Rapid Molecular Cloning of Rearrangements of the \textit{IGH} Locus Using Long-Distance Inverse Polymerase Chain Reaction


Clonal rearrangements of the Ig heavy chain (\textit{IGH}) locus consisting of either intrachromosomal (VDJ) rearrangements or interchromosomal translocations are a consistent feature of all B-cell malignancies and may be used both diagnostically and to monitor response to therapy. Many of these rearrangements are targeted to the \textit{IGHJ} segments, but only some can be amplified with regular polymerase chain reaction (PCR) techniques. To permit PCR amplification of potentially all \textit{IGHJ} rearrangements, we have devised a method incorporating self-ligation of restriction endonuclease-digested DNA fragments with long-distance PCR (long-distance, inverse PCR [LDisPCR]). We show here, using only 4 nested oligonucleotide primers, the successful amplification and DNA sequencing of all \textit{IGHJ} rearrangements up to 5.4 kb in length from a panel of 13 cases and cell lines of various types of B-cell malignancy. In all cases, both VDJ and DJ \textit{IGH} rearrangements and translocation breakpoints were amplified. Six cases exhibited t(14;18)(q32;q21). All translocation breakpoints were cloned and sequenced. Three cases exhibited a rearrangement to the \textit{BCL2} major breakpoint region (MBR). However, 2 other cases exhibited rearrangements between the MBR and the minor cluster region (mcr). These 2 cases broke within 44 bp of each other, confirming the presence of an additional 3' \textit{BCL2} breakpoint cluster region. The final case fell immediately 3' of the 3' UTR of the \textit{BCL2} gene adjacent to an Alu repeat. No other \textit{BCL2} breakpoints within this region have been reported. Four cases exhibited t(11;14)(q13;q32). All 3 cases with translocations targeted to the \textit{IGHJ} segments were successfully amplified and sequenced, including 1 case in which the \textit{BCL1} translocation could not be detected by DNA blot using the currently available probes. All three translocation breakpoints fell outside the \textit{BCL1} major translocation cluster between 20 and 40 kb telomeric and showed no clustering. Two of the three fell within or adjacent to Alu repeat regions. LDisPCR is a simple and robust technique that allows PCR amplification of nearly all \textit{IGHJ} rearrangements.

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THE \textit{IGH} LOCUS LIES immediately adjacent to the telomere of the long arm of chromosome 14 and comprises 51 functional \textit{VH} segments (and at least 30 pseudogenes), some 30 to 50 \textit{DH} segments, 6 \textit{JH} segments, and 9 \textit{CH} segments.\(^1\) In normal B-cell development, these gene segments undergo regulated recombination to produce a functional VDJ gene.\(^2\) B cells that fail to undergo this process correctly lack surface Ig and die by programmed cell death within the bone marrow.\(^3,4\) Because VDJ rearrangement occurs very early in B-cell differentiation, all malignancies of the B-cell lineage exhibit clonal rearrangement and/or deletion of the \textit{IGH} locus.\(^5,7\) Clonal \textit{IGH} rearrangements can be detected either by DNA blot using a \textit{JH} probe or by polymerase chain reaction (PCR) using a consensus \textit{JH} primer in combination with primers designed within conserved regions of the \textit{VH} gene.\(^6,11\) PCR-based techniques can be used both diagnostically and to monitor the response to therapy with unprecedented sensitivity.\(^1,13\) However, a potential disadvantage of using consensus \textit{IGH} primers is that only a fraction (about 50% to 70% in most studies) of the rearranged \textit{IGH} alleles are amplified and some 20% of cases of B-cell precursor acute lymphoblastic leukemia (BCP-ALL) remain PCR-negative using these techniques.\(^14-16\) In the context of BCP-ALL, the most common cause of PCR failure is due to \textit{DJ} recombination and, because of the complex nature of the \textit{DHJH} recombinations, only a few \textit{DHJH} recombinations can be amplified by PCR.\(^16\) Prospective identification of cases with biallelic \textit{DHJH} recombination may be biologically important, because this subgroup of BCP-ALL may represent transformation of the earliest B-cell precursor before full activation of the VDJ recombinase.

In the mature B-cell malignancies, and notably in the B-cell non-Hodgkin lymphomas (B-NHL), the most common cause of PCR failure is the occurrence of chromosomal translocations in which segments of other chromosomes become juxtaposed with the \textit{IGH} locus, normally via either the Joining (\textit{J}) or the Switch (\textit{S}) segments.\(^17,18\) Translocations targeted to the \textit{IGH} locus occur in greater than 70% of all B-NHL; moreover, specific translocations are largely associated with specific histological subgroups of disease.\(^19\) In some cases, clustering of the breakpoints within both the incoming oncogene and the \textit{IGH} locus has allowed the development of PCR assays.\(^20,21\) More recently, long-distance PCR has extended the repertoire of PCR-detectable translocation breakpoints.\(^22\) However, in other cases, involving notably the \textit{MYC} and \textit{BCL1} loci, the breakpoints can be dispersed over several hundred kilobases, beyond the present capacity of PCR technology.\(^23,24\) The breakpoints in partner chromosomes involved in \textit{IGH} translocations are rarely within coding regions of putative oncogenes, and therefore the major consequence of the translocation is deregulated expression rather than a fusion protein. Thus, these translocations do not lend themselves to reverse transcriptase mediated methods of cloning such as the rapid amplification of cDNA ends (RACE).

To address these problems, we have sought to develop a PCR-based assay that would allow rapid amplification of all recombination events within the \textit{IGH} locus, at both \textit{J} and \textit{S} segments. Inverse PCR, a method involving the amplification of restriction endonuclease-digested DNA ligated at low con-
centrations to form monomeric circles, represents a simple strategy for amplifying DNA sequences flanked on one side by a region of known sequence. By combining this technique for long-distance PCR and using oligonucleotide primer pairs complementary to regions within IGH, we have developed a method for the rapid molecular cloning of IGH rearrangements that we have termed long-distance inverse PCR (LDI-PCR). We report here the development and preliminary use of LDI-PCR and show the successful amplification of nearly all IGH rearrangements using only 4 nested oligonucleotide primers.

**MATERIALS AND METHODS**

**Cell lines and samples.** Details of the samples and cell lines studied in this report are shown in Table 1. The translocation breakpoints in many of the cell lines have been characterized previously by DNA blot and/or bacteriophage cloning and sequencing. References to the derivation of the cell lines used in this study are either given in the text or are available on request.

**Southern blot analysis.** DNA blot was performed as previously described. Briefly, high molecular weight DNA was prepared by digestion with restriction endonucleases for 3 to 4 hours at 37°C. Restriction digests included Bgl II, HindIII; Pst I; S; Sac I; Xba I; Mc-NHL, mantle-cell lymphoma.

Table 1. List of Cell Lines and Patient Samples, Abbreviated Karyotype, Results of Southern Blot With JH Probe, and Corresponding LDI-PCR Products for Given Enzyme Digest

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Karyotype</th>
<th>Digest</th>
<th>Size of IGHJ Fragments</th>
<th>Size of LDI-PCR Products</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BCL2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Case no. 1</td>
<td>B-CLL</td>
<td>t(14;18)(q32;q21)</td>
<td>H</td>
<td>2.5</td>
</tr>
<tr>
<td>DoHH2</td>
<td>B-NHL</td>
<td>t(8;14)(18)(q24;q32;21)</td>
<td>H</td>
<td>4.7</td>
</tr>
<tr>
<td>Granta 452</td>
<td>Transformed B-NHL</td>
<td>t(9;14)(18)(p13;q32;21)</td>
<td>P</td>
<td>4.7</td>
</tr>
<tr>
<td>Karpas 231</td>
<td>B-ALL</td>
<td>t(14;18)(q32;21)</td>
<td>Bg</td>
<td>3.9</td>
</tr>
<tr>
<td>Karpas 353</td>
<td>B-ALL</td>
<td>t(14;18)(q32;21)</td>
<td>H</td>
<td>3.6</td>
</tr>
<tr>
<td>SU-DUL5</td>
<td>Lymphoblastic lymphoma</td>
<td>t(8;18)(14)(q24;21;q32)</td>
<td>X</td>
<td>3.7</td>
</tr>
<tr>
<td><strong>BCL1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Case no. 2</td>
<td>Mc-NHL</td>
<td>t(11;14)(q13;q32)</td>
<td>H</td>
<td>3.3</td>
</tr>
<tr>
<td>Case no. 3</td>
<td>B-NHL</td>
<td>t(11;14)</td>
<td>P</td>
<td>2.8</td>
</tr>
<tr>
<td>Granta 519</td>
<td>Transformed Mc-NHL</td>
<td>t(11;14)(q13;q32.3)</td>
<td>Bg</td>
<td>3.2</td>
</tr>
<tr>
<td>NCEB-1</td>
<td>Mc-NHL</td>
<td>t(11;14)(q13;q32)</td>
<td>Bg</td>
<td>3.7(G)</td>
</tr>
<tr>
<td><strong>BCL1A</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wien 133</td>
<td>Burkitt’s</td>
<td>t(8;14)(12)(q24;q32;24)</td>
<td>S</td>
<td>2.3(U)</td>
</tr>
<tr>
<td>Others</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Case no. 4</td>
<td>B-NHL</td>
<td>t(1;14)(p22;q32), t(9;14)(p13;q32)</td>
<td>P</td>
<td>4.2</td>
</tr>
<tr>
<td>Case no. 5</td>
<td>B-CLL</td>
<td>t(10;14)(q22;q32), t(14;19)(q32;q32)</td>
<td>Bg</td>
<td>3.4</td>
</tr>
</tbody>
</table>

Abbreviations: G, germline band; g, weak germline band observed in clinical cases due to contaminating normal cells; U, unamplified band; Bg, Bgl II; H, HindIII; P, Pst I; S, Sac I; X, Xba I; Mc-NHL, mantle-cell lymphoma.
Fig 1. Restriction map of germline IGHJ region showing restriction sites used in digests together with site and orientation of oligonucleotide primers. Sequence information used to design the oligonucleotide primers was derived from published sequences. The position of the 5’ end of the oligonucleotide primers is indicated with respect to the 5’ BamHI site. Xho I and Sal I sites within J6I are underlined. B, BamHI; Bg, Bgl II; H, HindIII; P, Pst I; S, Sac I; X, Xba I.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Relative Position</th>
<th>Nucleotide sequence (5’-3’)</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>J6E</td>
<td>3950</td>
<td>CCCACAGGCAGTAGCAGAAAAAACA</td>
<td>External J6 primer</td>
</tr>
<tr>
<td>J6I</td>
<td>3924</td>
<td>TCTGGAGCTCGAGCTCGAGAACAAAAACAGCTAGGG</td>
<td>Internal J6 primer</td>
</tr>
<tr>
<td>JBE</td>
<td>4894</td>
<td>GAAAGCCGTCACCCGGAGGAGT</td>
<td>External primer for Bgl II digests</td>
</tr>
<tr>
<td>JBI</td>
<td>4956</td>
<td>CTTCGTTTGTGAAGGTTGTTTG</td>
<td>Internal primer for Bgl II digests</td>
</tr>
<tr>
<td>JHE</td>
<td>5687</td>
<td>TGGGATCGGTGCTCTCTGCT</td>
<td>External primer for HindIII digests</td>
</tr>
<tr>
<td>JHI</td>
<td>5735</td>
<td>GCCCTTTAATGGGATTTGGAGA</td>
<td>Internal primer for HindIII digests</td>
</tr>
<tr>
<td>JXE</td>
<td>6978</td>
<td>CACCTCGCTCGCCCTTTCTCTAA</td>
<td>External primer for XbaI/PstI digests</td>
</tr>
<tr>
<td>JXI</td>
<td>6140</td>
<td>CCCATGCCTTCAAAGCGATT</td>
<td>Internal primer for XbaI/PstI digests</td>
</tr>
</tbody>
</table>

RESULTS

Details of the cases and cell lines studied are shown in Table 1. These were selected because previous studies had shown that many of the translocation breakpoints would not be amenable to amplification by regular PCR techniques (Dyer et al., 27 Kiem et al., 34 Jadayel et al., 35 and M.J.S.D., unpublished observations).

In this preliminary study, all cases were analyzed by DNA blot, first to determine the sizes and number of the IGHJ rearrangements with various restriction enzymes that cut within the IGHJ-Cμ intron and second to determine the approximate location of breakpoints within the BCL1 and BCL2 loci. However, subsequent studies have shown that this step is not necessary, because LDI-PCR will detect nearly all IGHJ rearrangements within any given sample (T.G.W. and M.J.S.D., unpublished observations).

LDI-PCR was performed on the 13 cases. The restriction enzyme used in each digestion/ligation reaction is shown in Table 1. Twenty-five of the 26 rearranged IGHJ alleles and three germline IGHJ alleles seen on DNA blot were successfully amplified. Moreover, the sizes of the PCR products corresponded precisely to those anticipated from the DNA blot data, indicating that no major artifacts had occurred during LDI-PCR amplification (Fig 2). Subsequent sequencing data were compared with database sequences and those obtained from phage cloning and also showed no major artifacts (see below). Both alleles were amplified in the one PCR reaction even though they were sometimes of widely differing sizes. There was no preferential amplification of the shorter allele to the exclusion of the larger. Large amounts of PCR products of up to 5.4 kb were routinely obtained. Only one rearranged IGHJ allele failed to amplify and this was in the cell line Wien 133, which exhibited a complex, three-way translocation t(8; 14; 12)(q24.1; q32.3; q24.13). In this cell line there was an additional breakpoint between the JH5 and JH6 segments that precluded amplification with the primers used in this series of experiments.

All PCR products including both VDJ and DJ rearrangements and chromosomal translocations were cloned and sequenced.

IGHJ rearrangements. All cases showed clonal IGHJ rearrangements by DNA blot. In 10 cases, these were biallelic. However, the cell line NCEB-1, which exhibited t(11; 14) (q13; q32), showed only one IGHJ rearrangement with the other allele retaining germline configuration in all digests. Additionally, case no. 5, which exhibited t(10; 14)(q22;q32), showed only one IGHJ rearrangement in several digests, indicating that one IGHJ allele had been deleted. Subsequent studies indicated that the translocations in both NCEB-1 and case no. 5 had occurred to downstream switch regions and were therefore not amenable to LDI-PCR amplification using the set of primers described here (data not shown). The Wien 133 cell line exhibited 3 IGHJ rearrangements.

VDJ and/or DJ rearrangements were amplified in all cases. Sequence data for selected cases are shown in Table 2. VDJ
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Fig 2. Results of Southern blot with JH probe and LDI-PCR in cases studied. For each case, LDI-PCR products correspond in size to rearranged bands on Southern blot once unamplified distance between primers is allowed for. (A) Southern blot analysis of 10 µg of genomic DNA. Asterisks refer to germline bands. (B) Five microliters of LDI-PCR product was run on 0.8% agarose stained with ethidium bromide. A 1-kb ladder was used as a molecular weight marker.

rearrangements consisted of germline VH genes as well as VH genes that had undergone somatic mutation. Case no. 4, a B-NHL in leukemic phase that exhibited a t(1;14)(p22;q32) cytogenetically, was found to have a productive VDJ rearrangement on one IGHJ allele and a nonproductive DJ rearrangement on the other. The latter consisted of a D4-23/JH5 rearrangement with germline DH sequences 5’ of this (Table 2). This case, like NCEB-1 and case no. 5, presumably had undergone translocation to one of the downstream IGH switch regions.

Unusually, NCEB1 retained a germline IGHJ allele. This was confirmed by sequencing of the amplified allele.

BCL2 translocations. Most BCL2 translocations in follicular and diffuse NHL cluster to two defined regions of the 3’ region of the BCL2 gene termed the major breakpoint region (MBR) and the minor cluster region (mcr).37 The distribution differs in other B-cell malignancies such as B-cell chronic lymphocytic leukemia (B-CLL), in which rearrangements to the 5’ region are common.27 Six cases with translocation t(14;18)(q32;q21) were studied using the BCL2 probes shown in Fig 3. Cell lines DoHH2, Granta 452, Karpas 231, and Karpas 353 exhibited rearrangement of the BCL2 MBR probe, pFL-1. Case no. 1 was a case of B-CLL with t(14;18)(q32;q21) that showed a 3’ BCL2 breakpoint between the MBR and mcr as detected by probe pMW-1. Cell line SU-DUL5, derived from a case of transformed high-grade NHL, exhibited a complex three-way translocation involving MYC, BCL2, and IGHJ (Kiem et al34 and M.J.S.D., unpublished observations), but also showed rearrangement with this probe. The position of the BCL2 probes relative to one another and the position of the BCL2 breakpoints as determined by LDI-PCR and sequencing are shown in Fig 3A.

All six BCL2 translocation breakpoints were cloned and sequenced. On the translocated allele, there was loss of homology with IGH sequences upstream of JH6. In DoHH2, Granta 452, and Karpas 353, upstream sequences showed identity with the BCL2 MBR, with the BCL2 breakpoints
occurring at nucleotides 3231, 2999, and 3053, respectively, of the cDNA sequence. The sequences juxtaposed to JH6 in case no. 1 and in the cell line SU-DUL5 showed homology with a 108-bp breakpoint sequence previously cloned from a cell line (380) derived from a patient with B-cell ALL with t(14;18) (q32;q21) in which the breakpoint fell between the MBR and the mcr. Further sequencing around the breakpoints showed that all three clustered within a 300-bp region, with two falling within 44 bp of each other (Fig 3B).

In the cell line Karpas 231 (amplified from a BglII digestion), the sequence of 70 bp immediately 5' of JH6 showed no homology to any other sequence on the database but thereafter contained Alu repetitive elements. However, sequence from the other end of the 2.9-kb clone showed identity with BCL2 cDNA from the BglII site at nucleotide 4686 onwards, showing that the breakpoint in this cell line fell some 4.5 kb downstream of the MBR 70 bp 3' of an Alu region.

**BCL1 translocations.** Four cases with t(11;14) (q13;q32) were studied consisting of two cell lines (Granta 519 and NCEB-1) and two fresh cases of mantle-cell lymphoma in leukemic phase (Table 1, cases no. 2 and 3). In 1 of these (case no. 3), the t(11;14)(q13;q32) was not clearly identified cytogenetically. Rearrangements of the BCL1 locus in these 4 cases were sought by DNA blot with a panel of probes spanning the BCL1 locus as previously described (Fig 4A and Jadayel et al28). NCEB-1 showed rearrangement with a BCL1 major translocation cluster (MTC) probe, case no. 2 with probe p94PS, and case no. 3 with probe q13-7. However, no rearrangement of any of the available BCL1 probes was detected in the cell line Granta 519. Fluorescent in situ hybridization (FISH) of this cell line was therefore performed with the BCL1 cosmids I4, R4B, and pHS11; the breakpoint fell between cosmids I4 and pHS11, although none of the probes detected a split signal (data not shown).

**BCL1 translocation breakpoints from cases no. 2 and 3 as well as from the Granta 519 cell line were cloned and sequenced (Fig 4B).** In case no. 2, the translocated allele showed loss of homology with IGHJ immediately upstream of JH5. The breakpoint in this case occurred 170 bp downstream of an Alu repetitive region. Because DNA blot showed comigration of JH and the BCL1 (p94PS) probes, no further analysis was conducted. In case no. 3, sequences beyond JH6 showed homology with Alu repetitive elements. Given that this case showed rearrangement with the BCL1 q13-7 probe, the breakpoint in this could be mapped to approximately 20 kb telomeric of the MTC. In the cell line Granta 519, sequences beyond JH4 showed no homology with any sequence on the databases. Because both DNA blot and FISH failed to localize the breakpoint within the BCL1 region, a 0.5-kb EcoRI-SacI fragment single-copy probe was prepared from the 5.4-kb cloned PCR product and was hybridized with cosmids I4, R4B, and pHS11, which span a 100-kb region 3' of the BCL1 MTC. There was a strongly positive hybridization with pHS11, showing that the breakpoint fell within this cosmid. Analysis of the restriction map for this region and hybridization with DNA di-
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Fig 3. t(14;18) translocations. (A) Restriction map of the 3' region of the BCL2 gene showing position of breakpoints cloned in this study. Note the clustering of breakpoints in the region of the pMW-1 probe and the variant breakpoint in Karpas 231. *Leukemia 380, breakpoint not characterized in this study but cloned previously. (B) Sequence of pMW region and breakpoint sequences in case no. 1, SU-D7UL5, 380. JH sequences are underlined and n regions are in lowercase. JH segment involved in translocation is shown. B, BamHI; E, EcoRI; H, HindIII.

**DISCUSSION**

We have sought to develop a method for the rapid PCR amplification and cloning of all rearrangements and translocations involving the IGHJ locus. Preliminary results reported here for the IGHJ segments indicate that LDI-PCR is a simple and robust technique allowing amplification of all but one IGHJ allele in 13 cases of B-cell malignancy. Amplification of sequences up to 5.4 kb in length was readily observed. Whether longer PCR products can be routinely ob-
Fig 4. BCL1 breakpoints. (A) Partial restriction map of the BCL1 locus illustrating cosmid and probe locations and breakpoints cloned in this study. Breakpoint of Granta 519 marked with its derived probe (p519ES, a 500-bp EcoRI/Sac I fragment). Note that this breakpoint falls within a window not covered by other probes. Map taken from Williams et al and Raynaud et al. (B) Sequences of breakpoints cloned in cases with t(11;14); JH sequences are underlined and JH segment involved in breakpoint is shown. Bold type refers to Alu repeats. B, BamHI; E, EcoRI; H, HindIII; S, Sac I.

Six BCL2 translocations were cloned by LDI-PCR. As anticipated from the DNA blot results, three breakpoints fell within the MBR situated within the 3* untranslated region of the BCL2 gene. However, 2 further cases (case no. 1 and SU-DUL5) were shown to cluster within 44 bp of one another in a region lying between the MBR and the mcr. This region has been shown to contain the BCL2 breakpoint in 2 other cases of CLL with t(14;18)(q32;q21). Also, further sequencing showed that the BCL2 breakpoint in the B-cell ALL cell line 380 fell 240 bp centromeric. Together, these data indicate the presence of a third translocation cluster within the 3* region of the BCL2 gene. Finally, one other 3* breakpoint fell outside of any recognized clusters. To our knowledge, no other BCL2 breakpoints have been mapped.

In contrast to the defined clusters of BCL2 translocations, BCL1 translocations are dispersed over a region of at least 350 kb with only about 25% of cases clustering to the recombinase machinery.

Regarding chromosomal translocations, BCL1, BCL2, and BCL7A breakpoints were readily amplified and sequenced.

Intrachromosomal IGH rearrangements and interchromosomal translocations were cloned and sequenced. Both VDJ and DJ rearrangements were amplified. The latter are difficult or impossible to amplify using conventional PCR methods due to the number and complexity of DH rearrangements. Nevertheless, in the context of BCP-ALL, it may be important to detect cases with biallelic DJ rearrangement because these cases may represent the transformation of the earliest B-cell precursors that have failed to activate the full recombinase machinery.16
cations involving IGHJ were cloned by LDI-PCR. Mapping of these relative to other BCL1 probes showed dispersed breakpoints between 25 and 40 kb telomeric of the BCL1 MTC. Two of the three BCL1 translocations fell in or adjacent to Alu sequences. The breakpoint in the Granata 519 cell line could not be detected by Southern blot using the currently available panel of BCL1 probes and the p519ES probe derived from LDI-PCR may prove useful in defining other breakpoints between probes p210 and pHS11. The BCL1 translocation in the cell line NCEB-1 did not involve the IGHJ segments and it is likely that this translocation was targeted to a centromeric IG switch region, as has been reported in two multiple myeloma cell lines. Further development of the LDI-PCR method is being performed to allow the amplification of these translocations.

LDI-PCR allows the rapid cloning of all IGHJ translocations in B-cell malignancies. Analysis of such breakpoints by bacteriophage cloning continues to allow the isolation of new genes and the recognition of new pathogenic mechanisms. The benefits of LDI-PCR are first the speed and ease with which this cloning can now be performed and second that cloning can be performed when only small amounts of material are available. We have successfully amplified IGHJ rearrangements without prior DNA blot using only 10 ng of DNA. Therefore, this technique will be useful when material is limited.

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