# Production of Patient Specific Probes for the Detection of Minimal Residual Disease in Acute Lymphoblastic Leukemia

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**Abstrak**

*Dua puluh dua penderita ALL (acute lymphoblastic leukemia) pada anak-anak, setelah didiagnosis lalu dianalisis konfigurasi Ig (immunoglobulin) dan TcR-δ (T-cell receptor-delta) gennya dengan metode Southern blot. Pendeteksian gene rearrangements ini menunjukkan apakah junctional region konfigurasi IgH (immunoglobulin heavy chain) atau TcR-δ akan dianalisis dengan PCR (polymerase chain reaction). Urutan nukleotida Junctional regions dari rearranged IgH (immunoglobulin heavy chain) dan TcR-δ genes ditentukan dengan direct sequencing hasil produk PCR daerah ini. Berdasarkan data sekuens ini, maka pelacak yang spesifik terhadap pasien tersebut (patient-specific oligonucleotide probes) akan didesain. Selanjutnya, sumsum tulang dan darah tepi yang diambil selama dan setelah pengobatan, akan dianalisis dengan teknik PCR menggunakan pelacak yang spesifik terhadap pasien tersebut untuk mendeteksi sel leukemik. Lima pelacak yang spesifik telah didesain dan diuji spesifisitas dan sensitivitasnya.*

**Abstract**

*Twenty-two patients with childhood ALL were analyzed for the configuration of their Ig (immunoglobulin) and TcR-δ (T cell receptor delta) genes by Southern blot analysis. The detection of gene rearrangements indicated whether IgH (immunoglobulin heavy chain) or TcR-δ junctional regions should be analyzed by PCR (Polymerase chain reaction). The nucleotide sequence of junctional regions of the rearranged IgH and TcR-δ genes are determined by direct sequencing of PCR products of these regions. Based on the sequence data, patient-specific oligonucleotide probes were designed. Subsequently, bone marrow and / or peripheral blood samples taken during and after treatment, will be analyzed with the PCR technique, using patient - specific oligonucleotide probes for the detection of leukemic cells. Five patient specific probes have been designed and tested in specificity and sensitivity.*

***Keywords :*** *ALL (Acute Lymphoblastic Leukemia), MRD (Minimal Residual Disease), IgH (Immunoglobulin Heavy Chain) gene, TcR-δ (T-cell receptor-delta) gene.*

## INTRODUCTION

Approximately 20-30 % of children with ALL showed a relapses, in spite of major improvements in the treatment achieved during the last two decades. Apparently, the current treatment is not adequate to kill all the leukemic cells, although the vast majority appear to reach complete remission according to the current morphological detection technique. The detection limit of this technique is about 1-5% (1-5 leukemic cells in 100 normal cells). It is obvious that this technique only provides superficial information on the result of leukemia treatment.[1,2,3]

Therefore, more sensitive techniques are required for the detection of lower numbers of leukemic cells to determine whether the tumor load can be decreased during treatment.

The terminology of MRD (minimal residual disease) means that the leukemic cells are present in the peripheral blood or bone marrow at a level below the detection limit of conventional technique.[4]

Now by using recombinant DNA technology it is possible to recognize MRD in ALL by analyzing the rearrangements of the Ig and TcR genes, as illustrated in Figure 1 and Figure 2.

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The IgH genes are rearranged in 98% of cases of B-cell ALL, and 45-50% rearranged Ig light chain (IgL) genes.[5,6,7,8] The rearrangement of IgH genes are detectable with **a JH probe** (see Appendix 1) after digestion with the restriction enzymes of *Bgl* II, *Bam* HI, and *Hind* III. The rearrangements of TcR-δ genes occur in more than 90% of T-ALL, 65%-70% of precursor B-ALL which is detectable by **Jδ1 probe** (see Appendix 1).

The general aim of the project was detection of MRD in childhood of ALL, by studying the junctional region of IgH and TcR-δ gene rearrangements by the PCR. Therefore, first of all the configuration of their IgH and TcR-δ genes were analysed by use of Southern blot. Subsequently, the sequence of junctional regions of rearranged IgH and TcR-δ genes will be determined by direct sequencing of PCR products of these regions. From the sequence data can produce patient specific probes. Finally, this information will be used to detect leukemic cells after treatment and evaluate whether the treatment of ALL can be improved.

## MATERIALS AND METHODS

### *Cases*

The biological specimens that was studied consisted of peripheral blood (PB) and bone marrow (BM) samples obtained from 22 children with immunophenotypic common ALL, B-cell ALL and T- cell ALL. They aged between 2 and 9 years old. These children were treated with different multidrug regimens.

### *Isolation of lymphocytes*

DNA was extracted from fresh cells or frozen cells which were isolated from PB or BM according to the method described by Maniatis et al.[9]

### *Southern Blot*

Ten ug of this DNA was digested with the restriction enzymes (Pharmacia, Uppsala, Sweden), electrophoresed in 0.8% agarose gel, and transferred onto a Hybond $N^+$ membrane (Amersham, Tokyo, Japan). Then hybridization of Southern blots consisted of prehybridization step, probe labelling, hybridization with $^{32}$P-labelled probe and post-hybridization washes.[9] The availability of detailed information about the sizes of germline and rearrangement restriction fragments in Kb is a prerequisite for an accurate result (see Appendix 2).

### *Polymerase chain reaction*

PCR was carried out as described by Saiki et al[10] using the denaturation step at 94°C for 30 seconds, an annealing step at 55°C for 30 seconds, and an extension at 72°C for 1 minute 20 seconds. This was done for 40 cycles (for IgH). For Vδ2-Dδ3 rearrangements, the samples were subjected to the following cycles : the denaturation step at 95°C for 5 minutes, the annealing step at 66°C for 2 minutes, and the extension at 72°C for 40 seconds. This was done for 40 cycles in Thermocycler (Bio-Med, B.Braun, F.R.G). As negative control sample, a reaction was performed with $H_2O$ instead of template DNA. Oligomers used in the PCR is described in Appendix 3.

### *Direct sequencing*

Direct sequencing step consisted of purification of amplified DNA (Promega Corporation, USA), preparation of Dynabeads M-280 streptavidin (Dynal$^{(R)}$, Norway Co As), separation of DNA strands, annealing mixture, labeling reaction and termination reaction. The biotinylated primer was used for direct sequencing.

### *Dot Blot Hybridization*

The PCR product was serially diluted $10^0$-$10^4$ times starting from 5 µl (1:10 of the total volume of PCR product), then denaturated by adding 4 µl of 5N NaOH + 1 µl of 0.5 M EDTA (pH 8.2) and incubated at 95°C for 10 minutes. The samples were then chilled on ice and 50 µl of 2N $NH_4$ acetate pH 7.O was added. Subsequently, the samples were immobilized on a piece of Zeta Probe Membrane (Biorad membranes No: 162-0153), using the Bio-rad dot blot apparatus (Biorad). After application of the DNA on the membrane, the wells were rinsed with 500 µl of 0.4 N NaOH. After removing the membrane, it was washed in 2X SSC for 5 minutes (3 times). Subsequently, the membrane was air dried and stored or hybridized immediately.

## RESULTS

### Configuration of the TcR-δ and IgH genes by Southern blot

The rearrangements of the IgH gene were detected with a JH specific probe. Whereas, the rearrangements of the TcR-δ gene were detected with a Jδ probe. An example of such a Southern blot experiment is shown in Figure 3. Four ALL samples were hybridized with

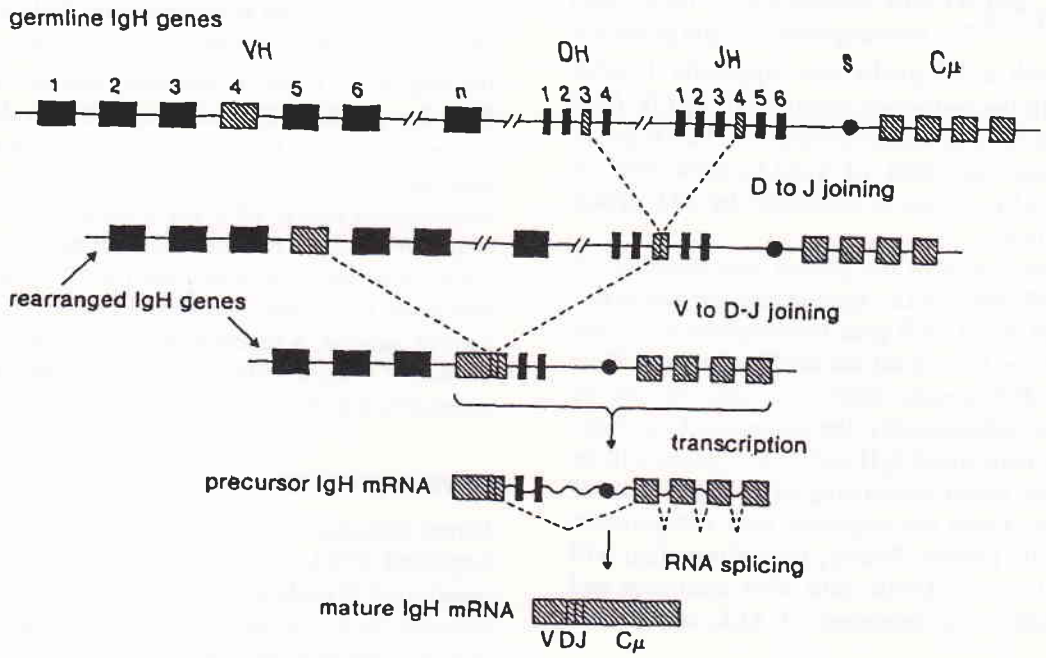


*Figure 1. Rearrangement of immunoglobulin heavy chain gene. First D to J joining occurs, followed by V to D-J joining. The rearranged genes can be transcribed into a precursor IgH mRNA; which becomes a mature IgH mRNA after splicing.*[11,12]

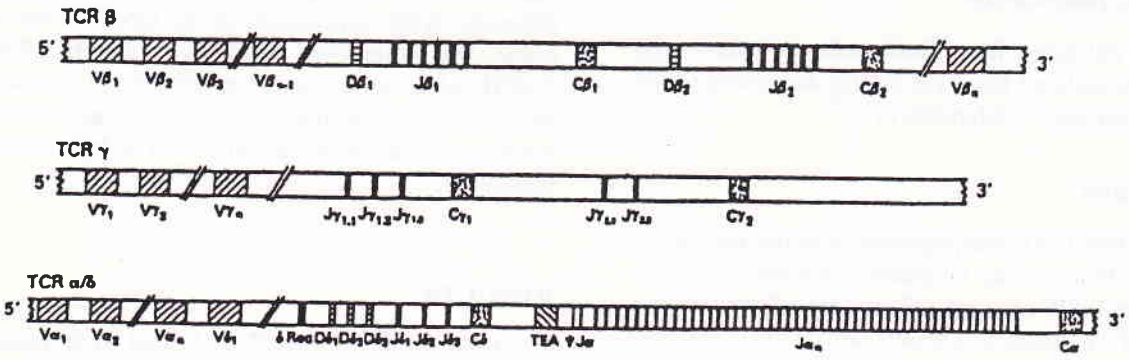


*Figure 2. The structure of the germline TcR genes in human.*[12]

the Jδ1 probe. BM and / or PB DNA of 4 ALL samples was cut with *Eco* RI (Figure 3A) or *BgL* II (Figure 3B). In *BgL* II and *Eco* RI, these samples showed rearrangements of the TcR-δ gene (Vδ2- Dδ3 rearrangement, Dδ2-Dδ3 rearrangement) and germline pattern.

Twenty two ALL patients were analyzed by Southern blotting. The results are shown in Table 1. Analysis of all these data indicated that rearrangement of TcR-δ region (see Table 2) and rearrangement of IgH gene region have occurred (see Table 3).

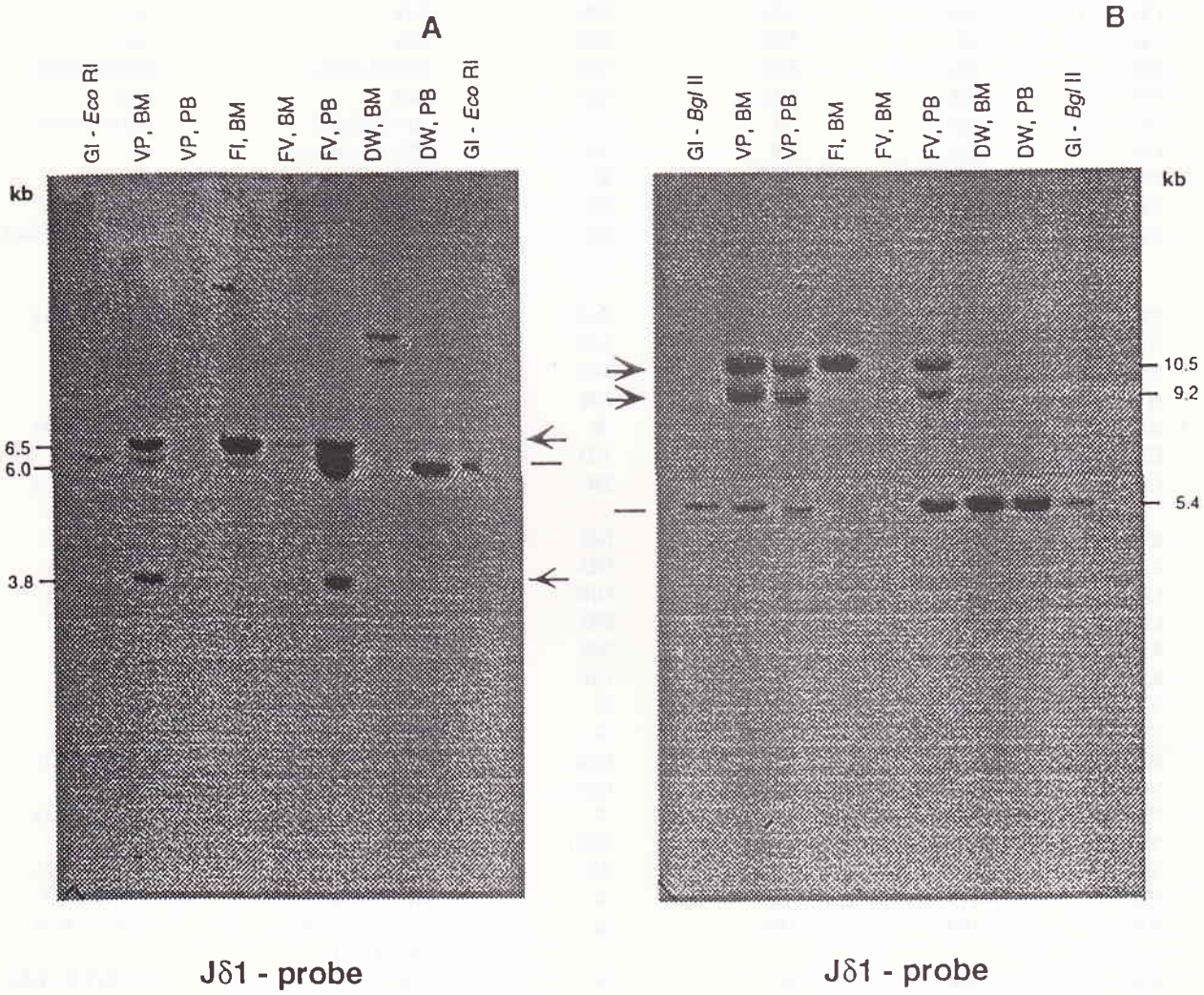


*Figure 3. TcR-δ gene configuration in 4 ALL patients. DNA samples from BM/or PB samples were digested with Eco RI (A) and Bgl II (B). The membrane was hybridized with $^{32}P$ labeled Jδ1 probe. Arrows indicated TcR-δ rearrangements. Solid bar indicated germline. * : unknown TcR-δ rearrangements.*

Table 1. Configuration of IgH and TcR-δ genes rearrangements in childhood ALL by Southern blot analysis.

| Name of patients | Source* | IgH | | TcR-δ | |
|---|---|---|---|---|---|
| | | *Eco* RI | *Bg*/II | *Eco* RI | *Bg*/II |
| BF | BM | R | R | D/R(Vδ2-Dδ3) | D/R(Vδ2-Dδ3) |
| BN | BM | ** | ** | R(Vδ2-Dδ3) | R(Vδ2-Dδ3) |
| BN | PB | NR | G | G | G |
| BC | BM | ** | ** | R(Vδ2-Dδ3) | R(Vδ2-Dδ3) |
| BC | PB | ** | ** | R(Vδ2-Dδ3) | R(Vδ2-Dδ3) |
| CW | BM | NR | NR | ND | ND |
| CW | PB | NR | NR | G | G |
| CK | BM | ND | ND | ND | ND |
| CK | PB | ND | ND | ND | ND |
| DB | BM | ND | ND | R(Dδ2-Dδ3) | R(Dδ2-Dδ3) |
| DB | PB | ND | ND | ND | ND |
| DC | BM | G | G | R(Vδ2-Dδ3) | R(Dδ2-Dδ3) |
| DW | BM | 2R | 2R | 2R* (unknown) | G |
| DW | PB | R | R | G | G |
| FV | BM | 2R | 2R | R(Vδ2-Dδ3) | NR |
| FV | PB | 2R | 2R | G/2R (Vδ2-Dδ3, Dδ2-Dδ3) | G/2R (Vδ2-Dδ3, Dδ2-Dδ3) |
| FI | BM | R | D/R | G/R(Vδ2-Dδ3) | R(Vδ2-Dδ3) |
| GI | BM | ND | ND | ND | ND |
| GL | PB | ND | ND | ND | ND |
| HT | PB | NR | NR | NR | NR |
| HB | BM | R | R | R(Vδ2-Dδ3) | R(Vδ2-Dδ3) |
| HB | PB | ND | ND | ND | ND |
| JA | BM | 2R | 2R | 2R(Vδ2-Dδ3, Dδ2-Dδ3) | 2R(Vδ2-Dδ3, Dδ2-Dδ3) |
| KY | BM | ND | ND | ND | ND |
| KY | PB | ND | ND | ND | ND |
| LN | BM | G | G/R | G | G |
| LN | PB | ND | ND | G | G |
| RM | BM | NR | NR | G | G |
| RM | PB | R | G/R | G | G |
| VB | BM | ND | R | ND | ND |
| VB | PB | R | R | ND | ND |
| VC | BM | NR | G/R | R(Vδ2-Dδ3) | R(Vδ2-Dδ3) |
| VC | PB | G | G/R | NR | NR |
| VU | BM | R | R | R(Vδ2-Dδ3) | R(Vδ2-Dδ3) |
| VU | PB | ND | ND | ND | ND |
| VS | BM | 2R | 2R | R(Vδ2-Dδ3) | R(Vδ2-Dδ3) |
| VS | PB | R | R | R(Vδ2-Dδ3) | R(Vδ2-Dδ3) |
| VP | BM | D/R | R | G/2R(Vδ2-Dδ3, Dδ2-Dδ3) | R(Vδ2-Dδ3) |
| VP | PB | R | R | NR | G/2R(Vδ2-Dδ3, Dδ2-Dδ3) |

Abbreviation:

NR : no result due to technical problem.
R : gene segment in rearrangement configuration of Jδ1.
ND : not determined because not enough sample.
G : gene segmen in germline configuration of Jδ1.
D : deletion of the involved gene (segment).
* Source samples from BM (bone marrow) or PB (peripheral blood).
** Not yet done.

Table 2. TcR-δ gene rearrangements in childhood ALL.**

| T cell receptor delta | *Eco* RI | *Bg*/II |
|---|---|---|
| Rearrangements of TcR-δ | 14/22 (63.63%)* | 14/22 (63.63%)* |
| One allele rearranged | 10/22 | 10/22 |
| Both alleles rearranged | 3/22 | 3/22 |
| One allele deleted / one rearranged | 1/22 | 1/22 |

* Number of cases demonstrating rearrangements / number of cases studied.
** Calculation data were obtained from table 1.

Table 3. Immunoglobin gene rearrangements in childhood ALL.**

| Immunoglobulin Heavy chain | *Eco* RI | *Bgl* II |
|---|---|---|
| Rearrangements of IgH genes | 14/22 (63.63%)* | 15/22 (68.18%)* |
| One allele rearranged | 9/22 | 10/22 |
| Both alleles rearranged | 4/22 | 4/22 |
| One allele deleted / one rearranged | 1/22 | 1/22 |

* Number of cases demonstrating rearrangements / number of cases studied
** Calculation data were obtained from table 1.

## The detection of IgH and TcR-δ rearrangements in childhood ALL by PCR technique

Most of the ALL patients had a Vδ2-Dδ3 rearrangement as judged by Southern blot analysis. Therefore, first of all the Vδ2-Dδ3 junctional region was amplified. The amplification products were separated in a 1.5% agarose gel (Figure 4). The sizes of these junctional region were 400 bp.

The amplification of the IgH junctional region was performed by PCR using the primers FR3-5' and LJH-3'. The sizes of the junctional regions were between 180-200 bp.

Fourteen cases out of the 22 ALL patients (63.64%) indicated amplification of Vδ2-Dδ3 rearrangements by PCR technique. Eight cases did not show Vδ2-Dδ3 rearrangements.

Seventeen cases out of the 22 ALL patients (77.27%) gave amplification products when the IgH junctional regions were amplified. In 4 cases no band was obtained. One case was not yet analyzed.

## The nucleotide sequences of junctional regions of rearranged TcR-δ genes.

The 14 cases of ALL with a Vδ2-Dδ3 rearrangement were chosen for direct sequencing. The biotinylated primer was used for direct sequencing after gel purification. The PCR products were sequenced. The sequence data were compared to published sequences confirming that the PCR products derived from Vδ2-Dδ3 genes rearrangements as shown in table 4. Sequences which are identical to Vδ, Dδ and Jδ1 germline sequences were identified. Other nucleotides in this region were judged a N region. In 7 of the 14 patients unique sequences are obtained. In 5 cases no monoclonal sequence was obtained. Two cases showed unreadable sequences. The sequences of patients KY and VP showed a deletion of Dδ3 gene.

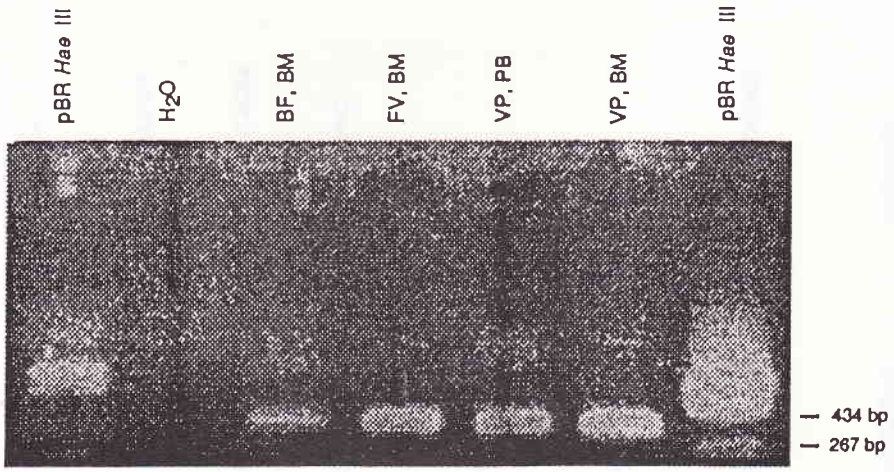


*Figure 4. PCR analysis of the Vδ2-Dδ3 gene rearrangements in 3 ALL patients (lane 3-6). The control is $H_2O$ (Lane 2). As a marker is used pBR Hae III (lane 1 and 7).*

Table 4. Junctional region sequences of TcR-δ rearrangement in ALL.

| | Vδ2 | N1 | Dδ1 | N2 | Dδ2 | N3 | Dδ3 | N4 | Jδ1 |
|---|---|---|---|---|---|---|---|---|---|
| germline | GCCTGTGACACC | | GAAATAGT | | CCTTCCTAC | | ACTGGGGGTACG | | ACACCGATAAAC |
| BN | GTGCCTGTG | CCGCCGG | | | | | CTGGGGGATACG**CACAGTG**CTACAAAACC TACAGAGACCTGTACAAAAA**CTGCAGGGC** **CAAAAGTGCA** * | | |
| KY | GTGCCTGTGA | GGGGGGCCGT | | | | | ..... **CAGT**GCTACAAAACC TACAGAGACCTGTACAAAAA**CTGCAGGGG** **CAAAAGTTGCCATT** * | | |
| VP | GTGCCTGTGACACC | GG | | | | | .....G**CACAGTG**CTACAAAACC TACAGAGACCTGTACAAAAA**CTGCAGGGG** **CAAAAGTTGCCATT** * | | |
| HB | GTGCCTGTGACACC | TGGT | | | | | ACTGGGGATACG**CACAGTG**CTACAAAACC TACAGAGACCTGTACAAAAA**CTGCAGGGG** **CAAAAGTGCA** * | | |
| VS | GTGCCTGTGACACC | GGGACAAC | | | | | ..... ATACGC**CACAGTG**CTACAAAAC CTACAGAGAC**CTGTACAAAAAGTGCA** * | | |
| BC | GTGCCTGTGACACC | GCCTACTTCTT | | | | | ACTGGGGGATACG**CACAGTG**CTACAAAAC CTACAGAGACCTGTACAAAAA | | |
| JA | GTGCCTG | TGACGTCTGA | | | | | .... GGGGGATACGCTACAAAACCTACAGA GACCTGTACAAAAA**CTGCAGGGGCAAAAG** **TGCA** * | | |

Abbreviation :
Underline and bold : heptamer.
Underline and * : Dδ3 primer.
Underline : germline sequences.
Point : deletion of Dδ3 gene.
Bold sequences : patient specific probe.

### Dot blot hybridization

Five patient specific probes were used to hybridize the DNA samples, except 2 patient specific probes (VP and JA). To test the specificity of patient specific oligo's, the oligo's were end labeled with gamma-$^{32}$P-dCTP's and used in a dot blot hybridization experiment.

The oligo's only hybridized to the DNA samples from which they derived (Figure 5). The detection sensitivity was variable. Probes HB, VB and BC only hybridized weakly probably due to the length of the oligo and consequently the low hybridization temperature (32°C). Probes KY and BN gave a stronger signal, both these probes had a Tm (temperature melting) of 42°C.

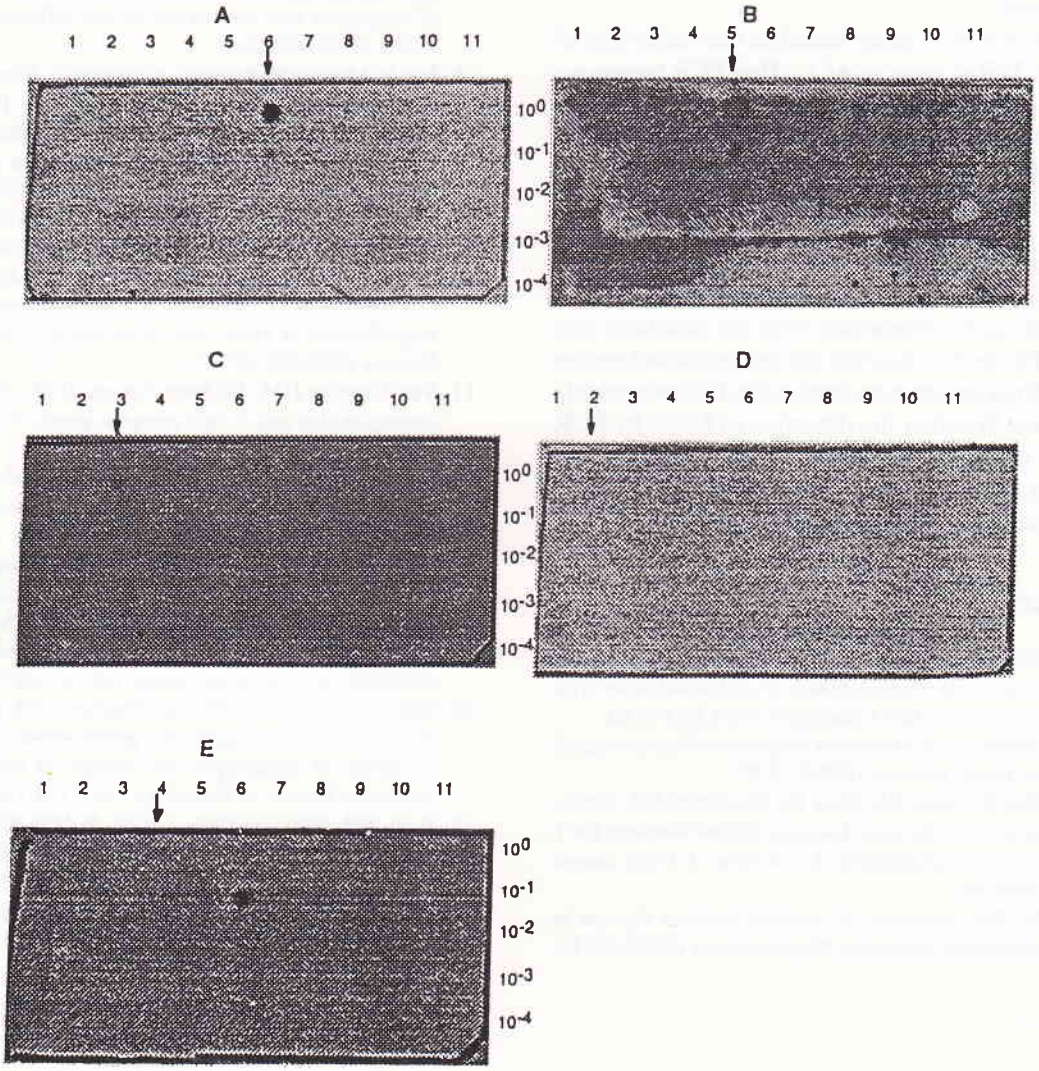


*Figure 5. Each set of ALL DNA samples was amplified by PCR (VB2- DB3), blotted and hybridized with leukemia specific oligonucleotide probe. Arrow indicates the detection of leukemic cells.*

Lane 1 : normal control DNA.
Lane 2 : patient VB.
Lane 3 : patient HB.
Lane 4 : patient BC.
Lane 5 : patient BN.
Lane 6 : patient KY.
Lane 7 : patient VS.
Lane 8 : patient V4
Lane 9 : patient VP
Lane 10 : patient DB
Lane 11 : patient GL

A : the membrane was hybridized to probe KY.
B : the membrane was hybridized to probe BN.
C : the membrane was hybridized to probe HB.
D : the membrane was hybridized to probe VB.
E : the membrane was hybridized to probe BC.

The samples were diluted starting from 5 µl of amplification DNA in ten fold serial dilution.

## DISCUSSION

Most of the ALL patients have TcR Vδ2-Dδ3 rearrangements and IgH rearrangement as detected by Southern blot. Therefore, in most cases it is not necessary to perform Southern blot analysis, but only perform the PCR with the Vδ2-5'/Dδ3-3' primers and the FR3/LJH primers. Only in the case of negativity Southern blot can be useful to judge the TcR and IgH rearrangement.

We set up a PCR assay based on the variability of the IgH and TcR-δ gene in ALL. This PCR technique allows the detection of MRD in ALL. By PCR, the junctional region of TcR-δ rearrangement could be amplified in 14 cases out of 22 (63.64%). Seventeen cases out of 22 (77.27%) gave amplification product of the IgH rearrangement. One of the advantages of PCR are that the technique requires less DNA than the Southern blot technique. It is more sensitive and less time consuming in comparison with the Southern blot technique. The probes specific for the junctional region of the TcR-δ region are now used in the follow up study these patients. Whether the detection of MRD by PCR technology will lead to decrease of the morbidity and mortality in ALL can only be answered after multicentre evaluation of the technique.

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Appendix 1. The probes required for southern blot analysis of TcR-δ gene rearrangements and IgH rearrangement

| | | |
|---|---|---|
| J δ1 probe | Clone | : pJ δS16 |
| | Vector | : pUC |
| | Resistance | : Ampicillin |
| | Insert (total) | : *Sac* I- *Sac* I ~ 1.5 kb |
| | Bacteria | : DH5αF (11,14) |
| JH probe | Clone | : *Pvu* II fragment of the JH gene segment |
| | Vector | : in pBR 322 |
| | Resistance | : Ampicillin |
| | Insert (total) | : *Bam* HI-*Hind* III ' 6 kb |
| | Insert (probe) | : *Sau* 3A I ~ 2.0 kb |
| | Bacteria | : DH1 (15) |

Apeendix 2. Approximate sizes of germline and rearrangement restriction fragments in kb (exon content of restriction fragment TcR-δ gene)

| | *Eco* RI | *Bgl* II | *Hind* III | *Bam* HI | *Kpn* I |
|---|---|---|---|---|---|
| germline Jδ1 | 6.0 | 5.4 | 6.0 | 17.5 | 17.5 |
| $Vδ_1$-$Jδ_1$ | 3.3 | 5.6 | 11.0 | > 30 | 9.4 |
| $Vδ_2$-$Jδ_1$ | 5.5 | 10.2 | 6.1 | 20 | 8.4 |
| $Vδ_2$-$Dδ_3$ | 6.5 | 10.5 | 7.0 | 21 | 9.4 |
| $Dδ_2$-$Dδ_3$ | 3.8 | 9.2 | 5.8 | 11.0 | 6.7 |
| $Dδ_2$-$Jδ_1$ | 2.8 | 8.2 | 4.8 | 10.0 | 5.7 |

Appendix 3. Oligomers used in the PCR and / or direct sequencing analysis of TcR-δ and IgH genes rearrangements

| The name of code | PCR / Sequence | Sequence |
|---|---|---|
| TcR-δ genes | | |
| Vδ1-5' backward | PCR | *Xba* I<br>GAAGATCTAGACTCAAGCCCAGTCATCAGTATCC<br>*Bgl* II |
| Vδ1-3' backward | PCR/Seq | *Sal* I<br>CGCGTCGACGCCTTAACCATTTCAGCCTTAC |
| Vδ2-5' backward | PCR | *Sal* I<br>CGCGTCGACCAAACAGTGCCTGTGTCAATAGG |
| Vδ2-3' backward | Seq | CGCGTCGACCTGGCTGTACTTAAGATACTTGC<br>*Sal* I |
| Dδ3-3' forward | PCR/Seq* | GTAGATCTAGAAATGGCACTTTTGCCCCTGCAG<br>*Bgl* II |
| Jδ1-3' forward | PCR | GAAGATCTAGACCTCTTCCCAGGAGTCCTCC<br>*Bgl* II |
| IgH genes | | |
| FR3-5' backward | PCR/Seq | ATGGAATTCACACGGC(CT)(GC)TGTATTACTGT<br>*Eco* RI |
| LJH-3' forward | PCR/Seq* | CACCTGAGGAGACGGTGACC |

5' and 3' extension of the nucleotide names indicates the location of the oligonucleotide primer within the gene segment. The restriction sites are indicated. Oligonucleotide primers used in PCR or sequence analysis are indicated PCR and sequence, respectively[16].
* : The blotinylated primer was used for direct sequencing.