qPCR Validation of Candidates for Modulators of the MAPkinase Signaling Pathway

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Introduction

With the completion of the human genome sequence, the detailed understanding of gene functions by analyzing the structure, biological activities, and dynamics of the encoded proteins has become the next challenge. The technology of RNA interference (RNAi), which allows the silencing of genes by introduction of small interfering RNA (siRNA), is recognized as an extremely powerful tool for systematic high-throughput functional genomic screening.

Mitogen-activated protein kinase (MAPK) signaling pathways are implicated in the regulation of major cellular functions such as growth, differentiation, immune-mediated inflammatory responses, stress response, and apoptosis. As such, they are the focus of intensive research to define their roles in physiology and human disease.

We carried out a whole-genome RNAi screen in HeLa cells for modulators of MAPkinase signaling. A set of 63 candidate genes were selected from the hit-list to validate endogenous expression via qRT-PCR in the course of the validation process. Gene expression levels were determined in dual-color assays using FAM-labeled Universal ProbeLibrary probes in combination with Universal ProbeLibrary Reference Gene Assays labeled with LightCycler® Yellow 555 on the LightCycler® 480 Instrument. Each of the 63 target genes was analyzed in combination with the reference gene recommended by the Universal ProbeLibrary ProbeFinder software. For some genes different reference gene assay combinations were tested in addition. The same genes were tested employing single-color Universal ProbeLibrary assays to confirm gene expression levels measured with the dual-color approach. Further, we tested the performance of the same dual- and mono-color assays using a real-time PCR instrument from Applied Biosystems (7900HT).

Materials and Methods

Cell culture

HeLa cells (ATCC® CCL2™) were maintained in DMEM supplemented with 1% L-glutamine, 1% penicillin/streptomycin, and 10 % FBS (GIBCO-BRL) at 37°C in 5% CO_2.

RNA isolation and reverse transcription

Total RNA was extracted from 5 x 10^7 HeLa cells in five biological replicates using a commercial RNA extraction kit. First–strand cDNA synthesis was performed in 10 technical replicates using 1 µg of total RNA each and the Transcriptor First Strand cDNA Synthesis Kit according to the manufacturer's instructions.

Assay design

Primer and probes for the Universal ProbeLibrary assays were designed with ProbeFinder software via the Universal ProbeLibrary Assay Design Center at www.universal-probelibrary.com using the dual-color mode option.

qRT-PCR

Single-color qRT-PCR performed with the LightCycler® 480 instrument contained a final concentration of 400 nM of each primer, 200 nM of Universal ProbeLibrary probe and 1x LightCycler® 480 Probes Master Mix in a reaction volume of 20 µl. For the dual-color qRT-PCR, the reactions were supplemented with 400 nM of the respective reference gene primers and 200 nM of the suitable Universal ProbeLibrary probe. Reaction conditions for the LightCycler® 480 were: 95°C for 10 minutes, 45 cycles of 95°C for 10 seconds, 60°C for 30 seconds, 72°C for 1 second, followed by 40°C for 30 seconds final cooling. Single-color qRT-PCR performed with the 7900HT (Applied Biosystems) consisted of 500 nM of each primer, 250 nM of Universal ProbeLibrary probe and 1 x FastStart Universal Probe Master (Rox) in a final reaction volume of 20 µl. For the dual-color qRT-PCR, the reactions were supplemented with 500 nM of the reference gene primer and 250 nM of the respective UPL probe. Amplification parameters for the 7900HT were: 95°C for 10 minutes, 45 cycles of 95°C for 15 seconds, 60°C for 60 seconds.

Results and Discussion

In total, the expression of 63 different candidate genes from our screen were tested in different setups (Table 1). First, we used the ProbeFinder software to design assays for the whole set in combination with the reference genes that the ProbeFinder software ranked as the best combination in dual-color assays. Four of these genes were analyzed with two, another two genes with three assays, to evaluate the success rate of assay design for the second and third choice. Eleven genes were tested in combination with two different reference genes to find a potential impact of the reference

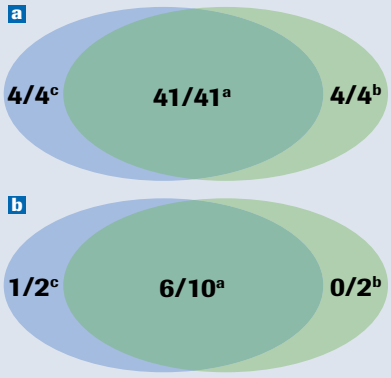

Figure 1: Overlap of number of genes and assays detected in dual- and single-color assays using the LightCycler® 480 instrument. Genes tested positive are indicated in front of the slash, assays thereafter. [a] Genes/assays positive with dual- and single-color mode. [b] Positive only with single-color mode. [c] Positive only with dual-color mode. A gene was defined positive when at least one assay had worked (see also Table 1).

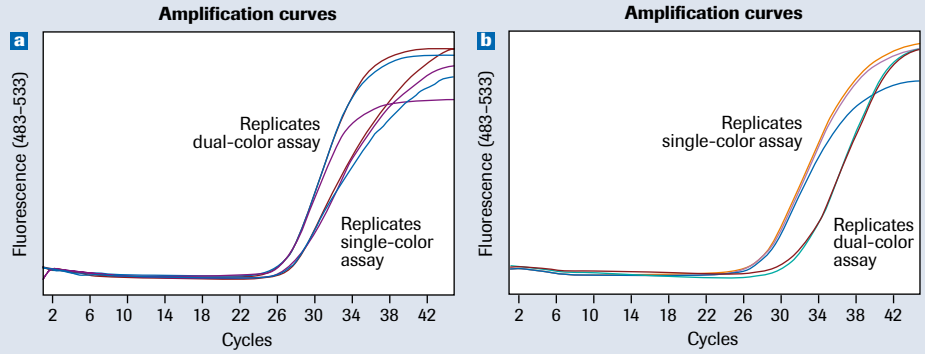

Figure 2: Matching of data from dual- and single-color assays: (a) While results were similar with dual- and single-color assays, **(b)** few assays showed differences where either the single-color assay resulted in a lower Cp value than the dual-color assay or vice versa (not shown).

gene on the measured expression of the target gene. For one gene we even tested three different primer/probe combinations in an array with three different reference genes (Table 1). Second, we employed cDNA of the same cell line that had been used in our cell-based screen and compared the performance of the single- and dual-color assay systems on two different real-time PCR instruments (LightCycler® 480 System and 7900HT).

Using the LightCycler® 480 Instrument, the expression of 45 genes was detected with the Universal ProbeLibrary dual-color assay when testing only one primer and probe combination. These results were confirmed with the single-color approach, except for four genes for which no expression was detected there (Figure 1). For these four genes Cp-values of 22.4, 32.63, 37.43, and 39.13 had been calculated with the dual-color approach, indicating a very low endogenous expression level of most. No expression was measured for 11 genes with the Universal ProbeLibrary dual-color assays. These results were confirmed for seven of the genes with the single-color assays, indicating the lack of endogenous expression of the respective genes in the cell line under investigation. For the four other genes Cp values of 25.31, 33.2, 36.54 and 37.11 were detected with the single-color approach, indicating a low-level expression of three of these genes. For those seven genes where more than one primer-probe combination was used for the respective gene (16 primer-probe combinations in total), expression was detected with at least one Universal ProbeLibrary dual-color assay in all cases. In the single-color mode the expression of all but one of these genes was observed with all assays (Figure 2).

In general, most of the Universal ProbeLibrary dual-color assays worked well on the LightCycler® 480 Instrument. For 84% of the assays the detected Cp values differed by no more than 0 to 2.0 cycles from those detected with the single-gene approach (Table 2 and Figure 3).

With the 7900HT instrument the expression level of 45 different genes could be detected using one dual- as well as one single-color assay (Figure 3). For the genes where more than one primer-probe combination and/or several reference genes were tested, most of the assays (14 of 16) worked. In the two unsuccessful cases, both assay types failed. This might suggest an inappropriate primer design, as the same genes had been positively tested with other primer/probe combinations. For about half of the genes where the dual-color assays failed, also no expression was detected with the single-gene assays. For the other genes Ct values ≥ 35 were calculated in the single-color mode, indicating a very low expression level of the respective genes. Comparable Ct values (range: 0–2 Ct values)

Table 1: Experimental design. A total of 63 genes were tested with one to three primer probe combinations and in combination with up to three reference genes. This table explains the distribution of genes and the different combinations.

	Total	One primer/ probe combination	Two primer/ probe combinations	Three primer/ probe combinations
Number of genes/assays	63/72	56/56	5/10	2/6
Combination with one reference gene	52	48	3	1
Combination with two reference genes	10	8	2	0
Combination with three reference genes	1	0	0	1

Table 2: Performance of single- and dual-color assays.

Differences in Cp/Ct values between single- and dual-color assays	LightCycler® 480 Instrument (%)	7900HT instrument (%)
≤1	54	41
> 1 ≤ 2	30	36
> 2 ≤ 3	5	2
> 3	11	21

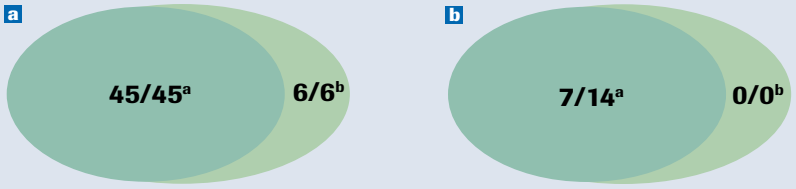

Figure 3: Overlap of number of genes and assays detected in dual- and single-color assays using the 7900HT instrument. Genes tested positive are indicated in front of the slash, assays thereafter. [a] Genes/assays positive with dual- and single-color mode. [b] Positive only with single-color mode. A gene was defined positive when at least one assay had worked (see also Table 1).

were detected with the single- and the dual-color assays for 77% of the assays with this instrument (Table 2).

For half of the target genes comparable results were obtained with both real time PCR instruments when different reference gene assays were combined with the same target gene primers and probes (Table 1). However, the LightCycler® 480 instrument performed better with the Universal ProbeLibrary mono- and dual-color assays when results were broken down to the individual assays (Table 2). In several cases differences in the Cp/Ct values were seen with the dual-color assays depending on the reference genes used, indicating an influence of the reference gene on the detection (level) of the target gene.

Conclusions

The Universal ProbeLibrary dual-color assays performed well with both real-time PCR instruments, and the results also compared well with the respective mono-color assays in most cases. Almost identical Cp/Ct values were detected for about half of the analyzed genes when comparing the LightCycler® 480 instrument to the 7900HT instrument. Another third of the detected values differed by <2 cycles with both assay types.

In view of the minimal, albeit observable differences of results that were obtained with the mono- and dual-color assays irrespective of the instruments, we make the following recommendations: The same chemistry (either mono- or dual-color assays) should be used throughout a study (*i.e.*, for all genes tested in one set-up). The same reference gene should be used in dual-color assays when a quantitative comparison of expression levels is envisioned for several genes in the same sample. The same instrument should be used as well, to avoid instrument specific variations.

We conclude that the mono- and the dual-color assays are both efficient tools for the quantitative analysis of gene expression. The major advantage of the dual-color assay is its intrinsic property of providing an internal expression control of a reference gene, permitting to directly correlate the expression of different target genes. Hence the number of controls and references can be reduced in setups where this kind of analysis is envisioned, saving costs and efforts. Internal controls also reduce plate and position effects, as they allow for a direct measurement of the reference in the same sample. Further, the dual-color assay drastically enhances the reproducibility and comparability of results, and thus the quality of data.

The validation of gene expression is one of the most important steps in the validation procedure of RNAi experiments, as false-positive hits can be immediately identified. Use of the dual-color chemistry substantially reduces the number of reactions that need to be performed in order to permit a qualitative and quantitative comparison of gene expression-levels, which is a key element in systems biology. ■

References

Sahin O *et al.* (2007) Proc Natl Acad Sci USA 104:6579–6584

Order INFO

Product	Pack Size	Cat. No.
Human PBGD Gene Assay	200 x 50 µl assays	05 046 149 001
Human HPRT Gene Assay	200 x 50 µl assays	05 046 157 001
Human ACTB Gene Assay	200 x 50 µl assays	05 046 165 001
Human PGK1 Gene Assay	200 x 50 µl assays	05 046 173 001
Mouse ACTB Gene Assay	200 x 50 µl assays	05 046 190 001
Mouse GAPD Gene Assay	200 x 50 µl assays	05 046 211 001
Rat GAPD Gene Assay	200 x 50 µl assays	05 046 220 001
Rat ACTB Gene Assay	200 x 50 µl assays	05 046 203 001
Universal ProbeLibrary Set, Human	library of 90 probes	04 683 633 001
Universal ProbeLibrary Set, Mouse	library of 90 probes	04 683 641 001
Universal ProbeLibrary Set, Rat	library of 90 probes	04 683 650 001
Universal ProbeLibrary Extension Set, Probes #91 – #165	set of 75 probes	04 869 877 001
LightCycler® 480 Probes Master	500 x 20 µl reactions	04 707 494 001
FastStart Universal Probe Master (Rox)	2.5 ml	04 913 949 001
Transcriptor First Strand cDNA Synthesis Kit	1 kit for 50 reactions	04 379 012 001