ProbeLibrary: Faster Design & Execution of Quantitative Real-Time PCR (1)

Peter Mouritzen, Peter S. Nielsen, Nana Jacobsen, Mikkel Noerholm, Christian Lomholt, Henrik M. Pfundheller, Niels B. Ramsing, Sakari Kauppinen, and Niels Tolstrup

Abstract

ProbeLibrary, a fast, specific and flexible format for quantitative real-time PCR, is described. The concept is based on the fact that just 90 short probes provide transcriptome-wide coverage in most organisms. The short probes have the high T_M and specificity required for real-time PCR due to the incorporation of locked nucleic acid (LNA). The probes are dual-labelled fluorogenic probes that are used in standard real-time PCR protocols, on standard instrumentation. ProbeLibrary probes are used in assays designed using free, web-based assay design software, called ProbeFinder. In seconds, ProbeFinder designs highly specific intron-spanning assays for a target transcript. The system allows multiple design options, including designs for transcript variants and gene family members. Assay design success rate is 96 %.

Principles Behind the ProbeLibrary Concept

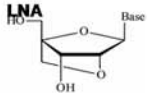
Principle 1

Real-time PCR probes of 8-9 nucleotides incorporating locked nucleic acid (LNA) have equivalent T_m and functionality to standard (>18 nt) real-time PCR probes and are compatible with real-time PCR methodology

Table 1: Short LNA probes have high T_m and improved single mismatch discrimination features compatible with real-time PCR methodology

Probe	Target		
	Perfect match 3'-acgaccac-5'	Single mismatch 3'-acggccac-5'	ΔT_m
DNA 8-mer 5'-tgctggtg-3'	T_m=35°C	T_m=25°C	10°C
LNA 8-mer 5'-TGCTGGTG-3'	T_m=71°C	T_m=45°C	26°C

About LNA



- Bicyclic RNA/DNA analogue
- High affinity duplex formation
- Improved base-pairing specificity
- Obeys Watson-Crick base pairing rules

Principle 2

Selected 9-mer sequences occur with sufficient frequencies within a species' transcriptome to enable transcriptome-wide targeting by just 90 probes

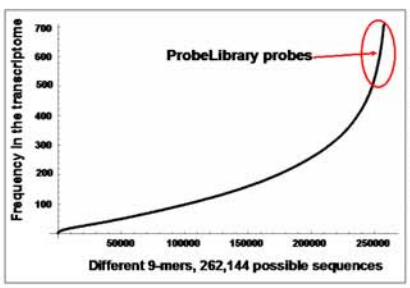


Figure 1. The frequency distribution of all possible 9-mer sequences in the human RefSeq transcriptome at NCBI. The total number of different 9-mers is equal to 4^9 = 262,144. The different 9-mers were plotted against their frequency of occurrence in the human RefSeq transcriptome. The probes in the ProbeLibrary™ were selected from the upper part of this curve.

Real-Time PCR assay design using short LNA-based probes

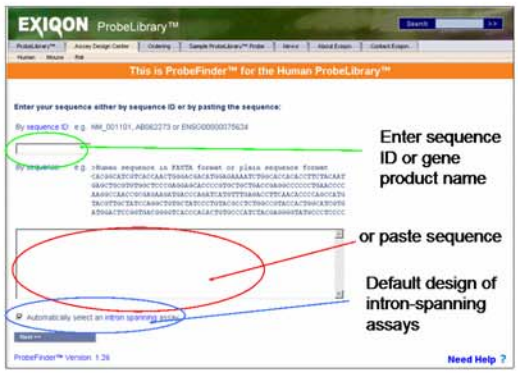


Figure 2. ProbeFinder entry page. Target genes are entered here. From one to ten target genes can be entered per round of assay design

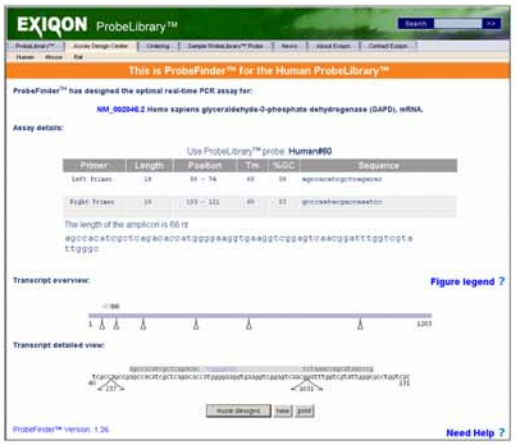


Figure 3. Assay Design results page. Selection of ProbeLibrary probe and optimal primer design.

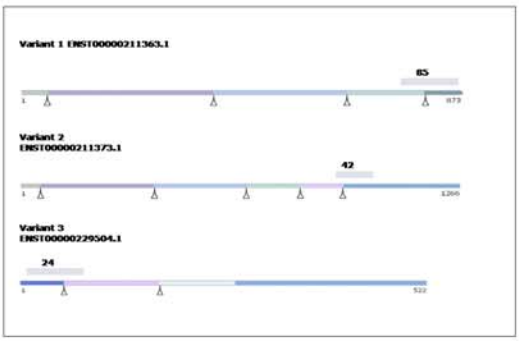


Figure 4. Extra design options. An example of assay design for transcript variants.

Exiqon A/S
Bygstubben 9
DK-2950 Vedbaek
Denmark

tel: (+45) 45 66 08 88
fax: (+45) 45 66 18 88
www.probelibrary.com

ProbeLibrary: Faster Design & Execution of Quantitative Real-Time PCR (2)

Peter Mouritzen, Peter S. Nielsen, Nana Jacobsen, Mikkel Noerholm, Christian Lomholt, Henrik M. Pfundheller, Niels B. Ramsing, Sakari Kauppinen, and Niels Tolstrup

How ProbeFinder designs assays

Locate Introns

Introns are identified by:
- prediction algorithm
- a look-up in Ensembl
- user-defined positions marked in the submitted sequence

Identify target sites

Input sequence is searched for all possible target sites matching probes in the ProbeLibrary

Design of PCR primers

A primer pair is designed for each target site using Primer3

In silico PCR

Each primer pair is checked for cross-hybridization to areas unrelated to the target within the relevant genome and transcriptome

Rank Assays

Assays are ranked in order to:
- favour unique assays without cross-hybridizations to other areas of the genome
- favour intron-spanning amplicons
- favour small amplicon size

Optional Design Extras

Designs for transcript variants
Common assays for multiple variants

Assay Validation on 175 most cited Human RefSeq mRNAs

Table 2. Performance of 175 human ProbeLibrary-based real-time PCR assays designed by the ProbeFinder software at www.probelibrary.com

Assay Category	Number of Assays
Successful Assays[1]	168[2]
Failed assays[3]	7[4]
Assay Success Rate	96 %

[1] Successful assay: classified as having sigmoid-shaped real-time PCR amplification plot, ample fluorescence signal, single amplicon of the correct size.
[2] 21 of these assays were successful in a second test round, when the second ranked assay was selected from the list designed by ProbeFinder software.
[3] Assays were deemed to have failed, if both the first and second ranked assays designed by ProbeFinder were not successful
[4] Low or insignificant amount of amplicon or no increase in fluorescence – for 5 transcripts no PCR amplicon could be generated

Table 3: ProbeLibrary Species Coverage Based on Transcripts Recorded at the Ensembl Database

Species Genome	Coverage	Average Probes per Transcript	Real Time PCR Assays Available	
			Intron Spanning Assays	Total Assays
Human	99%	19	155,114	639,956
Mouse	99%	16	140,422	509,789
Rat	98%	13	128,998	364,405
Arabidopsis	98%	8	59,775	199,251
Drosophila	99%	14	65,840	253,742
Chimpanzee	96%	14	174,699	519,513
C. Elegans	95 %	6	38,864	134,356

Conclusions

The ProbeLibrary reduces the time spent on assay design significantly,taking just seconds in many cases. There is a high probability that the designed assay will work first time, enabling a faster ability to move from target identification to real-time PCR assay. In practice, this leads to higher throughput. The system is very flexible, instantly generating multiple assay options for many transcripts. The system also posseses the specificity associated with probe-based assays. The ProbeLibrary is very useful for wide-ranging gene expression studies, because it gives the flexibility to design and carry out assays for many genes fast.

EXIQON

www.ProbeLibrary.com

Exiqon A/S
Bygstubben 9
DK-2950 Vedbaek
Denmark
tel: (+45) 45 66 08 88
fax: (+45) 45 66 18 88
www.probelibrary.com