*Roche Molecular Biochemicals*
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**LightCycler**

# Selection of Hybridization Probe Sequences for Use with the LightCycler

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## 1. Introduction

**Purpose of This Note**

Detection of specific amplicons with hybridization probes provides real-time, sequence-specific analysis of amplified target sequences. For instance,this method can, during an amplification reaction, detect specific single copy sequences in genomic DNA or identify single-base mutations.

This Technical Note gives specific guidelines for selecting sequences that will make suitable hybridization probes (see Section 3). In addition, we feel that selection can be even more successful if the role of the probe is understood fully (see Section 2).

***Note:*** Selecting sequences that will make suitable hybridization probes for LightCycler applications is not as difficult as the length of this Technical Note would indicate. In fact, most selected sequences will function satisfactorily as hybridization probes, even if specific selection rules are not followed.

**In This Note**

Use this Technical Note to learn more about the following topics:

# 2. Role of Hybridization Probes in Sequence-Specific Analysis

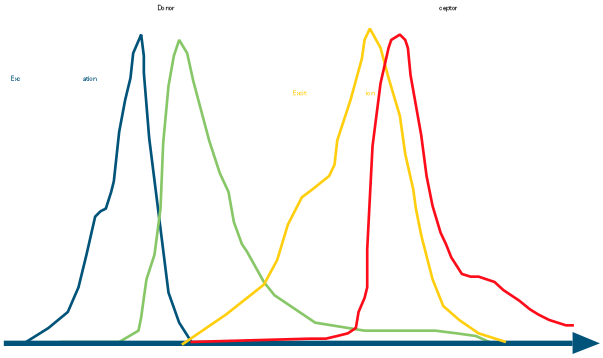
**Overview**

The LightCycler hybridization probe method uses two oligonucleotide probes that hybridize, in a head-to-tail arrangement, to adjacent sequences on the target DNA. Each probe is labeled with a different marker dye. Interaction of the two dyes can only occur when both are bound to their target.
When the two probes are hybridized to their target sequences,the marker dyes are in close proximity and there can be a fluorescence resonance energy transfer (FRET) between them.

**Fluorescence Resonance Energy Transfer (FRET)**

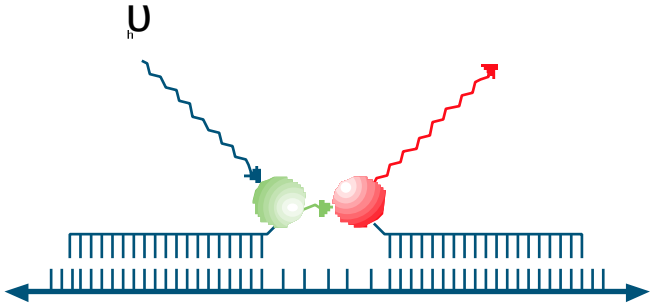
Fluorescence resonance energy transfer (FRET) is a distance-dependent transfer of energy between two adjacent fluorophores without the emission of a photon. Primary conditions for FRET are:

- Donor and acceptor molecules must be in close proximity.
- Excitation spectrum of the acceptor must overlap fluorescence emission spectrum of the donor (see figure below).
- Donor and acceptor transition dipole orientations must be approximately parallel.



**FRET with Hybridization Probes**

The following schematic illustrates how two adjacent oligonucleotide probes produce a measurable fluorescence emission.

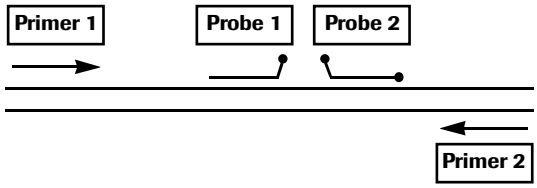


During the PCR annealing step, two different oligonucleotides hybridize, head-to-tail, to adjacent regions of the target DNA. The fluorophores, which are directly coupled to the oligonucleotides, are very close in the hybrid structure. The “F1” donor fluorophore (e.g., fluorescein) is excited by an external light source, then passes on part of its excitation energy to the adjacent “F2” acceptor (e.g., LightCycler-Red 640 or 705) (FRET) via dipole-dipole interactions. The excited F2 fluorophore emits measurable light (at a different wavelength).

# 3.1. Choice of Suitable Probe Targets

**Location of Probe Target**

The probe target should be near the 3' end of the target strand (i.e.,as far as possible from the 5' primer-binding site on the same strand). We recommend choosing a target that is close to, but not overlapping the primer-binding site on the opposite strand (see diagram below). This placement allows time for fluorescent measurements to be made before the primers are completely extended, and before the probes are displaced by Taq polymerase.




**Suitable Target Sequences**

Target sequences should have the following characteristics:

| Characteristic | Explanation |
|---|---|
| Occurs in a "balanced" sequence region (i.e., a region that has a nearly equal distribution of the four bases) | "Balanced" sequences tend to bind probes tightly, but not too tightly. |
| Does not contain monotonous or repetitive sequences | Monotonous and repetitive sequences tend to form hybrids which "slip". |
| Are not self-complementary | Self-complementary sequences can form loops and thus be less accessible to hybridization. |

## 3.2. Characteristics of Suitable Hybridization Probes,

Continued

**Choice of Fluorescent Labels**

The adjacent ends of the hybridization probes should be labeled with fluorophores. Use the table below to choose appropriate labels for the probes.

| For this probe | Label this end | With this dye |
|---|---|---|
| **Probe 1** ("donor" or 5' probe) | 3' end | Fluorescein (FITC, 3FL) |
| **Probe 2** ("acceptor" or 3' probe) | 3' end | • LightCycler-Red 640, or<br>• LightCycler-Red 705 |

***Caution about Probe Labels:***

When labeling probes, observe the following cautions:

- Do not invert the labeling of the probes. For instance, do not label probe 1 (the 5' hybridization probe) with a LightCycler-Red dye and probe 2 (the 3' hybridization probe) with fluorescein.
- Due to the properties of the optical unit, only FITC derivatives of fluorescein (containing a sulfur linkage) produce reliable results in the LightCycler.

**Preventing Probe Extension**

Because the probes have a higher melting temperature than the primer pairs, these probes could also serve to prime de novo synthesis during amplification. To prevent extension of the probes during PCR, phosphorylate the 3' end of the acceptor probe (This probe will be labeled with a LightCycler-Red dye at its 5' end; see above topic.)

***Note:*** The 3' end of the donor probe will be labeled with fluorescein (see above topic) and thus cannot be extended during PCR.

**Leave a Gap between Hybridized Probes**

When annealed to the target, the hybridization probes must sit close enough to each other to allow at least 50 % of the energy to be transferred between the fluorophores. Leave a gap — optimally between 1 and 5 bases — between the 3' end of probe 1 (donor probe) and the 5' end of probe 2 (acceptor probe). This leaves space for the fluorophores at the ends of the probes.
**Recommendation:** For initial experiments, choose targets with a gap of only one base between them.

# 4. Software Tools for Designing Hybridization Probes

**Introduction**

Software tools are available to help you design hybridization probe sequences. This section gives a brief overview of some of these tools.

**Software for Selecting Probe Sequences**

- There are many programs available for designing PCR primers (For examples,see Section 2.3.2, Chapter C of the LightCycler Operator's Manual). Some of these can also be used to help design hybridization probes.
- If you are carrying out a sequence analysis without the help of a sophisticated software package, you may find that the search function of a text processor such as Microsoft Word is quite sufficient. In this case, search for the final four bases of the target sequence (or their complementary sequence) within the sequence of the amplicon or the probe.

**Software for Calculating Probe Melting Temperatures**

For calculation of the melting points of the primers and probes, we strongly recommend using a program that can calculate Tms thermodynamically, i.e. a program that takes into account all neighboring bases and not just the GC content. For example, three excellent programs are:

- OLIGO, from Molecular Biology Insights, Inc., Cascade, CO, USA (http://oligo.net), or MedProbe, Oslo, Norway (www.medprobe.com)
- Program (JAVA script) available from TIB MOLBIOL, Berlin, Germany (www.TIB-MOLBIOL.de/oligo_ag.html)
- On-line oligonucleotide $T_m$ calculator (http://alces.med.umn.edu/rawtm.html)
  ***Note:*** You can also access this $T_m$ calculator from the home page of the Virtual Genome Center (http://alces.med.umn.edu/VGC.html).

**Cautions About Calculated $T_m$ Values**

The $T_m$ values calculated with software programs are reasonably accurate (relative error, 1°–2°C). However, as the table below shows, you should be careful how you apply that data to the design of hybridization probe systems.

| Operation | Potential problem | Suggested solution |
|---|---|---|
| Comparing the relative $T_m$ values of probes and primers | Each software program assumes different default parameters for DNA concentration and salt content. | Compare only $T_m$s calculated with the same program. |
| Setting PCR annealing temperatures for primers and probes | • Calculated $T_m$s do not correspond to PCR annealing temperatures.<br>• Primer annealing generally occurs at much higher temperatures than the calculated primer $T_m$ (because primers are present in considerable excess). | To optimize a reaction, determine true annealing temperatures experimentally, as follows:<br>• For primers, use a gradient cycler.<br>• For hybridization probes, use the LightCycler Melting Curve Analysis Module. |

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