shown to be significantly improved when carrier is used (1, 15). The reverse transcription yields for the RNA MultiStandard varied more than 100-fold. The lowest yield (0.4%) was obtained with AMV for 10^6 RNA molecules, and the highest yield (90%) was obtained with SuperScript III for 10^4 RNA molecules (Table 1). The latter was overall the most efficient reverse transcriptase, with a mean yield of 83%. MMLV and MMLVH gave mean yields of 44% and 40%, respectively, whereas the mean yields of the other reverse transcriptases were <25%. The yield obtained with MMLVH was comparable to that reported in a previous study (15).

In conclusion, we show that reverse transcription yields vary up to 100-fold with the choice of reverse transcriptase and that the variation is gene dependent. Previously, we also reported a dependence on priming strategy (1). Hence, for quantitative gene expression measurements based on reverse transcription to be comparable among laboratories, the same enzyme, priming strategy, and experimental conditions must be used.

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### Table 1. Absolute reverse transcription yields for RNA.

<table>
<thead>
<tr>
<th>Mean (SD) yields (%) at external RNA input (in molecules)</th>
<th>Mean (SD) yield for RNA MultiStandard, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>10^6</td>
<td>10^5</td>
</tr>
<tr>
<td>MMLVH</td>
<td>22</td>
</tr>
<tr>
<td>Omniscrypt</td>
<td>7.2</td>
</tr>
<tr>
<td>AMV</td>
<td>0.4</td>
</tr>
<tr>
<td>MMLV</td>
<td>32</td>
</tr>
<tr>
<td>Improm-II</td>
<td>32</td>
</tr>
<tr>
<td>cAMV</td>
<td>6.3</td>
</tr>
<tr>
<td>ThermoScript</td>
<td>1.1</td>
</tr>
<tr>
<td>SuperScript III</td>
<td>87</td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>24 (29)</td>
</tr>
</tbody>
</table>

*a Reverse transcription yields of RNA prepared from liver and spleen. The samples were diluted 30-fold before QPCR measurements, giving initial copy numbers of 33–333 molecules/sample. Note the markedly higher yields at an input of 10^7 RNA molecules.

*b Reverse transcription yield for samples containing 10^5-10^6 RNA MultiStandard molecules.

#### References


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#### Multiplexed Real-Time PCR Using Universal Reporters, Andreas M. Rickert, Hans Lehrach, and Silke Speirling (MaxPlanck-Institute for Molecular Genetics, Berlin, Germany; *address correspondence to this author at: Max-Planck-Institute for Molecular Genetics, Ihnestrasse 73, 14195 Berlin, Germany; fax 49-30-8413-1128, e-mail speirling@molgen.mpgh.de)

Real-time quantitative PCR is a sensitive and accurate method for gene expression studies (1). The detection chemistries of all real-time PCR procedures are based on one of two principles for monitoring amplification products: binding to double-stranded DNA or hybridization to single-stranded DNA. Small molecules bind to double-stranded DNA either as intercalators or as minor groove binders, e.g., ethidium bromide (2), Hoechst 33258 (3), or SYBR® Green I (4). Several approaches using target-specific hybridization to single-stranded DNA have been introduced, including Molecular Beacons (5), Scorpions (6, 7), the TaqMan or hydrolysis/5’-nuclease assay (8, 9), the AEGIS probe system (10), labeled primers (11, 12), and light-up probes (13). In contrast to binding of dyes to double-stranded DNA, these methods are suitable for multiplexing approaches because they use differentially labeled fluorescent dyes. However, as they require a unique probe or modified primer for each target, currently used hybridization-based methods for real-time quantitative PCR have high reagent costs and require large developmental efforts.

Here we present a real-time PCR assay that uses universal hybridization-based probe sets suitable for any target. Because the assay uses tagged locus-specific non-modified amplification primers, PCR products can be
monitored via common reporters (cr) hybridizing to the common tails. The general principle of combining tailed PCR primers with universal probes has been introduced for other genetic applications, such as single-nucleotide polymorphism genotyping (14, 15) and in situ amplification (16), but these methods have not been applied to quantitative gene expression studies. Our system, which is similar to the method developed by Whitcombe et al. (15), leads to a more flexible and low-cost setup than conventional hybridization-based approaches. Using differentially labeled universal reporting reagents, we have developed a multiplex setup for simultaneous analysis of target gene and internal control (housekeeping gene), which is an accepted method for normalizing sample-to-sample variation. We then compared the cr-real-time PCR assay with SYBR Green I assays with respect to robustness and sensitivity. As a proof of principle, we applied the new assay to a study of three previously published, differentially expressed candidate genes for congenital heart defects (17).

The principle of the cr-real-time PCR assay is as follows: Target DNA is subjected to PCR in the presence of four oligonucleotides; one low-concentration, tailed, locus-specific primer; one locus-specific primer without a tail; one common primer annealing to a common sequence stretch of the tail; and one universal TaqMan probe corresponding to another common part of the tail (Fig. 1 and Table 1). The use of locus-specific primers that span at least one intron of the genomic sequence minimizes problems associated with DNA contamination. During the first amplification cycle, the tailed locus-specific primer initiates the polymerase reaction, leading to the synthesis of a fragment with the tail at the 5’ end. In the second cycle, the complement of the tail is synthesized. Starting from cycle three, the common amplification primer primes synthesis on the fragment including the tail sequence. From this step on, the TaqMan probe anneals to the products resulting from the amplification with the common primer and the locus-specific primer without a tail. The TaqMan probe bound to the tailed target amplicon is hydrolyzed by the 5′-nuclease activity of the DNA polymerase, leading to a physical separation of the reporter and quencher dye and release of fluorescence emission (8).

Introduction of further common tails and corresponding, differentially labeled TaqMan probes opens up the possibility for multiplexing (Fig. 1), although the degree of multiplexing is limited by the number of different dyes and the restrictions of currently available instruments (1). The universal tails and TaqMan probes can be easily transferred to other targets in combination with two locus-specific primer sequences. In cases in which locus-specific primers are already designed, ligation to their corresponding common tails by use of the ligation-based synthesis method (18) could be envisaged. Furthermore, the amplification of primer-dimers or pseudogenes can be
tested by a melting analysis using SYBR Green I instead of the TaqMan probe for amplicon detection.

Because the cr-real-time PCR assay requires three interacting amplification primers per target gene (Fig. 1), the optimum ratio had to be adjusted. For separate analysis, the 20-μL reactions contained 1× TaqMan Universal PCR Master Mix (Applied Biosystems), 0.3 μM appropriate TaqMan probe, 0.4 μM each of the reverse amplification primer (R1/2-primer) and common forward primer (L-primer), 0.004–0.2 μM tailed forward primer (L1/2-primer), and various amounts of template. In the multiplex analysis, the 20-μL assay mixture contained 1× TaqMan Universal PCR Master Mix (Applied Biosystems), 0.3 μM each of both universal TaqMan probes, 0.4 μM each of both locus-specific reverse primers (R-primers), 0.04–0.2 μM both tailed forward primers (L1/2-primer), 0.4–1.0 μM the common forward primer (L-primer), and various amounts of plasmid or cDNA templates. Cr-real-time PCRs were performed and measured on an ABI Prism 7900HT system. The thermocycling protocol consisted of an initial denaturation at 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 s and 60 °C for 1 min. Subsequently, a dissociation curve was generated in the range of 60–95 °C.

A concentration of 0.016 μM of the tailed locus-specific primer was sufficient as this primer is required only during the initial steps. No signal improvement was achieved with higher concentrations, whereas amplifications were less efficient with lower concentrations. The concentrations of the other two amplification primers were optimally kept at 0.4 μM each. Transferring these assay conditions for separate analyses to multiplexing approaches revealed preferential amplification of one target gene over the other. Given the competitive nature of multiplexed reactions, an increase in concentration of the universal primer necessary for all targets (L-primer) to 0.8 μM appeared crucial. When we used these conditions, the same amplification results were obtained independently from the uniplex or multiplex background of the reaction (see the Data Supplement that accompanies the online version of this Technical Brief at http://www.clinchem.org/content/vol50/issue9/).

The same assay conditions could be transferred to all loci of interest without any further optimization effort (see the online Data Supplement). This successful application of the same reaction conditions demonstrated the robustness and flexibility of the cr-real-time PCR assay. Because each reaction was performed in triplicate, showing only marginal variations (see the online Data Supplement), the cr-real-time PCR demonstrated high reproducibility and accuracy, which were in the same range as the results obtained here (see the online Data Supplement) and the results reported previously (19, 20) for the SYBR Green I assay.

Two different setups were performed using SYBR Green I for real-time detection of amplification products. The two-primer setup contained 1× SYBR Green PCR Master Mix (Applied Biosystems), 0.4 μM forward and reverse primer (L1/2-primer without common tail and R1/2-primer), and various amounts of plasmid or cDNA templates. To simulate the amplification conditions of the cr-real-time PCRs, the three-primer setup was performed with an amplification mixture containing 1× SYBR Green PCR Master Mix, 0.4 μM each reverse primer (R1/2-primer) and common forward primer (L-primer), 0.016 μM tailed forward primer (L1/2-primer), and various amounts of template. All 20-μL amplification reactions were carried out and measured on an ABI Prism 7900HT system (Applied Biosystems), using the same thermal profile as described for the cr-real-time PCR assay.

To evaluate the sensitivity and dynamic range of the cr-real-time PCR assay, we prepared two serial dilutions and subjected various amounts to cr-real-time PCRs as well as to two- and three-primer SYBR Green I assays. A dilution series of total cDNA ranging from 1 (corresponding to 100 ng of reverse-transcribed RNA) to 1:50 000

**Table 1. Oligonucleotides used for cr-real-time PCR assays.**

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence, 5’–3’</th>
<th>Gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1_PIPPIN_F</td>
<td>[tail-L1]-ACCCAGGACTTACCGGACA</td>
<td>PIPPIN</td>
</tr>
<tr>
<td>PIPPIN_R</td>
<td>AACTGCTTACAGCGGCTTTG</td>
<td></td>
</tr>
<tr>
<td>L1-FLJ10350_F</td>
<td>[tail-L1]-CTCAGGTGAGTCTCCCAAGCA</td>
<td>FLJ10350</td>
</tr>
<tr>
<td>FLJ10350_R</td>
<td>TGTCGGTCTGACCTTGTCC</td>
<td></td>
</tr>
<tr>
<td>L1_TNNL1_F</td>
<td>[tail-L1]-TGGATGAGGAGCGATACGACAT</td>
<td>TNNL1</td>
</tr>
<tr>
<td>TNNL1_R</td>
<td>GTTCTTAATCTCCGCTTTGGTGTTG</td>
<td></td>
</tr>
<tr>
<td>L2_B2M_F</td>
<td>[tail-L2]-TGCGTGTCTGATGTTGATGCT</td>
<td>B2M</td>
</tr>
<tr>
<td>B2M_R</td>
<td>TCTCTGCTCCCCACCTCTAGGT</td>
<td></td>
</tr>
<tr>
<td>tail-L1</td>
<td>TGCAAAATTCAGACTAGATCCACAGGTCCTGCGACTGGACAGG</td>
<td></td>
</tr>
<tr>
<td>tail-L2</td>
<td>TGGCAAAATTCAGACTAGATCATCGCGTCCTGACAGGACAGG</td>
<td></td>
</tr>
<tr>
<td>TaqMan-fam</td>
<td>FAM-CCACACGGTCTGCGACTGGACAGG</td>
<td></td>
</tr>
<tr>
<td>TaqMan-tet</td>
<td>TET-CATCCCGCTCCGACAGGACAGG</td>
<td></td>
</tr>
<tr>
<td>L-primer</td>
<td>GCACAAATTCAGACTAGGA</td>
<td></td>
</tr>
</tbody>
</table>

*Each assay uses two locus-specific amplification primers, one common primer, and one universal TaqMan probe (see Fig. 1). Common primers and their corresponding sequence stretches of the tails are underlined, TaqMan probes and their corresponding sequence stretches of the tails are in italics. TaqMan probes were labeled with the reporter dyes 6-carboxyfluorescein (FAM) or tetrachloro-6-carboxyfluorescein (TET) at the 5’ end and with the quencher dye 6-carboxytetramethylrhodamine (TAMRA) at the 3’ end.*
the cDNA samples. We thank Maike Tribbels and Chris-
FLJ10350
FLJ10350

We are grateful to Martin Lange for providing the

We analyzed expression of three genes that had been
found, by SYBR Green I analyses, to be differentially
expressed in patients with congenital heart defects (17).

The results confirmed the previously published data, with
FLJ10350 and TNNI2 being significantly up-regulated and
PIPPIN being significantly down-regulated (see the online
Data Supplement). Throughout our assays, we saw no
amplification of the no-template controls (see the online
Data Supplement).

For normalization of the target genes analyzed in the
course of this study, the housekeeping gene B2M was
simultaneously assayed with the genes of interest. The
obvious sample-to-sample variations (see the online Data
Supplement) stress the importance of effective systems for
normalization, as achieved with the multiplexed cr-real-
time PCR assay.

In summary, we have described a single-step method
for real-time PCR that is sensitive, robust, and requires
minimal optimization effort. Because the system uses
nonmodified, tailed amplification primers and universal
reporting reagents, it is characterized by a flexible and
low-cost format. The use of differentially labeled
reporting reagents enables multiplexing approaches for moni-
toring of more than one target per well, e.g., both a
candidate and housekeeping gene. Therefore, the cr-real-
time PCR assay appears suitable for the broad spectrum
of real-time PCR applications.

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the cDNA samples. We thank Maike Tribbels and Chris-
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ACE2 Gene Polymorphisms Do Not Affect Outcome of
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Severe acute respiratory syndrome (SARS) is the first
pandemic of the 21st century (1). Since its recognition,
8437 individuals have been affected and 813 have died
(2). Approximately 20–30% of patients required intensive
care admission (1). Although there was a slight predom-