RealTime *ready* Focus Panels

Functionally tested qPCR assays based on the Universal ProbeLibrary for gene expression analysis on LightCycler® Platform

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Introduction

The Universal ProbeLibrary is a fast, specific and flexible format for quantitative real-time PCR experiments. This technology consist of just 165 short (8-9mer) probes providing transcriptome-wide coverage in most organisms. The short probes are highly specific and possess a high melting temperature (Tm) required for real-time qPCR due to the incorporation of locked nucleic acid (LNA).

The major advantages of the Universal ProbeLibrary technology are:

- Complete transcriptome coverage of many organisms with only 165 different probes.
- Fast and easy assay design process by web-based ProbeFinder software.
- Fast turn-around time from assay design to experiment.
- Compatibility of UPL assays with many master mixes on any real-time PCR instrument.
- Universal PCR conditions for all UPL assays to eliminate the need to optimize cycling conditions or primer/probe concentrations.

RealTime *ready* Assay Supply Concept

The Universal ProbeLibrary is now extended to offer functionally tested qPCR assays for gene expression profiling. The assays are supplied dried-down and ready-to-use in LightCycler® 480 microwell plates in 96-, 384- and later 1536-format. RealTime *ready* Focus Panels contain pre-plated qPCR assays targeting selected genes from specific pathways or functional groups.

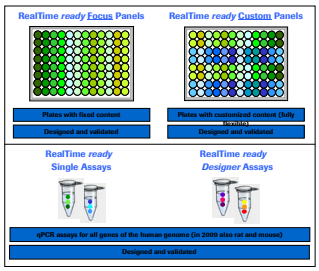

Fig.1 Product concept for RealTime *ready* assay supply.
Focus Panels contain a fixed selection of genes, while the content of the Custom Panels is fully flexible. Additionally, all assays are available as single assays in tubes, with 300 rxns.

RealTime *ready* Focus Panels

The defined content of the Focus Panels which results of a cooperation with experts allows fast and easy analysis of all relevant genes for a given pathway or gene/protein family. The following RealTime *ready* Focus Panels are now available as pre-plated qPCR assays for the LightCycler® 480 instrument. Only master mix and cDNA have to be added.

- **Human Reference Gene Panel, 96**
  with 19 housekeeping genes in quadruplicate
- **Human Cell Cycle Regulation Panel, 96**
  with 84 target genes
- **Human GPCR Panel, 96**
  with 84 target genes
- **Human ABC Transporter Gene Panel, 96**
  with 42 target genes in duplicate
- **Human Nuclear Receptor Panel, 96**
  with 84 target genes
- **Human Apoptosis Panel, 96**
  with 84 target genes
- **Human Apoptosis Panel, 384**
  with 372 target genes

All RealTime *ready* Focus Panels will be available in a 384-well format and more panels will be launched in 2009.

Control Concept of RealTime *ready* Focus Panels

All RealTime *ready* Focus Panels include 7 of the most common housekeeping genes and 5 control assays for RNA quality check (RT positive and RT minus control).

The control assays are:
- 3 RT positive controls: Check for degradation of the initial RNA and the quality of the RT step (3 assays targeting different positions of the same transcript at the 5'-end, in the middle and at the 3'-end of the transcript). Delta of Cp values should be within 1 Cp.
- 2 RT minus controls: Check for residual genomic DNA contamination (2 identical assays targeting the same transcript position, the sample in the first well is the transcribed cDNA, the sample in the second well is the untranscribed RNA). Delta of Cp values should be more than 10 Cps.

All RealTime *ready* qPCR Assays fullfil stringent specifications

A standard curve is done for all assays to guarantee a high degree of performance. All assays are tested under the following conditions:
- Sample material: 10-fold dilution series of commercially available MAQC material (2:1-mixture of Universal Human Reference RNA (Stratagene) and FirstChoice Human Brain Reference Total RNA (Ambion)
- RT-step: Reverse-transcription with Oligo-dT and random hexamer priming and Transcriptor First Strand cDNA Synthesis Kit (Roche)
- PCR: LightCycler®480 Instrument with LightCycler® 480 Probes Master in a 384 MWP with a 10 µl PCR setup

The following criteria have to be fulfilled:
- PCR efficiency (2.0 +/- 0.2)
- Specificity (single band in gel analytic)
- Sensitivity (max. cDNA conc. Cp ≤ 34)
- Dynamic range (tested against 5 orders of magnitude)
- r^2-value of standard curve ≥ 0.99
- Fluorescence height (fluorescence of max. cDNA conc.: 10 – 30 units)

Performance Data of RealTime *ready* Focus Panels

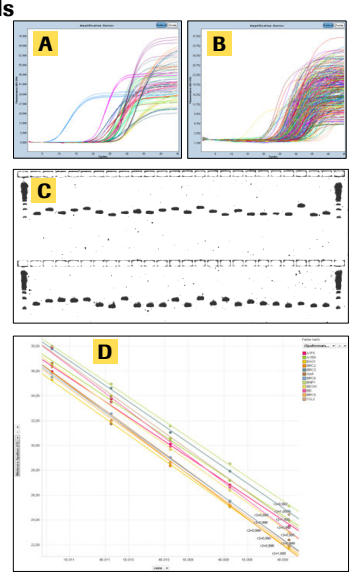

Fig. 2 Showcases of RealTime *ready* Focus Panels
Serial 1:10 dilution of cDNA derived from a 2:1 mixture of Universal human Reference RNA (Stratagene) and FirstChoice Human Brain Reference Total RNA (Ambion), reverse-transcribed to cDNA with Transcriptor First-Strand cDNA Synthesis Kit (Roche).
A: Amplification curves of Human Reference Gene Panel, 96;
B: Amplification curves of Human Apoptosis Panel, 384;
C: Subset of 48 assays from apoptosis panel on a gel;
D: Linearity of amplification for 12 Apoptosis Assays over 5 orders of magnitude.

Application: Gene silencing effects on apoptosis

Data kindly provided by U. Tschulena, DKFZ Heidelberg

HeLa-derived cells were transfected with siRNA targeting GFP as a control, and HLR-CHOP-cells with siRNAs targeting GDF3 and Donson and with siRNAs targeting C24ORF19 and Bard1. All of the transfections were carried out in duplicate and the samples were treated with TNF-alpha one day after transfection, followed by RNA isolation one day later. Knock-down effects were tested on RealTime *ready* Reference Gene Panel, 96 (GDF3, Donson) and Apoptosis Panel, 384 (C24ORF19, BARD1).

Correlation between biological replicates
A nearly perfect correlation between the biological replicates was demonstrated.

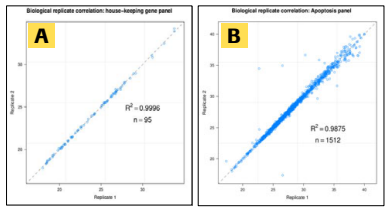

Fig. 3 **A**: Human Reference Gene Panel; **B**: Human Apoptosis Panel

Regulation status of the 19 targets of the Reference Gene Panel in siRNA tranfected cells
This shows the importance of analyzing the regulation status of a number of reference genes before normalizing to one or more of them. Consequently normalization was always to an average of all reference genes on the panels.

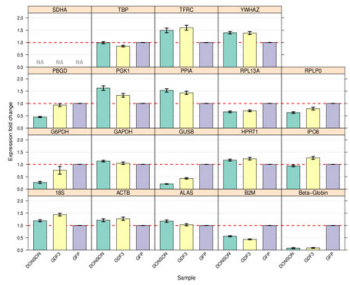

Fig. 4 Effects of knock-down of GDF3 and Donson on expression levels of reference genes, compared to control cells (GFP knock-down).

Changes in the expression level of selected genes of the Apoptosis Panel after siRNA transfection
Expression level of detectors in each sample were calculated using ΔΔCt method.

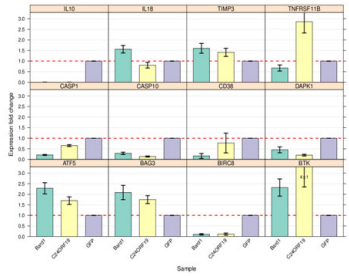

Fig. 5 Ct-values were normalized to the reference genes and then compared to relative changes of GFP-treated control cells (GFP is always set to 1.0).

RAS Assay Supply – Outlook to future developments

The assay supply concept for customized real-time PCR assays is planned to be extended to other applications such as gene expression assays for additional organisms (mouse, rat).