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Evaluation of a Homemade SYBR® Green I Reaction Mixture for Real-Time PCR Quantification of Gene Expression

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Abstract

Real-time PCR is an accurate method that can be used for the quantification of specific DNA molecules. Here we provide a protocol for SYBR® Green I in real-time PCR applications using plastic reaction tubes. We report that SYBR Green I is alkali labile and once degraded inhibits the PCR. In our optimized protocol, diluted aliquots of SYBR Green I remain stable for at least two weeks. We also evaluated different cDNA synthesis protocols for the quantification of multiple genes from the same cDNA preparation. The best result was obtained with cDNAs synthesized by OmniScript™ reverse transcriptase from 2.5 μg total RNA using oligo $d(T)_{18}$ primers. The cDNA reactions could be diluted 1:25, allowing the quantification of up to 125 different medium expressed genes of Arabidopsis. Extension times ranged between 20 and 40 bp/s for accurate quantification of PCR products up to approximately 400 bp in the Rotor-Gene 2000 system. Using our optimized real-time PCR protocol, the reproducibility and amplification efficiency was high and comparable to a commercially available SYBR Green I kit. Furthermore, the sensitivity allowed us to quantify 10–20 copies of mRNA and dsDNA. Thus, the protocol eliminates the need for expensive pre-made kits.

INTRODUCTION

Real-time PCR has developed into one of the most powerful analytical tools for the quantification of defined nucleic acid sequences. Since only minute amounts of template are required, applications include the evaluation of gene copy number and mRNA expression and the diagnosis of pathogens and mutations. The basic principle of real-time PCR is the recurring measurement of a fluorescent signal, which is proportional to the amount of amplification product. The amplification cycle at which the emission intensity of the amplification product rises above an arbitrary threshold level (Ct cycle) is inversely proportional to the log of the initial number of target sequences (6). Thus, the fewer amplification cycles are needed for the emission to exceed the threshold level, the more target molecules are in the sample.

Several detection systems are now available and are either based on hybridization probes such as Taqman™ probes (7), Molecular Beacons (13), and Scorpions™ (14) or on

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intercalation by fluorescent dyes, such as the ds-DNA-binding dye SYBR® Green I (Roche Diagnostics, Penzberg, Germany) (9,15). While hybridization probes offer the advantage of target sequence specificity, a specific probe is required. Given that the synthesis of fluorescence-labeled probes is still a major cost factor, the availability of a general detection method is of great benefit to those studies that aim at the quantification of a high number of genes. The SYBR Green I-based quantification method is the method of choice for these applications. It has been repeatedly argued that the SYBR Green I method results in more precise and sensitive assays (4,8,12). Until now, SYBR Green I was offered in ready-to-use kits, mainly for the glass capillary PCR system distributed by Roche Diagnostics (LightCycler™). Thus, one aim of our study was to provide an inexpensive and universal SYBR Green I-based, real-time PCR mixture that is suitable for use in standard polypropylene reaction tubes.

The ABI PRISM® 7700 and Taqman 5700 (both from Applied Biosystems, Foster City, CA, USA) and the LightCycler are well-established real-time thermal cyclers. Some recently launched machines are the Rotor-Gene 2000 (Corbett Research, Sydney, Australia), the iCycler (Bio-Rad Laboratories, Hercules, CA, USA), the Smart Cycler® (Cepheid, Sunnyvale, CA, USA), and the Mx4000 (Stratagene, La Jolla, CA, USA). Here we present data generated on the Rotor-Gene 2000 because this system combines the fast cycling time of the LightCycler with the use of standard 0.2-mL polypropylene PCR tubes. Additionally, the Rotor-Gene 2000 allows for immediate real-time monitoring and melting curve analysis.

The efficient reverse transcription reaction is one of the essential steps in real-time quantification, and buffer incompatibilities greatly affect the outcome of subsequent cDNA quantifications. Therefore, we evaluated various reverse transcription systems for their influences on the efficiency of the real-time PCR. Our optimization efforts allow the reproducible quantification of up to 125 different genes from a single cDNA synthesis reaction with 2.5 µg total RNA of *Arabidopsis*.

MATERIALS AND METHODS

Plant Strains and Growth Conditions

Arabidopsis thaliana accession Columbia (Col) was obtained from the Arabidopsis Stock Center (Columbus, OH, USA). Plants were grown as described previously (2). For massive root production, plants were cultivated on liquid medium containing 1× MS salt mixture (10) supplemented with 2% sucrose.

DNA and RNA Isolation

Genomic DNA was isolated from seedlings as described previously (5). Isolation of total RNA was performed essentially as described previously (1). Total RNA was prepared from root tissue using the RNeasy™ Plant Mini kit (Qiagen, Hilden, Germany). Plant material was ground in liquid nitrogen, mixed with RLT buffer (Qiagen), and further treated following the manufacturer's instructions. The quality and quantity of RNA were evaluated by photometric measurements and on denaturing agarose gels.

cDNA Synthesis

To remove traces of genomic DNA before cDNA synthesis, total RNA (2.5 μg) was treated with 3 U RNase-free DNase I (Roche Diagnostics) in the presence of 1 mM $MgCl_2$ for 30 min at 37°C. DNase treatment was stopped by incubation at 75°C for 10 min, and the denatured RNA was chilled on ice. For reverse transcription, we compared the following reverse transcriptases: two from Moloney murine leukemia virus (MMLV)—SuperScript™ I (Invitrogen, Carlsbad, CA, USA) and Revert-Aid™ (Fermentas AB, Vilnius, Lithuania)—one avian myeloblastosis virus (AMV) (Promega, Madison, WI, USA), and the OmniScript (Qiagen). cDNA was synthesized using the following concentrations of reverse transcriptases: 200 U SuperScript or RevertAid, 4 U OmniScript, or 8 U AMV. In addition, the enzyme reactions were supplemented with 2 μM oligo $d(T)_{18}$ primers and dNTPs at 0.7 mM for SuperScript or 0.5 mM for the others. Ribonuclease inhibitor (20 U; Fermentas AB) was added to the OmniScript, RevertAid, and AMV reactions. The final volume was 15 μL for SuperScript and 20 μL for the other enzymes. The synthesis of cDNAs was performed at 37°C for 60 min, except for the AMV reaction, which was incubated at 42°C. Reverse transcriptase activities were stopped by heating at 95°C for 5 min. For comparison, 5 μL sterile water was added to the SuperScript reaction. Dilutions of 1:5 (25 ng/μL), 1:10 (12.5 ng/μL), 1:25 (5 ng/μL), 1:50 (2.5 ng/μL), 1:100 (1.25 ng/μL), and 1:1000 (125 pg/μL) were prepared with sterile water, and real-time PCR was performed with 1 μL undiluted and diluted cDNAs.

Preparation of PCR Standards for Quantification

Serial dilutions of purified and quantified PCR products were used as standard templates. Templates were obtained by amplification of genomic DNA with three different sets of primers (Table 1), yielding fragments of 195, 426, and 709 bp, respectively. PCR conditions were as follows: 3 min initial denaturation at 94°C and 35 cycles of 15 s at 55°C, 30 s at 72°C, and 15 s at 94°C in an OmnE cycler (Hybaid, Teddington, UK). The reaction mixture of a 20-μL final volume contained 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mg/mL BSA (Fermentas AB), 0.1% Triton® X-100 (Sigma, St. Louis, MO, USA), 200 μM each dNTP (Fermentas AB), 5 pmol each primer, 0.5 U *Taq* DNA polymerase (Appligene Oncor, Illkirch, France) and 20 ng genomic *Arabidopsis* DNA. The reaction was overlaid with 30 μL mineral oil (Sigma). PCR products were purified by PEG precipitation to remove salts, nucleotides, and unincorporated PCR primers. Briefly, 1/10 volume 3 M NaOAc, pH 5.2, and 1 volume of 30% PEG-8000 (Fluka) were added, incubated for 15 min on ice, and centrifuged at 12 000× *g* for 30 min at 4°C. The supernatant was removed with a pipet, and pellets were washed with 70% ethanol, dried, and dissolved in TE buffer. An aliquot of the PCR products was used for the quantification on a 1.5% agarose gel by measuring and comparing the ethidium bromide-stained fragment intensities with a 100-bp DNA size marker (Fermentas AB) of known concentration with the UVP® Image Store 7500 system (UVP, San Gabriel, CA, USA). After calculation of approximate copy number, the quantified PCR products were standardized to about 10^9 molecules/μL, and serial 10-fold dilutions were made with TE buffer, pH 8.0. From these dilutions, 1 μL was used as template for the real-time optimization.

Real-Time PCR Conditions

One microliter of a 1000-fold dilution of SYBR Green I, 1 µL template, and 2 µL of the 10× PCR mixture (100 mM Tris-HCl, pH 8.5, 500 mM KCl, 15–30 mM $MgCl_2$, ±0.2 mg/mL BSA, ±1.5% Triton X-100), 5 pmol each primer, 0.5 U *Taq* DNA polymerase were added and adjusted with sterile water to a final volume of 20 µL in 0.2-mL tubes (Greiner Bio-One GmbH, Kremsmuenster, Austria). After an initial denaturation step for 90 s at 94°C, 35–40 cycles followed by 10 s at 94°C, 5 s at 55°C, and 10, 15, 20, or 30 s at 72°C (for the 195, 426, or 709 bp fragments, respectively). The fluorescence was measured three times at the end of the extension step at 72°C, 81°C, and 83°C. Subsequently, a melting curve was recorded between 65°C and 99°C with a hold every 2 s. A typical real-time quantification including the melting curve analysis took less than 70 min. The identities of the PCR products were examined by agarose gel electrophoresis and melting curve analysis.

RESULTS AND DISCUSSION

Optimizing the cDNA Synthesis for Real-Time PCR

For accurate real-time PCR analysis, the quantity of cDNA must represent the original RNA amounts, and the reverse transcription buffer has to be compatible with subsequent PCRs. Thus, we compared four reverse transcription systems and used the synthesized cDNA as templates for PCR with *UBQ5*-specific primers (Table 1) and the SYBR Green I detection system. The onset of fluorescence (Ct cycles) varied between different reverse transcription systems. As depicted in Table 2, the amplification of undiluted cDNA templates was delayed when compared to the corresponding 1:5 and 1:10 dilutions. Dilutions of 1:5 were sufficient to eliminate the inhibitory effects of cDNA reactions for SuperScript and OmniScript but not for RevertAid (Table 2). The Ct values were similar for 1:10 and higher dilutions of SuperScript, OmniScript, and RevertAid, whereas the AMV-generated cDNA templates resulted in fewer cDNAs (Table 2).

Melting curve analysis reveals the ratio between specific and nonspecific products. Whereas the specific *UBQ5* product peaks at 92°C, nonspecific products exhibited a broad melting curve starting at 71°C. Nonspecific products were amplified from the undiluted cDNA templates of the AMV, SuperScript, and RevertAid systems. Only specific products were detected with the undiluted cDNA templates of the OmniScript system and the 1:10 dilutions of the SuperScript and RevertAid systems. Thus, for all MMLV systems, we recommend dilutions of at least 1:5 (SuperScript and OmniScript) and 1:10 (RevertAid).

The advantage of the cDNA dilution strategy is that expression analyses of multiple genes can be carried out from the same sample RNA. Our results show that no differences in the accuracy of the PCRs were obtained with 1:25 dilutions for the different MMLV cDNA synthesis systems (Table 2). Furthermore, all MMLV cDNA synthesis systems could be diluted up to 1:100 (1.25 ng/µL total RNA) without the occurrence of nonspecific PCR products. The lowest dilution used was 1:1000 (125 pg/µL total RNA). At such high dilutions, only medium-to-high expressed genes reached the detection limit. Thus, by using an optimized cDNA synthesis system, it is possible to dilute the cDNA reaction from 1:5 up to 1:1000 times and to quantify as few as 20 cDNA copies with SYBR Green I.

Establishment of a Homemade SYBR Green I Reaction Mixture

Commercially available kits usually contain the buffer, nucleotides, $MgCl_2$, *Taq* DNA polymerase, and other compounds, which in part are kept secret. Some kits include SYBR Green I in the master mixture, but others do not. During the optimization of the homemade SYBR Green I master mixture, we initially got variable results that were traced to differences of individual BSA lots. The other suspect component of the reaction buffer was Triton X-100. Thus, we tested the effect on BSA with and without Triton X-100 on the amplification efficiency and reproducibility of the real-time PCR and compared our buffers with the SYBR Green I master mixture from Roche Molecular Biochemicals. In summary, the master mixture greatly depended on the BSA origin, and omitting BSA improved the amplification reaction (Table 3). Excluding both Triton X-100 and BSA or only Triton X-100 was detrimental to the reaction. Thus, our homemade 10× SYBR Green I reaction buffer (100 mM Tris-HCl, pH 8.5, 500 mM KCl, 20 mM $MgCl_2$, and 1.5% Triton X-100) performed closely to the Roche buffer (Table 3). We determined also the reproducibility by repeated quantification of *UBQ5* standard dilutions. After 230 repetitions, the mean standard curve [conc= 10^(−S*Ct + y)] had a slope of −0.269 ± 0.017 and a y (Y intercept*S) of 9.13 ± 0.27. Since the slope(s) correlates with PCR efficiency ($E = 10^{-S}$-1) and 1 would be the optimum, the mean efficiency of the 230 repetitions was 0.86 ± 0.08. To test if the reaction volume could be down-scaled to 10 μL, we analyzed dilution series of the *UBQ5* template using the 10× reaction buffer without BSA. Although the overall fluorescence was reduced, the sensitivity and efficiency were similar to those of 20-μL reactions (Table 3).

Optimizing the $MgCl_2$ Concentration

Nath et al. (11) have shown that the addition of SYBR Green I influenced the optimal concentration of $MgCl_2$. Furthermore, the magnesium concentration affects primer annealing, product specificity, and enzyme activity. Thus, we tested the effect of $MgCl_2$ concentration of 1.5–3 mM on each gene and primer (data not shown). In summary, all templates and primers gave good results with 2 mM $MgCl_2$. Since these primers were optimized for 1.5 mM $MgCl_2$ in conventional PCR, the effect of SYBR Green I can be compensated by the addition of 0.5 mM $MgCl_2$.

Stability of SYBR Green I

In initial experiments, we diluted the SYBR Green I in TE (pH 8.0) and stored aliquots at −20°C. After thawing, the SYBR Green I aliquot was kept at 4°C and protected from light. Freshly thawed aliquots had reduced fluorescence and after a few days, the intensities and product synthesis decreased significantly. Apparently, SYBR Green I degradation products are potent inhibitors of PCR. It has been observed that SYBR Green I, which is used to stain DNA in agarose gels, is most stable within a pH range from 7.5 to 8.3. The pH of Tris-based buffers is temperature sensitive, as Tris-buffered solutions of pH 8.0 at room temperature will change to about pH 8.5 when stored at 4°C. This increase is sufficient to render the dye unstable. Therefore, we made dilutions of SYBR Green I with TE, pH 7.5. Freshly suspended aliquots were stored at −20°C. In a stability test, one SYBR Green I aliquot was thawed, kept at 4°C, and protected from light. After 3, 5, 7, 11, 18, and 21 days, *UBQ5* standard dilutions were quantified with real-time PCR using the same SYBR Green I

aliquot. We found that the dye remained stable for as long as 18 days. After 21 days, SYBR Green I exhibited its inhibitory effect on PCR (data not shown).

SYBR Green I Concentration

To assess the influence of the SYBR Green I concentration, we started with 1 µL of a 1000-fold dilution for a 20 µL reaction volume (1:20 000 final dilution). With this concentration, we obtained good results for every primer pair tested. To find out whether changes in the final concentration would lead to a deterioration (or improvement) of reaction kinetics, we included 1:10 000, 1:30 000, and 1:40 000 dilutions in our experiments with real-time PCR buffers containing BSA and Triton X-100. Increasing SYBR Green I concentrations lead to lower Ct values and higher reaction efficiencies (Table 4).

Amplification Efficiency and Sensitivity Depend on Fragment Length

The processivity of different *Taq* DNA polymerases varies between 30 and 80 bp/s. Consequently, small fragments need short cycle times. Some companies recommend for real-time PCR quantification product sizes of less than 150 bp. Betzl et al. (3) have proposed an extension time of about 25 bp/s for the LightCycler system. We tested various extension times for different product sizes in 10-fold dilutions covering a range from 10^8 to 1 molecules/µL. As summarized in Table 5, the amplification of fragments between 200 and 400 bp exhibits a similar efficiency irrespective of the extension times of 20 or 40 bp/s. However, PCR efficiencies declined if larger fragments (i.e., 709 bp) were amplified.

Optimal Acquisition Temperature

As SYBR Green I intercalates every dsDNA, target product quantification is most accurate when the occurrence of nonspecific byproducts, including primer dimers, is minimized. As seen in the melting curve analyses, nonspecific DNA products do not melt completely at 72°C. On the other hand, the SYBR Green I fluorescence declines at higher temperatures. It is therefore important to acquire the fluorescence (acquisition temperature) at the lowest temperature where most nonspecific products are melted. For most of the genes we quantified so far with the homemade SYBR Green I master mixture, the acquisition temperatures of 81°C and 83°C appeared as optimal.

In conclusion, our homemade 10× SYBR Green I master mixture of 100 mM Tris-HCl, pH 8.5, 500 mM KCl, 20 mM $MgCl_2$, and 1.5% Triton X-100 is a low-cost alternative for real-time PCR systems that use polypropylene reaction tubes. The reproducibility and sensitivity of gene expression analyses by quantitative real-time PCR was increased by the evaluation and prevention of inhibitory effects of different cDNA synthesis systems and the optimization of SYBR Green I stability.

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Table 1

Primer Sequences, Expected PCR Product Sizes, and Melting Temperatures for Real-Time PCR Analysis

Gene	MIPS[a] Accession No.	Primer Pairs (5′→3′)	Fragment Length (bp)	T_m[b]
DRT100	At3g12610	TTGTTGGCATCGCCGTTTAGTTCCGCAGCTGATACCGTACCATTCCT	195	90°C
UBQ5	At3g62250	AACCCTTGAGGTTGAATCATCCGTCCTTCTTTCTGGTAAACGT	426	92°C
ARIR	At1g05880	AATGCGGTCCCTTGTCCCAAGGGATGAACTTAAGCTGCGATACGT	709	87.5°C

[a] Munich Information Center for Protein Sequences

[b] Empirically determined with SYBR Green I

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Table 2

The Effect of Threshold Cycles to Dilutions on Different cDNA Systems

	Mean Threshold Cycle ± SD			
Dilutions	**SuperScript**	**OmniScript**	**RevertAid**	**AMV**
undiluted	21.21 ± 0.08	18.86 ± 0.06	no product	20.94 ± 0.04
1:5	16.08 ± 0.14	15.81 ± 0.13	17.41 ± 0.01	
1:10	17.64 ± 0.39	18.19 ± 0.55	17.51 ± 0.27	22.65 ± 0.04
1:25	18.93 ± 0.13	19.15 ± 0.06	19.19 ± 0.08	
1:50	19.75 ± 0.06	20.01 ± 0.08	19.98 ± 0.07	
1:100	21.19 ± 0.4	21.29 ± 0.42	21.5 ± 0.4	23.42

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Table 3
Evaluation of Different SYBR Green I Reaction Mixtures and Volumes

Reaction Mixtures and Volumes	PCR Efficiency E = 10^{-S} -1	Mean Threshold Cycles			
		10^6	10^5	10^4	10^4
BSA, Triton X-100	0.76	11.10	15.00	18.65	23.45
Triton X-100	0.85	10.62	14.94	18.48	21.97
Roche	0.92	11.07	15.07	18.35	21.75
10 µL	0.82	11.07	15.03	18.72	22.65

Standard curves of serial diluted *UBQ5* templates were generated with buffers containing ± 0.2 mg/mL BSA and 1.5% Triton X-100 in final reaction volumes of 20 and 10 µL.

S, slope of the standard curve

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Table 4

The Effect of SYBR Green I Dilutions on Threshold Cycles and Efficiency of Real-Time PCR

		Copy Number of *UBQ5*			
Dilutions	**PCR Efficiency**	**10^7**	**10^6**	**10^5**	**10^4**
1:40 000	0.70	7.61	11.98	16.45	20.55
1:30 000	0.74	7.55	11.25	17.24	19.41
1:20 000	0.75	7.06	10.77	15.42	19.30
1:10 000	0.78	6.62	10.89	14.78	18.59

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Table 5
Effect of the Extension Time on the PCR Efficiency

Fragment Length (bp)	Extension Time (s)	Base Pair/Extension Time (bp/s)	Standard Curve conc = 10^(−S*Ct + y)	PCR Efficiency E = 10^{-S}-1
195	10	20	conc = 10^(−0.272*Ct + 9.188)	0.87
426	10	43	conc = 10^(−0.27*Ct + 9.2)	0.86
	20	21	conc = 10^(−0.266*Ct + 9.323)	0.85
709	15	47	conc = 10^(−0.157*Ct + 9.8)	0.44
	30	24	conc = 10^(−0.23*Ct + 8.9)	0.68

S, slope of the standard curve

y, Y intercept*S