Quantification of Bcr-Abl transcripts in chronic myelogenous leukemia (CML) using standardized, internally controlled, competitive differential PCR (CD-PCR)

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ABSTRACT

The quantification of Bcr-Abl transcript numbers in chronic myelogenous leukemia (CML) patients described here uses simultaneous competitive PCR amplification of the target gene (Bcr-Abl) and a reference gene (porphobilinogen deaminase; Pbgd) together with a single composite competitor molecule for both targets based on heterologous sequences. Using this technique, Bcr-Abl transcript numbers could be reproducibly determined even in clinical samples known to harbour poor quality RNA.

Quantification of gene transcript numbers is of major interest in biology and clinical medicine, e.g., disease monitoring (1,2). In an attempt to study the expression of Bcr-Abl transcript numbers in CML we first adopted a published protocol using homologous competitor fragments carrying an insert and amplifying with the same primers as the target sequence (3,4). However, heterodimeric formation between target and competitor sequence and variability of RNA quality in clinical samples led us to focus on three different goals: (i) construction of competitor fragments based on heterologous sequences (5); (ii) parallel amplification of a second gene and its competitor as internal reference (6); (iii) equimolar presence of both competitors in the reaction.

We introduced the amplification of the ‘housekeeping’ form of the porphobilinogene deaminase (Pbgd) reference gene that allowed us to estimate the amount of cDNA present in the reaction. This ‘housekeeping’ gene was favoured over others because of its sequence characteristics and because no pseudogene has been described (7). With the addition of two competitor fragments we were able to evaluate equivalence points for both targets in one test tube. The Bcr-Abl competitor fragment contained a second primer binding site for nested amplification. All sequences were confirmed by automated sequencing. In order to assure an equimolar ratio both competitors were ligated and cloned into the pCR1 vector (Invitrogen, Leek, NL). The competitor plasmid was constructed such that a restriction digestion between the two competitor fragments yielded a linear 5.1 kb construct (Fig. 1). After photometric quantitation a serial dilution in the range from 10⁷ to 10⁴ molecules was prepared with steps at every half order of magnitude on a logarithmic scale, i.e. 10⁷, 3.2×10⁶, 10⁶, 3.2×10⁵, etc. Our standard setup for a given clinical sample is four to five reactions over a minimum range of four logarithmic steps that will detect 80% of the equivalence points for both targets. A 50 µl reaction mix containing at least 100–200 ng randomly transcribed RNA, 25 nmol each of outer Bcr-Abl primers and 2.8 nmol of Pbgd primers (Table 1) was subjected to 32 cycles of the first PCR. Samples visibly negative for Bcr-Abl after the first PCR are subjected to a second PCR with the internal Bcr-Abl primers. After 25 cycles of second PCR the equivalence points for Bcr-Abl were detectable in samples with even fewer copy numbers. Amplification products were separated on a 3% agarose gel and stained with ethidium bromide for densitometric analysis. Calculation of integrated optical density (IntOD) was performed with the ONE-Dscan software (Scanalytics, Billeria, USA).

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Figure 2. Changes of Bcr-Abl transcript numbers during interferon treatment in a patient with CML. Three different samples are displayed: (A) obtained at diagnosis, (B) at 2 months, (C) at 8 months. Data are summarized in (D). Note that the equivalence points between Bcr-Abl and Bcr-Abl competitor (■, left y axis) do not change significantly when appreciated alone. However, upon consideration of the Pbgd reference gene (▲, left y axis), the ratio (∇, right y axis) is dramatically different.

IntOD data were plotted logarithmically and equivalence points (EqP) were determined. A correction for the different size of products in the gel for target and competitor sequences was made to evaluate the number of molecules in the sample. The correction factors are given in Table 1. The relative changes in Bcr-Abl copy numbers were expressed as the ratio of corrected EqP for Bcr-Abl divided by the corrected EqP for the Pbgd reference gene.

Table 1. Lengths of amplification products and primer sequences

<table>
<thead>
<tr>
<th>Primer</th>
<th>Target (bp)</th>
<th>Competitor (bp)</th>
<th>Correction factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bcr-Abl b3a2</td>
<td>nb1-abl3</td>
<td>532</td>
<td>608</td>
</tr>
<tr>
<td>b2a2</td>
<td>nb1-abl3</td>
<td>457</td>
<td>608</td>
</tr>
<tr>
<td>Pbgd</td>
<td>pbgd8-pbgd3</td>
<td>324</td>
<td>387</td>
</tr>
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2. PCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>Target</th>
<th>Competitor</th>
<th>Correction factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bcr-Abl b3a2</td>
<td>b2a-ca3</td>
<td>460</td>
<td>557</td>
</tr>
<tr>
<td>b2a2</td>
<td>b2a-ca3</td>
<td>385</td>
<td>557</td>
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REFERENCES