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Quantitative analysis of DNA aberrations amplified by competitive polymerase chain reaction using capillary electrophoresis

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Abstract

We compared the use of capillary electrophoresis (CE) in a polymer network with the use of slab gel electrophoresis for the quantitative analysis of polymerase chain reaction (PCR)-amplified DNA samples. We quantified residual lymphoma cells carrying a translocation between chromosomes 14 and 18, in consecutive patient peripheral blood samples that were amplified by competitive PCR. For CE analysis we used a 4% linear polyacrylamide network. Results show that the calculated number of translocations in patient samples using both analyses were comparable. We conclude that CE is a sensitive, non-radioactive, fast and accurate method for quantitation of competitive PCR products.

1. Introduction

Tumour-specific markers can be used to monitor residual disease in patients during treatment. One of these markers is the reciprocal chromosomal translocation t(14;18), frequently found in follicular lymphomas. Because of this translocation the *bcl*-2 gene on chromosome 18 is coupled to the immunoglobulin heavy-chain locus on chromosome 14. Random nucleotides are inserted between the two genes providing a patient specific DNA pattern [1]. The polymerase chain reaction (PCR) is a sensitive method to detect the presence of lymphoma cells carrying t(14;18) by amplification of the translocation breakpoint [2]. Since, as a result of exponential amplification, small variations in reaction conditions can lead to large differences in product yield, an internal standard is needed for accurate quantitation. This internal standard has to be coamplified in the same reaction as the target DNA using the same primers for equal amplification efficiency.

For this purpose a competitive PCR has been developed [3]. Serial dilutions of a known number of patient-derived translocation molecules are coamplified with a fixed number of competitor translocation molecules. These competitor molecules serve as an internal standard. Plotting of the ratios of the two PCR products

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against the initial number of patient-specific translocation molecules results in a patient-specific calibration curve. By comparison of the ratio of the PCR products of an unknown number of patient translocation molecules coamplified with the same fixed number of competitor molecules with the calibration curve, the unknown number of translocations in the patient samples can be calculated. This way the residual disease of a patient with a non-Hodgkin's lymphoma (NHL) could be monitored after allogeneic bone marrow transplantation [4]. In this procedure, the competitive PCR products were analyzed on conventional slab gels using radioactivity for detection.

The fast development of capillary electrophoresis (CE) prompted us to investigate whether this time-consuming and laborious slab gel procedure could be replaced by automated CE analysis. Recently a quantitative analysis using capillary electrophoresis was described by Lu et al. [5]. However, they analyzed products of a non-competitive PCR, e.g. without the use of an internal standard. We analyzed competitive PCR products of consecutive samples from a patient with lymphoma cells carrying t(14;18) using capillary electrophoresis as well as the traditional slab gel electrophoresis. The unknown numbers of translocation molecules in the patient samples, determined using the two methods, were compared.

2. Experimental

2.1. DNA isolation

DNA was isolated [6] from a lymph node sample of patient 1 and from a lymph node sample and consecutive peripheral blood samples of patient 2. All samples contained lymphoma cells carrying t(14;18).

2.2. Oligonucleotides

Oligonucleotides were synthesized on a 391A DNA synthesizer (Applied Biosystems, Warrington, UK). The names and the sequences of the oligonucleotides used for the *bcl-2* region were: MBR3-Xhol; 5′-CCC TCG AGG AGC TTT GTT TCA ACC AAG TC-3′ (position 2752 for the 7th nucleotide [7]) and MBR2; 5′-TCC CTT TGA CCT TGT TTC TTG A-3′ (position 2826 [7]). Oligonucleotides used for the immunoglobulin heavy-chain region were: JH-CON; 5′-ACC TGA GGA GAC GGT GAC C-3′ and JH-HindIII; 5′-TCA AGC TTA CCT GAG GAG ACG GTG ACC-3′ (identical to JH-CON but contains 8 additional bases at the 5′ site) [2].

2.3. Preparation of patient-derived molecules

Several polymerase chain reactions were amplified in a Perkin-Elmer Cetus thermocycler 480 (Norwalk, CT, USA). Each reaction contained 1 μg lymph node DNA of patient 1 or patient 2, 500 μM dNTP's, 30 pmol of oligonucleotides MBR3-Xhol and JH-HindIII and 2.5 U *taq* DNA polymerase (Life Technologies, Gaithersburg, MD, USA) in a total volume of 100 μl, overlaid with 80 μl mineral oil. Amplification started with an initial denaturation step at 94°C for 5 min followed by 35 cycles at 94°C for 1.5 min, at 55°C for 2 min and at 72°C for 2 min. The PCR products were pooled, separated from oligonucleotides on a 2% agarose gel, excised and electro-eluted by Biotrap (Schleicher and Schuell, Dassel, Germany). After precipitation the DNA was dissolved in 800 μl water. The concentration was determined by absorbance measurement and from this, the number of patient-derived translocation molecules was calculated. The translocation molecules of patient 1 were used as competitor molecules in the competitive PCR.

2.4. Competitive polymerase chain reaction

The competitive PCR was performed as previously described [3]. Serial dilutions of a known number of patient 2 derived translocation molecules were coamplified with a fixed number of patient 1 derived competitor translocation molecules in addition of 1 μg K562 DNA [contains no t(14;18) and serves as a negative control]. Amplification was performed with 30 pmol of oligo-

nucleotides MBR2 and JH-CON for 55 cycles with an annealing temperature of 58°C. Samples which were analyzed using slab gel electrophoresis were amplified in the presence of 1.85 MBq/ml [a-^{32}P]dCTP (Amersham, Buckinghamshire, UK). Other conditions were as described above.

One microgram of several consecutive peripheral blood DNA samples of patient 2, with an unknown number of translocation molecules, were also coamplified with the same number of competitor molecules.

2.5. Capillary

A fused-silica capillary [310 mm (L_{eff}) × 0.075 mm I.D.] (SGE, Ringwood, Vic., Australia) was precoated with 3-methacryloylpropylmethoxysilane (Sigma, St. Louis, MO, USA). Additional coating was performed by purging with a 4% acrylamide (4% T, 0% C) solution containing 8 μl of 10% N,N,N′,N′-tetramethylethylenediamine (TEMED) and 4 μl of 10% ammonium persulfate (APS) per millilitre of acrylamide solution [8]. The acrylamide, TEMED and APS were all obtained from Bio-Rad Laboratories (Richmond, CA, USA). The acrylamide solution was left in the capillary for 18 h to polymerize at 15 bar. The filled capillary was mounted in an assembly cartridge and placed in a Bio-Rad BioFocus 3000 CE instrument (Bio-Rad, Hercules, CA, USA). After each run the capillary was rinsed with water for 5 min and filled again with the polymerized 4% linear polyacrylamide by purging for 5 min at 8 bar.

2.6. Analysis and quantitation of PCR products using capillary electrophoresis

The competitive PCR products were purified with the Magic PCR Preps DNA purification system (Promega, Madison, WI, USA) and dissolved in 50 μl of water. All samples were injected electrokinetically at reversed polarity (cathode at the injection side) of 280 V/cm for 10 s and separated under constant voltage of 425 V/cm, in a TAE running buffer (40 m*M* Tris-acetate pH 8.3, 2 m*M* EDTA). UV absorbance was measured at 260 nm. During the runs the carrousel was kept at 15°C and the capillary was kept at 25°C.

Using the BioFocus 3000 Integrator program (Bio-Rad), the areas under the curves were determined (AUC). Ratios of the AUC of the serial dilution reactions were plotted logarithmically against the initial number of known patient 2 derived translocation molecules in the serial dilution reactions. This resulted in a patient specific calibration curve. Using this calibration curve, the number of unknown t(14;18) translocation molecules in the patient 2 samples could be determined.

2.7. Analysis and quantitation of PCR products using slab gel electrophoresis

A 5-μl volume of 10-fold diluted PCR product in formamide was loaded on a 7% PAA (acrylamide:bisacrylamide = 24:1), 7 *M* urea gel. Separation was performed in a 0.5 × TBE running buffer (45 m*M* Tris-borate pH 8.3, 0.1 m*M* EDTA). The gel was exposed to an X-ray film (Kodak) and the autoradiogram was analyzed by densitometric scanning on a LKB laser densitometer. Band intensities were determined using Gelscan XL software and the ratios were plotted logarithmically against the initial number of patient 2 derived translocation molecules.

3. Results

We used a competitive PCR to quantify residual lymphoma cells carrying a t(14;18). Competitive PCR amplification of patient 1 derived translocation molecules resulted in a 362 bp PCR product. Amplification of patient 2 derived translocation molecules resulted in a 312 bp PCR product. The 362 bp fragments were used as internal standard.

We analyzed the competitive PCR products using capillary electrophoresis as well as slab gel electrophoresis. Because of electrokinetic injection, the samples analyzed using capillary electrophoresis were desalted and purified using the Magic PCR Preps DNA purification system. To

investigate whether the Magic PCR Preps DNA purification system had a predilection for the 312 bp or the 362 bp PCR product we analyzed different radioactive samples before and after purification (Fig. 1). The results from densitometric scanning of the band intensities showed no predilection (data not shown).

Fig. 2 shows the quantitative analysis by capillary electrophoresis. Determination of the curve areas and plotting of the ratios resulted in a patient-specific calibration curve with a correlation coefficient $r = 0.993$. The corresponding equation is $\ln(y) = 0.983\ \ln(x) - 8.031$, where y is the ratio and x is the initial number of known translocation molecules.

Fig. 3 shows the analysis using slab gel electrophoresis. Scanning of the band intensities and plotting of the ratios against the initial number of known patient 2 derived translocation molecules resulted in a patient-specific calibration curve with a correlation coefficient $r = 0.970$. The corresponding equation is $\ln(y) = 1.220\ \ln(x) - 9.895$.

The results of the calculated number of unknown translocation molecules in the consecutive samples of patient 2 using capillary electrophoresis and slab gel electrophoresis are summarised in Table 1.

To check the reproducibility of CE analysis using electrokinetic injection, we divided a sample into three portions which were separately used for electrokinetic injection. The standard deviation of the ratio of the AUC was 3.2% (data not shown).

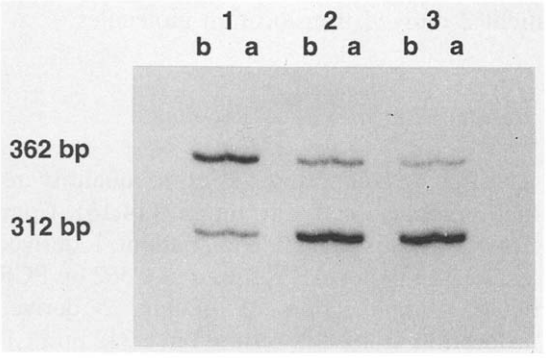

Fig. 1. Autoradiogram of 3 different competitive PCR products before and after purification with the Magic PCR Preps DNA purification system. Lane 1: 1000 competitor molecules and 6000 patient-derived translocation molecules before (b) and after (a) purification; lane 2: 1000 competitor molecules and 1000 patient-derived translocation molecules before (b) and after (a) purification; lane 3: 1000 competitor molecules and 600 patient-derived translocation molecules before (b) and after (a) purification.

4. Discussion

The PCR is a sensitive method to determine the presence or absence of malignant cells carrying t(14;18). In the competitive PCR used in this study, translocation molecules from a patient were used as competitor molecules. Because the length of the amplified t(14;18) breakpoint differs in each patient, the internal standard molecules can be distinguished from the target molecules by size. This competitive PCR method can also be used to quantify other DNA or RNA molecules. Celi et al. have recently described a rapid and versatile method to synthesize internal standards [9], which can be used as competitor molecules in the competitive PCR.

The products of the competitive PCR are usually analyzed on conventional slab gels. The preparation of these gels is time consuming, laborious and they can only be used once. We therefore used capillary electrophoresis in a polymer network. The preparation of the capillary is easy and it can be used for several automated analyses because the capillary can be filled again [10]. Another advantage of CE compared to slab gel electrophoresis is the detection. The detection of DNA molecules using UV absorption is direct and the data are stored in an electropherogram, which can be immediately used for evaluation. Detection of DNA molecules using radioactivity is hazardous, the gel has to be exposed which takes additional time and the band intensities have to be scanned after exposure to obtain information about the quantity. Scanning and subsequent computer analysis is time consuming and only part of the band is scanned. This means that any irregularity in the band can lead to different densitometric results.

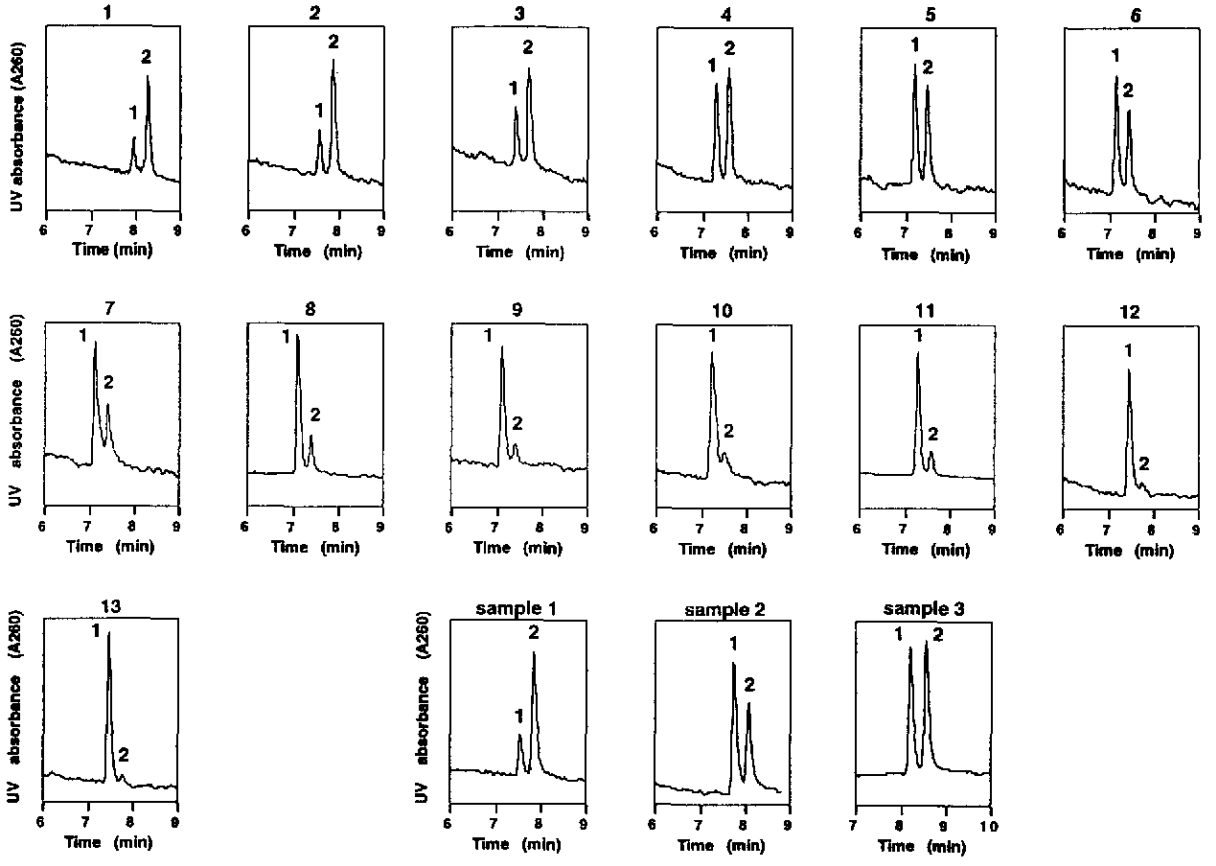

Fig. 2. Electropherograms of analysis using capillary electrophoresis. 1–13: as described for lane 1–13 in Fig. 3, sample 1–3: as described for lane 17–19 in Fig. 3. Peak 1 represents the 312 bp PCR product and peak 2 represents the 362 bp PCR product.

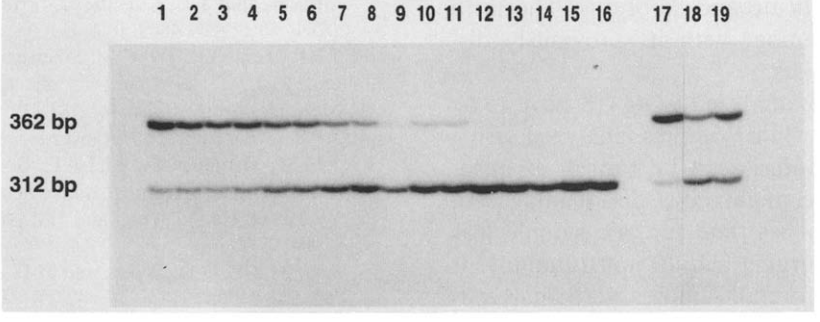

Fig. 3. Autoradiogram of analysis using slab gel electrophoresis. Lane 1–14: coamplification of serial dilutions of a known number of patient-derived translocation molecules (10 000, 8000, 6000, 5000, 4000, 3000, 2000, 1000, 900, 800, 600, 400, 200 and 100) and 1000 competitor molecules in addition of 1 μg K562 DNA; lane 15: control, 1000 competitor molecules; lane 16: control, 1000 competitor molecules and 1 μg K562 DNA; lane 17–19: coamplification of consecutive patient blood samples (resp. Feb '91, Aug '91 and Jan '92) with an unknown number of translocation molecules and 1000 competitor molecules.

Table 1
Calculation of an unkown number of translocation molecules in consecutive samples of patient 2, using the patient-specific calibration curves

Sample	Date	t(14;18)/μg DNA	
		Slab gel	Capillary
1	Feb '91	9964 ± 1566	12 027 ± 987
2	Aug '91	2854 ± 274	3195 ± 155
3	Jan '92	4084 ± 444	5038 ± 290

Another disadvantage of radioactivity and autoradiography is the non-linear dose–response relationship, e.g. the band intensities on an autoradiogram can reach a maximum. If the intensity of one of the bands on the autoradiogram reaches its maximum, the ratio of the band intensities is disturbed. Detection using UV absorbance does not suffer from this problem because there is a linear relationship between the number of molecules and the area under the curve.

Previously described methods for the analysis of competitive PCR products are: counting the radioactivity present in bands excised from agarose gel [11], or excised from polyacrylamide gel [12] and densitometric scanning of the bands on a negative film of an ethidium bromide stained agarose gel [13]. The first two methods use radioactivity and have the same disadvantages as using slab gel electrophoresis. Furthermore, excising bands is delicate and laborious. The third method uses scanning, with its previously discussed disadvantages.

Because samples analyzed using CE have to be run sequentially while samples analyzed using slab gel electrophoresis can be run all at once, total analysis time might be a disadvantage. In our study the analysis time for one sample was 20 min (10 min purging and 10 min running). It took about 6 h before all samples were analyzed. The total electrophoresis time using slab gel electrophoresis was about 2 h, but regarding the additional time needed for preparing the gel, exposure and time consuming scanning and subsequent computer analysis we conclude that CE is much faster. Besides, the analysis using capillary electrophoresis is fully automated.

Results show that the number of translocation molecules calculated using both methods are within each others range. The correlation coefficients and the standard deviation of the ratio of the AUC (3.2%), obtained by injecting a sample 3 times, provide sufficient accuracy for screening of DNA or RNA molecules.

We conclude that CE is a non-radioactive, fast, accurate and sensitive method for the quantitation of competitive PCR products and because CE is automated it opens possibilities for genetic routine analysis.

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