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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 target sequences 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 pCRII 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 photometrical quantitation a serial dilution in the range from 10^7 to 10^1 molecules was prepared with steps at every half order of magnitude on a logarithmic scale, i.e. 10^7, 3.2×10^6, 10^6, 3.2×10^5,

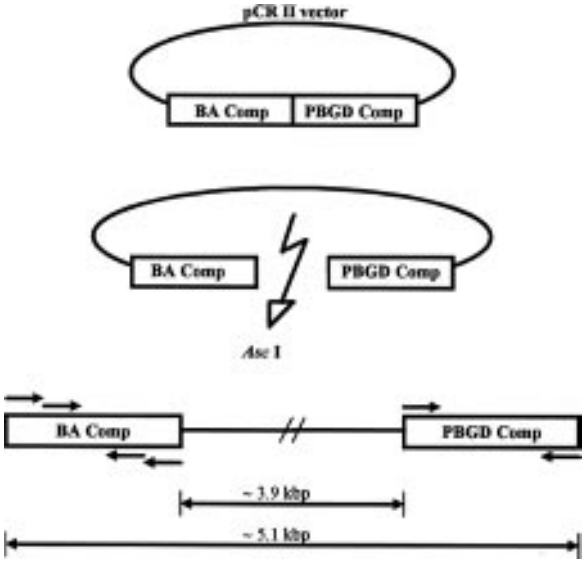

Figure 1. Construction of the Bcr-Abl competitor fragment. After restriction digestion with the appropriate restriction enzyme (*Asc*I), a linearized fragment results which carries the Bcr-Abl competitor on one side (608 bp) and the Pbgd competitor (431 bp) on the other. Full length amplification over the backbone of the molecule (5.1 kb) was excluded by the chosen reaction conditions. BA Comp, Bcr-Abl competitor fragment; PBGD Comp, Pbgd competitor fragment; *Asc*I, restriction endonuclease; arrows, position of primers.

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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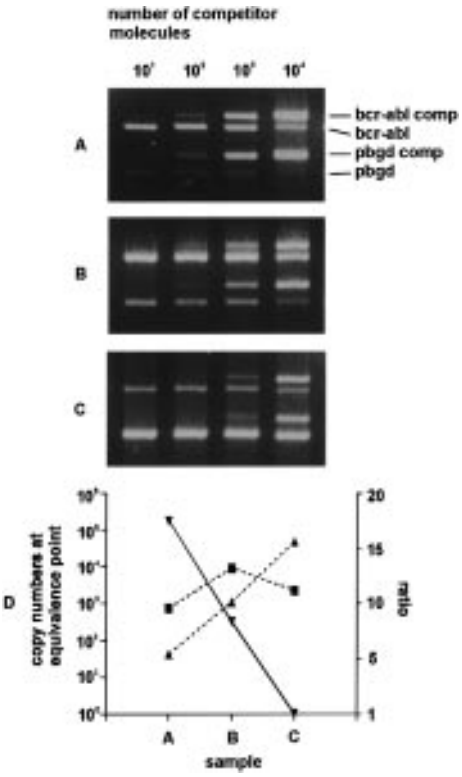

Figure 2. Changes of Bcr-Abl transcript numbers during interferon treatment in a patient with CML. Three different samples are displayed: (**A**) obtained at diagnosis, (**B**) at 2 months, (**C**) at 8 months. Data are summarized in (**D**). Note that the equivalence points between Bcr-Abl and Bcr-Abl 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

		Primer	Target (bp)	Competitor (bp)	Correction factor
1. PCR					
Bcr-Abl	b3a2	nb1-abl3	532	608	1.14
	b2a2	nb1-abl3	457	608	1.33
Pbgd		pbgd8-pbgd3	324	387	1.19
2. PCR					
Bcr-Abl	b3a2	b2a-ca3	460	557	1.21
	b2a2	b2a-ca3	385	557	1.45

name	**sequence**
nb1	5′-gAg CgT gCA gAg Tgg Agg gAg AAC A
abl3	5′-ggT ACC Agg AgT gTT TCT CCA gAC Tg
pbgd8	5′-ggC TgC AAC ggC ggA AgA AAA C
pbgd3	5′-TTg CAg ATg gCT CCg ATg gTg AAg
b2a	5′-TTC AgA AgC TTC TCC CTg gCA TCC gT
ca3	5′-TgT TgA CTg gCg TgA TgT AgT TgC TTg g

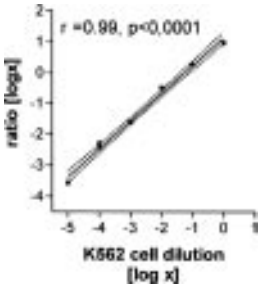

Figure 3. Determination of Bcr-Abl copy numbers in a cell dilution series. CD-PCR was performed in duplicate for each dilution. Bcr-Abl expression as the computed ratio of $EqP_{Bcr-Abl}/EqP_{Pbgd}$ is shown over five orders of magnitudes. Linear regression analysis (r) with calculation of the level of significance (p) was performed. The dotted lines indicate the 95% confidence interval.

This novel approach of an internally standardized, competitive differential PCR not only allows us to determine the relative changes (ratio) in gene expression, but also to determine the absolute amounts of Bcr-Abl cDNA copy numbers per microgram of RNA. To underline this hypothesis we scanned three subsequent clinical blood samples of a patient with CML and found that the changes of the absolute amounts did not necessarily correspond to the ratio in Bcr-Abl transcript copy numbers (Fig. 2). These data show that, in quantitative PCR, determination of reference gene expression is important, in particular for clinical samples. Further, in follow-up studies comparison of the calculated ratio is more reliable. To assure accuracy and reproducibility of the assay, negative controls were included at all stages and a control cell dilution series of the Bcr-Abl positive cell line K562 in Bcr-Abl negative Molt4 cells was prepared (Fig. 3). A detection threshold was reached such that one positive cell in 10^5 negative cells could routinely be evaluated. Linearity of detection was shown over five orders of magnitude. The coefficient of variation (CV) calculated for the relative changes in Bcr-Abl cDNA copy numbers was less than 5% for several samples when repeated five times. These findings confirm the results of the assay to be reproducible.

This technique should easily be transferred to other target sequences.

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