Quantitative Real-Time PCR Method for Detection of B-Lymphocyte Monoclonality by Comparison of $\kappa$ and $\lambda$ Immunoglobulin Light Chain Expression

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Background: An abnormal IgL$\kappa$:IgL$\lambda$ ratio has long been used as a clinical criterion for non-Hodgkin B-cell lymphomas. As a first step toward a quantitative real-time PCR-based multimarker diagnostic analysis of lymphomas, we have developed a method for determination of IgL$\kappa$:IgL$\lambda$ ratio in clinical samples.

Methods: Light-up probe-based real-time PCR was used to quantify IgL$\kappa$ and IgL$\lambda$ cDNA from 32 clinical samples. The samples were also investigated by routine immunohistochemical analysis and flow cytomtery.

Results: Of 32 suspected non-Hodgkin lymphoma samples analyzed, 28 were correctly assigned from real-time PCR measurements assuming invariant PCR efficiencies in the biological samples. Four samples were false negatives. One was a T-cell lymphoma, one was a diffuse large B-cell lymphoma, and one was reanalyzed and found lymphoma-positive by in situ calibration, which takes into account sample-specific PCR inhibition. Twelve of the samples were fine-needle aspirates, and these were all correctly assigned.

Conclusions: This work is a first step toward analyzing clinical samples by quantitative light-up probe-based real-time PCR. Quantitative real-time PCR appears suitable for high-throughput testing of cancers by measuring expression of tumor markers in fine-needle aspirates.

The rapid expansion in knowledge of the human genome and the development of techniques for analysis of nucleic acids have opened new possibilities for diagnostics. In our first attempt to use quantitative real-time PCR (Q-PCR) for detection of malignant tumors, we have focused on the analysis of non-Hodgkin lymphomas (NHLs).

The vast majority of NHLs develop from a B-lymphocyte lineage. B-lymphocytes produce immunoglobulins with a heavy chain and either a $\kappa$ (IgL$\kappa$) or a $\lambda$ (IgL$\lambda$) light chain. Which light chain will be produced is determined early in the development of each B-lymphocyte. In healthy humans, ~60% of B-cells produce $\kappa$ chains and the rest produce $\lambda$ chains. Normal lymphoid tissues therefore contain a mixture of B-cells with an IgL$\kappa$:IgL$\lambda$ ratio of ~60:40 (1, 2).

Lymphomas, like all malignant tumors, are clonal and arise from one transformed cell. Lymphoma tissues are dominated by the tumor cells; consequently, the IgL$\kappa$:IgL$\lambda$ ratio is changed. With $\kappa$-producing tumors, the IgL$\kappa$:IgL$\lambda$ ratio is higher, whereas with $\lambda$-producing tumors, the ratio is lower. Assuming that the translation efficiency and stability of the IgL$\kappa$ and IgL$\lambda$ mRNAs are similar, clonality may be detected by measuring the IgL$\kappa$:IgL$\lambda$ expression ratio.

Real-time PCR can detect and quantify target nucleic acids in biological samples with light-up probes (3–5). We have used this approach to assess the expression of IgL$\kappa$ and IgL$\lambda$ in B-cell lymphoid tumors. Although it cannot yet replace traditional immunohistochemistry (IHC) and flow cytometry in diagnosis, this assay, which takes into account sample-specific PCR inhibition.

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account reaction efficiency, demonstrates the potential of quantitative reverse transcription-PCR in cancer diagnostics.

**Material and Methods**

**Sample Collection**

Surgical lymph node biopsies from previously untreated patients were transported from the operation theatre in ice-water-chilled boxes and handled in the laboratory within 30 min. Material for the study was rapidly frozen in dry ice/isopentane and stored at −70 °C. Samples BR101–BR112 were fine-needle aspirates suspended in RNAlater (Ambion) directly at the time of sample collection. The cell suspension was stored in RNAlater at 4 °C for 1 or 2 days before the cells were collected by centrifugation and stored frozen at −80 °C. Parts of the tissues were fixed in formalin and used for routine diagnostic analysis. Diagnosis was reached by a combination of microscopic histologic evaluation; immunostaining of several markers, including the κ and λ chains (IHC); and in some cases, flow cytometry. The samples were classified as lymphadenitis or malignant lymphoma according to the Revised European-American Lymphoma classification system (6).

**RNA Extraction and cDNA Synthesis**

RNA was extracted by use of the Fast Prep System (FastRNA Green; Qbiogene). We mixed 10 μg of total RNA with 2 μg of poly(dT) oligomers (Pharmacia) and incubated the mixture at 65 °C for 5 min. First-strand cDNA synthesis was then performed by adding 0.05 mol/L Tris-HCl (pH 8.3), 0.075 mol/L KCl, 3 mmol/L MgCl₂, 0.01 mol/L dithiothreitol, 10 U/mL Moloney murine leukemia virus reverse transcriptase (Life Technologies), 0.05 units/mL RNA guard (Life Technologies), 0.075 mol/L KCl, 600 nM each of the primers, and 600 nM IgL sequences for IgL PCR systems were designed for a 231-bp fragment of human IgLκ (GenBank accession no. AK024974) and a 223-bp fragment of human IgLλ (GenBank accession no. X51755), which comprise the IgLxLUP and IgLλLUP target sequences, respectively.

**Reaction conditions** were optimized as described elsewhere (10). IgLκ and IgLλ PCs both contained 75 mM Tris (pH 8.8), 20 mM (NH₄)₂SO₄, 1 mL/L Tween 20, 1 U of JumpStart™ Taq DNA polymerase (with antibody; Sigma-Aldrich), and 200 ng/μL bovine serum albumin (Fermentas). Specific components for the IgLκ PCR were 5 mM MgCl₂, 0.2 mM deoxyribonucleotides (Sigma-Aldrich), 800 nM each of the primers (MedProbe Inc.), and 800 nM IgLxLUP; for the IgLλ PCR, specific components were 3.5 mM MgCl₂, 0.4 mM deoxyribonucleotides, 600 nM each of the primers, and 600 nM IgLλLUP. Primer sequences for IgLκ were 5’-TGA CCA ACG ACT AGA-3’ (forward) and 5’-GGG GTG AGG TGA AAG ATG AG-3’ (reverse); for IgLλ, primer sequences were 5’-GAG CCT GAC GCC TGA G-3’ (forward) and 5’-ATT GAG GGT TTA AGT GCA G-3’ (reverse). Real-time PCR was performed in a LightCycler (Roche Diagnostics) using the thermocycler program: 3 min of preincubation at 95 °C followed by 50 cycles for 0 s at 95 °C, 10 s at 55 °C, and 11 s at 74 °C. Fluorescence was monitored (excitation at 470 nm and emission at 530 nm) at the end of the annealing phase (the LightCycler F1 channel). All amplification curves were baseline-adjusted by subtracting the arithmetic average of the five lowest fluorescence read-out values in each sample (arithmetic baseline adjustment in the LightCycler software). The threshold was set to a value of 1.00, which was significantly above background noise, and the number of cycles required to reach this values, CT, was determined (11).

**Model for the Quantification of Relative Expression of Two Genes**

A mathematical model was developed to determine the ratio of the expression of two genes by real-time PCR. The model is general and applied here to the IgLκ and IgLλ genes. The basic equation describing real-time PCR amplification is:
\[ N_{CT} = N_0 \cdot (1 + E)^{CT} \]  

(1)

where \( N_0 \) is the number of cDNA molecules, \( E \) is the PCR efficiency (\( E = 1 \) corresponds to 100% efficiency and is expressed in percentage throughout this work), \( CT \) is the threshold cycle number, and \( N_{CT} \) is the number of template copies present after \( CT \) PCR cycles. \( E \) is assumed to be independent of \( N \) in the particular amplification range. It is determined by performing a dilution series of mRNA or cDNA calibrator and is calculated from the slope of a plot of \( CT \) vs \( \log N_0 \):

\[ I = k \cdot N_{CT} \]  

(3)

where \( k \) is a system and instrument constant, and \( N_{CT} \) is the number of target DNA molecules present at threshold. The relative expression of the IgLk and IgLa genes is obtained as:

\[ N_{CTigk} = N_{0igk} \cdot (1 + E_{igk})^{CT_{igk}} \]  

(4)

\[ I_{igk} = k_{igk} \cdot N_{CTigk} \]  

(5)

\[ N_{CTigl} = N_{0igl} \cdot (1 + E_{igl})^{CT_{igl}} \]  

(6)

\[ I_{igl} = k_{igl} \cdot N_{CTigl} \]  

(7)

At threshold, \( I_{igk} = I_{igl} \). Equating Eq. 5 with Eq. 7 and rearranging we obtain:

\[ K_{RS} = \frac{k_{igl}}{k_{igk}} \cdot \frac{N_{CTigk}}{N_{CTigl}} \]  

(8)

where the relative sensitivity, \( K_{RS} \), reflects the difference in the probes’ fluorescence and binding efficiencies in the two assays. Inserting Eqs. 4 and 6 and rearranging we get:

\[ \frac{N_{0igk}}{N_{0igl}} = K_{RS} \cdot \frac{(1 + E_{igk})^{CT_{igk}}}{(1 + E_{igl})^{CT_{igl}}} \]  

(9)

This is the central equation to calculate the ratio between the numbers of copies of two cDNA molecules. \( CT_{igk} \) and \( CT_{igl} \) are the CT values obtained from the PCR amplifications of the IgLk and IgLa cDNAs; \( E_{igk} \) and \( E_{igl} \) are the efficiencies of the two PCRs, which are obtained as slopes of plots of \( CT \) vs \( \log N_0 \) in dilution series of the samples; and \( K_{RS} \) is the relative sensitivity constant of the two PCR assays, determined using test samples with known cDNA concentrations.

The fractions of IgLk and IgLa mRNA, expressed as percentages, are:

\[ IgLk = 100 \cdot \frac{K_{RS} \cdot (1 + E_{igl})^{CT_{igl}}}{(1 + E_{igk})^{CT_{igk}}} \]  

(10)

\[ IgLa = 100 \cdot \frac{1}{1 + K_{RS} \cdot \frac{(1 + E_{igk})^{CT_{igk}}}{(1 + E_{igl})^{CT_{igl}}}} \]  

(11)

**Results**

**Experimental Uncertainty**

To classify a sample as either lymphoma-negative with 60:40 IgLk:IgLa expression ratio or positive with a deviating expression ratio, we must know with what accuracy \( CT \) can be determined. We therefore designed experiments to measure the variation in \( CT \) attributable to experimental error and biological variability. We first studied the reproducibility of the PCR by splitting a sample into aliquots that were analyzed in parallel runs (intraassay). We then also included variation attributable to sample handling by analyzing the same sample in independent runs (interassay). To minimize variation in template concentration between the two assays being compared, we prepared a master mixture containing template and all common PCR components and split it into two aliquots to which the unique components for the IgLk and the IgLa reactions were added. Each experiment was performed eight times with patient sample BR0 (Fig. 1).

In most reports, PCR reproducibility is expressed as the SD in \( CT \). The variance, \( SD^2 \), is:

\[ SD^2 = \frac{\sum_{i=1}^{n} (CT_i - \langle CT \rangle)^2}{n - 1} \]  

(12)

where \( \langle CT \rangle \) is the mean of the measured \( CT \), and \( SD \) is the square root of the variance. However, because we are interested in determining the amount of cDNAs in the sample, the SD of \( (1 + E)^{-CT} \), which is proportional to the number of cDNA molecules (Eq. 1):

\[ N_0 = N_{CT} \cdot (1 + E)^{-CT} \]  

(13)

is more relevant. The variance in \( (1 + E)^{-CT} \) is:

\[ SD^2 = \frac{\sum_{i=1}^{n} \left[ (1 + E)^{-CT_i} - \langle (1 + E)^{-CT} \rangle \right]^2}{n - 1} \]  

(14)

where \( \langle (1 + E)^{-CT} \rangle \) is the mean of \( (1 + E)^{-CT} \). To obtain the relative uncertainty in the number of cDNA molecules, we normalize the SD with the average value to obtain the CV, which we express as a percentage:

\[ CV = 100 \times \frac{SD}{\langle (1 + E)^{-CT} \rangle} \]  

(15)

CV is the uncertainty in the determination of the number of cDNA molecules in the sample attributable to experimental factors.

The intraassay CV, which reflects the reproducibility of
the PCR, was 3.0% for the IgLκ reaction and 4.9% for the IgLλ reaction (Table 1). The interassay CVs, to which experimental errors also contribute, were only slightly larger: 8.1% for the IgLκ reaction and 5.0% for the IgLλ reaction. Although it was not possible to calculate a CV for the ratio of the two cDNAs, we can estimate how much the IgLκ:IgLλ expression ratio in a negative sample could deviate from 60:40 because of experimental uncertainty in a bad case \([CV(a/b)]^{1/2}\) as is frequently inferred in PCR user bulletins. If we suppose that the number of IgLκ cDNA molecules is overestimated, because of experimental error, by 2 SD and that the number of IgLλ cDNA molecules is also underestimated by 2 SD, then the measured ratio would be \((60/40) \times (1 + 0.162)/(1 - 0.100) = 1.94 = 66/34\). If instead the amount of IgLκ cDNA is underestimated and that of IgLλ cDNA is overestimated, then the measured ratio would be \((60/40) \times (1 - 0.162)/(1 + 0.100) = 1.14 = 53/47\).

**Table 1. Variations in CT values for the IgLκ and IgLλ reactions in eight repeated measurements of sample BR0 run either in parallel (intraassay) or separately (interassay).**

<table>
<thead>
<tr>
<th></th>
<th>IgLκ</th>
<th>IgLλ</th>
</tr>
</thead>
<tbody>
<tr>
<td>CT(<em>{\text{max}})–CT(</em>{\text{min}})</td>
<td>0.15</td>
<td>0.28</td>
</tr>
<tr>
<td>CV (CT)(^b)</td>
<td>0.23</td>
<td>0.39</td>
</tr>
<tr>
<td>CV (copy)(^c)</td>
<td>3.0</td>
<td>4.9</td>
</tr>
<tr>
<td>Intraassay</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Interassay</td>
<td>0.37</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>0.62</td>
<td>0.37</td>
</tr>
<tr>
<td></td>
<td>8.1</td>
<td>5.0</td>
</tr>
</tbody>
</table>

\(^a\) Difference between the highest and lowest measured CT values.

\(^b\) CV in CT values.

\(^c\) CV in copy number.
Hence, because of experimental uncertainty and variation in PCR efficiency attributable to the added components, we expect negative samples to display an IgLc:IgLα expression ratio of 53:47 < N0IgLc/N0IgLα < 66:34.

**DETERMINATION OF PCR EFFICIENCIES IN PATIENT SAMPLES**

Biological samples frequently contain PCR inhibitors, such as heme (12), heparin (13), and IgG (14), and inhibition may vary substantially among samples (15, 16). To correctly interpret results of real-time PCR measurements, it is crucial to estimate the PCR efficiency with high precision in the particular biological sample studied; even a small error in the assumed PCR efficiency may cause large errors in the estimated number of cDNA molecules. We have therefore designed an approach to determine the efficiencies of PCR reactions in situ in biological samples. By diluting the test sample in steps and measuring the CT value at each dilution, we could determine the efficiencies of PCR reactions in situ in patient samples. By diluting the test sample in steps and measuring the CT value at each dilution, we could construct an intrinsic calibration curve from which the biological samples were characterized this way, as well as purified template that should not contain any inhibitors.

The PCR efficiencies obtained when we amplified purified template were E(IgLc) = 95% and E(IgLα) = 93%, signifying that both reactions proceeded with very high efficiencies as expected for optimized PCR assays. Six of the patient samples exhibited efficiencies that were ~10% lower; the IgLα PCR efficiency was 75% < E(IgLα) < 86% with (E(IgLα)) = 79%, and the IgLc efficiency was 79% < E(IgLc) < 91% with (E(IgLc)) = 85% (Table 2). The seventh sample, BR17, exhibited typical IgLc efficiency (83%), whereas the IgLα efficiency was only 59%. The reason for the extremely low efficiency of the IgLα reaction in this sample is unclear. It was considered an outlier and was not included in the calculation of average efficiencies.

When comparing the yields of two reactions, the efficiency ratio:

$$X_{ER} = \frac{(1+E_{IgLc})}{(1+E_{IgL\alpha})}$$  \hspace{2cm} (16)

is the relevant parameter (see Eq. 9). For the six samples, $1.01 < X_{ER} < 1.07$ with $\langle X_{ER} \rangle = 1.03$ (Table 2). Hence, after ~25 amplification cycles, which was typically required to reach threshold with the patient samples (Fig. 1), twice $(1.03^{25} = 2)$ as many κ DNA molecules had been formed.

<table>
<thead>
<tr>
<th>Patient sample</th>
<th>CT&lt;sup&gt;a&lt;/sup&gt;</th>
<th>E&lt;sup&gt;a&lt;/sup&gt;</th>
<th>CT&lt;sup&gt;b&lt;/sup&gt;</th>
<th>E&lt;sup&gt;b&lt;/sup&gt;</th>
<th>1+E&lt;sub&gt;IgLc&lt;/sub&gt;</th>
<th>1+E&lt;sub&gt;IgLα&lt;/sub&gt;</th>
<th>$K_{rs}$&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>BR0</td>
<td>21.0</td>
<td>79.4</td>
<td>21.7</td>
<td>68.3</td>
<td>1.014</td>
<td>1.42</td>
<td></td>
</tr>
<tr>
<td>BR18</td>
<td>14.2</td>
<td>86.6</td>
<td>19.6</td>
<td>72.1</td>
<td>1.065</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BR37</td>
<td>22.2</td>
<td>84.2</td>
<td>20.8</td>
<td>75.7</td>
<td>1.048</td>
<td>1.84</td>
<td></td>
</tr>
<tr>
<td>BR42</td>
<td>18.7</td>
<td>90.4</td>
<td>17.9</td>
<td>84.4</td>
<td>1.033</td>
<td>1.41</td>
<td></td>
</tr>
<tr>
<td>BR46</td>
<td>17.1</td>
<td>82.8</td>
<td>16.4</td>
<td>77.9</td>
<td>1.028</td>
<td>1.42</td>
<td></td>
</tr>
<tr>
<td>BR52</td>
<td>21.9</td>
<td>88.7</td>
<td>17.9</td>
<td>85.8</td>
<td>1.015</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>21.2</td>
<td>86.4</td>
<td>17.9</td>
<td>84.5</td>
<td>1.034</td>
<td>1.52</td>
<td></td>
</tr>
<tr>
<td>Template&lt;sup&gt;d&lt;/sup&gt;</td>
<td>94.7</td>
<td>93.2</td>
<td>1.008</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BR17</td>
<td>23.1</td>
<td>83.0</td>
<td>23.3</td>
<td>58.9</td>
<td>1.152</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Undiluted samples.  
<sup>b</sup> PCR efficiencies calculated from the slopes of plots CT vs log(cDNA concentration) (Fig. 2).  
<sup>c</sup> Relative sensitivity ($K_{rs}$) from Eq. 9 assuming N0IgLc/N0IgLα = 1.5.  
<sup>d</sup> Purified template.

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![Fig. 2. Efficiencies of IgLc (A) and IgLα (B) PCR assays.](image_url)

The lines are normalized at maximum template concentrations. PCR efficiencies are obtained from the slopes of the fitted lines as: $E = 10^{-\text{slope}} - 1$. The outlier, sample BR17, is indicated with a dotted line. Purified template is shown with a dashed line. For all lines, $R^2 > 0.99$. 
compared with DNA because of the difference in PCR efficiencies.

Finally, to relate the measured CT values of the two real-time PCRs to the ratio between the numbers of corresponding CDNA molecules, we must also determine the relative sensitivity, $K_{RS}$, of the two probing systems (Eq. 8).

$$K_{RS} = \frac{N_{0_{IgL}}}{N_{0_{IgL}}} \cdot \frac{(1 + E_{IgL})^{CT_{IgL}}}{(1 + E_{IgL})^{CT_{IgL}}}. \quad (17)$$

$K_{RS}$ can be determined by use of IgLx and IgLA calibrators. For our purpose, however, it is better to calculate $K_{RS}$ from the CT values ($CT_{IgLx}$ and $CT_{IgLa}$) and PCR efficiencies ($E_{IgLx}$ and $E_{IgLa}$) determined for the four negative samples (Table 2), assuming an IgLx:IgLα expression ratio of 60:40. The reason is that the assumption of a particular IgLx:IgLα expression ratio will cancel when calculating the relationship between $CT_{IgLx}$ and $CT_{IgLa}$ for negative samples (see below). This gave $1.4 \leq K_{RS} \leq 1.9$ (Table 2). Hence, assuming an IgLx:IgLα expression ratio of 60:40, the probing of IgLx DNA is ~50% more sensitive than the probing of IgLA DNA with the probes and conditions here.

**CHARACTERIZATION OF PATIENT SAMPLES**

A total of 32 patient samples were analyzed for B-cell lymphoma by the Q-PCR assay. Twelve of the samples were collected as fine-needle aspirates. All samples were run in duplicate, including negative controls. The data are summarized in Table 3 and in Table 1 of the Data Supplement (available with the online version of this article at http://www.clinchem.org/content/vol49/issue1/) and are plotted in Fig. 3. In the plot, each symbol represents one sample and is positioned on the coordinates $CT_{IgLx}$, $CT_{IgLa}$. The corresponding number of CDNA molecules of purified template, calculated assuming $E_{IgLx} = 95\%$ and $E_{IgLa} = 93\%$, is indicated in logarithmic scale on the opposite axes. Samples considered negative by IHC analysis are shown as circles, and positive samples are shown as squares.

Negative samples with an IgLx:IgLα gene expression ratio of 60:40 are expected to lie on a straight line. Rewriting Eq. 9, we get:

$$N_{0_{IgL}} \cdot (1 + E_{IgL})^{CT_{IgL}} = K_{RS} \cdot N_{0_{IgL}} \cdot (1 + E_{IgL})^{CT_{IgL}}. \quad (18)$$

**Table 3. Summary of patient sample classification by real-time PCR.**

<table>
<thead>
<tr>
<th>Sample</th>
<th>No. of samples</th>
<th>Correctly classified samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphadenitis</td>
<td>16</td>
<td>16 (100%)</td>
</tr>
<tr>
<td>B-cell lymphoma</td>
<td>15</td>
<td>13 (87%)</td>
</tr>
<tr>
<td>T-cell lymphoma</td>
<td>1</td>
<td>0 (0%)</td>
</tr>
</tbody>
</table>

*Information about individual patient samples is provided in Table 1 of the Data Supplement.

BR17 was found lymphoma-positive after in situ calibration.

converting it to logarithmic form:

$$CT_{IgLx} \cdot \log(1 + E_{IgLx}) = \log(K_{RS} \cdot \frac{N_{0_{IgL}}}{N_{0_{IgL}}})$$

and rearranging, we obtain:

$$CT_{IgLx} = \log \left( \frac{K_{RS} \cdot N_{0_{IgL}}}{N_{0_{IgL}}} \right) \cdot \frac{\log(1 + E_{IgLx})}{\log(1 + E_{IgLx})} = \frac{b \cdot CT_{IgLx} + m}{b} \cdot CT_{IgLx} + m$$

(20)

This describes a linear relationship between $CT_{IgLx}$ and $CT_{IgLa}$ with slope $b$ and intercept $m$. Inserting ($E_{IgLx} = 0.85$, $E_{IgLa} = 0.79$, and ($K_{RS} = 1.5$), which are the average values determined for the six samples above (Table 2), and $N_{0_{IgL}}/N_{0_{IgL}} = 60:40 = 1.5$, we obtain $b = 0.95$ and $m = 0.021$. Note that the relative sensitivity, $K_{RS}$, was calculated from measurements on negative samples assuming a 60:40 expression ratio (Eq. 17). This cancels the $N_{0_{IgL}}/N_{0_{IgL}}$ ratio in the second term. Hence, the calculated slope and intercept of the relationship between $CT_{IgLx}$ and $CT_{IgLa}$ for negative samples is independent of the assumption of a particular IgLx:IgLα expression ratio. A line with $b = 0.95$ and $m = 0.021$ is shown in Fig. 3.

Some negative samples were slightly off the line representing 60:40 expression (Fig. 3). This may be attributable to variations in PCR efficiencies among the samples. Such variations can cause an error in the estimation of the
number of cDNA molecules from the measured CT values when mean PCR efficiencies are assumed. If the efficiencies of the two PCR assays in a sample deviate from the mean values to about the same degree, the measured CT values will still correctly reflect the expression ratio and negative samples will fall on the 60:40 line, although they will be displaced diagonally from where they would be if their efficiencies were \( (E_{iLx} \) and \( E_{iL\alpha} \). However, if the efficiency of one of the reactions deviates more than the other from the mean values, a negative sample may be off the 60:40 line. For the seven samples characterized by in situ calibration (Table 2), the measured CT values could be corrected for the differences between their specific PCR efficiencies and the mean efficiencies:

\[
CT_{corr} = \frac{\log(1 + E) \cdot \log(1 + E)}{\log(1 + E)}
\]  

The corrected CT values are shown in Fig. 3 with open symbols, and they are connected to the measured CT values by arrows. Indeed, most arrows, sample BR17 being the only exception, are diagonal, indicating that the two reactions are inhibited to the same degree, which does not affect classification.

To account for experimental error and variations in PCR efficiencies in classification of samples, we estimated limits within which negative samples should be found. Keeping the intercept fixed in Eq. 20:

\[
CT_{iLx} = \frac{\log(1 + E_{iLx})}{\log(1 + E_{iLx})} \cdot CT_{iL\alpha} + \log\left(\frac{N_{iLx}}{N_{iL\alpha}}\right)
\]

we calculated the SD of the slope, \( b = \log\left(\frac{1 + E_{iLx}}{\log(1 + E_{iLx})}\right) \), from the efficiencies determined for the six samples (BR17 was excluded) characterized by in situ calibration. This gave an SD of 0.031. For a gaussian distribution, the 95% confidence region is given by the mean \( \pm \) 1.96 SD. In Fig. 3, the dashed lines enclose the region defined by:

\[
CT_{iLx} = (0.95 \pm 0.06) \cdot CT_{iL\alpha} + 0.021
\]

Although the confidence region takes into account most of the experimental variation, it accounts for neither the variance in the intercept nor the natural variation in the \( iLx:IGLA \) expression ratio among healthy individuals. These factors would broaden the confidence region further. Hence, the region indicates where negative samples are expected to be found with at least 95% probability. All negative samples in this study fell within this region (Fig. 3).

Positive samples with \( iLx \) clonality were below the 60:40 line, whereas those with \( IGLA \) clonality were above it. This separation of clonal and normal samples was as good for fine-needle aspirates as for the surgical biopsies, although the former contained less material and had higher CT values. Most positive samples fell outside the confidence region. However, samples BR5 and BR17 were within the region although they were positive. BR17 had anomalous \( IGLA \) PCR efficiency, and when corrected for that, it fell outside the region. We do not know why sample BR5 was within the region. It may have also been attributable to anomalous PCR efficiencies, or it could have been the result of limited clonality. Three samples, indicated by * in Fig. 3, were classified as diffuse large B-cell lymphomas by IHC analysis. One of them, BR29, was close to the 60:40 line. The other two were correctly assayed as positive; one with \( IGL\alpha \) clonality and the other with \( IGLA \) clonality. BR23 was a T-cell lymphoma, as judged by IHC analysis, and the sample therefore exhibited a normal \( IGLx:IGLA \) ratio. An interactive Excel sheet to analyze \( IGL\alpha \) and \( IGLA \) expression data has been made available as part of the Data Supplement accompanying the online version of this article (http://www.clinchem.org/content/vol49/issue1/) and at http://www.molbiotech.chalmers.se/research/mk/anders/index.htm.

**Discussion**

We have developed an approach to determine the relative expression of two genes by real-time PCR. We used it to assay for B-cell lymphomas in human samples by measuring the relative expression of \( IGL\alpha \) and \( IGLA \) mRNA. The ratio between \( IGL\alpha \) and \( IGLA \)-producing cells in healthy individuals was \( \sim \)60:40, and we expect a similar \( IGL\alpha:IGLA \) ratio for negative samples in our real-time PCR assay. In lymphoma-derived samples, the \( IGL\alpha:IGLA \) ratio is expected to deviate substantially from 60:40 because of clonal expansion. In contrast to most other assays based on relative gene expression measurements, this one does not rely on comparison with the expression of housekeeping genes, which has been seriously questioned (17–19).

**PCR IN BIOLOGICAL SAMPLES IS SUBSTANTIALLY INHIBITED AND THE DEGREE OF INHIBITION VARIES AMONG SAMPLES AND DEPENDS ON TEMPLATE**

In most studies of gene expression by Q-PCR, the CT values determined for test samples have been related to CT values of standard samples containing known amounts of cDNA. This approach relies on the crucial assumption that PCR efficiencies in the test and standard samples are the same. If this is not true, a CT value measured in a test sample will correspond to a different number of cDNA copies than the same CT value measured in the standard sample. The error introduced by such an assumption may be substantial because of accumulation effects. For example, 80% efficiency in the test sample and 85% efficiency in the standard sample produces a 50% difference in the number of DNA copies after 25 cycles (Eq. 1). The common procedure to account for differences in PCR efficiencies between test and standard samples is to amplify a reference gene, usually a housekeeping gene, in parallel and to relate the expression of...
the gene being studied to the expression of the housekeeping gene. This, of course, relies on the assumption that expression of the housekeeping gene is constant among the samples being compared, which has also been questioned (17–19). Furthermore, which is rarely acknowledged, it also assumes that the efficiencies of the two reactions, i.e., the PCR of the studied gene and the PCR of the housekeeping gene, are inhibited to the same degree in the standard sample as well as in the test sample (20):

\[
\frac{(1 + E_{\text{test sample}})}{(1 + E_{\text{housekeeping}})} = \frac{(1 + E_{\text{standard sample}})}{(1 + E_{\text{housekeeping}})}
\]

(24)

This critical assumption has, to our knowledge, not been scrutinized. Although one might be inclined to think that inhibitory components that may be present in biological samples should have the same effect on all PCRs, it is not necessarily so. The degree of inhibition may depend on features that are particular for the different PCR systems, such as the length and sequence of template, template tertiary structure, and lengths and sequences of primers. Inhibition may also be indirect through competition for critical elements such as ions and deoxynucleotide triphosphates (dNTPs). The two PCR systems designed here obtain optimum efficiencies at different concentrations of Mg\(^{2+}\), dNTPs, primers, and probe (5 mM MgCl\(_2\), 0.2 mM dNTPs, 800 nM primers, and 800 nM probe vs 3.5 mM MgCl\(_2\), 0.4 mM dNTPs, 600 nM primers, and 600 nM probe), and elements in biological samples that interact with these PCR components are expected to interfere with the reactions to different degrees.

The PCR efficiencies of both reactions in seven samples and of purified template were determined in situ from CT values measured in dilution series. The total dilution was 64 times, which was sufficient to make the effect of experimental variation in CT values on the slopes of the CT-vs-log(cDNA concentration) plots negligible. Significant PCR inhibition in the biological samples was evident from the significantly higher PCR efficiencies obtained with purified template compared with those obtained in patient samples: purified template had efficiencies >93% compared with efficiencies in the range 75–95% for 13 of the 14 reactions in the patient samples. The IgLa reaction in sample BR17 was extensively inhibited, with an efficiency of only 59%. The IgLx reaction in the same sample had the expected efficiency (83%; see Table 2), suggesting that the sample contained components that predominantly interfered with the IgLa PCR. We did not investigate the cause of inhibition but considered the sample an outlier and did not include it when calculating average efficiencies.

**CLASSIFICATION OF SAMPLES**

The most accurate classification of samples as lymphoma-negative or -positive is expected when sample-specific inhibition of both PCR reactions is determined by in situ calibration and accounted for. For the seven samples characterized this way, the fractions of IgLx expression calculated directly from the measured CT values, i.e., without correcting for sample-specific effects, were 52% (BR0), 59% (BR46), 60% (BR42), and 63% (BR37) in the negative samples and 24% (BR18), 43% (BR17), and 89% (BR52) in the positive samples (Table 1 in the Data Supplement). Because lymphoma-negative samples should express IgLx to ~60% (1, 2), BR46, BR42, and BR37 are clearly negative, whereas BR18 and BR52 are positive. Classification of BR0 and BR17 is less clear, although the negative BR0 sample expressed IgLx to a higher extent than the positive BR17 sample (52% vs 43%). When sample-specific efficiencies, as determined by in situ calibration, were taken into account, IgLx expression in the negative samples was 56%, 62%, 62%, and 62%, whereas it was 6%, 16%, and 93% in the positive samples. The negative samples are thus clearly distinguished from the positive ones, as also seen in Fig. 3, where they are clearly separated by the confidence region based on at least 95% probability.

We did not optimize the in situ calibration protocol in this work, but decided rather arbitrarily to dilute 64 times in steps of 4 and to choose samples containing sufficient material to avoid any statistical uncertainty (21, 22). Note that the in situ calibration protocol is economic with sample because it is based on dilution; less material is required, for example, than when running regular duplicate or triplicate samples. We performed the dilution in four steps, which means that four measurements were made on each sample. Although the samples were run in parallel, some additional hands-on time was required. For high-throughput analyses, it may therefore be time-efficient to first perform a standard single or duplicate measurement on each sample, do a gross classification, and then further characterize uncertain samples by in situ calibration. The gross classification can be quite accurate if any differences in average efficiencies and sensitivities of the two assays are considered. Fig. 3 shows such classification of the samples studied here. All negative samples are within the confidence region, whereas most positive ones are outside it. Three samples in Fig. 3 are false negatives. Two of them are at the periphery of the confidence region and therefore candidates for reinvestigation by the more accurate in situ calibration protocol. Indeed, BR17 was reinvestigated and found to be positive. The positive sample that appears as false negative and is not at the periphery is a diffuse large B-cell lymphoma. The reason for its misclassification has not been identified. It may be attributable to tumor sampling error or highly anomalous PCR efficiencies. However, some diffuse large B-cell lymphomas express very low amounts of immunoglobulins, and the monoclonal immunoglobulin production of the tumor cells may not be detected against the background of polyclonal immunoglobulin produced by nonlymphoma B-lymphocytes in the sample. The classification presented takes into account experimental uncer-
tainty, but to evaluate the individual variation that may be present in various types of NHL a larger series of tumors must be characterized.

The described real-time PCR assay compares the expression of IgLκ and IgLλ and is designed to detect B-cell monoclonality in NHL. This is not sufficient for general tumor screening of patient samples, but demonstrates the potential, accuracy, and effectiveness of real-time PCR-based assays. When more markers are available, real-time PCR-based methods may have great potential as an important complement to morphologic and immunologic methods in clinical tumor diagnostics. Accurate analysis of fine-needle aspirates with a yield of 1000–100,000 representative cells is clearly sufficient for successful analysis. Indeed, we estimate that the material in a fine-needle aspirate will be sufficient for up to 50 different tests to detect various tumor cell characteristics, including B-cell monoclonality.

We thank our colleagues at the TATAA Biocenter for valuable discussions. A patent application is pending for the method to measure the IgLκ/IgLλ expression ratio. This work was supported by the Swedish Research Council and the Swedish Cancer Society.

References
Table 4. Classification of patient samples and fraction of IgLκ expression.

<table>
<thead>
<tr>
<th>Sample</th>
<th>R.E.A.L.</th>
<th>IHC</th>
<th>Real-time PCR</th>
<th>Flow Cytometry analysis</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Classification</td>
<td>clonality</td>
<td>% κ expression</td>
<td>% κ expression</td>
</tr>
<tr>
<td>BR0</td>
<td>Lymphadenitis poly</td>
<td>62 (52)</td>
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<tr>
<td>BR1</td>
<td>NHL-CLL</td>
<td>IgLλ</td>
<td>(6)</td>
<td>1</td>
</tr>
<tr>
<td>BR2</td>
<td>NHL-DLBCL</td>
<td>IgLλ</td>
<td>(0.4)</td>
<td>3</td>
</tr>
<tr>
<td>BR4</td>
<td>NHL-CLL</td>
<td>IgLκ</td>
<td>(99)</td>
<td>95</td>
</tr>
<tr>
<td>BR5</td>
<td>NHL-Foll</td>
<td>IgLκ</td>
<td>(76)</td>
<td>65</td>
</tr>
<tr>
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<td>NHL-DLBCL</td>
<td>IgLκ</td>
<td>(98)</td>
<td>ND</td>
</tr>
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<td>NHL-Foll</td>
<td>IgLλ</td>
<td>(8)</td>
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<tr>
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<td>IgLλ</td>
<td>(2)</td>
<td>32</td>
</tr>
<tr>
<td>BR13</td>
<td>NHL-CLL</td>
<td>IgLκ</td>
<td>(96)</td>
<td>96</td>
</tr>
<tr>
<td>BR17</td>
<td>NHL-Foll</td>
<td>IgLλ</td>
<td>6 (43)</td>
<td>32</td>
</tr>
<tr>
<td>BR18</td>
<td>NHL-Foll</td>
<td>IgLλ</td>
<td>16 (24)</td>
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<tr>
<td>BR22</td>
<td>NHL-Foll</td>
<td>IgLκ</td>
<td>(83)</td>
<td>ND</td>
</tr>
<tr>
<td>BR23</td>
<td>T-cell lymphoma</td>
<td>-</td>
<td>(39)</td>
<td>ND</td>
</tr>
<tr>
<td>BR29</td>
<td>NHL-DLBCL</td>
<td>IgLλ</td>
<td>(54)</td>
<td>26</td>
</tr>
<tr>
<td>BR37</td>
<td>Lymphadenitis poly</td>
<td>56 (63)</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>BR42</td>
<td>Lymphadenitis poly</td>
<td>62 (60)</td>
<td>ND</td>
<td></td>
</tr>
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<td>BR43</td>
<td>Lymphadenitis poly</td>
<td>(68)</td>
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</tr>
<tr>
<td>BR46</td>
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<td>62 (59)</td>
<td>46</td>
<td></td>
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<tr>
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<td>IgLκ</td>
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<tr>
<td>BR52</td>
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<td>IgLκ</td>
<td>93 (89)</td>
<td>95</td>
</tr>
<tr>
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</tr>
<tr>
<td>BR102&lt;sup&gt;d&lt;/sup&gt;</td>
<td>NHL-Foll</td>
<td>IgLκ</td>
<td>(94)</td>
<td>ND</td>
</tr>
<tr>
<td>-----------------</td>
<td>----------</td>
<td>------</td>
<td>------</td>
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<tr>
<td>BR103&lt;sup&gt;d&lt;/sup&gt;</td>
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<td>poly</td>
<td>(81)</td>
<td>ND</td>
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<td>poly</td>
<td>(54)</td>
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</tr>
<tr>
<td>BR105&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Lymphadenitis</td>
<td>poly</td>
<td>(34)</td>
<td>ND</td>
</tr>
<tr>
<td>BR106&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Lymphadenitis</td>
<td>poly</td>
<td>(57)</td>
<td>ND</td>
</tr>
<tr>
<td>BR107&lt;sup&gt;d&lt;/sup&gt;</td>
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<td>poly</td>
<td>(33)</td>
<td>ND</td>
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<td>BR108&lt;sup&gt;d&lt;/sup&gt;</td>
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<td>poly</td>
<td>(43)</td>
<td>ND</td>
</tr>
<tr>
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<td>Lymphadenitis</td>
<td>poly</td>
<td>(57)</td>
<td>ND</td>
</tr>
<tr>
<td>BR110&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Lymphadenitis</td>
<td>poly</td>
<td>(47)</td>
<td>ND</td>
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<td>BR111&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Lymphadenitis</td>
<td>poly</td>
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</tr>
<tr>
<td>BR112&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Lymphadenitis</td>
<td>poly</td>
<td>(43)</td>
<td>ND</td>
</tr>
</tbody>
</table>

<sup>a</sup> IHC, immunohistochemical analysis; NHL, non-Hodgkin lymphoma; DLBCL, diffuse large B-cell lymphoma; Foll, follicular lymphoma; CLL, chronic lymphatic leukemia; ND, not determined; poly, polyclonal.

<sup>b</sup> Fraction IgLκ expression calculated by equation 10 either assuming average PCR efficiencies (within brackets) or taking into account sample-specific PCR efficiencies determined by in situ calibration (Table 2).

<sup>c</sup> Fraction IgLκ expression calculated by flow cytometry.

<sup>d</sup> Fine needle aspirates.