Fast and Convenient Primer Probe Design for Multiplex Assays with the LightCycler Probe Design Software 2.0

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Introduction

There is growing demand in the research community for an optimized system that will perform multiplex experiments covering a wide range of applications such as single nucleotide polymorphism (SNP) screening, or evaluation of small interfering (si) RNA efficiency. Our solution is the new LightCycler 2.0 Instrument in combination with the LightCycler Multiplex DNA Master HybProbe. The LightCycler Probe Design Software 2.0 completes this system allowing the design of primers and probes for multiple targets in just one step.

Additional features offer more flexibility for the design and evaluation of oligonucleotides in silico:

- Regions for each primer and probes can be specified individually.
- Primers designed by the software can be submitted directly to a BLAST search at the National Center for Biotechnology Information (NCBI).
- Existing primers and/or probes can be defined as “Fixed Oligos”.
- Cross-Complementarity Tool gives information about melting temperature (Tm) of oligonucleotides and possible dimers of all combinations of oligonucleotides in the multiplexing assay.

LightCycler Probe Design Software 2.0

The LightCycler Probe Design Software 2.0 has three main screens based on the steps in the design process. The sequence screen is used to import the sequence and specification of search and reaction parameters (Figure 1a). On the analysis screen a good region for the automatical search for primers and probes is proposed, optionally the user can specify different search ranges either for forward primer, reverse primer, and probes, or for the entire amplicon. The result screen lists primer and probe sets proposed for the experiment. Different sets span a range of amplicon locations on the target and are sorted (tabulated) according to a score summarizing information on general oligonucleotide quality (specificity, cross-complementarity) and to the match of users requirements, such as Tm or amplicon size (Figure 1b).

Additional tools allow more specific input for existing oligonucleotides, or further analysis of primers and probes:

- Fixed Oligos
  Any existing oligonucleotide for a given target can be included as Fixed Oligo for a new design. The LightCycler Probe Design Software 2.0 will automatically search for the oligonucleotides missing to complete a primer probe set for this target.
- Cross-Complementarity Tool
  All primers and probes of a design (i.e., 16 oligonucleotides for a quadruplex) can be checked for the probability of dimerization. Additionally, any other existing oligonucleotide can also be added and checked against all others.

Figure 1:
(a) Sequence screen for input of sequences and selection of search and reaction parameters (i.e., melting temperatures, labeling dyes for the probes, amplicon size and buffer used for the PCR). (b) Result screen with a listed view of different primer probe sets (in blocks of four lines per assay; one line for each target).
Performing a Multiplex Experiment

The workflow for designing and performing a multiplex assay is shown in Figure 2:

1. Import the sequences for your genes of interest into LightCycler Probe Design Software 2.0.
2. Specify the parameters (dye, probe T_m, etc.) for each target.
3. Design primers and probes for all genes of the multiplex experiment in one step.

After synthesis of the oligonucleotides:

1. Run your multiplex assay on the LightCycler 2.0 Instrument.
2. Evaluate PCR performance by real-time online detection and/or melting-curve analysis and agarose gel analysis.

Application and Results

The sequence information on each of the target genes (factor V, prothrombin, HFE845 and HFE187) was retrieved from the GenBank database at NCBI and saved as txt file using GenBank file format.

After specification of the experiment type (mutation analysis or quantification mode), the sequences were imported into LightCycler Probe Design Software 2.0. Only the labeling dyes and T_m's for the probes of each target were specified. For all other parameters we used the software defaults.

After initial sequence analysis of each target sequence, without further restrictions on search ranges for primers and probes, the multiplex design was performed for all targets in one mouse click (multiplex initial search).

Figure 2: Workflow for a multiplex experiment

Figure 3: Real-time online detection of the four targets in monoplex (blue), duplex (green), triplex (orange), and quadruplex (pink) amplification. There is no effect on PCR efficiency for a given target, regardless of whether monoplex or multiplex reactions were performed.
The first selection set of a standard primer probe search (16 primers and probes) for each assay was synthesized and evaluated in multiplexing experiments utilizing human DNA samples and plasmid DNA.

**Quantification mode**

The software’s quantification mode was evaluated as part of an experimental setup to monitor the impact of siRNA on the corresponding mRNA levels. The test addressed the question of whether there is a shift in crossing points (that are an indicator of the initial amount of target in the reaction and the efficiency of amplification) for multiplex reactions compared with monoplex reactions.

A shift in crossing points could appear due to competition of amplification reactions (such as false amplification products if any of the primer probe sets had cross-complementarities). Thus, it is a main feature of the LightCycler Probe Design Software 2.0 to check all oligonucleotides of a multiplexing assay against each other and against all target sequences for cross-complementarities.

As shown in Figure 3, there was no difference in crossing points, regardless of whether monoplex or multiplex PCRs were performed. This allowed the combination of primers and probes for the target of interest and of all primers and probes for the housekeeping genes within one reaction vessel, and the performance of quadruplex experiments.

**Mutation analysis mode**

The performance of primers and probes designed in the mutation analysis mode was evaluated in a quadruplex PCR for plasmids with inserts for factor V, prothrombin, HFE845, and HFE187, followed by melting-curve analysis. The test included combinations of homozygous wild-type and mutant samples as well as heterozygous and negative controls in multiplex reactions (Figure 4).

For all targets there was a clear discrimination of the different alleles. The melting points were identical (within 1°C) for the respective alleles for both the homozygous and the heterozygous sample. The deviation of predicted Tₘ (by LightCycler Probe Design Software 2.0) compared with measured Tₘ was within 2°C.

The same experiment was reproduced with human DNA samples and yielded the same results (data not shown).

**Summary**

The LightCycler Probe Design Software 2.0 is an all-around tool reducing the time and effort needed to design and optimize primers and probes for multiplexing experiments. Predefined settings for all buffers and probe dyes from Roche Applied Science help to reduce the number of parameters required for starting a primer probe search. Primers and probes can be designed within minutes in just three simple steps. Existing, well-performing primers and probes from previous experiments can be included as part of a new search and can be applied in subsequent multiplex assays.