. In this example, various amounts of primer-dimer signal contribute to the amplification curves (see samples 1, 2 and 3).

Limitation

Since SYBR Green I binds to any ds DNA, like *e.g.* ethidium bromide, the SYBR Green I format cannot discriminate between different dsDNA species. The specific product, non-specific products and primer-dimers are detected equally well. Any double-stranded PCR artifact contributes to signal intensity, which may result in overestimation of the concentration of the target sequence. However, a melting curve analysis is an appropriate tool to discriminate between product and primer-dimer (or other artifact) and should always be included in the SYBR Green I program. For quantification, the PCR must be optimized and artifact-free. (See Technical Note LC1/update 2002.)

3. Sequence-Specific Probe Binding Assays

Introduction

A PCR assay that uses sequence-specific probes labeled with fluorophores can be highly specific, since fluorescence increases only if the specific target is present in the reaction. Product identification by melting curve analysis is usually not required.

Due to this sequence specificity, artificial by-products (such as primer-dimers or PCR by products) will not be detected. However, they may be present in the reaction and affect the sensitivity and efficiency of the PCR.

3.1 SimpleProbe Format

SimpleProbe Format for SNP Genotyping and Mutation Detection

Single-labeled probes are a special type of simplified hybridization probe that can detect mutations and SNPs (single nucleotide polymorphisms). The so-called SimpleProbe format requires only one hybridization probe, labeled with only one fluorophore (Fluorescein), to achieve sequence specificity. Typically such a probe is designed to specifically hybridize to a target sequence that contains the SNP of interest. Once hybridized to its target sequence, the SimpleProbe probe emits more fluorescence than it does when it is not hybridized. As a result, changes in fluorescence are based solely on the hybridization status of the probe.

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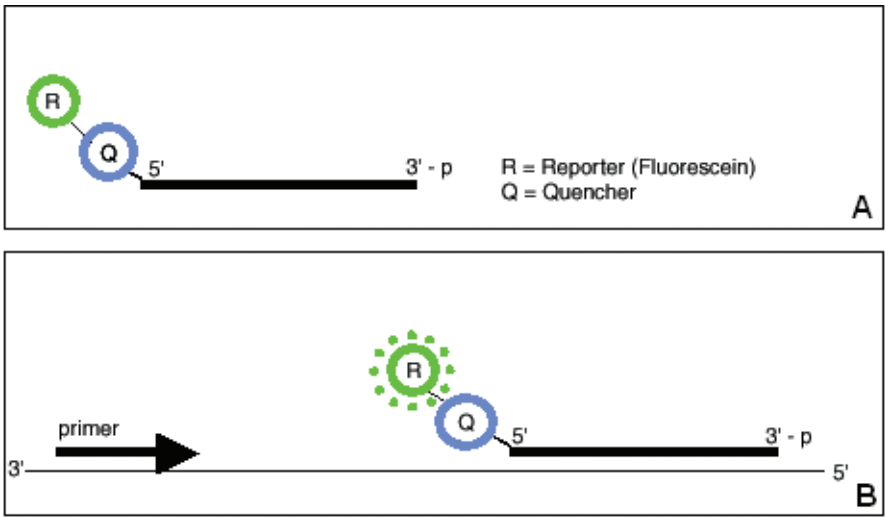
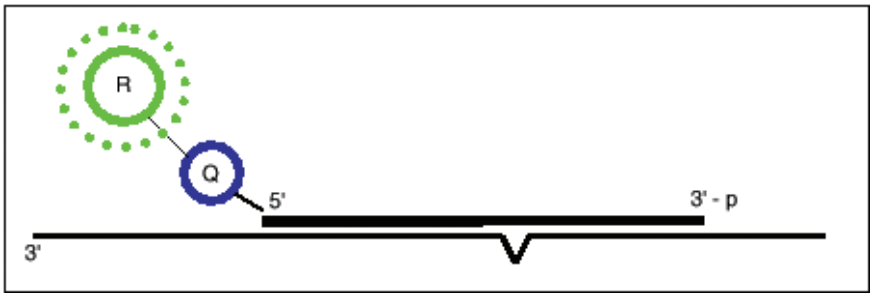
3.1 SimpleProbe Format continued

SimpleProbe probes are an excellent tool for SNP genotyping and mutation detection because they readily identify wild-type, mutant, and heterozygous samples with only a single short probe.



△ **Figure 3:** Principle of SimpleProbe Detection

Figure 3 illustrates how SimpleProbe detection works. A SimpleProbe probe may be labeled at either its 3´- or 5´-end. If a SimpleProbe is free in solution, emission of the reporter dye is quenched by a specific, non-fluorescent quencher (situation A). When the probe hybridizes to its target, quenching is reduced and Fluorescein, when excited by the LED of the LightCycler Instrument, emits green fluorescence (situation B). However, even when the probe is not hybridized, background fluorescence is detectable at 530 nm, so the signal-to-noise ratio is low.



△ **Figure 4:** SNP Detection with SimpleProbe Format

For SNP analysis, the LightCycler Instrument monitors the melting behavior of the Simple Probes (Fig. 4). By measuring the fluorescence, the instrument can detect melting of the probe-target hybrids as the temperature increases. The more stable the hybridization between SimpleProbe and target sequence, the higher the melting temperature. Mutations like SNPs weaken the stability of SimpleProbe binding.

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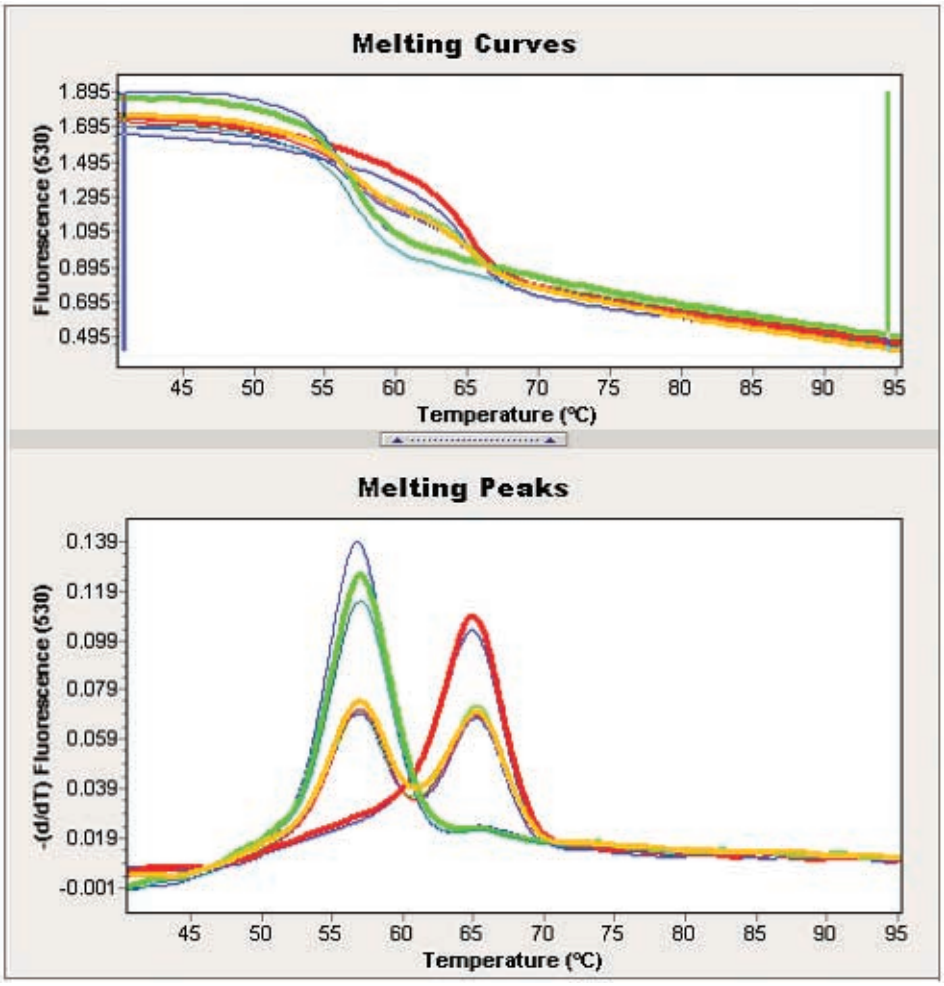
3.1 SimpleProbe Format continued

SimpleProbe – Application Example: Melting Curve Analysis

5A



5B

△ **Figure 5A:** Graph A shows sample fluorescence versus temperature (melting curve data). In each sample, signal fluorescence clearly decreases as temperature increases, reflecting the quenching of the SimpleProbe signal as the probe is displaced from its target.

△ **Figure 5B:** Graph B plots the first negative derivative of the sample fluorescence versus temperature, which shows the melting temperature of each sample as a peak. In this example, the assay can clearly differentiate the wild-type samples (higher melting peaks that reflect a perfect match between target sequence and probe) from the mutant samples (lower melting peaks that reflect sequence mismatches between target and probe).

3.1.1 Design Considerations for SimpleProbe Probes

Design of SimpleProbe Probes

To design a successful SimpleProbe, pay particular attention to the reverse complementary region (DNA region that the probe binds). Use an artificial reverse complementary oligo to test the detection capabilities of the probe. The interior of the SimpleProbe probe should always cover the mutation site; do not let the sequence that covers the mutation be near either the 3´- or 5´-end of the probe. The region of the target that is directly below the fluorescence label (positions -1 and -2) should contain C or T; avoid having a G at position -1 or -2 (figure 6). Do not allow wobble bases at positions 1 – 4 to create a stable alignment of the fluorescence dye with the double-stranded DNA.

Note: In some cases, secondary structure may affect proper functioning of single-labeled probes. In such cases we recommend using Hybridization Probes rather than SimpleProbe probes.

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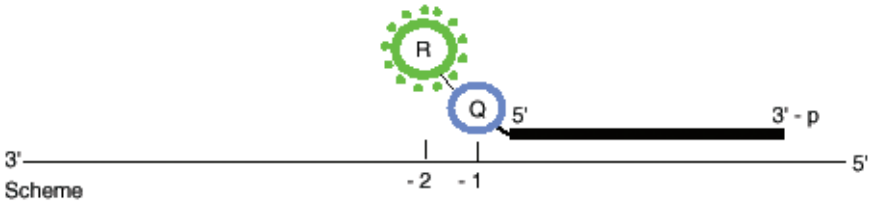
3.1.1 Design Considerations for SimpleProbe Probes continued



△ **Figure 6**: Design of SimpleProbes

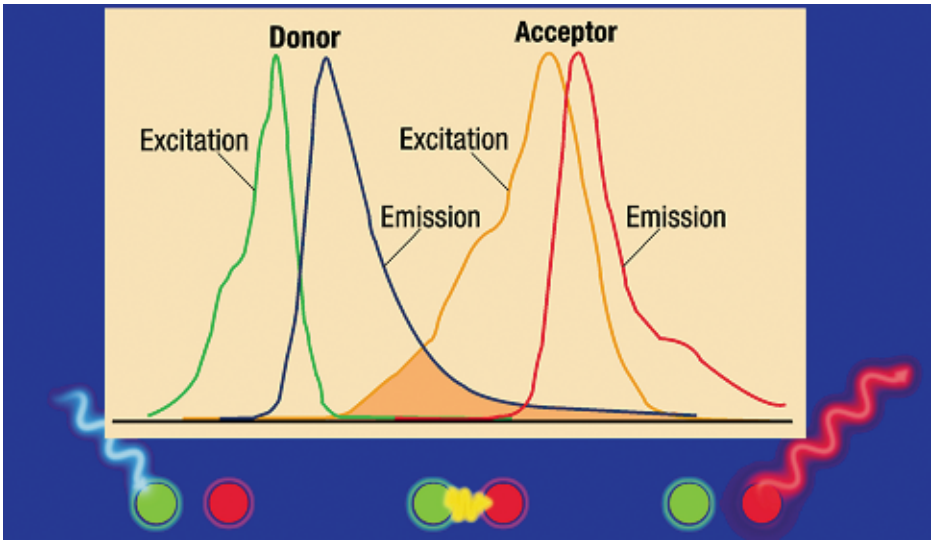
3.2 Sequence-Specific Probe Binding Assays Using FRET

Fluorescence Resonance Energy Transfer (FRET)

Most sequence-specific probe formats use the so-called FRET principle. Fluorescence Resonance Energy Transfer (FRET) is based on the transfer of energy from one fluorescent molecule (fluorescein) to another adjacent fluorescent molecule (*e.g.*, LightCycler Red 640). When the blue LED of the instrument excites fluorescein (at 470 nm), it leads to a transfer of energy and subsequent excitation of the LightCycler Red 640 molecule (which is not directly affected by the blue LED). The light emitted by the LightCycler Red 640 molecule is measured at 640 nm.

Primary conditions for FRET are:

- Donor and acceptor molecules must be close to each other
- Excitation spectrum of the acceptor must overlap fluorescence emission spectrum of the donor (Figure 7)
- Dipole orientations of donor and acceptor must be approximate parallel



△ **Figure 7:** FRET Process

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3.2 Sequence-Specific Probe Binding Assays Using FRET continued

The donor dye (*e.g.*, fluorescein) is excited by a photon from the blue light source. This excites certain electrons in the donor molecule from ground level to a higher energy level. This energy may be released by

- emitting fluorescence (*e.g.* fluorescence light of different, longer wavelength)
- transfer of energy to an acceptor dye (*e.g.*, LightCycler Red 640)

When the energy is released, the electrons return to ground level.

This FRET process can be used in various ways to generate a sequence-specific signal during PCR. One format is based on quenching the fluorescence of the donor dye (*e.g.*, Hydrolysis Probes see chapter 3.2.2), another uses the fluorescence emission of an acceptor dye (Hybridization Probes see chapter 3.2.1). In either case, the two fluorophores have the characteristics described above.

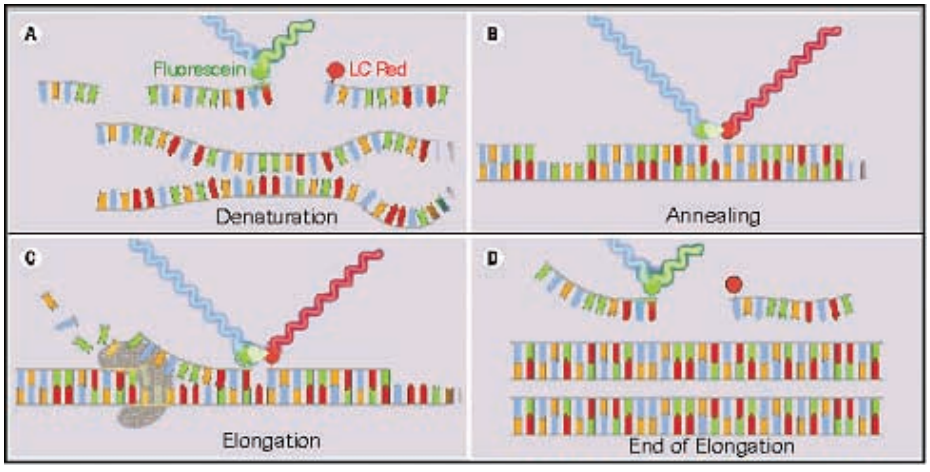
3.2.1 Hybridization Probe Format

Monitoring PCR with HybProbe Probes

The Hybridization Probe format uses two specially designed oligonucleotides that hybridize, side by side, to an internal sequence of the amplified fragment during the annealing phase of PCR. One probe (donor) is labeled at the 3´-end with fluorescein; the second probe (acceptor) is labeled at the 5´-end with a LightCycler Red fluorophore. (To keep it from being extended, the acceptor probe is also phosphorylated at the 3´-end.) These labels are close to each other only when the two oligonucleotides anneal to adjacent regions on the target, making fluorescence resonance energy transfer (FRET) possible. The energy of the donor dye on the first probe excites the acceptor dye on the second HybProbe probe, which then emits fluorescent light at a different wavelength. The instrument detects this emitted light.

The LightCycler 2.0 Instrument can detect HybProbe probes that are labeled with LightCycler Red 610, LightCycler Red 640, LightCycler Red 670 or LightCycler Red 705. These labeled HybProbe probes can be used separately or in combination, which permits either single- or multiple-color detection.

The energy transfer from the donor to the acceptor depends greatly on the spacing between the two dye molecules. Energy is only transferred efficiently if the molecules are in close proximity (between 1 – 5 nucleotides). The amount of fluorescence emitted is directly proportional to the amount of target DNA generated during PCR.



△ **Figure 8:** HybProbe Probes during PCR

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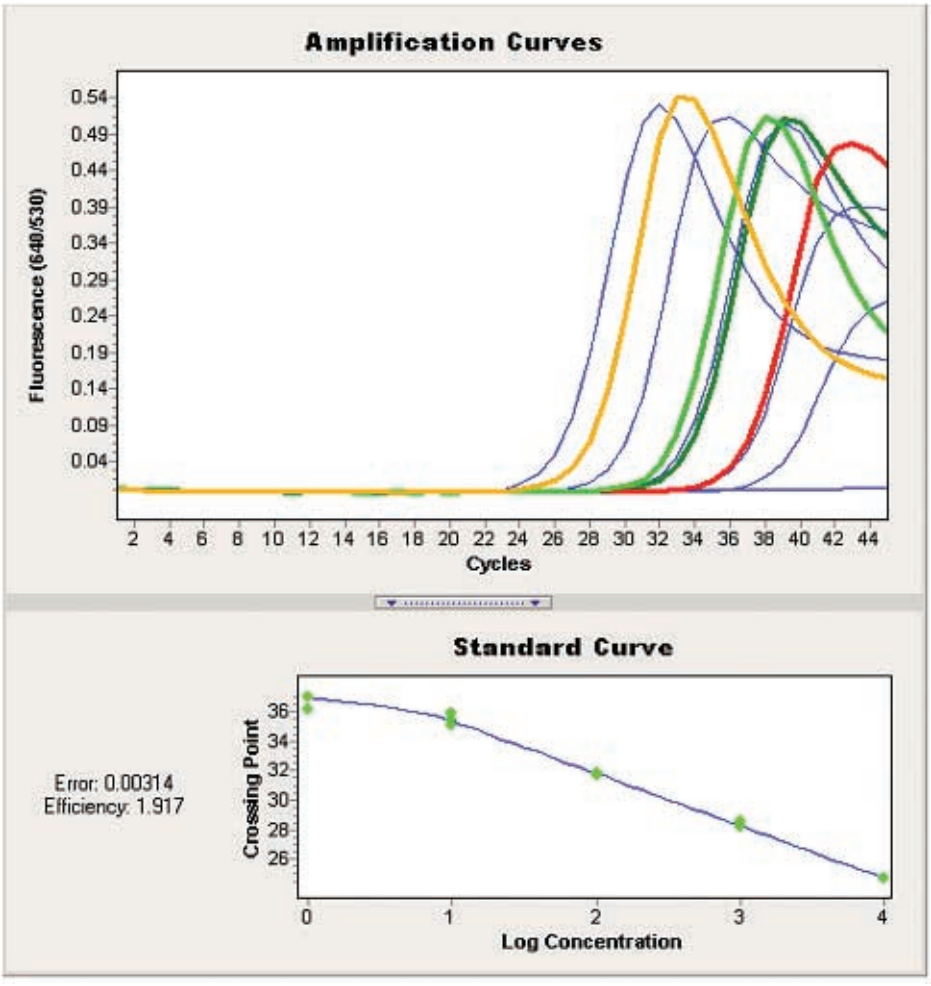
3.2.1 Hybridization Probe Format continued

Figure 8 shows the behavior of HybProbe Probes during different stages of PCR. The donor dye probe has a fluorescein label at its 3´-end, and the acceptor dye probe has a LightCycler Red label at its 5´-end. Hybridization does not occur during the denaturation phase of PCR (Figure 8A). Since the distance between the unbound dyes prevents energy transfer, no fluorescence will be detected from the red acceptor dye during this phase. In Figure 8B, the probes hybridize to the amplified DNA fragment in a head-to-tail arrangement, thereby bringing the two fluorescent dyes into close proximity. Fluorescein is excited by the LED, which causes it to emit green fluorescent light. The emitted energy excites LightCycler Red. The red fluorescence emitted by the second probe is measured at the end of each annealing step, when the fluorescence intensity is greatest. After annealing, an increase in temperature leads to elongation and displacement of the probes (Figure 8C). At the end of this step, the PCR product is double-stranded, the displaced HybProbe probes are back in solution and too far apart to allow FRET to occur (Figure 8D).

Note: At the end of amplification, both HybProbe probes are still intact and may be used in a melting curve experiment, *e.g.*, for mutation detection or SNP analysis.

HybProbes – Application Example 1: Quantification



△ **Figure 9:** Using hybridization probes to detect amplification of ß-actin from different starting amounts of human genomic DNA. This analysis is highly sequence-specific and does not detect any by-products or primer-dimers. Accurate quantification of the target depends on a standard curve that correlates crossing points (cycle numbers) with the concentration of known standard dilutions (blue amplification curves).

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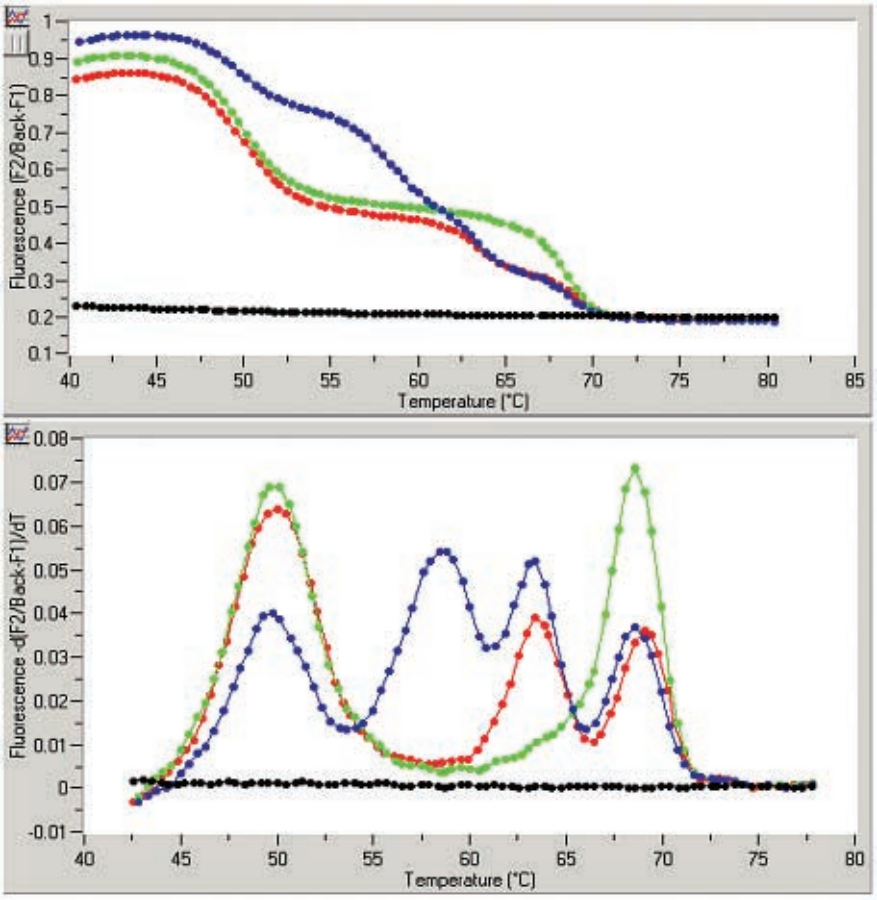
3.2.1 Hybridization Probe Format continued

HybProbes – Application Example 2: Melting Curve Analysis



△ **Figure 10:** Combining a melting curve analysis with the hybridization probe format produces a powerful tool for mutation detection (*e.g.*, genotyping single nucleotide polymorphisms, SNPs). The upper graph shows melting curve data (fluorescence versus temperature) generated with LightCycler Red 640 as reporter dye; the lower graph shows the melting peaks of two different ApoE genotypes. A single mismatch between the hybridization probes and their target sequences changes the melting temperatures of the bound probes by more than 5°C.

Design of HybProbe Probes

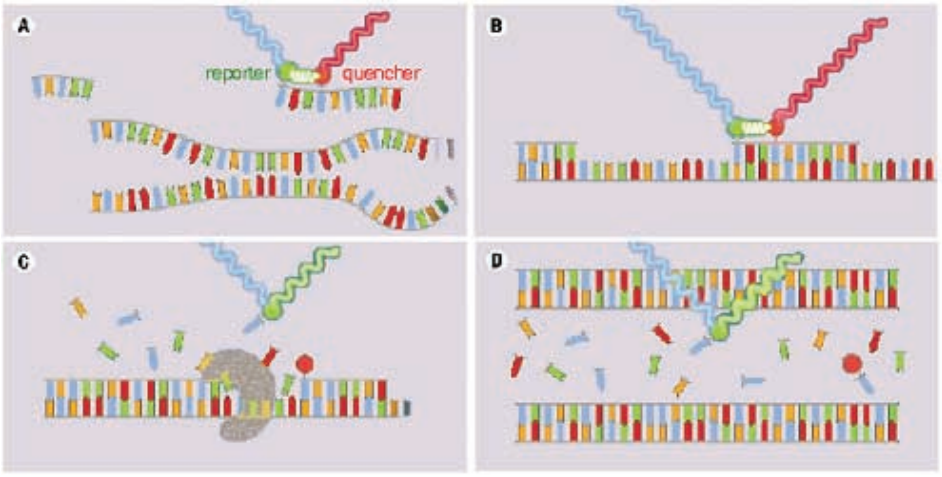
Guidelines for selecting sequences that will make suitable hybridization probes, along with special considerations for mutation analysis, are given in Technical Note No. LC 6/99 "Selection of Hybridization Probe Sequences for Use with the LightCycler" and LC 14/2000 "General Recommendations for LightCycler Analysis of Multiple Mutations Using Hybridization Probe Color and Melting Temperature."

Note: We highly recommend using LightCycler Probe Design Software 2.0 to design HybProbe probes, since this software was specially developed for SYBR Green I, SimpleProbe and HybProbe assays, including multiplex or multicolor applications.

3.2.2 Hydrolysis Probes

Monitoring PCR with Hydrolysis Probes

Hydrolysis probes emit fluorescence when the 5′ → 3′ exonuclease activity of Taq polymerase hydrolyzes them. These assays, conventionally called "TaqMan" assays, can technically be described as homogeneous 5′ nuclease assays, since the assay uses cleavage of a single 3′-non-extendable probe to indicate the accumulation of a specific target DNA sequence. This single probe contains both a fluorescence reporter and a fluorescence quencher, which are close to each other. When the probe is intact, the quencher dye is close enough to the reporter dye to suppress the reporter fluorescence signal (fluorescence quenching via FRET). During PCR, the 5′ nuclease activity of the polymerase cleaves the probe, separating the reporter and quencher, and permits the reporter dye to emit fluorescence.



△ **Figure 11:** PCR with Hydrolysis Probes

Figure 11 shows a PCR run in the presence of hydrolysis probes. The probe carries two fluorescent dyes that are close enough to cause quenching of the green (reporter) fluorescence signal. In the denaturation phase (Figure 11A), the strands of the target DNA separate as the temperature increases. During the annealing phase (Figure 11B), primers and probes specifically anneal to the target sequence. The Hydrolysis Probe is phosphorylated at the 3′-end, so it cannot be extended. As the DNA polymerase extends the primer, it will cleave the probe with its inherent 5′ nuclease activity (Figure 11C). The probe fragments are then displaced from the target, and polymerization of the new amplicon continues (Figure 11D). The DNA polymerase will separate the reporter and quencher only if the probe has hybridized to the target. After probe cleavage, the reporter dye is now unquenched and able to emit measurable fluorescent light. Accumulation of PCR products is detected directly by monitoring the increase in fluorescence of the reporter dye.
As with SYBR Green I, the fluorescent signal of the reporter dye is measured at the end of extension phase (Figure 11C).

Note: After amplification in a TaqMan PCR assay, all the probes are digested (Figure 11D). Melting curve analysis cannot be performed. Therefore, this type of assay requires a different experimental approach for detecting a mutation or SNP.

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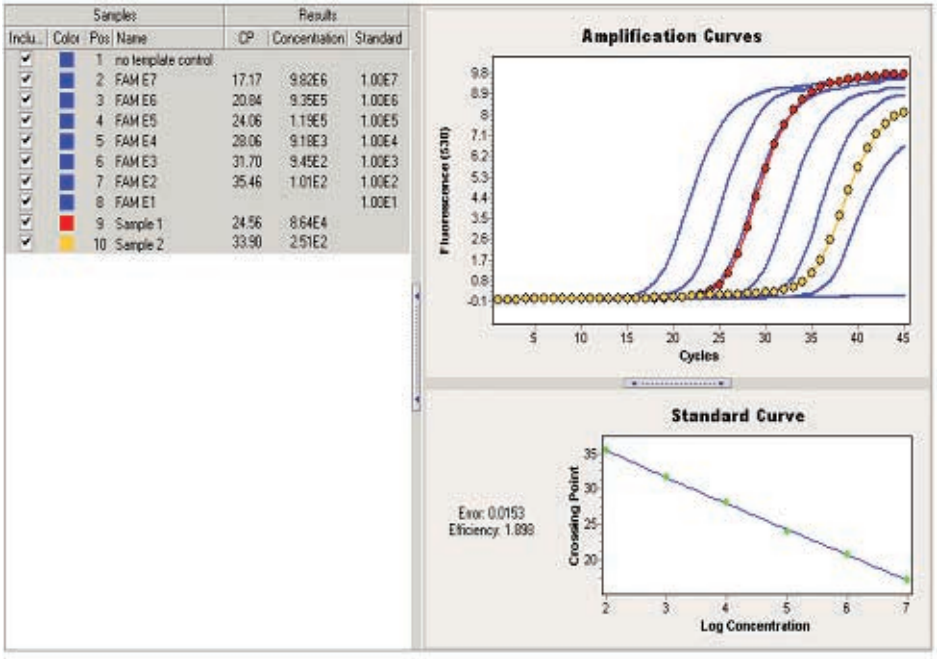
3.2.2 Hydrolysis Probes continued

Hydolysis Probe – Application Example: Quantification



△ **Figure 12:** Using the sequence-specific Hydrolysis Probe format to quantify two unknown samples. The standard curve (crossing point versus concentration) was generated with a serial dilution of a known standard (blue amplification curves).

Special Considerations for Hydrolysis Probe Assays

The quality of a Hydrolysis Probe depends on

- The quenching of the intact probe
- Hybridization efficiency
- Cleavage activity of the enzyme.

For a digestible hybridization complex to form correctly, the Hydrolysis probe must anneal to the target before primer extension. The Tm of the Hydrolysis Probe should be only slightly higher than the Tm of the PCR primer, so the hybridization complex is stable. Furthermore, the probe sequence must account for mismatches in the DNA template, since these will also affect the annealing temperature.

There are also several different ways to program the PCR. Either two-step or three-step PCR programs will provide suitable experimental results. Higher $MgCl_2$ amounts in the amplification solution may be necessary to ensure stable hybridization of the TaqMan probe and favor the hydrolysis event. For best results, the amplicon should be short (approx. 150 bp) and the annealing/elongation temperature should be 60°C.

4. Summary: LightCycler Assay Formats

Assay Format	Detection Channel	Reporter Dye	Application
SYBR Green I	530	SYBR Green I	Product Characterization Quantification
Hybridization Probes (HybProbe)	610 640 670 705	LightCycler RED 610 LightCycler RED 640 LightCycler RED 670 LightCycler RED 705	Quantification Mutation Analysis
Hydrolysis Probes (TaqMan)	530 560	FAM VIC or HEX	Quantification (Mutation Analysis)
Single Label Probes (SimpleProbe)	530	Fluorescein	Mutation Analysis

△ **Table 13** summarizes the four different assay formats, their reporter dyes and detection channels, and recommends the types of applications for which each is suitable.

Analyzing Multicolor Experiments

The LightCycler Instrument can simultaneously detect signals from two or more dyes, which make it possible to obtain more information from a single reaction. The channels chosen for analysis depend on the fluorescent dyes used in the experiment.

In a multicolor reaction, the wavelengths of light emitted by the dyes overlap, causing one channel to pick up signals from more than one dye. This so-called cross-talk can cause misleading data. See Technical Note LC No. LC 19 "Color Compensation" to learn how to use color compensation to correct for this bleed-over between channels in multicolor experiments.

Other Fluorescence Formats

Note: In addition to the formats listed above, the LightCycler Instrument also supports other fluorescence-detection formats, such as performing a FRET reaction with Molecular Beacons (by using fluorescein as the FRET donor dye). However, it is essential that any fluorescent dyes used in an analysis be compatible with the optical unit of the LightCycler Instrument.

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