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Short Communication

Different real-time PCR systems yield different gene expression values

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ABSTRACT

Most polymerase chain reaction (PCR) systems employ pre-determined settings and proprietary master mixes that differ from one system to another. It is not known whether these differences may affect gene expression values. We compared two major real-time PCR technologies, from Life Technologies (formerly Applied Biosystems; ABI7500) and Roche Applied Science (LC480), using their default settings, proprietary reagents and other potential variables such as ramp rates and magnesium concentrations. We analyzed four genes (IL-8, COX2, ID-1 and CXCR2) in a human breast cancer cell line and found that two of them, though readily detected by ABI, were not detected using the Roche system. By altering some of the parameters and reagents used in the Roche protocol, we were able to detect expression of these two genes, but the level remained far below that detected by ABI, particularly for ID-1. When we tested three additional ID-1 primer pairs, two of these primer pairs yielded higher expression values in the LC system, yet still significantly lower than the values obtained in ABI. These results suggest critical differences in these two PCR systems, which could result in significant discrepancies in results reported by different laboratories.

1. Introduction

Real-time PCR is a major technology used in quantitative gene expression studies. Recently, technical problems that affect real-time PCR performance have been identified that result in under/overestimation of input template copy numbers [1]. This raises the question of whether different PCR machines will yield similar gene expression values when analyzing the same biological system. Several studies have addressed this issue [2–4], including one that compared seven different PCR machines [4], and concluded that it is not a problem. To the best our knowledge, however, no such study has investigated more than one or two genes. Clearly, we cannot extrapolate from such data to conclude that expression values of all or even most genes will not significantly differ as determined using different PCR systems.

We analyzed relative expression (ΔC_T) values of twelve genes (IL-8, CD44, PPAR-δ, COX2, ID-1, p21, CyclinD, Gro-γ, CXCR2, OPN, PPAR-α, and c-myc) in a human breast cancer cell line (MDA-MB231), using two PCR systems, from Life Technologies, Inc. (former Applied Biosystems) and Roche Applied Science. We report here the results of four of these genes (IL-8, COX2, ID-1, and CXCR2; β-actin as a reference gene) obtained with ABI7500 and LC480, two of the more widely used instruments for real-time PCR. We found very significant differences in the expression of two of these genes, ID-1 and CXCR2, in the two systems. Attempts at optimizing PCR conditions did not solve this problem. Comparison of several different ID-1 primer pairs demonstrated that some primers yielded higher expression values than others, but three primer sets yielded significantly lower values for ID-1 in the LC system. We conclude that the differences between these two systems are not easily addressed and are serious enough to be reported in this short communication.

2. Material and methods

We used a human breast cancer cell line (MDA-MB231). Cells were harvested and washed with PBS and suspended immediately in RNAprotect® Cell Reagent (Qiagen, Valencia, California). RNA was then isolated from the cells by MagNA Pure Compact RNA Isolation Kit (Roche Applied Science, Indianapolis, Illinois, USA). 200–400 ng total RNA was reverse transcribed to cDNA with 250 U Superscript III reverse transcriptase (Invitrogen, Carlsbad, California, USA) in a volume of 30 μl, using 1 μg random hexamer primers and 1 μg oligo dT_{12-18} primers (Invitrogen, Carlsbad, California, USA). We report our results for four genes, IL-8 (forward primer: AGATATTGCACGGGAG AATATACAAA; reverse primer: TCAATTCCTG-AAATTAAAGTTCGGAT), COX2 (forward primer: TCTGCAGAGTTGGAAGCA-CTCTA; reverse primer: GCCGAGGCTTTTCTACCAGAA), ID-1 (forward primer: GCT GGACGAGCAGCAGGTA; reverse primer: GCGTGAGTAACAGCCGTTCA)

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and CXCR2 (forward primer: CATGGCTTGATCAGCAAGGA; reverse primer: TGGAAG-TGTGCCCTGAAGAAG). β-actin (forward primer: CTGGCACCCAGCACAATG; reverse primer: GCCGATCCACACGGA GTACT) is the internal control gene in this study. Primers were designed by Primer Express 3.0 Software (PE Applied Biosystems, Foster City, CA). Primer length was 21–27 nucleotides, with a theoretical Tm of 58–60 °C. The amplicon size ranged from 66 to 110 bp. For the comparison of different ID-1 primer sets, "ID-1 143 bp" (forward primer: GTAAACGTGCTGCTCTACGACATGA; reverse primer: AGCTCCAACTGAAGGTCCCTGA) and "ID-1 330 bp" (forward primer: CCAGTGGCAGCACCGCCACC; reverse primer CGGATTCCGAGTTC AGCTCC) were referred by previous publications [5,6]. The primer "ID-1 89 bp" (forward primer: GGCTGTTACTCACGCCTCAAG; reverse primer: TCGATGACGTGCTGGAGAATC) was newly designed by Primer Express 3.0 Software. Real-time PCR was carried out in 96-well plates using either the Applied Biosystems 7500 Fast Real-Time PCR System (ABI7500) or the Roche LightCycler 480 (LC480). The PCR mix for 24 μl was prepared by adding to 4 μl cDNA template (4.5–9.0 ng), 8 μl of forward and reverse primers (final concentration is 167 nM/each primer) and 12 μl of SYBR Green Master Mix (with ROX, for ABI7500) (2× Concentration). The PCR running conditions were: one cycle at 95 °C for 10 min, 40 cycles of amplification at 95 °C for 15 s, 60 °C for 1 min. Subsequently, a dissociation program was applied with one cycle at 95 °C for 15 s, 60 °C for 1 min and 95 °C for 15 s.

Gene expression data were initially expressed as C_T (threshold cycle; sometimes referred to as Cp or cross point in LC480 system) values, the number of cycles required for the quantity of DNA to reach some pre-set value. We also determined a ΔC_T value for each gene, as the difference between its C_T value and that of the reference gene, β-actin.

We first compared the two different instruments using the "optimal" reagent mix for each one; we then ran the two systems using the same ramp, temperature and time settings for each. Next, we compared them using the same reagents for each machine. The fourth step, we adjusted the concentration of magnesium chloride in one of that reagent mixes to determine its effect on PCR amplification so as to affect gene expression levels. Finally, we compared the effect of three additional pairs of ID-1 primers; two of them were reported effective by other teams with LightCycler system.

All statistical tests were carried out after ΔC_T was determined. We used analysis of variance to study the effects of PCR system on gene expression as measured by ΔC_T. PCR values that were beyond detectable threshold were omitted from the analyses. First we tested whether gene and calculation system had an effect based on an overall F-test. If this global test was significant, we tested for pairwise differences among the three C_T calculation methods using pairwise *t*-tests with a Holm correction for multiple comparisons. These three methods are described in the following section. We also investigated the effects of dose of magnesium chloride on ΔC_T values using linear regression. All tests were conducted at a 5% level of significance, after adjustment of multiple comparisons and were carried out in *Stata version 10*.

3. Results and discussion

3.1. ABI7500 vs LC480 using respective reagents

The two PCR technologies differ in several aspects, including the software, light sources and the approach to acquisition of fluorescence data, so they employ different reagents. The ABI7500 uses the "ABI Mix" (FastStart SYBR Green Master with ROX) and LC480 uses the "LC480 Mix" (LightCycler® 480 SYBR Green I Master). So we first performed analyses using the reagent mix which is proprietary for each system. Since the Roche LC480 system provides two different ways to determine the cross point (C_p) value, Second Derivative Maximum and Fit Points methods, the relative gene expression results (ΔC_p values) from each of these two methods were compared to ABI7500 (ΔC_T values), using the same set of cDNA.

Significant differences of ΔC_T were observed for all four genes between the two methods of LC480 and ABI7500 ($P < 0.01$) with the same samples and the same conditions (Fig. 1A). Particularly surprising was that using the Second Derivative Maximum Method of LC480, no C_p value could be determined for ID-1; that is, its expression level, as determined by this method, was below the limit of detection. When we used the Fit Points method, we obtained a ΔC_p value of ID-1 of 17.31 ± 0.69, as determined from a C_p value of 31.44 ± 0.64 of this gene, and a C_p value of β-actin of 15.20 ± 0.65. So using the Fit Points method, we were able to detect the ID-1 gene, but its level was still much lower than that found using ABI technology ($\Delta C_T = 9.82 \pm 0.64$). The difference in expression level was about seven cycles, or 2^{-7} fold, i.e. 128-fold reduced ($P < 0.001$).

3.2. Amplification efficiency of ABI7500 vs LC480

Since the ID-1 gene is clearly high-expressed in this breast cancer cell line [7], the inability of LC480 to detect it, even over a large number of cycles, suggested to us a significant discrepancy between two systems. One possible factor that could account for this discrepancy is amplification efficiency. The ΔC_T values that we determined in these experiments may not be necessarily representative of actual message levels, because the two systems may amplify at different efficiencies, i.e. cycle numbers may represent different levels of amplification, though the same primers were used.

To address this possibility, we first tested PCR efficiency (E) of all four genes plus β-actin using both instruments and their respective master mixes by analyzing a standard curve ($E = 10^{-1/\text{slope}}$). An ideal amplification reaction would produce a standard curve with an efficiency of 2.0 (fold of PCR product increase per cycle), indicative of a doubling of target DNA during the exponential phase of each cycle. We found that the PCR efficiency using the ABI7500 system ranged from 1.80 to 2.09. However, when the same template and the same primers were used on the LC480, we observed that the PCR efficiencies of most of the genes were either <1.80 or >2.20 (Table 1, "default setting" column). Although no hard and fast rules determine when efficiency values are similar enough to use ΔC_T method, a rough guide is that they should be within 10% of the ideal values of 2.0 (1.8× to 2.2×) [8]. Indeed, only β-actin was within 1.8× to 2.2× range for the LC480 using either procedure, as well as COX2 using the Fit Points method (Table 1, "default setting" column).

This low efficiency in the LC480 might account for at least some of the difficulty of detecting these genes, and was quite surprising, given that we were using the proprietary reagents and protocol. In addition to the great variability of efficiency values seen with the LC480, the values of two genes (ID-1 and CXCR2) were "undetectable" because we were able to obtain C_T values only for the first two serial dilutions. The other dilutions resulted in C_p values recorded as ">35", so the standard curve could not be calculated. This further indicated that expression of these genes was not efficiently detected using the LC480 instrument. In principle, detection should be possible by extending cycle number. However, in practice, at C_T values greater than 35, non-specific hybridization becomes a problem. Thus, 40 cycles were the limit in this study.

One way to avoid problems related to amplification efficiency in gene expression studies is to express results in terms of $\Delta\Delta C_T$ values, rather than ΔC_T values. The $\Delta\Delta C_T$ values are determined by comparing the ΔC_T value of a particular gene under two or more conditions, for example, in the presence or absence of a drug treatment, or in the presence or absence of some disease state. Under these conditions, differences in amplification efficiency

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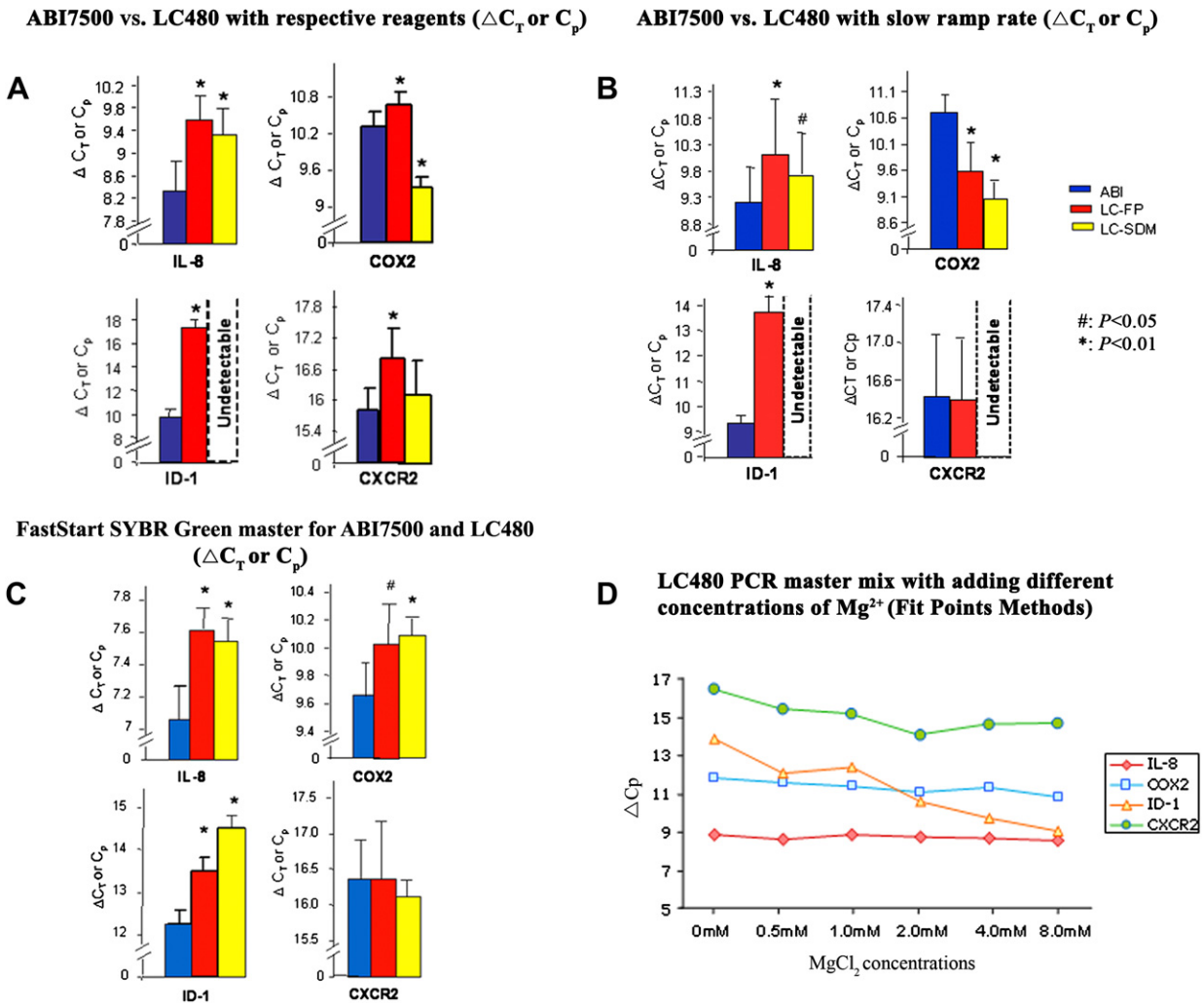

Fig. 1. Comparison of gene expression analysis between ABI machine (ABI7500) and LightCycler (LC480). Blue bar: the mean of ΔC_T values obtained from ABI7500; Red bar: from LC480 Fit Points Method (LC-FP); Yellow bar: from LC480 Second Derivative Maximum Method (LC-SDM). A: ABI7500 vs. LC480 using respective reagents (ΔC_T or C_p). ABI7500 used "ABI Mix" (FastStart SYBR Green Master with ROX), threshold (0.2) was assigned manually. LC used "LC480 mix" (LightCycler 480 SYBR Green I Master) with Fit Points method's threshold = 0.6406. Each ΔC_T (or C_p) value is the mean of 18 determinations, samples stored in RNAProtect® Cell Reagent after collecting cells. Real-time PCR assays were done in a reaction volume of 24 μl containing 1x SYBR Green Master mix, 167 nM each primer, 4 μl cDNA under the following conditions: 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. Comparison of ΔC_T values of four genes between the two systems: For COX2: ABI *vs* LC-FP ($P < 0.001$); ABI *vs* LC-SDM ($P < 0.001$); FP *vs* SDM ($P < 0.001$). For CXCR2: ABI *vs* LC-FP ($P < 0.001$); ABI *vs* LC-SDM ($P = 0.02$); FP *vs* SDM ($P = 0.003$). For ID-1: ABI *vs* LC-FP ($P < 0.001$); Undetectable data for SDM. For IL-8: ABI *vs* LC-FP ($P < 0.001$); ABI *vs* LC-SDM ($P < 0.001$); FP *vs* SDM ($P = 0.09$). B: ABI vs. LC480 with slow ramp rate setting (ΔC_T or C_p). LC480 was set up at slow ramp rate: temperature increasing at ramp rate = 2.6 °C/second; temperature decreasing at ramp rate 1.6 °C/second. ABI7500 was default ramp setting (auto). Fit Points method threshold = 1.6322. Each ΔC_T or C_p value is the mean of 18 samples was treated the same way as that in Fig. 1A. Real-time PCR assays were done in the same reaction volume and same reagent as those in Fig. 1A. Comparison of ΔC_T values of four genes between the two systems: for COX2: ABI *vs* LC-FP ($P < 0.001$); ABI *vs* LC-SDM ($P < 0.001$); FP *vs* SDM ($P < 0.001$). For CXCR2: ABI *vs* LC-FP ($P = 0.88$); undetectable data for SDM. For ID-1: ABI *vs* LC-FP ($P < 0.001$); undetectable data for SDM. For IL-8: ABI *vs* LC-FP ($P = 0.007$); ABI *vs* LC-SDM ($P = 0.15$); FP *vs* SDM ($P = 0.16$). C: FastStart SYBR Green Master Mix for both ABI7500 and LC480 (ΔC_T or C_p). Both machines used "ABI Mix" with default ramp rate setting. Threshold setting on ABI7500 was 0.2. LC480 Fit Points method threshold = 2.2194. Each ΔC_T (or ΔC_p) value is the mean of 6 samples treated the same way as that in Fig. 1A after collecting cells. Real-time PCR assays were carried out in the same reaction volume and same reagent as those in Fig. 1A. Comparison of ΔC_T values of four genes between the two systems: for COX2: ABI *vs* LC-FP ($P = 0.03$); ABI *vs* LC-SDM ($P = 0.016$); FP *vs* SDM ($P = 0.61$). For CXCR2: no significant differences. For ID-1: all pairwise comparisons significance at $P < 0.001$. For IL-8: ABI *vs* LC-FP ($P < 0.001$); ABI *vs* LC-SDM ($P < 0.001$); FP *vs* SDM ($P = 0.48$). D: The effect of magnesium concentrations on gene expression (ΔC_p). LC480 PCR master mix with different concentration of added Mg^{2+} ($MgCl_2$) using LC480 Fit Points Method. The *X* axis represents the added $MgCl_2$ final concentrations, and the *Y* axis represents the mean of ΔC_p values from 6 samples treated the same way as that in Fig. 1A after collecting cells. Differences in intercepts and slopes were observed for each gene ($P < 0.001$). The dose effect was strongest for ID-1 expression (slope = −0.53, $P < 0.001$) and least for IL-8 (slope = −0.03, $P = 0.09$). Dose effect was significant for COX2 (slope = −0.10, $P < 0.001$) and for CXCR2 (slope = −0.15, $P = 0.003$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

cancel out, since the efficiency of amplification for a particular gene using a particular PCR system should be the same regardless of how it is treated. We did not compare $\Delta\Delta C_T$ values of ID-1 in the two PCR systems, however, because we were not concerned about different expression levels between different experimental conditions. We focused on the differences of PCR efficiency between two systems when using the same templates and primers, and found the efficiencies, in some cases, were unacceptable low — much less than the ideal value of 2-fold amplification per cycle. This suggests that the proprietary conditions used may not be ideal for some genes and surely this ought to be of major concern to researchers.

3.3. Comparison of instrument settings

When data indicate that PCR efficiency is not adequate, a possible response is to try to optimize PCR conditions. We took

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Table 1
PCR efficiencies of four Genes plus β-actin by three methods.

	ABI7500			LC480 Fit Points				LC480-SDM		
	Default setting	Slow ramp	"ABI Mix"	Default setting	Slow ramp	"ABI Mix"	Added 2 mM MgCl2	Default setting	Slow ramp	"ABI Mix"
β-actin	2.09	2.09	1.95	1.92	1.86	1.97	1.76	1.85	1.91	1.87
IL-8	1.90	1.86	1.97	1.69	1.84	1.81	1.71	1.57	1.97	1.95
COX2	1.80	1.86	2.10	1.80	2.02	1.77	1.75	1.74	1.95	1.99
ID-1	1.83	1.83	1.73	1.67	1.64	1.58	1.74	Undet	Undet	1.68
CXCR2	1.85	1.75	1.80	2.29	Undet	1.48	1.95	Undet	Undet	1.66

"Defaulting setting": default ramp rate setting and proprietary reagent for each instrument. (For ABI7500 the default ramp rate setting is "Auto" and for LC480 that is 4.4 °C/s for heating up and 2.2 °C/sec for cooling down. The proprietary reagent for ABI7500 is FastStart SYBR Green Master with ROX and the LightCycler® 480 SYBR Green I Master is for LC480.) "Slow ramp": the test was to compare PCR efficiencies when LC480 was set up to slower ramp rate: temperature increasing at ramp rate = 2.6 °C/second, temperature decreasing at ramp rate = 1.6 °C/second. "ABI mix": FastStart SYBR Green Master with ROX. "Undet": undetectable data.

note of the fact that ABI7500 and LC480 differ in their ramp rate settings. This could be significant, because theoretically, a fast ramp rate may not allow sufficient time for DNA denaturing or for primer annealing, which could have dramatic effects on PCR performance, even causing reaction failures. This could conceivably account for at least some of the generally greater expression levels determined using the ABI, because its overall running time was much longer (by more than 1 h) than LC480 when using the same protocol, suggesting a slower ramping (on "auto" setting). While we do not know and were unable to learn the ramping time on ABI7500 setting, we lowered as follows the ramping time in the LC480 from the default ramp rate (4.4 °C/sec for heating up and 2.2 °C/sec for cooling down) on the premise that the PCR conditions would match better than those used by ABI7500:

Denaturation (1 cycle): 95 °C, 10 min, ramp rate = 2.6 °C/sec.
Amplification (40 cycles): 95 °C, 15 s, ramp rate = 2.6 °C/sec.
60 °C, 60 s, ramp rate = 1.6 °C/sec.

We found that changing the ramp rate conditions in the LC480 to match more closely those of the ABI did not have much effect on improving the match of expression values; in fact, levels of ID-1 and CXCR2 using the Second Derivative Maximum Method were again undetectable (Fig. 1B). As shown in Table 1 "slow ramp" column, this was reflected in very low or undetectable efficiency values. Thus use of the Second Derivative Maximum Method consistently failed to detect C_T values for our two genes.

3.4. ABI7500 vs LC480 with "ABI Mix"

Other than PCR platform, the PCR reagent might also play an important role in optimization. To test this, we next compared the two systems using the same reagent mix: "ABI Mix" i.e. FastStart SYBR Green Master (ROX) for both instruments. We did not use the "LC480 Mix" for ABI machine because the Roche LC480 proprietary master mix (LightCycler 480 SYBR Green I Master) does not contain an internal fluorescence reference (ROX), so it is not appropriate for the ABI 7500 machine, on which the reporter dye should be normalized by ROX before generating C_T. This is necessary to correct for fluorescent fluctuations due to changes in concentration or volume in the wells. Since LC480 has a different thermal-cycling system and software, normalization can be done without any passive reference dye.

The expression values of two genes – IL-8 and COX2 – statistically differed between ABI7500 and LC480 systems (though the difference was within 0.5 ΔC_T). The use of "ABI Mix" in the LC480 system brought ΔC_T values of ID-1 into detectable range, but there was still a significant difference in the levels determined by the three methods: 12.25 ± 0.33 in ABI *vs* 13.48 ± 0.31 in Fit Points method and 14.46 ± 0.31 in Second Derivative Maximum (Fig. 1C). Thus, there remained as much as two PCR cycles difference, i.e. four fold discrepancy (2^{-2}), using LC480 *vs* ABI. This difference was statistically significant ($P < 0.01$). The use of the "ABI Mix" also improved efficiency values for the LC480 system. As shown in Table 1, "ABI Mix" column, the values for IL-8 and COX2 were increased compared with the values in "Default setting" column, though those for ID-1 and CXCR2 were still very low.

3.5. Critical effect of magnesium concentration

Since magnesium is required and critical for Taq polymerase activity, we next focused on the concentration of $MgCl_2$ in the PCR master mix, by adding 0.5 mM, 1 mM, 2 mM, 4 mM, and 8 mM (final concentration) of $MgCl_2$ into LightCycler 480 SYBR Green I Master mix. The ramp rate for this test was the default setting for LC480. When the shapes of amplification curves and PCR efficiency of the target and reference gene are very similar among the samples to be compared, the Fit Points method is more suitable, since we can manually set the cross point so it fits all curves at the same height, so as to avoid any false value from noise-band. Thus, in this experiment, we determined C_p values using only the Fit Points method to investigate the effect of adding extra magnesium.

As shown in Fig. 1D, though the ΔC_p values of IL-8 and COX2 were largely unaffected by different concentrations of added Mg^{2+} in LightCycler 480 SYBR Green I Master mix, it appeared the added Mg^{2+} reduced the ΔC_p values of ID-1 and CXCR2 (representing increased expression levels), which indicated the amplification sensitivity improved for these two genes after adding extra Mg^{2+}. But while 2 mM added concentration appeared to be optimal for CXCR2, it did not completely reduce differences for ID-1.

We then used the standard curves to test PCR efficiency in 2 mM added $MgCl_2$ condition only by Fit Points method of LC480. As shown in Table 1, "2 mM $MgCl_2$" column, the PCR efficiency of β-actin, IL-8, and COX2 in added 2 mM Mg^{2+} condition was similar or slightly lower than default setting, while it became detectable for ID-1 (1.74) and CXCR2 (1.95). Thus using higher concentrations of Mg^{2+}, we were able to improve the efficiency of amplification of these two genes and reduce the differences in ΔC_T values in a complex fashion relative to the proprietary master mix between two instruments. This suggests that Mg^{2+} concentration is different in the two different master mixes. Apparently the Mg^{2+} concentration in LightCycler 480 SYBR Green I Master mix is not best suited for some genes (in this study, ID-1 and CXCR2), making them difficult to amplify under these conditions. After adding Mg^{2+}, the previously undetectable gene expression level became detectable for those genes. But this additional Mg^{2+} reduced PCR efficiency for other genes (IL-8, COX2 and β-actin). So it is clearly difficult to find an ideal condition for amplifying all genes at the same time.

3.6. Comparison of different primers

A remaining parameter that might account for poor PCR efficiencies is the primer pair. It is possible that the systems differ with

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Table 2
Average ΔC_T of ID-1 gene amplified by four different primer pairs (Mean ± SD).

	37 °C			4 °C		
	ABI7500	LC-FP	LC-SDM	ABI7500	LC-FP	LC-SDM
ID-1	8.83 ± 0.18	14.84 ± 0.42*	15.56 ± 0.51*	9.16 ± 0.20	13.52 ± 1.20*	14.47 ± 0.79*
ID-1 89 bp	3.80 ± 0.74	5.77 ± 0.04*	5.46 ± 0.09Δ	4.13 ± 0.44	5.56 ± 0.17*	5.38 ± 0.10*
ID-1 143 bp	4.12 ± 0.70	4.77 ± 0.44	4.70 ± 0.37	4.36 ± 0.16	4.71 ± 0.16	4.69 ± 0.10Δ
ID-1 330 bp	8.77 ± 0.47	13.00 ± 0.84*	13.28 ± 0.76*	8.57 ± 0.30	13.00 ± 0.25*	13.38 ± 0.17*

*$P < 0.01$, compared with the values obtained by ABI7500.
Δ$P < 0.05$, compared with the values obtained by ABI7500.

respect to the optimal primers for amplification. To test this possibility, we compared the effect of three additional primers for ID-1. One of these was newly designed by ourselves (named "ID-1 89 bp"), while the other two ("ID-1 143 bp" and "ID-1 330 bp") were reported effective by other groups using the LightCycler system [5,6]. All four primers amplify the same region of the gene and in principle should be specific for the same transcript variants.

Six samples (cDNA of MDA-MB231) were tested. Samples were stored at 37 °C and 4 °C in RNAprotect® Cell Reagent for 6 h after collecting cells, with three cell preparations at each temperature. According to the PCR protocol in the reference publications, the temperature of PCR extension for "ID-1 143 bp" and "ID-1 330 bp" is 72 °C, while for "ID-1" and "ID-1 89 bp", it is 60 °C, that is, the same as in our previous reactions. So we analyzed β-actin at either 72 °C or 60 °C, according to the ID-1 primer pair used, to obtain accurate ΔC_T values. All other conditions were identical for every primer pair.

Table 2 shows average ΔC_T values of ID-1 gene amplified by these four different primer pairs. We found statistically significant differences in ΔC_T values between ABI and LC480, using either method of analysis for the latter system ($P \leq 0.02$, usually ≤ 0.01) for each of the primer pairs except "ID-1 143 bp". Amplification using "ID-1 330 bp" resulted in a ΔC_T value difference between ABI7500

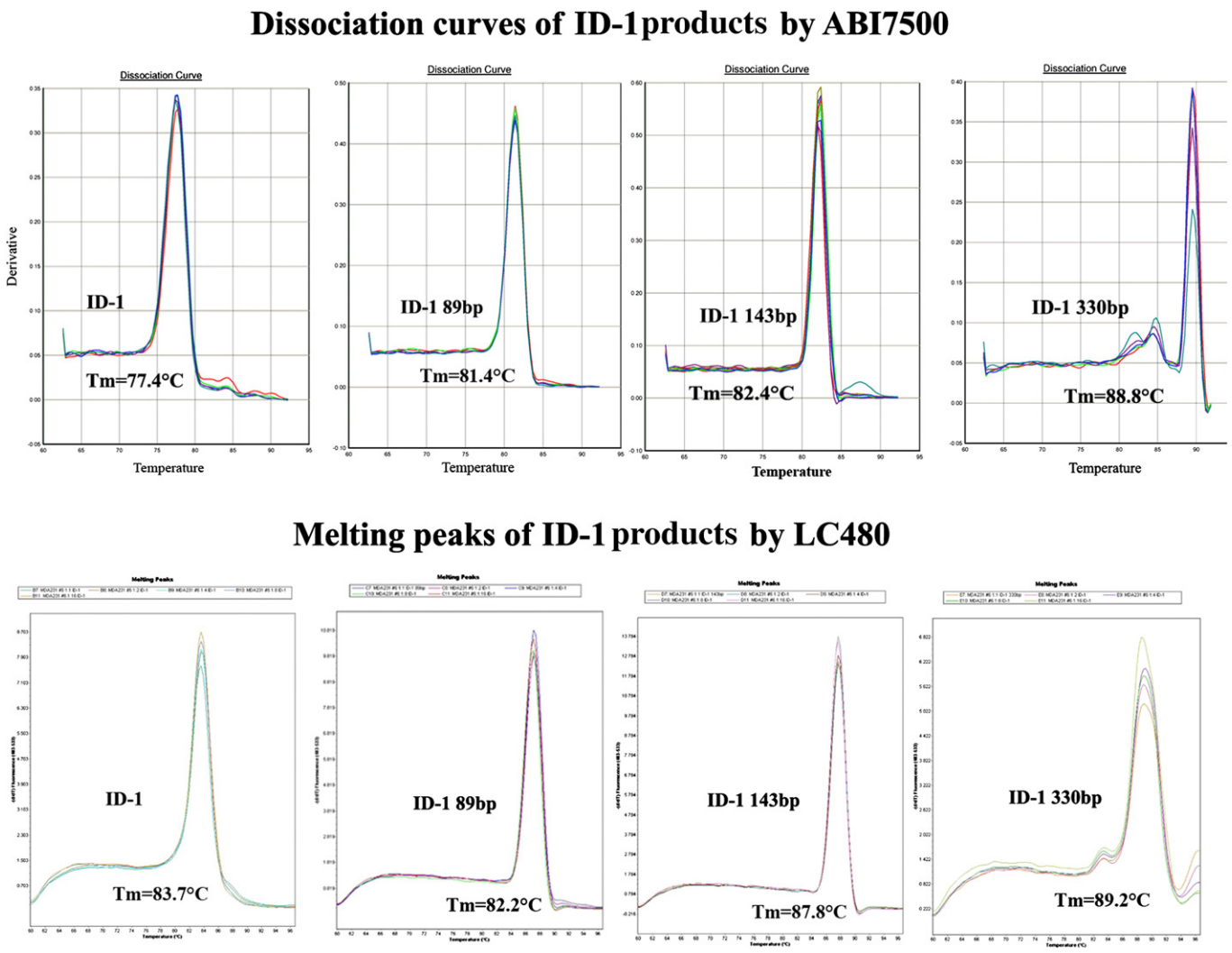

Fig. 2. Dissociation curves or melting peaks for four ID-1 PCR products. Each pair of primer's name and Tm is labeled near the curve. Upper panel: dissociation curves from the PCR reaction reports of ABI7500. Lower panel: melting peaks from the PCR reaction reports of LC480.

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(8.77 or 8.57) and LC480 (13.00/13.28 or 13.38) that was about as great as that obtained using the original ID-1 primer (8.83 or 9.16 *vs* 14.84 or 13.52/15.56 or 14.47) (Table 2). "ID-1 89 bp" in contrast, yielded expression levels on LC480 that were much closer to those on ABI7500 system, though the differences, about 1–1.5 cycles, were still statistically significant ($P < 0.05$ and <0.01) (Table 2). For "ID-1 143 bp", difference between the two PCR systems was significant only when analyzed using LC-SDM method at 4 °C ($P < 0.05$). The PCR efficiencies of these three new primer pairs were all acceptable (1.83–2.07) with both systems, while our original "ID-1" primer had poorer efficiencies in the LC480 system (1.87 with ABI *vs* 1.71/1.64 with LC). The dissociation curves of all four amplicons showed a single peak, indicating no non-specific PCR product was present, regardless of PCR efficiencies (Fig. 2).

These results suggest that much of the differences in expression values of ID-1 between the two systems that we observed using our original primer pair may be reduced by selection of different primer pairs. In fact, the two primer sets that gave fairly similar expression values for the ABI and LC systems, ID-1 89 bp and 143 bp, also yielded expression values in the ABI system that were about 4 cycles (16-fold) higher than the expression values of the other two primers in the same system (Table 2). This suggests that either of these two primer sets may be optimal for ID-1 amplification in either system.

However, the fact remains that much lower expression values in either system resulted from the use of two other primer sets, including a primer pair that was previously used by another group to amplify ID-1 in the LightCycler system (ID-1 330 bp). So there is a discrepancy that cannot be accounted for simply by our original primer pair. Other primer pairs that, according to the primer design software should have been valid, did not give the same expression values in the two different systems, nor did they give values in one system as high as those of another primer pair in the same system.

Primer design software allows the user to select factors like GC content, Tm, length, and where on the template the primer binds. Our results clearly indicate, though, that the software cannot guarantee that a particular primer pair provides the maximum level of expression using a particular platform and analyzing a particular system. The only way to be certain of this is to test numerous primer pairs, all of which may pass the tests of the primer design software. We believe that this problem may account for differences reported in the literature for expression levels of the same genes in the same tissues, using different PCR systems and primers [9,10].

In summary, we found major differences in the expression level for ID-1, one of our 12 target genes, in the ABI and LC systems. These differences could not be entirely resolved by optimizing PCR conditions. The proprietary conditions specified by the manufacturer of the PCR system are supposed to produce optimal results. We show here that, for at least one gene out of a dozen we tested, they do not always do so. While further work would be necessary to confirm this, it seems unlikely that ID-1 is the only gene for which this problem arises. Since we studied only four genes in detail in this study, there may be other genes which show discrepant expression values if more genes were analyzed.

While respecting proprietary issues, we wish to emphasize the potential benefits of companies such as ABI and Roche specifying exactly the composition and concentrations of all their reagent mixes. A further recommendation we make is that the parameters of default and other fixed settings be specified, and that users be allowed to change these settings so as to standardize or at least compare their procedures with others who may be using a different apparatus. Users should also publish all these values, so that others can evaluate whether their approaches can be fairly compared.

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References

[1] Hilscher C, Vahrson W, Dittmer DP. Faster quantitative real-time PCR protocols may lose sensitivity and show increased variability. Nucleic Acids Res 2005;33:e182.
[2] Exner MM, Lewinski MA. Sensitivity of multiplex real-time PCR reactions, using the LightCycler and the ABI PRISM 7700 sequence detection system, is dependent on the concentration of the DNA polymerase. Mol Cell Probes 2002;16:351–7.
[3] Bolufer P, Colomer D, Gomez M, Martinez J, Gonzalez S, Gonzalez M, et al. Quantitative assessment of PML-RARa and BCR-ABL by two real-time PCR instruments: multiinstitutional laboratory trial. Clin Chem 2004;50:1088–92.
[4] Silvy M, Mancini J, Thirion X, Sigaux F, Gabert J. Evaluation of real-time quantitative PCR machines for the monitoring of fusion gene transcription using the Europe against cancer protocol. Leukemia 2005;19:305–7.
[5] Iwatsuki M, Fukagawa T, Mimori K, Nakanishi H, Ito S, Ishii H, et al. Bone marrow and peripheral blood expression of ID1 in human gastric carcinoma patients is a bona fide indicator of lymph node and peritoneal metastasis. Br J Cancer 2009;100:1937–42.
[6] Xu B, Sun Y, Tang G, Xu C, Wang L, Zhang Y, et al. Id-1 expression in androgen-dependent prostate cancer is negatively regulated by androgen through androgen receptor. Cancer Lett 2009;278:220–9.
[7] Lin CQ, Singh J, Murata K, Itahana Y, Parrinello S, Liang SH, et al. A role for Id-1 in the aggressive phenotype and steroid hormone response of human breast cancer cells. Cancer Res 2000;60:1332–40.
[8] Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C (T) method. Nat Protoc 2008;3:1101–8.
[9] Ahmed FE, Vos P, iJames S, Lysle DT, Allison RR, Flake G, et al. Transcriptomic molecular markers for screening human colon cancer in stool and tissue. Cancer Genomics Proteomic 2007;4:1–20.
[10] Lu S, Chiu YS, Smith AP, Moore D, Lee NM. Biomarkers correlate with colon cancer and risks: a preliminary study. Dis Colon Rectum 2009;52:715–24.

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