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Identification and characterisation of clonal incomplete T-cell-receptor Vδ2–Dδ3/Dδ2-Dδ3 rearrangements by denaturing high-performance liquid chromatography and subsequent fragment collection: implications for minimal residual disease monitoring in childhood acute lymphoblastic leukemia

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Abstract

Incomplete T-cell-receptor δ (TCR-δ) rearrangements are widely used for detection of minimal residual disease in childhood acute lymphoblastic leukemia. In a substantial number of cases both alleles are rearranged and polymerase chain reaction (PCR) products have either to be cloned or excised and reamplified from acrylamide gels. Here we describe a novel HPLC-based method for identification and characterization of clonal TCR-δ targets. Clonality of PCR amplified TCR-δ products was examined on a high-resolution micropellicular DNASep matrix (WAVE Nucleic Acid Fragment Analysis System, Transgenomic) and subsequently classified into clonal, biclonal or negative samples. Vδ2-Dδ3 and Dδ2-Dδ3 rearrangements were analyzed by denaturing high-performance liquid chromatography (DHPLC), using triethylammonium acetate as an ion-pairing reagent, with a linear acetonitrile gradient at 50 °C. Biclonal elution profiles consisted of two individual homoduplex peaks and one heteroduplex peak unique for each patient sample. For characterization of biclonal rearrangements DHPLC separated samples were subjected to a second run and individual clonal peaks were collected. A software-controlled fragment collector was arranged in tandem with the HPLC system for this purpose and purified PCR products were collected in a time-dependent manner. Fractions were air dried and subsequently sequenced directly. The specificity of the observed patient specific sequences was tested via real time quantitative PCR on a LightCycler system.

Keywords: Incomplete T-cell-receptor δ rearrangements

1. Introduction

Clonal antigen receptor rearrangements are widely used as patient-specific markers for detection of

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minimal residual disease (MRD) in acute lymphoblastic leukemia (ALL). Since ALL blasts are derived from one single transformed lymphoid precursor cell, junctional regions of immunoglobulin- or T-cell receptor (TCR) rearrangements are unique for each leukemia patient. Most of the TCR-δ gene complex is located on chromosome 14q11 embedded within the V and J segments of the TCR-α gene locus. The complex included several V, D and J segments separated by long stretches of DNA. Only after a recombination event these segments rearrange to functional V–(D)–J segments (Fig. 1). Clonal, incomplete TCR-δ rearrangements are detectable in about 50% of B-cell precursor (BCP) ALL [1,2]. These Vδ2-Dδ3 or Dδ2-Dδ3 rearrangements result in a nonproductive gene not capable of forming a functional δ-gene. Nevertheless the junctional region between the rearranged segments display suitable

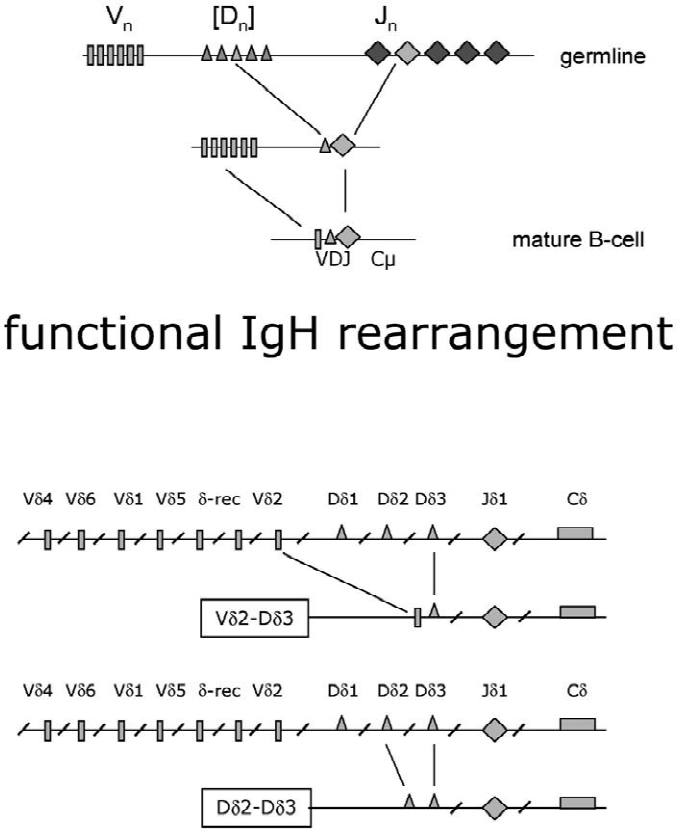


Fig. 1. Schematic diagram of functional IgH rearrangements or non-functional TCR-δ rearrangements in BCP-ALL. Germline IgH V–D–J gene segments are rearranged via D–J and V–(D–J) recombinations into a mature, functional B-cell receptor complex. At the recombination sites from each V–D- or J-segment nucleotides are inserted or deleted to increase the antigen recognition variability. The non-functional TCR-δ complex first rearranges V- and D- segments or D- and D-segments with insertion or deletion of nucleotides. This may proceed into a deletion of the TCR-δ gene segment due to rearangements within the surrounding TCR-α complex.

targets for the detection of MRD due to an extensive insertion and/or deletion of nucleotides [2–5]. Several studies demonstrated the applicability of these targets for detection of MRD [6–9].

Southern blot and/or polymerase chain reaction (PCR)-based techniques allow the identification and characterization of unknown leukemic samples from the time point of diagnosis. Southern blotting identifies only the type of rearrangement without further information about the junctional region. Therefore, only PCR-based approaches generally allow for the subsequent analysis of the product by direct sequencing or by sequencing after cloning into a plasmid vector. Direct sequencing is often limited due to impurities from polyclonal background amplification or due to biclonal rearrangements on both alleles of the leukemic sample. Heteroduplex induction followed by acrylamide gel electrophoresis has been described to circumvent these pitfalls [10,11]. Here, PCR products have to be excised and re-amplified for successful downstream analysis. In cases where the resolution of the method used was insufficient, the samples had to be cloned and several clones had to be sequenced.

We recently described the use of partially denaturing high-performance liquid chromatography (DHPLC) for identification of clonal TCR-γ rearrangements [12]. This method permits the analysis of unknown products in a semi-automated way within 10 min without further hands-on work and is highly reproducible using a target specific analysis protocol. The DHPLC method has originally been described for detection of known and unknown mutations reviewed in Refs. [13,14]. Based on the different stability of wildtype and mutated PCR products single or multiple [15,16] base pair (bp) exchanges are detectable as heteroduplex peaks at different column oven temperatures.

The aim of our recent study was to test the feasibility of DHPLC analysis for identification and characterization of incomplete TCR-δ rearrangements in childhood BCP-ALL. In a substantial number of cases both alleles are rearranged for Vδ2-Dδ3 or Dδ2-Dδ3, respectively. These biclonal leukemic samples (with two distinct clonal products within one PCR) were separated by DHPLC and fractionated for subsequent direct sequencing. The determined sequences were then tested for detection of MRD on the LightCycler system [17,18] using a germline TaqMan probe. The method presented here allows us to combine the preparative advantages of DHPLC for characterization of patient specific targets with the analytical properties of real time PCR for quantification of MRD.

2. Experimental

2.1. Patients

Bone marrow (BM) or peripheral blood (PB) from several children with BCP-ALL at the time of diagnosis was analyzed. Ficoll separated cells were frozen in 10% dimethylsulfoxide (DMSO)–50% fetal calf serum (FCS)–RPMI 1640 and stored at −80 °C until use. DNA was isolated using the Wizard genomic DNA purification kit (Promega, Madison, WI, USA). Patients were treated according to the COALL 92 or 97 protocol [19,20]. Cell samples were collected after informed consent from the patients or their parents with approval from the local ethics committee. The diagnosis was based on morphological and immunological examination of BM or PB. Cell samples contained between 40 and 95% malignant cells.

2.2. PCR

PCR analysis was performed with standard primers as described previously in Ref. [21]. A Vδ2-Dδ3 or Dδ2-Dδ3-gene rearrangement (without any nucleotide insertions or deletions flanking the junctional region) results in the amplification of a 150-bp PCR product. For subsequent analyses of initial leukemic DNA samples 50-μl reactions were performed containing 1 U Taq polymerase (Gibco) and 100 ng DNA as well as final concentrations of 2 μ*M* of each primer, 3 m*M* $MgCl_2$, and 200 n*M* of each dNTP. The PCR buffer used was recommended by the provider of the Taq polymerase. The PCR cycling protocol included a 5-min denaturation step at 95 °C, followed by 35 cycles of 30 s at 95 °C, 30 s

at 55 °C, 30 s at 72 °C followed by a final extension step of 2 min. PCR was performed in 200-μl, ultra-thin PCR tubes (Biozym, Germany) in a T-gradient thermocycler (Biometra, Göttingen, Germany).

2.3. Acrylamide gel electrophoresis

An aliquot of the final PCR product was subjected to electrophoresis on an acrylamide gel followed by a rapid silver staining protocol [22]. In brief, a 0.3 mm ultra-thin polyacrylamide was cast onto Gelbond (FMC) films. Samples were run in a discontinuous buffer system. Gels contained 7.1% glycerol and 33 m*M* Tris–sulfate buffer, pH 9.0, and were run with a buffer strip containing 140 m*M* Tris–borate, pH 9.0, onto a cooled (8 °C) Pharmacia Multiphore System (Amersham Pharmacia, Germany). The whole procedure takes about 1 h and allows the staining of up to 50 samples. Up to 2 μl of the 50 μl PCR product was used for separation.

2.4. DHPLC analysis

An 8-μl volume of each PCR product was analyzed by DHPLC on a WAVE System (Transgenomic, Crewe, UK). The shortest and best suitable gradient for analysis of the targets was determined by running universal gradients at different temperatures, starting with 50 °C, for several products (data not shown). An optimized gradient was then used for analysis of the BCP-ALL samples. Buffer A consists of 0.1 *M* triethylammonium acetate (TEAA), 0.0025% (v/v) acetonitrile (ACN), pH 7.0; buffer B: 0.1 *M* TEAA–25% (v/v) ACN, pH 7.0; buffer C (wash buffer): 75% ACN. Buffer B starts at 45% and ends at 60% with a flow-rate of 0.9 ml/min and 1.5%/min increase within the analysis portion of the gradient.

2.5. Fragment collection and sequencing

Multiple consecutive HPLC fractions were collected using a Transgenomic FCW-200 in-line fragment collector [23]. Fragment collection was performed in a two-step procedure. In an initial DHPLC run the time window for the elution of the peaks of interest was determined. During a second injection these peaks were collected during the previously defined time interval. Usually, 80 to 100 μl of eluent containing the peak of interest was collected. Collection volume is determined by the product of DHPLC buffer flow-rate (0.9 ml/min) and collection time. In detail, the fragment collection software was programmed as follows: using the first run as a reference for specific peak collection, the detection type was set to ''whole window modus''. The number of collected fractions then depends on the vial volume and corresponds to the time window in the chromatogram over which the fragments are collected. Collected fractions were analyzed via a ''result chart'' in the WAVEMAKER software. A collection table displays each collected vial relative to the corresponding segment of the peak of interest in the elution diagram. Positive fractions were dried under vacuum (Eppendorf Concentrator 5301; Eppendorf, Hamburg, Germany) until complete removal of the eluent. The latter DNA served without further handling as template for direct sequencing (Abi Prism BigDye terminator cycle sequencing kit) with the target specific primers used for amplification (40 cycles) and analysed on an Abi Prism 310 sequencer.

2.6. Real time PCR

Real time PCR using the LightCycler System (Roche, Germany) was employed to perform quantitative PCR analyses. A germline Dδ3 Taqman probe (5′-6FAM-ATATCCTCACCCTGGGTCCC-ATGCCXT-P; X=TAMRA) was used in combination with patient specific ASO primers and a Dδ3 specific PCR primer (5′-CTGCTTGCTGTGTTTGT-CTCCT). Quantification of residual disease was performed in 20-μl reactions using 500 ng template DNA, 1 U Platinum Taq DNA polymerase (Invitrogen) and 2 μl 10× buffer, 5 m*M* $MgCl_2$, 200 μ*M* of each dNTP, 100 n*M* TaqMan probe, 5 μg bovine serum albumin (BSA), 500 n*M* antisense primer, and 500 n*M* ASO primer and 5% DMSO. Amplification conditions for quantification were as follows: 5 min initial denaturation at 94 °C followed by 50 cycles with 8 s denaturation and 23 s annealing/extension at the appropriate temperature between 60 and 68 °C. Single point fluorescence measurements were per-

formed during the extension step. Serial 10-fold dilutions from 10^{-1} to 10^{-6} of initial leukemic cell DNA in DNA from healthy controls was used for the determination of sensitivity limits and for optimization of the real time quantitative (RQ) PCR reaction conditions.

3. Results

3.1. DHPLC analysis

DHPLC analysis was performed for Vδ2-Dδ3 and Dδ2-Dδ3 rearrangements on patient samples with a known type of TCR-δ rearrangement based on PCR and polyacyrlamide gel electrophoresis (PAGE) analysis. In order to determine the optimal time window for analysis a 20-min ACN gradient from 40 to 72% buffer B, with an increase of 2% buffer B per min was chosen. The positive control elutes under these conditions between 45 and 60% buffer B. Based on these results a 10-min gradient was chosen as a general analysis condition for both targets. Higher column oven temperatures do not lead to a better quality of separation. The length of the gradient was selected within a relative broad time window, which allows the detection of clonal or biclonal samples with different junctional regions. These junctional regions sometimes differ by more than 30 nucleotides (due to deletions and insertions). Characteristic clonal elution profiles with extremely different junctional region sequences are shown in Fig. 2. For characterisation of the specific junctional region, clonal samples were sequenced directly. Biclonal rearrangements elute with three distinct peaks: one sharp heteroduplex peak 1–2 min followed by two homoduplexes (Fig. 2). Biclonal samples lead to negative sequencing reactions due to overlapping junctional regions. In order to circumvent cloning or gel excision of these bands we used a fragment collector based on time-dependent, single-peak collection of DHPLC separated products. Oligoclonal rearrangements (Fig. 3) consist of more than three elution peaks. They were not further analyzed due to their relative instability during therapy and the risk of false negative MRD results [24]. Polyclonal rearrangements elute with a broad smear and are not suitable for further downstream applications (Fig. 3).

3.2. Fragment collection

Biclonal samples were subjected to a second DHPLC run. After a first run, elution profiles were analyzed on the WAVE system and results were evaluated with the WAVEMAKER software. A typical biclonal fragment reveals three peaks. The heteroduplex fraction elutes from the column at a lower acetonitrile concentration than the corresponding homoduplexes. The resulting elution profile serves as a matrix for subsequent fragment collections. The first homoduplex peak of interest was marked with a time window for initialization of the collection software during the next run. In our application, we started fragment collection from the beginning of the first clonal/homoduplex peak to the end of the second clonal/homoduplex peak with an individual fraction volume of 80 μl. The collected fractions were dried under vacuum and sequenced directly after reconstitution. A typical chromatogram and the corresponding sequences from collected fractions are shown in Fig. 4. Whereas sequencing of vials 1 and 2 leads to a clear chromatogram corresponding to clone A overlapping reactions were detected in vials 3 and 4 due to a coelution with peak B. Vial 5 then presents a single sequence corresponding to peak B only.

Table 1 summarizes the results from 10 collected biclonal samples. Sequence variations in the junctional regions are listed and compared to germ line sequences. Finally we tested the received patient specific junctional regions via RQ-PCR for specificity and sensitivity.

3.3. Real time quantitative PCR

For RQ-PCR 10-fold dilution series of patient DNA (time point of diagnosis) in DNA derived from five healthy unrelated donors was used. The described RQ-PCR approach allowed unequivocal amplification and quantification of both alleles for detection of MRD in each patient (Fig. 5). Sensitivities of 10^{-3} to 10^{-5} (one leukemic cell in 1000 or 100 000 normal cells) were achieved in all

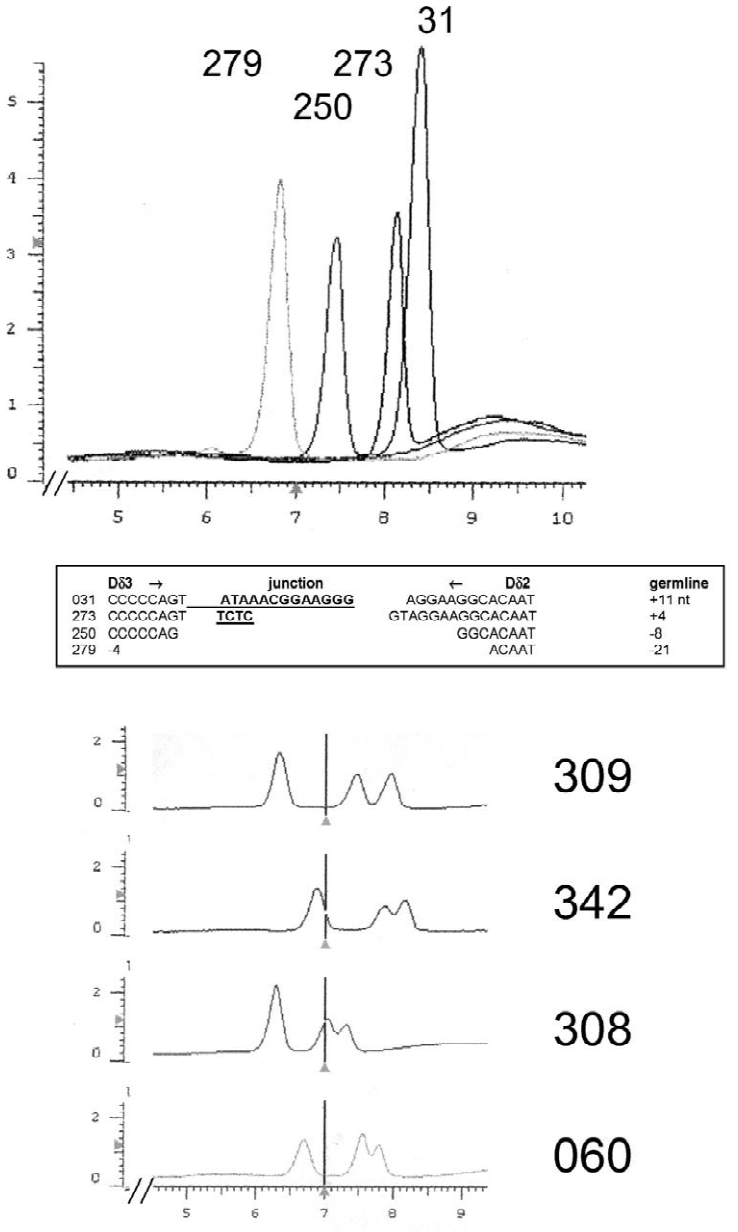


Fig. 2. Identification of clonal (upper panel) or biclonal (lower panel) incomplete TCR-δ rearrangements by DHPLC. An 8–10-μl volume of PCR product was analyzed on a WAVE System equipped with a DNASep Cartridge at an oven temperature of 50 °C. Four different clonal Dδ2-Dδ3 rearrangements were subjected to DHPLC analysis. Length differences result into different elution times for each sample (a). Biclonal samples eluted with a proceeding heteroduplex peak and two homoduplex peaks representing two clonal PCR products. Length variations resulted also into different elution pattern. Numbers in the figure represent individual DNA samples used for PCR.

samples tested. Thus, both targets from a biclonal rearrangement can be used for a quantitative analysis at different time points during therapy. Taken together, we suppose a step by step analysis of clonal incomplete TCR-δ rearrangements in BCP-ALL as described in Fig. 6.

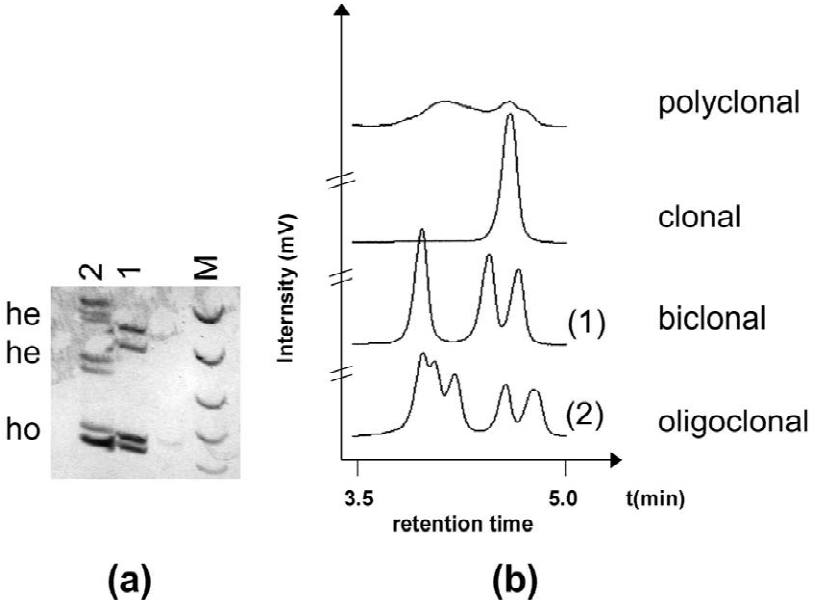


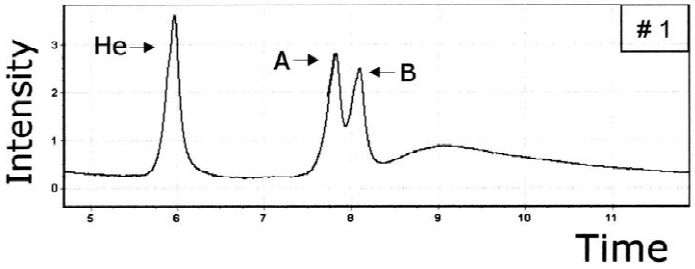
Fig. 3. (a) Typical acrylamide gel separation of two samples (biclonal and oligoclonal) followed by silver staining, (b) characteristic DHPLC elution pattern for clonal, biclonal, oligoclonal and polyclonal samples.

4. Discussion

The purpose of our study was to evaluate the feasibility of DHPLC for the detection and discrimination of several forms of incomplete TCR-δ rearrangements (clonal, biclonal and oligoclonal) in childhood BCP-ALL. Furthermore, we tested the application of a tandem arranged fragment collector for purification of biclonal rearrangements for subsequent sequencing reactions.

Incomplete TCR-δ rearrangements are detectable in about 40–50% of BCP-ALL [1–4,25–27] and display suitable targets for detection of MRD [9]. In a substantial number of cases both alleles were positive for one rearrangement (either Vδ2-Dδ3 or Dδ2-Dδ3) that gives rise to two clonal PCR products within one PCR reaction. These biclonal PCR products were detectable in about 30% of our Vδ2-Dδ3 positive samples (data not shown). Comparable to acrylamide gel electrophoresis and heteroduplex analysis all biclonal samples gave a characteristic elution profile and were unequivocally distinguishable from clonal or oligoclonal samples (Fig. 3). Whereas clonal rearrangements were sequenced directly, overlapping junctional regions within a biclonal product prohibits this quite simple sequencing approach.

We recently described the feasibility of DHPLC for discrimination of polyclonal from clonal/biclonal TCR-γ rearrangements. From these experiments we know that a detection limit for this screening of 12.5% (leukemic cells in unrelated cells) is sufficient for identification of clonal rearranged TCR genes [12]. We applied and extended this methodology to the identification of incomplete TCR-δ rearrangements. Vδ2-Dδ3/Dδ2-Dδ3 rearrangements are the most common rearrangements (except IgH) in BCP-ALL [26,28] and therefore widely used for clone specific detection of minimal residual disease. These clone- and patient-specific sequences allows a highly specific and sensitive detection of leukemic cells during therapy. Direct sequencing can identify single rearranged fragments [5]. PCR fragments with double rearranged alleles render sequencing inconclusive just at the beginning of the region of interest, i.e., the junctional region. Compared to the heteroduplex analysis of several other investigators [10,11] DHPLC has an automated setup with no gel preparation or post PCR handling, as we performed no heteroduplex induction in our samples. Additionally,



Vial #	Start [min]	End [min]	Volume [µl]	Sequencing
1	7.65	7.74	79	A
2	7.74	7.83	77	A
3	7.84	7.92	75	Overlapping
4	7.92	8.01	75	-
5	8.01	8.10	77	B
6	8.10	8.19	75	B
7	8.19	8.28	75	B
8	8.28	8.35	60	-

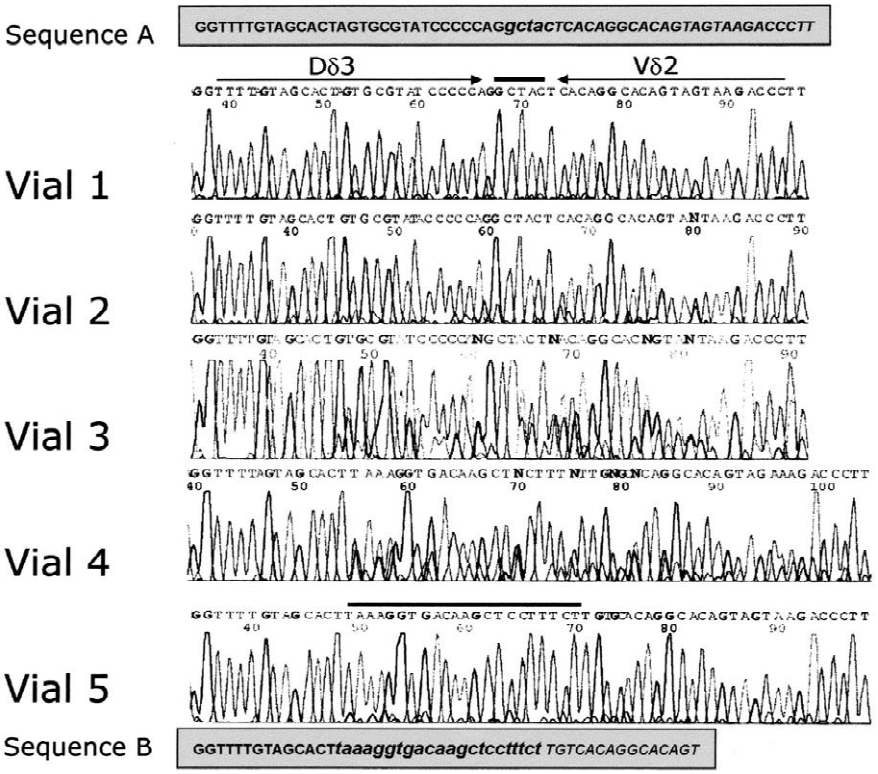


Fig. 4. Typical elution pattern of a biclonal TCR-δ Vδ2-Dδ3 rearrangement. (1) A heteroduplex peak (He) elutes prior to the two homoduplex peaks, A and B, in the case of a biclonal sample. The latter are resolved according to the lengths of the respective PCR fragments. During the second/collection run eight individual fractions were collected in the time window from 7.65 (7.32)–8.35 (8.19) min. Up to 80 µl eluent was collected per vial. Five fractions were sequenced. Sequencing results are assigned to specific peaks observed in the DHPLC chromatogram. Individual sequencing results for peaks A and B are shown including the patient specific junctional region (marked line). The two collected fractions from 7.84 to 8.04 min included both sequences due to overlapping junctional regions.

Table 1
Examples of direct sequencing results for biclonal samples after DHPLC analysis and fragment collection

DNA-No.	Dδ3 deletions	''N''-insertion	Vδ2 deletions	Length to germ line	RQ-PCR sensitivity 10 (exp)
1	−1	GCTAC	−4	0	n.d.
	−16	TAAAGGTGACAAGCTCCTTTCT	−2	+4	
24	−3	GGGGG	−8	−6	n.d.
	−1	GACCC	−5	−1	
218	−3	GAG	−13	−13	n.d.
	−3	TCGGTGTCA	−6	0	
220	−23	ACAGGTCGACCCCCCGC	−3	−9	n.d.
	−1	CCTGGAGTC	−7	1	
241	−8	TCCCCTGACAC	−5	−2	−4
	0	TTCCGCCCGGGGG	−5	−8	−5
273	−4	TAAGGAGGC	−8	−3	n.d.
	−2	CCG	0	1	
371	−16	AGGA	−4	−16	−4
	0	ACGATTGGGCC	0	11	−4
430	−4	AAGGT	−14	−13	n.d.
	0	GAGA	−3	−1	
481	−5	TCTCGGG	−12	−10	−3
	−1	ATA	−3	−1	−4
526	−5	TCGCGGA	−3	−1	−4
	0	CCC	0	+3	−4

All samples were sequenced with a Dδ3 specific primer. Sensitivity was tested via a dilution series on the LightCycler system. 10^{-4} means a detection sensitivity of one leukemic cell in 10 000 normal cells. N.D.—Not done.

the short and reproducible DHPLC running conditions allow the analysis of 96 samples overnight.

For a detailed molecular analysis of biclonal products, i.e., sequencing, we performed two DHPLC runs for each biclonal PCR product. The first run serves as a positioning step for the second collection run. Five to 10 fractions were collected on a microtiter plate, two to three best matching fractions from each peak were prepared for direct sequencing. This procedure allows for a complete analysis of biclonal samples without further PCR or cloning steps. In a recent paper, Emmerson and colleques described the DHPLC based fragment collection of low-level mosaicisms [29]. They collected the heteroduplices that resulted from two different (mutated) alleles. In contrast, we are only interested in the two clonal products in order to generate patient specific (i.e., allele/clone specific) oligonucleotides for the detection of MRD.

A detailed molecular analysis of incomplete TCR-δ PCR products seems to be absolutely necessary for these rearrangements [30,31]. In contrast to Southern blot analysis, PCR also amplifies minor clonal rearrangements. The resulting small sub-clones are relatively frequent in BCP-ALL [31,32]. Steenbergen et al. [32] point out that in BCP-ALL this may be a common feature. Sczcepanski et al. [24] clarified this in their comparative study of T- and B-cell receptor rearrangements between initial and relapse diagnosis. Because of the relative instability of oligoclonal targets, incomplete TCR-δ rearrangements should only be used if they are monoclonal or biclonal. Especially in oligoclonal samples the response to chemotherapy may vary within different (sub)-clones leading to false negative measurement of minimal residual disease during therapy [33].

In order to circumvent or to minimize these problems we established a novel analytical and preparative procedure for identification and characterization of biclonal rearrangements prior to MRD

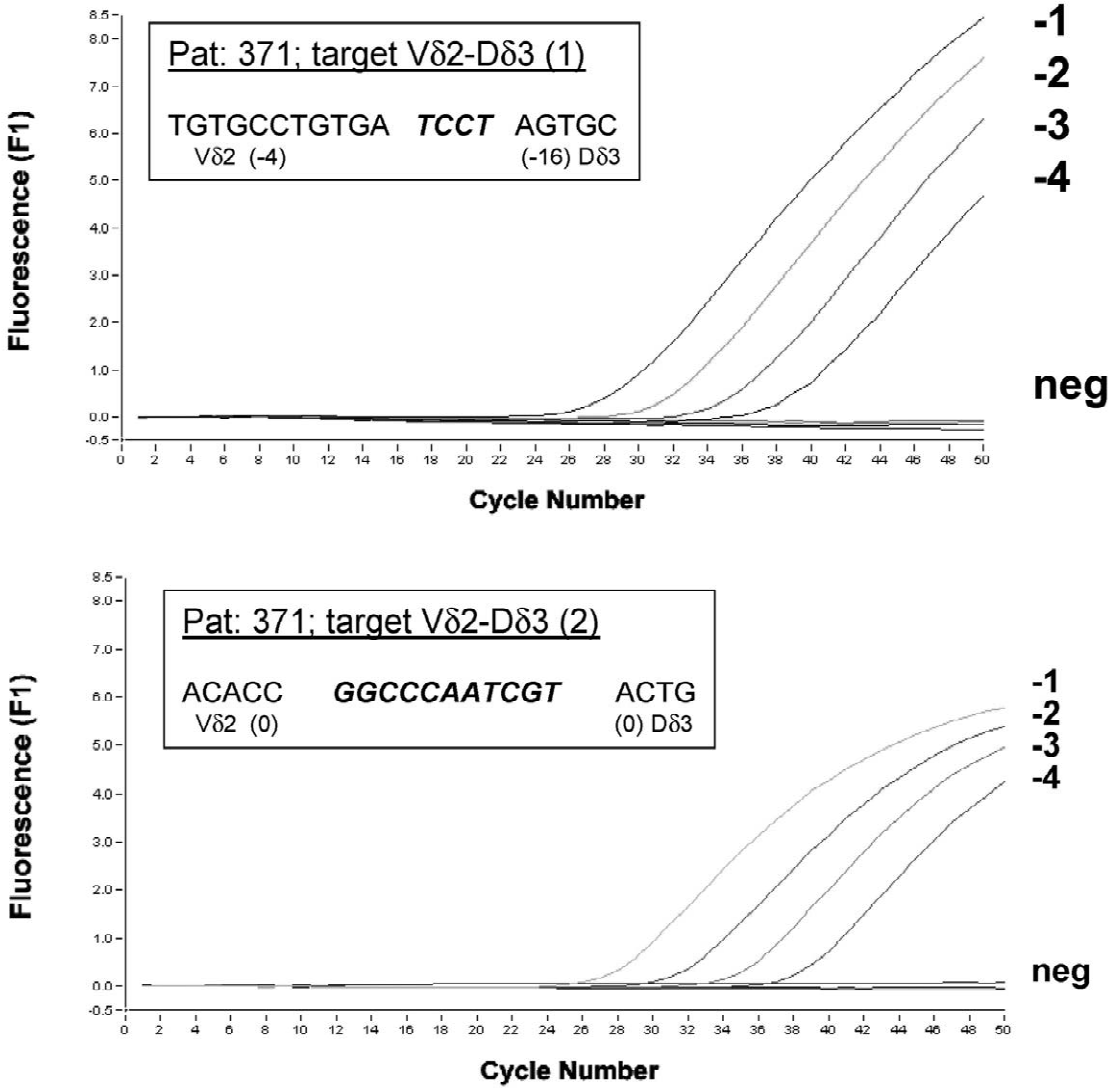


Fig. 5. RQ-PCR on the LightCycler system. PCR was performed with a germ line Dδ3 PCR primer, a germ line Dδ3 TaqMan probe and a unique ASO patient specific primer. DNA from time point of diagnosis was 10-fold serial diluted in DNA from unrelated samples. A 500-ng amount of DNA was used in a 20 μl PCR setup. Patient number and corresponding junctional regions contained in the ASO primers (see Table 1) are indicated. Diagrams show the fluorescence intensity vs. cycle number. Abbreviations: -1, 10^{-1}; -2, 10^{-2}; -3, 10^{-3}; -4, 10^{-4}.

analysis. To test the specificity of the received sequences, RQ-PCR was performed with allele specific oligonucleotides (ASO primer) corresponding to each allele. This allows one to use both allelic variants for the exact examination of minimal residual disease during therapy.

We suppose that after DHPLC screening monoclonal samples should be sequenced directly, biclonal samples should be run twice for fragment collection and oligoclonal samples should be disregarded due to their instability. The above described protocol allows the exact definition of the clonality status as well as the direct sequencing of biclonal samples utilizing only one PCR per sample.

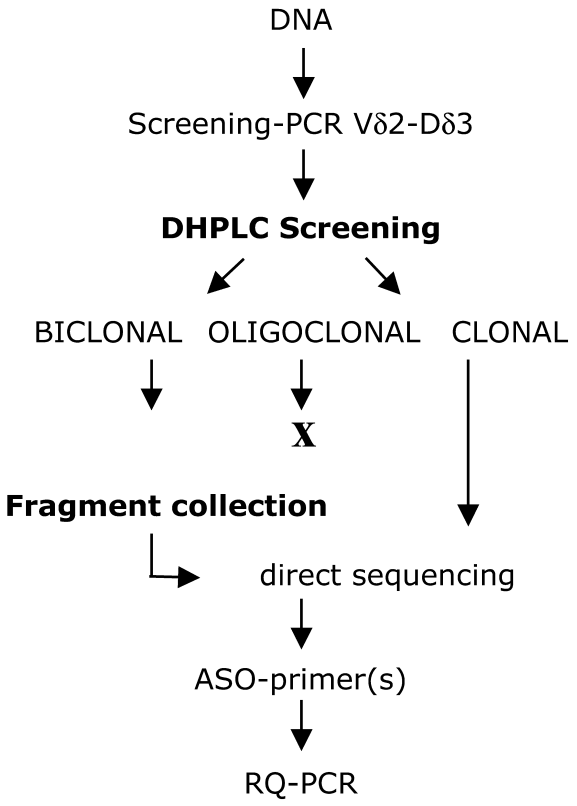


Fig. 6. Flow chart for the detection, identification, and characterization of incomplete TCR-δ targets by means of DHPLC and RQ-PCR.

Our results show that DHPLC analysis for clonality assessment is a rapid and reliable tool for identification of MRD targets in newly diagnosed leukemias under standardized conditions, that fragment collection simplifies the identification of single alleles within biclonal rearrangements and that subsequent real time PCR on the LightCycler enables us now to measure MRD quantitatively. The described strategy of DHPLC followed by RQ-PCR will standardize the molecular detection of MRD in childhood BCP-ALL.

References

[1] A. Biondi, P. Francia di Celle, V. Rossi, G. Casorati, G. Matullo, G. Giudici, R. Foa, N. Migone, Blood 75 (1990) 1834.
[2] T.M. Breit, I.L. Wolvers-Tettero, A. Beishuizen, M.A. Verhoeven, E.R. van Wering, J.J. van Dongen, Blood 82 (1993) 3063.
[3] T. Yano, A. Pullman, R. Andrade, M. Uppenkamp, J.P. de Villartay, G. Reaman, S. Crush-Stanton, D.I. Cohen, M. Raffeld, J. Cossman, Br. J. Haematol. 79 (1991) 44.
[4] S. Yokota, T.E. Hansen-Hagge, C.R. Bartram, Blood 77 (1991) 141.
[5] K. Langlands, O.B. Eden, P. Micallef-Eynaud, A.C. Parker, R.S. Anthony, Br. J. Haematol. 84 (1993) 648.
[6] H. Cave, C. Guidal, P. Rohrlich, M.H. Delfau, A. Broyart, B. Lescoeur, C. Rahimy, O. Fenneteau, N. Monplaisir, L. d'Auriol et al., Blood 83 (1994) 1892.
[7] H. Cave, J. van der Werff ten Bosch, S. Suciu, C. Guidal, C. Waterkeyn, J. Otten, M. Bakkus, K. Thielemans, B. Grandchamp, E. Vilmer, New Engl. J. Med. 339 (1998) 591.
[8] J.J. van Dongen, T. Seriu, E.R. Panzer-Grumayer, A. Biondi, M.J. Pongers-Willemse, L. Corral, F. Stolz, M. Schrappe, G. Masera, W.A. Kamps, H. Gadner, E.R. van Wering, W.D. Ludwig, G. Basso, M.A. de Bruijn, G. Cazzaniga, K. Hettinger, A. van der Does-van den Berg, W.C. Hop, H. Riehm, C.R. Bartram, Lancet 352 (1998) 1731.
[9] T. Seriu, D. Erz, Y. Stark, C.R. Bartram, Leukemia 11 (1997) 759.
[10] M. Bottaro, E. Berti, A. Biondi, N. Migone, L. Crosti, Blood 83 (1994) 3271.
[11] A.W. Langerak, T. Szczepanski, M. van der Burg, I.L. Wolvers-Tettero, J.J. van Dongen, Leukemia 11 (1997) 2192.
[12] U. zur Stadt, J. Rischewski, R. Schneppenheim, H. Kabisch, Clin. Chem. 47 (2001) 2003.
[13] W. Xiao, P.J. Oefner, Hum. Mutat. 17 (2001) 439.
[14] P.J. Oefner, C.G. Huber, J. Chromatogr. B 782 (2002) 27.
[15] P.A. Doris, P.J. Oefner, B.S. Chilton, A. Hayward-Lester, J. Chromatogr. A 806 (1998) 47.
[16] A. Hayward-Lester, P.J. Oefner, S. Sabatini, P.A. Doris, Genome Res. 5 (1995) 494.
[17] C. Eckert, O. Landt, T. Taube, K. Seeger, B. Beyermann, J. Proba, G. Henze, Leukemia 14 (2000) 316.
[18] M. Nakao, J.W. Janssen, T. Flohr, C.R. Bartram, Cancer Res. 60 (2000) 3281.
[19] G.E. Janka-Schaub, D.O. Harms, M.L. den Boer, A.J. Veerman, R. Pieters, Klin. Padiatr. 211 (1999) 233.
[20] U. zur Stadt, D.O. Harms, S. Schluter, M. Schrappe, U. Goebel, H. Spaar, G. Janka, H. Kabisch, Leukemia 15 (2001) 283.
[21] M.J. Pongers-Willemse, T. Seriu, F. Stolz, E. d'Aniello, P. Gameiro, P. Pisa, M. Gonzalez, C.R. Bartram, E.R. Panzer-Grumayer, A. Biondi, J.F. San Miguel, J.J. van Dongen, Leukemia 13 (1999) 110.
[22] B. Budowle, R. Chakraborty, A.M. Giusti, A.J. Eisenberg, R.C. Allen, Am. J. Hum. Genet. 48 (1991) 137.
[23] A. Kuklin, K. Munson, P. Taylor, D. Gjerde, Mol. Biotechnol. 11 (1999) 257.
[24] T. Szczepanski, M.J. Willemse, B. Brinkhof, E.R. van Wering, M. van der Burg, J.J. van Dongen, Blood 99 (2002) 2315.

[25] K. Hettinger, S. Fischer, S. Panzer, E.R. Panzer-Grumayer, Br. J. Haematol. 102 (1998) 1050.
[26] K. Seeger, T. Taube, C. Eckert, C. Hanel, M. Pogodda, G. Henze, Br. J. Haematol. 113 (2001) 318.
[27] C.A. Felix, D.G. Poplack, G.H. Reaman, S.M. Steinberg, D.E. Cole, B.J. Taylor, C.G. Begley, I.R. Kirsch, J. Clin. Oncol. 8 (1990) 431.
[28] T. Szczepanski, A. Beishuizen, M.J. Pongers-Willemse, K. Hahlen, E.R. Van Wering, A.J. Wijkhuijs, G.J. Tibbe, M.A. De Bruijn, J.J. Van Dongen, Leukemia 13 (1999) 196.
[29] P. Emmerson, J. Maynard, S. Jones, R. Butler, J.R. Sampson, J.P. Cheadle, Hum. Mutat. 21 (2003) 112.
[30] C.G. Steward, N.J. Goulden, F. Katz, D. Baines, P.G. Martin, K. Langlands, M.N. Potter, J.M. Chessells, A. Oakhill, Blood 83 (1994) 1355.
[31] L. Lo Nigro, G. Cazzaniga, A. Di Cataldo, A. Pannunzio, E. D'Aniello, G. Masera, G. Schiliro, A. Biondi, Leukemia 13 (1999) 190.
[32] E.J. Steenbergen, O.J. Verhagen, E.F. van Leeuwen, H. van den Berg, A.E. von dem Borne, C.E. van der Schoot, Blood 86 (1995) 692.
[33] V. de Haas, W.B. Breunis, R. Dee, O.J. Verhagen, W. Kroes, E.R. van Wering, J.J. van Dongen, H. van den Berg, C.E. van der Schoot, Br. J. Haematol. 116 (2002) 87.