A Novel Method to Compensate for Different Amplification Efficiencies between Patient DNA Samples in Quantitative Real-Time PCR

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Quantification of residual disease by real-time polymerase chain reaction (PCR) will become a pivotal tool in the development of patient-directed therapy. In recent years, various protocols to quantify minimal residual disease in leukemia or lymphoma patients have been developed. These assays assume that PCR efficiencies are equal for all samples. Determining t(14:18) and albumin reaction efficiencies for sixteen follicular lymphoma patient samples revealed higher efficiencies for blood samples than for lymph node samples in general. However, within one sample both reactions had equivalent efficiencies. Differences in amplification efficiencies between patient samples (low efficiencies) and the calibrator in quantitative analyses result in the underestimation of residual disease in patient samples whereby the weakest positive patient samples are at highest error. Based on these findings for patient samples, the efficiency compensation control was developed. This control includes two reference reactions in a multiplex setting, specific for the β-actin and albumin housekeeping genes that are present in a constant ratio within DNA templates. The difference in threshold cycle values for both reference reactions, ie, the ΔCt2 value, is dependent on the amplification efficiency, and is used to compensate for efficiency differences between patient samples and the calibrator. The β-actin reference reaction is also used to normalize for DNA input. Furthermore, the efficiency compensation control facilitates identification of patient samples that are so contaminated with PCR inhibitory compounds that different amplification reactions are affected to a different extent. Accurate quantitation of residual disease in these samples is therefore impossible with the current quantitative real-time PCR protocols. Identification and exclusion of these inadequate samples will be of utmost importance in quantitative retrospective studies, but even more so, in future molecular diagnostic analyses. (J Mol Diag 2001, 3:55–61)

Disease-associated chromosomal markers facilitate specific detection of malignant cells in patient tissues and fluids, and have proved useful for diagnostic and prognostic purposes. By means of the polymerase chain reaction (PCR), malignant cells can be detected to a sensitivity limit of about a single malignant cell in a background of 10⁶ to 10⁷ normal cells. Monitoring the number of residual malignant cells during the course of disease may predict for early relapse, preceding apparent clinical manifestation, and may verify the efficacy of treatment regimens.²–⁴ Monitoring by quantitative PCR (QPCR) may become a fundamental tool to support patient directed treatment in future, at least for certain types of malignancies. Various QPCR analyses based on competitive PCR strategies have been developed in the past.⁵–⁸ However, the use of these assays is frequently hampered by a high risk for contamination and the labor-intensiveness of the technique. Real-time PCR⁹,¹⁰ based on Taqman chemistry¹¹,¹² has innovated PCR technology, allowing faster analysis of patient samples in a closed system, thereby lowering the risk of contamination. The use of an internal fluorescent probe warrants the specificity of the reaction and allows for the determination of sample positivity within the exponential phase of the reaction.

Various quantitative real-time PCR and RT-PCR protocols have recently been developed for the detection of disease associated chromosomal translocations,¹³–¹⁶ or disease-associated T cell receptor or Ig gene rearrangements.¹⁷,¹⁸ All protocols developed thus far are based on the assumption that patient samples and calibration samples have equal efficiencies during amplification. At least for the amplification of genomic DNA templates this assumption may not be true, since many patient samples seem contaminated with PCR inhibiting compounds. It is

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exactly for this reason that competitive PCRs have been developed in the past.\textsuperscript{5–8} Factors like DNA fragmentation,\textsuperscript{19} residual RNA in DNA preparations,\textsuperscript{20} the presence of PCR inhibiting compounds like residual anti-coagulant heparin\textsuperscript{21} or proteinase K-digested heme compounds like hemoglobin\textsuperscript{22} or myoglobin\textsuperscript{23} will negatively affect PCR efficiency.

In this report, we demonstrate that the amplification efficiency for different DNA templates isolated from whole blood or bone marrow samples is subject to extensive variation, leading to underestimation of minimal residual disease or false negative result in quantitative PCR analyses. We developed a general real-time PCR control, ie, the efficiency compensation control (ECC), to compensate for different efficiencies between patient samples. This approach comprises the inclusion of two different reference reactions, one of which is carried out with mutant primers. Both reference reactions have been developed to amplify with equivalent efficiencies on the same template. The exact difference in the threshold cycle values (\(\Delta Ct\)) between both reference reactions (\(\Delta Ct\) value) is dependent on the amplification efficiencies. This \(\Delta Ct\) value can, therefore, be used to calculate the exact efficiencies of both reactions for each sample in comparison to a calibrator sample, thus providing a way to compensate for differences in efficiencies between patient samples and the calibrator in quantitative analyses. In this way, accurate estimation of residual disease in patient samples becomes possible, regardless of DNA quality or the presence of PCR-inhibiting compounds.

**Materials and Methods**

**Real-Time PCR**

During PCR amplification, the number of molecules that is synthesized (\(X_n\)) depends on the number of template molecules present at the beginning of the reaction (\(X_0\)), the reaction efficiency (\(E\)) and the number of amplification cycles (\(n\)) (Eq. 1).\textsuperscript{24} In real-time PCR, sample positivity is defined as the cycle number at which emitted fluorescence exceeds the 10 times SD of baseline emissions, and is called the threshold cycle (\(Ct\)). The number of molecules synthesized at the threshold cycle (\(X_{Ct}\)) is specific but constant (\(C\)) for each type of reaction and may depend on reporter dye (6-carboxy-fluorescein (FAM) or 27-dimethyl-4,5-dichloro-6-carboxy-fluorescein (JOE)).

\[
X_{Ct} = X_0^* (1+E)^n \Leftrightarrow X_{Ct} = X_0^* (1+E)^{Ct} \Leftrightarrow X_{Ct} = C^*(1+E)^{-Ct} \quad (1)
\]

**Normalization**

To correct for differences in DNA or RNA load between patient samples, the target PCR, \(t(14;18)\) in this report, is normalized to a reference PCR, \(\beta\)-actin in this report, that is specific for an endogenous housekeeping gene. The \(t(14;18)\) and the \(\beta\)-actin reactions outlined in this report have equivalent amplification efficiencies, allowing direct normalization of the \(t(14;18)\) PCR to the \(\beta\)-actin PCR at the Ct level (Eq. 2; K Livak, personal communication and Applied Biosystems User Bulletin #2).

\[
\frac{X_{\text{arg, ref}}}{X_{\text{arg, ref}}} = \frac{X_{\text{arg}}} {Ct_{\text{arg}}} = C^*(1+E)^{Ct_{\text{arg}}} \Leftrightarrow \\
\quad \text{when } E_{\text{arg, ref}} = E_{\text{ref}} \Rightarrow X_{\text{arg}} = C^*(1+E)^{-\Delta Ct_{\text{1}}} 
\]

**PCR Efficiency**

The efficiency (\(E\)) of a reaction can be determined by a standard curve. \(Ct\) values for serial template dilutions in \(dH_2O\) are related to the logarithm of the dilution factor, and the slope is a measure for reaction efficiency (Eq. 3; K Livak, personal communication and Applied Biosystems User Bulletin #2):

\[
eff = E = 10^{-\frac{1}{\text{slope}}} - 1 \quad (3)
\]

**Quantification Using a Single Calibrator**

For quantification, patient samples can be related to a single calibration sample, facilitating the use of Eq. 4 (ie, the comparative \(Ct\) method; K Livak, personal communication and Applied Biosystems User Bulletin #2). However, this method demands equivalent amplification efficiencies between patient samples and the calibrator.

\[
X_{\text{ON, sample}} = \frac{C^* (1+E_{\text{Sample}})^{-\Delta Ct \text{S}}} {C^* (1+E_{\text{Cal}})^{-\Delta Ct\text{Cal}}} \Leftrightarrow \\
\quad \text{when } E_{\text{Sample}} = E_{\text{Cal}} \Rightarrow X_{\text{ON, sample}} = X_{\text{ON, Cal}} (1+E)^{\Delta Ct \text{S}} - \Delta Ct \text{Ct} \quad (4)
\]

whereby \(\Delta Ct\) = \(\Delta Ct \text{S} - \Delta Ct \text{Ct}\)

The calibrator (\(X_{\text{ON, Cal}}\)) used for the quantification of circulating lymphoma cells during the course of disease in follicular non-Hodgkin’s lymphoma (NHL) patients is a 100-fold dilution of \(t(14;18)\) positive cell line SUDHL6 DNA into a DNA pool obtained from four EBV-induced lymphoblastoid B cell lines, and equals 750 \(t(14;18)\)-carrying lymphoma cells per 0.5 \(g\) of DNA (equivalent to 75,000 cells).\textsuperscript{4,14}

**The Efficiency Compensation Control (ECC)**

The ECC outlined in this report is used to compensate for varying PCR efficiencies between patient samples and the calibrator. The ECC depends on two different reference reactions, ie, the multiplicity \(\beta\)-actin gene and the single copy albumin gene. Both reactions amplify with equivalent efficiencies on identical DNA templates (see results), but provide different \(Ct\) values. The difference between both \(Ct\) values, ie, the \(\Delta Ct_2\) value, is solely
dependent on the actual PCR efficiency for that template (Eq. 5):

$$\frac{X_{[\text{bact}]}(n+1)E_{\text{sample}}^{C_{t}^{(\text{bact})}}}{X_{[\text{Alb}]}(n+1)E_{\text{sample}}^{C_{t}^{(\text{Alb})}}} = C_{0}^{n}(1+E)^{-\Delta C2}$$

whereby $\Delta C2 = C_{t}^{(\text{bact})} - C_{t}^{(\text{Alb})}$

$\Delta C2$ values can be used to determine amplification efficiencies for patients relative to the efficiency of the calibrator, providing a correction factor for different efficiencies between patient samples and the calibrator (Eq 6):

$$\frac{X_{[\text{bact}]}(n)E_{\text{sample}}^{C_{t}^{(\text{bact})}}}{X_{[\text{Alb}]}(n)E_{\text{sample}}^{C_{t}^{(\text{Alb})}}} = C_{0}^{n}(1+C_{t}^{(\text{Alb})}E_{\text{sample}}^{\Delta C2})$$

This correction factor can be introduced into Eq. 4, facilitating the quantification of patient samples relative to a single calibrator with compensation for different efficiencies between patient samples and the calibrator sample (Eq 7):

$$X_{\text{ON,Sample}} = X_{\text{ON,Calibrator}}(1 + E_{\text{Calibrator}}^{\Delta C2})$$

DNA Isolation

Patient whole blood sample DNA was isolated by a standard high-salt lysis method as described by Miller et al. 25

$t(14;18)$ PCR Analysis

Amplification reactions are performed in the ABI/Prism 7700 sequence detector (Applied Biosystems, Foster City, CA). Five hundred nanograms of patient sample DNA is amplified in duplicate in the presence of 300 nmol/L oligonucleotides MBR-2 (5’-TGC TGA AAC ATT CAC CCT TCA GAG AGT CTG AGA GCC T-3’; bp 2816–3037 of GenEMBL database entry acc. 14745) and JH-con-1 160 nmol/L of dual-labeled fluorogenic MBR internal probe 5’-(FAM)-CAC AGA CCC ACC CAG AGC CC-(TAMRA)-p3’, 250 μmol/L dNTPs, 1.25 U of AmpliTag DNA polymerase, and 1 mmol/L MgCl$_2$ in Sample Buffer A in a total volume of 50 μl. Amplification conditions are the same as mentioned above.

Table 1. Definition of Symbols

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>$X_n$</td>
<td>number of molecules at cycle $n$</td>
</tr>
<tr>
<td>$X_0$</td>
<td>initial number of molecules</td>
</tr>
<tr>
<td>$E$</td>
<td>efficiency of amplification</td>
</tr>
<tr>
<td>$n$</td>
<td>number of cycles</td>
</tr>
<tr>
<td>$C_t$</td>
<td>threshold cycle</td>
</tr>
<tr>
<td>$C_t^{(C_t-C_d)}$</td>
<td>number of molecules at $C_t$</td>
</tr>
<tr>
<td>$X_{\text{ON}}$</td>
<td>normalization of target molecules to reference molecules</td>
</tr>
<tr>
<td>$\Delta C2$</td>
<td>$C_{t}^{(\text{sample})} - C_{t}^{(\text{calibrator})}$</td>
</tr>
</tbody>
</table>

Results

To study variation in amplification efficiencies among patient DNA samples, the efficiencies of our t(14;18) and albumin PCRs were first tested on cell line DNA. Genomic DNA from the t(14;18)-carrying cell line SUDDL6 was serially diluted in dH$_2$O and amplified in duplicate to construct t(14;18) and albumin standard curves for eight to ten independent experiments. R$^2$ values of all curves exceeded 0.98, and reaction efficiencies were calculated from the slopes (Eq. 3). The average efficiencies for the t(14;18) reaction ($n = 11$) and the albumin reaction ($n = 8$) were $E = 0.91 \pm 0.05$ and $E = 0.93 \pm 0.04$, respectively (Table 2). We then determined the efficiencies for both reactions on patient DNA samples that were isolated from lymph node biopsies or whole blood specimens (Table 2). Lymph node DNA samples most likely seem to contain higher contents of PCR inhibiting compounds resulting in lower amplification efficiencies compared to patient whole blood DNA samples. Both reaction efficiencies for each DNA sample compared, showed that a low efficiency for one reaction was accompanied by a low efficiency for the other reaction. For lymph node DNA samples, whole blood DNA samples have much lower efficiencies. The average efficiency difference ($\Delta E$ value) between both reactions is $\Delta E = 0.023$ for lymph node DNA samples ($n = 3$ pairs) and $\Delta E = -0.009$ for whole blood DNA samples ($n = 8$ pairs).

Based on these observed differences in reaction efficiencies between patient samples (Table 2), we have developed the “efficiency compensation control (ECC)”. This control uses two reference reactions that are specific for the $\beta$-actin and the albumin housekeeping genes. When testing both reaction efficiencies on serial B cell line DNA pool dilutions (DNA pool from four EBV-induced B cell lines), both reactions performed with efficiencies of $E = 0.95$ and $E = 0.94$ for the $\beta$-actin and albumin reaction, respectively. $\beta$-actin is a multicopy gene with
several pseudogenes and albumin is a single copy gene, and are present in a constant ratio for genomic DNA templates. Both PCRs will therefore produce different Ct values at identical template concentrations (Figure 1). For genomic DNA templates, the \( D_{Ct} \) value between these reactions is exclusively determined by the actual amplification efficiencies for both reactions (Figure 1), and is independent of initial DNA concentration. In the experiment as described above, the \( D_{Ct} \) value between the \( \beta \)-actin reaction and the albumin reaction remained constant over all serial B cell line DNA pool dilutions (500 ng - 8 ng) and was \( D_{Ct} = 3.86 \pm 0.08 \). Samples that amplify with high reaction efficiencies will provide lower \( D_{Ct} \) values in comparison to samples that are contaminated with PCR inhibiting compounds and consequently amplify with lower reaction efficiencies (Figure 1). This \( D_{Ct} \) value can therefore be used to compensate for efficiency differences between patient samples and the calibrator in quantitative minimal residual disease studies. The target and reference PCRs should be performed on the same template dilution to exclude differences in reaction efficiencies.

We then calculated the \( D_{Ct} \) value that is theoretically needed between two reference PCRs to distinguish between efficiency differences as low as \( E = 0.05 \). This will of course depend on the reproducibility of both Ct values for the reference reactions. The standard deviations in Ct values for both reference reactions performed on the serial B cell line DNA pool dilutions as described above ranged between 0.007 cycles for 0.5 \( \mu \)g DNA to 0.11 cycles for the highest dilution (0.8 ng DNA). Assuming a SD in Ct values of 0.11 cycles for both reference reactions, the \( D_{Ct} \) value between both reactions needs to be in the order of ten cycles to distinguish between efficiency differences among DNA samples as low as \( D_{E} = 0.05 \). No combinations of multicopy gene PCRs (\( \beta \)-actin, 18S ribosomal DNA) versus single copy gene PCRs (albumin, RNase-P) were identified that produced a sufficient \( D_{Ct} \) value. As an alternative, a mutant albumin primer pair was designed that amplified with an efficiency of \( E = 0.96 \) compared to the \( \beta \)-actin PCR (\( E = 0.95 \) as described above). The mutant albumin primer pair produced Ct values that were about 6 cycles higher Ct values for each template dilution in comparison to the wild-type albumin reaction. The \( D_{Ct} \) values between the \( \beta \)-actin PCR and this mutant albumin PCR was about 9 cycles over all template dilutions tested. This PCR com-

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### Table 2. \( t(14;18) \) and Albumin PCR Efficiencies for Follicular NHL Patient Lymph Node and Peripheral Blood DNA Samples

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Patient biopsy</th>
<th>( t(14;18) ) PCR product length (bp)</th>
<th>( E_{t(14;18)} )</th>
<th>( E_{Albumin} )</th>
<th>( \Delta E )</th>
</tr>
</thead>
<tbody>
<tr>
<td>SUDHL6</td>
<td>LN</td>
<td>385</td>
<td>0.91 ± 0.05</td>
<td>0.93 ± 0.04</td>
<td>-0.02</td>
</tr>
<tr>
<td>1</td>
<td>LN</td>
<td>312</td>
<td>0.75</td>
<td>0.70</td>
<td>0.05</td>
</tr>
<tr>
<td>2</td>
<td>LN</td>
<td>363</td>
<td>0.74</td>
<td>0.72</td>
<td>0.02</td>
</tr>
<tr>
<td>3</td>
<td>LN</td>
<td>350</td>
<td>0.59</td>
<td>0.59</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>LN</td>
<td>360*</td>
<td>0.67/0.59</td>
<td>ND</td>
<td>—</td>
</tr>
<tr>
<td>5</td>
<td>LN</td>
<td>267*</td>
<td>0.63/0.58</td>
<td>ND</td>
<td>—</td>
</tr>
<tr>
<td>6</td>
<td>LN</td>
<td>299</td>
<td>ND</td>
<td>0.95</td>
<td>—</td>
</tr>
<tr>
<td>7</td>
<td>LN</td>
<td>449</td>
<td>ND</td>
<td>0.65</td>
<td>—</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>343</td>
<td>0.66</td>
<td>0.72</td>
<td>0.023</td>
</tr>
<tr>
<td>8</td>
<td>PB</td>
<td>398</td>
<td>0.97</td>
<td>0.95/0.91</td>
<td>0.04</td>
</tr>
<tr>
<td>9</td>
<td>PB</td>
<td>367</td>
<td>1.03</td>
<td>0.99</td>
<td>0.06</td>
</tr>
<tr>
<td>10</td>
<td>PB</td>
<td>366</td>
<td>0.89</td>
<td>1.0/0.94</td>
<td>-0.08</td>
</tr>
<tr>
<td>11</td>
<td>PB</td>
<td>329</td>
<td>0.84</td>
<td>0.85</td>
<td>-0.01</td>
</tr>
<tr>
<td>12</td>
<td>PB</td>
<td>313</td>
<td>1.05</td>
<td>0.96</td>
<td>0.09</td>
</tr>
<tr>
<td>13</td>
<td>PB</td>
<td>343</td>
<td>0.91</td>
<td>0.99</td>
<td>-0.08</td>
</tr>
<tr>
<td>14</td>
<td>PB</td>
<td>520</td>
<td>0.91</td>
<td>0.92</td>
<td>-0.01</td>
</tr>
<tr>
<td>15</td>
<td>PB</td>
<td>337</td>
<td>0.89</td>
<td>0.97</td>
<td>-0.08</td>
</tr>
<tr>
<td>16</td>
<td>PB</td>
<td>336*</td>
<td>ND</td>
<td>0.87</td>
<td>—</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>368</td>
<td>0.94</td>
<td>0.94</td>
<td>-0.009</td>
</tr>
</tbody>
</table>

LN, lymph node; PB, peripheral blood.

\*Larger PCR products are also formed due to priming of JH-con primer to downstream JH-gene segments.

†Product size was determined from the breakpoint sequence and confirmed by gel-electrophoresis.

\( ^a-c \)Samples amplified within the same amplification reaction.

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**Figure 1.** Theoretical schematic illustration of \( \beta \)-actin and albumin amplification curves at high and low reaction efficiencies (E). The \( \beta \)-actin (black curves) and albumin (gray curves) amplification curves and Ct values at high (solid lines) and low (stippled lines) reaction efficiencies for a hypothetical genomic DNA template concentration. Low reaction efficiencies result in higher Ct values for both reactions in a non-linear relationship (Eq. 1). DNA templates with higher efficiencies for both reactions will have lower difference in Ct values between both reference reactions (low \( D_{Ct} \) values) than templates with lower reaction efficiencies (high \( D_{Ct} \) values).
The ECC. Analysis of therefore not be corrected for different efficiencies using These samples may suffer from severe PCR inhibition that samples with high efficiencies that were calculated from standard curves. Four samples with high efficiencies that were derived from standard curves. For seven out of eight samples, the ECC determined efficiencies match the efficiencies as determined from the standard curves of the t(14;18) and albumin PCR (Table 3). For five samples, the ECC based efficiencies fall within the SD of the efficiencies that were derived from standard curves.

### Discussion

In quantitative PCR studies monitoring for minimal residual disease, patient samples are compared to a golden standard that either consists of a well-defined calibrator sample (ie, the comparative Ct method) or consists of a calibrator serial dilution. All real-time quantitative PCR studies performed thus far are based on the assumption that PCR efficiencies are the same for patient samples and the calibrator. However, many factors affect PCR efficiency and consequently the outcome of quantitative PCR analyses. It is commonly believed that amplicon size plays an important role in this matter, as well as contaminating factors in the patient sample that will inhibit the amplification reaction and will therefore delay reaction efficiency. A few of these PCR inhibiting factors have been characterized that interfere with quantitative PCR analyses, especially within the hemat-oncology field.19-23

Testing the efficiencies for the t(14;18) target and albumin reference PCR for various patient DNA samples, efficiencies for both reactions varied between different patient samples probably due to contaminating PCR inhibiting compounds. PCR efficiency was not affected by amplicon size (Table 2), at least for amplicons ranging between 89 and 520 bp, nor was it due to DNA degradation. DNA templates isolated from patient lymph node biopsies amplify with lower efficiencies in comparison to patient whole blood DNA samples (Table 2). Most importantly, for each DNA sample the efficiencies for both PCRs were equivalent, indicating that a low t(14;18) reaction efficiency for a DNA template is accompanied by a similar low efficiency for the albumin reaction for that same template. This indicates that PCR inhibiting factors affect different reactions performed on the same template to a similar extent. For all t(14;18) and albumin standard curves, the deviations of Ct values for all template dilutions relative to the regression curve were equivalent, and no evidence was found for increasing deviations at higher template dilutions as should be expected in case of dilutable PCR inhibition (all R² values exceeded 0.98 but in general were in the order of 0.995). PCR inhibition for the samples evaluated in this study was non-dilutable, and is presumably caused by compounds that tightly bind to the DNA like hemoglobin degradation compounds.

Our results show that quantitative studies tend to suffer from efficiency differences between samples, leading to inaccurate results. For instance, lower reaction efficiencies for patient samples resulting in higher Ct values will lead to underestimation of residual disease content. This especially affects patient samples with low residual malignant cell numbers. Normalization of the target PCR (the t(14;18) reaction in this report) to a reference PCR, usually specific for an endogenous housekeeping gene, will only partially compensate for this problem since the number of reference gene copies is always relatively high in comparison to the number of target copies reflecting the amount of malignant cells. The efficiency compensation control (ECC) was developed to compensate for sample specific efficiency variation in comparison to a calibrator (equation 7), and consists of a multiplex PCR for two endogenous housekeeping genes, ie, β-actin and albu-

### Table 3. Comparison of Reaction Efficiency as Determined from the Efficiency Compensation Control (E<sub>ECC</sub>) and as Determined from the Slope of Standard Curves (E<sub>B</sub>)

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>ΔCt&lt;sub&gt;2&lt;/sub&gt;</th>
<th>E&lt;sub&gt;ECC&lt;/sub&gt;</th>
<th>E&lt;sub&gt;B&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>17*</td>
<td>9.88</td>
<td>0.83</td>
<td>0.92 ± 0.01</td>
</tr>
<tr>
<td>17†</td>
<td>9.47</td>
<td>0.88</td>
<td>0.84 ± 0.05</td>
</tr>
<tr>
<td>18†</td>
<td>9.41</td>
<td>0.89</td>
<td>0.89 ± 0.01</td>
</tr>
<tr>
<td>19†</td>
<td>9.47</td>
<td>0.88</td>
<td>0.92 ± 0.01</td>
</tr>
<tr>
<td>1</td>
<td>8.32</td>
<td>1.05</td>
<td>1.04 ± 0.02</td>
</tr>
<tr>
<td>4†</td>
<td>8.51</td>
<td>1.02</td>
<td>1.05</td>
</tr>
<tr>
<td>20†</td>
<td>8.66</td>
<td>1.0</td>
<td>1.06 ± 0.08</td>
</tr>
<tr>
<td>21†</td>
<td>8.56</td>
<td>1.01</td>
<td>0.96 ± 0.04</td>
</tr>
</tbody>
</table>

Patient sample DNA isolated from peripheral blood samples* or bone marrow aspirates.† The experimentally determined efficiency (E<sub>B</sub>) is the average efficiency for the β-actin and the mutant albumin reaction as calculated from the slope of the standard curves.
min, that are present in a constant ratio in DNA templates but that produce different Ct values (ΔCt2) for identical template. Both reactions performed with equivalent efficiencies on serial template dilutions, and it is reasonable to assume that both ECC reference reactions will be equally affected by contaminating PCR inhibiting substances as is the case for the t(14;18) and wild-type albumin PCR. The ΔCt2 value is therefore independent of initial template concentration, but depends on the exact amplification efficiencies (equation 6). The ECC can theoretically distinguish between patient samples that differ in amplification efficiencies as low as E = 0.05. Disease associated chromosomal aneuploidy involving the β-actin or albumin genes may affect results obtained by the ECC. This is unlikely for follicular NHL blood or bone marrow samples since the total number of circulating t(14;18) positive lymphoma cells during the course of disease is in general below five percent of total mononuclear cells.3,4,8 However, for leukemias with a high tumor cell percentage in blood and bone marrow samples during apparent disease in contrast to clinical remission phases, this could be a problem.

Patient DNA templates that had been stored for several years produced abnormal ECC ΔCt2 values for 32% of the samples compared to 4% of freshly isolated patient DNA samples. For these samples, the ECC will not be useful to correct for efficiency differences in comparison to a calibrator sample. These samples probably contain so many PCR inhibiting compounds that different PCRs are affected to a different extent. Quantitation of residual disease in these samples will therefore not be possible. The ECC seems very useful in identifying such patient samples in quantitative analyses.

In conclusion, the ECC serves three purposes: first, the β-actin Ct value for each sample is used to normalize for different DNA input. Second, the ECC ΔCt2 value is used to compensate for efficiency difference in comparison to the calibrator. Third, the ECC identifies patient DNA templates that may not be quantifiable for minimal residual disease content due to unequal inhibition of the various amplification reactions. Since the ECC uses two reference PCRs that are specific for endogenous housekeeping genes, it may be applicable as a general control in real-time quantitative studies using genomic DNA templates. In our opinion, the ECC approach is a powerful control for TAQMAN-based analyses that can be performed in the ABI PRISM 7700 system (Applied Biosystems) but needs further evaluation in other real-time PCR systems. For probe designs, other fluorescent reporter groups can be used, but FAM in combination with JOE or VIC proved to be a useful combination in a multiplex setting. Hydrolyzing probes may be replaced by molecular beacons or scorpion primers.26

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References

eloma patients is validated in a multicentre study. Blood 2000, 96:63–70
20. Pikaart M, Villeponteau B: Suppression of PCR amplification by high levels of RNA. Biotechniques 1993, 14:24–25