Technical Advance

Development and Validation of a Quantitative Polymerase Chain Reaction Assay to Evaluate Minimal Residual Disease for T-Cell Acute Lymphoblastic Leukemia and Follicular Lymphoma

Gregory A. Hosler,* Robert O. Bash,[†] Xin Bai,*
Vinay Jain,[‡] and Richard H. Scheuermann*

From the Department of Pathology and Laboratory of Molecular Pathology* and the Department of Pediatrics,[†] University of Texas Southwestern Medical Center, and the Baylor-Charles A. Sammons Cancer Center,[‡] Baylor University Medical Center, Dallas, Texas

The presence of occult disease in cancer patients after therapy is one of the major problems faced by oncologists. For example, although 95% of pediatric T-cell acute lymphoblastic leukemia (T-ALL) patients have a complete therapeutic response to multiagent chemotherapy, half will relapse, indicating that they must have harbored low levels of residual cancer cells at the end of therapy. Sensitive detection assays promise to help identify those patients that carry this minimal residual disease (MRD) and are at risk of relapse. We have developed and validated a quantitative polymerase chain reaction (PCR) assay targeting tumor-specific chromosomal rearrangements, including del(1) involving the *tal-1* locus in pediatric T-ALL and t(14;18) involving the *bcl-2* locus in follicular lymphoma. This quantitative PCR assay utilizes a synthetic internal calibration standard (ICS) that contains priming sequences identical to those found flanking the chromosomal rearrangement breakpoints. Using this ICS-PCR method, the limits of detection were 5 tumor cells at ratios of 1 tumor cell in 10⁵ normal cells and a linear range up to 100% tumor cells. This ICS-PCR method has also performed well in terms of precision and accuracy as indicated by low coefficients of variation, minimal random, proportional, and constant errors, and good clinical sensitivity and specificity characteristics. This technique will allow for the evaluation of parameters such as the rate of therapeutic response and the levels of MRD as predictors of patient outcome. (*Am J Pathol* 1999, 154:1023-1035)

Patients that appear tumor-free by standard evaluation methods after therapy are considered to be in clinical complete remission even though malignant cells may remain.^{1,2} Unfortunately, this minimal residual disease (MRD) may expand to life-threatening levels. For example, although the malignant cells in 95% of pediatric T-cell acute lymphoblastic leukemia (T-ALL) patients respond to initial chemotherapy, and the patients are considered in complete remission, 30% to 50% go on to relapse.³⁻⁶ These relapsing patients must have carried undetected MRD while in remission. It is hypothesized that a sensitive assay for the detection of MRD could identify patients at risk of relapse. Current detection assays, including histology and flow cytometry, although appropriate for aiding in diagnosis, often lack the objectivity and low limits of detection that might be necessary for evaluation of MRD.^{3,7-9} Conversely, the polymerase chain reaction (PCR) has been reported to detect as few as 1 tumor cell in a background of 10⁶ normal cells,^{9,10} lowering the limits of detection by several logs in comparison with other diagnostic methods.^{3,11} Thus, PCR may be useful in identifying patients at risk of relapse by providing an objective and sensitive means of detecting and evaluating MRD.

The use of PCR in the detection of tumor cells relies on the presence of a tumor-specific molecular aberration that correlates with a specific disease. These include point mutations, deletions, insertions, and translocations as well as excessive transcription of normal or novel genes.^{9,12} Examples of tumor-specific targets include the *bcr/abl*, *pml/rara*, and *aml/eto* novel fusion genes found in chronic myelogenous leukemia (CML), acute promyelocytic leukemia (APL), and acute myelogenous leukemia

Supported by a grant from Biosource International and grant CA74965 from the National Institutes of Health.

Accepted for publication January 15, 1999.

Address reprint requests to Dr. Richard H. Scheuermann, Department of Pathology, UT Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, Texas 75235-9072. E-mail: scheuerm@utsw.swmed.edu.

A

cgGCCACCAATACGTAACGGCGAacgtgctgcggctctctagcaccacccccgcctcctgac
tggCGAGGTTTCTGACCAGTCAGcaggcgtggcgcgcccttcagttcgcgagcttggtttg
ccgctcaggttcccgcgaccccaacgtcccagagcgggggccggagtcggcgggtggcgctcttgg
agccggctcccgctctaccctgcaaacagacctagctccgcggaagtgcggtgaagtggagctttg
tgtctctgtttcccttgagaggcggcagcgggggccctgggaaggttgggtgtaacattcaaaagc
ctgtagtgggttccgcccctccaggagcctggagcagatgacgagaaggggagctagtgggagaaatt
aagcagtcctgaaatccttgggtatcatctgagcctaaggtatgtaaaagaggttgcagtcgataacg
tgccattaaagtgtttttacgtgggaattcttggaggactgaaacctggaatgctcgccttgcattc
ctcacaattctgctcacactctgctacgtagtaagg...

5' breakpoint

tal-1

sil pr. →

90kb

5' 3'

1a 1b 2 3 4 5/6 exons

3' d2 breakpoint

intron

exon 1b

3' d1 breakpoint

tttggctaagtcacatctctgtgcttaattcttctct
aIGATGATAACCAGAAGTCCCATGcaagtgcata
tgtgttttctgggctgtggaactgagtgacgttccalac
attattatctcaccacaaccttttaaggtcatataagtta
gttctcataacttgtgacgtcgacattatctcccattta
caaatgagaaaaadtaggttcagagatcagtgactaaaat
tcgaacatgtgttcttccactccacatgaaaGAATGA
ACCCAGAGACCCTCggtgg...

....cattttccacggagcgcgcccgcccgctgcgcgcgccctccgcctcgagactcttctct
tcttccccctttctctacgaatatacagaaatgcgcgaggtgtgtgtgtttt cattttctctctgtgtgtgtgtgcatgctgg
GGGATTGTGAGAGTGC GTTCatga
gaactgaggcgccgtgtgctgtctttaa
gtgtgGTCTTCTTGGCGACAGGCT
CTgtgtccgagtggtgtgctgcgcg
gttgggtgtgtag...

[illegible]

(AML), respectively, the t(14;18) translocation involving the *bcl-2* gene in follicular lymphoma, and del(1) involving the *tal-1* gene in pediatric T-ALL.

The 90-kb del(1) deletion is found in transformed cells in 25% to 30% of pediatric T-ALL patients.¹³⁻¹⁶ This deletion brings the *tal-1* gene near the *sil* promoter, thus activating its expression¹⁷ (Figure 1A). Breakpoints at the 5' end of the deletion are clustered in a region spanning ~10 bp. Breakpoints at the 3' end of the deletion are also tightly clustered but generally occur in two regions. Depending on the cluster region used, two types of deletions are generated, *tal-1*^{d1} and *tal-1*^{d2}, occurring in 85% and 10% of del(1) cases, respectively.^{13,16,18} PCR detection of these deletions using rearranged genomic DNA as template is possible due to the presence of these tightly clustered breakpoint regions.

Another example of a tumor-specific target is the t(14;18) translocation seen in 80% to 85% of follicular lymphoma cases^{19,20} (Figure 1B). At the 3' end of the *bcl-2* locus on chromosome 18, the breakpoint usually occurs in one of two regions: the major breakpoint region (MBR) in approximately 60% of cases²⁰⁻²³ and the minor cluster region (mcr) in 25%.^{24,25} On chromosome 14, the breakpoint occurs at the 5' junction of any one of the six *J_H* segments in the immunoglobulin heavy chain locus.^{21,22,26,27} Again, the fact that the breakpoint regions are clustered makes these rearrangements amenable to detection by PCR using genomic DNA as a template.

PCR has now been used in several disease settings to detect MRD and evaluate the prognostic significance of a positive result.²⁸ The results are promising in certain disease settings. For example, detection of *pml/rarα* fusion transcripts in APL patients after therapy has been shown to be a good predictor of relapse.²⁹⁻³³ Likewise, detection of tumor-associated translocations and deletions in T-ALL during induction and maintenance therapy correlated with a poor outcome.^{14,34} Additionally, detection of t(14;18) translocations in the bone marrow of follicular lymphoma patients³⁵ and t(11;14) in the marrow of mantle cell lymphoma patients³⁶ has been shown to be an excellent prognostic indicator of relapse.

Although many groups have studied MRD detection by PCR and its relationship to prognosis in these disease settings, simple PCR has several drawbacks, including the problem of false negative results and the inability to accurately quantify tumor load. Therefore, some groups have begun to use quantitative PCR approaches to quantify tumor burden. Indeed, in recent analyses of ALL,³⁷⁻⁴² AML,^{43,44} APL,³² CML,^{45,46} T-ALL,^{14,47} and follicular lymphoma,^{48,49} quantitative analysis of tumor burden ap-

pears to more clearly define patient prognosis. Many of these preliminary studies, however, have relied on limiting dilution,⁵⁰ nested PCR, and semiquantitative assays, which can be problematic in terms of accuracy, precision, and variability in results between laboratories. Many so-called quantitative assays rely on quantification of the PCR products, but this is not necessarily quantitative PCR. Intensities of bands are affected by a multitude of factors that alter the amplification efficiency of the reaction and have little to do with the amount of starting material. For accurate quantification, an internal standard is needed to correct for differences in amplification efficiency. Some laboratories are beginning to use this concept, but little work has been done to validate such approaches as true quantitative assays.

Here we report the development of a quantitative PCR assay using an internal calibration standard (ICS), LQ1, with priming sites for del(1) found in T-ALL, t(14;18) in follicular lymphoma, t(11;14) in mantle cell lymphoma, and several control genes. This ICS-PCR technique was evaluated for amplification efficiency, detection limits, precision, accuracy, analytical range, sensitivity, and specificity⁵¹ with excellent results. The advantage of this assay over many other quantitative assays is that it is easy to perform and relatively inexpensive. Although we have focused on these diseases, the technique and evaluation approaches described can be applied to any tumor-specific rearrangement or any application in which specific nucleic acids can be targeted.^{52,53}

Materials and Methods

Positive Controls

The positive amplification controls for each rearrangement were as follows: MOLT-16, RPMI8402, HSB-2, and CCRF-CEM cell lines for the *tal-1*^{d1} in pediatric T-ALL; a known positive patient sample and a plasmid clone generated from this sample for *tal-1*^{d2}; SUDHL-6 cell line (provided by Ian Magrath) and a known positive patient sample for t(14;18) MBR in follicular lymphoma; SUDHL-16 cell line (provided by Alan Epstein), a known positive patient sample, and a plasmid clone generated from this sample for the t(14;18) mcr in follicular lymphoma.

DNA Isolation

For cell mixture samples, cells were counted using a hemocytometer, and then dilutions were made by mixing

Figure 1. Tumor-specific genomic rearrangement targets. **A:** The *tal-1* deletions. These 90-kb deletions bring the active *sil* promoter near the *tal-1* gene. The 5' breakpoints of these deletions (bracketed) are tightly clustered within a few base pairs. The 3' breakpoints (bracketed) are also tightly clustered but can occur at different sites. *tal-1*^{d1} accounts for 85% of the breakpoints in this region and *tal-1*^{d2} approximately 10%. All breakpoint regions occur within introns. The sequences in the regions of each breakpoint are indicated.^{18,60,61} Primer sequences used to amplify the respective rearrangements are capitalized and in boldface. Probe sequences used for hybridization are underlined. **B:** The t(14;18) translocation. The rearrangement (double arrow) of the *bcl-2* gene on chromosome 18 (top) with the IgH region on chromosome 14 (bottom) places the *bcl-2* promoter under the influence of the IgH enhancer. V, D, and J refer to segments that normally recombine to form the variable region of the immunoglobulin gene; C_μ marks the constant region. The breakpoint on chromosome 18 in individual tumors with t(14;18) occurs either at the major breakpoint region, MBR, or the minor cluster region, mcr. These regions encompass a length of 150 bp or 500 bp, respectively. The 5' ends of the MBR and mcr regions are indicated in the sequences by single-headed arrows. On chromosome 14, the break occurs at the 5' end of any one of the 6 *J_H* segments. The sequences adjacent to the MBR and mcr breakpoint regions are shown.⁶² Below, the entire sequence of the *J_H* region is included, with each *J_H* segment in italics, and a consensus sequence used as the 3' primer is capitalized and in boldface. Primer and probe sequences are indicated as in A.

known numbers of the cells that carry a desired translocation (eg, CCRF-CEM for *tal-1*^{d1} and SUDHL-6 for t(14;18) MBR) with cells that do not (K562). The cell mixtures were pelleted and resuspended in 200 μ l of PBS. DNA was extracted according to the manufacturer's protocol for the QIAamp Blood Kit from Qiagen (Qiagen, Valencia, CA) with the following important modifications. After application of the cell lysate to the isolation column, the column was washed with 400 μ l of 1X AL buffer (lysing solution). We have found that this improves amplification efficiency for PCR by removing PCR inhibitors carried over during the standard procedures (unpublished). The isolation columns were incubated with 200 μ l of AE elution solution at 70°C for 5 minutes before centrifugation, and then the eluate was re-applied to the column, re-incubated at 70°C, and re-centrifuged to maximize DNA yield.

The pediatric T-ALL patient samples were collected in conjunction with a Pediatric Oncology Group study. The follicular lymphoma samples were collected in conjunction with a Texas Oncology Physicians Association study.

PCR Primers and Hybridization Probes

Primers were designed to be 18 to 22 nucleotides long, 40% to 60% G/C with a G or C anchor in the 3' position. Primers with potential hairpins or 3' complementary regions were avoided. At least 10 primer pairs, either published or newly designed, were evaluated for each target. For each rearrangement, at least two primer sets were chosen outside the breakpoint cluster regions. Probe sequences, 35 to 40 nucleotides long, were also selected for each translocation PCR product. Primer and probe sequences are listed in Table 1.

PCR

PCR was performed under standard conditions. PCR reactions contained 20 pmol of each oligonucleotide primer (Biosource International, Camarillo, CA) in standard *Taq* buffer (50 mmol/L KCl, 10 mmol/L Tris/HCl, pH 8.3, 1.5 mmol/L MgCl₂), with 2.5 U of *Taq* polymerase, 200 μ mol/L dATP, dGTP, and dCTP, 150 μ mol/L dTTP, 100 μ mol/L dUTP (Perkin-Elmer, Branchburg, NJ), and template DNA as indicated. Amplification conditions were optimized for each pair, but generally consisted of a 95°C initial denaturation step for 2 minutes, 95°C for 0.5 minute, 60°C for 0.5 minute, and 72°C for 1 minute for 34 cycles and a 9-minute 72°C final extension. Reactions were run on a Perkin-Elmer 9600 or 2400 thermocycler.

To perform the quantitative assay, each reaction contained both ICS molecules and genomic DNA as templates. The amount of genomic DNA used in each reaction was maximized to provide for the detection of the lowest tumor levels. Typically 10⁵ to 10⁶ cell equivalents (0.7 to 7 μ g) were used. When the reaction was complete, the products were separated by gel electrophoresis and stained for 30 to 60 minutes using SybrGold (Molecular Probes, Eugene, OR), a sensitive DNA intercalating dye. The gel was subsequently scanned with a

FluorImager (Molecular Dynamics, Sunnyvale, CA), and the ratio of tumor target to ICS target was calculated by comparing band pixel values using ImageQuant software (Molecular Dynamics).

In our experience, quantification was best when the ratio of signals from the translocation and ICS products is close to 1:1. To achieve this ratio, the starting test DNA sample was titrated down several logs in separate PCR reactions by serial dilution. To maximize the linear range of the assay, we used the fewest number of ICS molecules per reaction that could be consistently amplified (20 to 100 molecules).

Southern Blot

DNA from the agarose gels was transferred to nylon filters using a vacuum blot apparatus and an alkaline transfer buffer (0.4 mol/L NaOH, 0.6 mol/L NaCl). The blots were air dried for 1 hour and then prehybridized for 1 hour at room temperature in 10 ml of prehybridization buffer (1% SDS, 1.5X SSPE, and 100 mg of nonfat dry milk). Translocation-specific oligonucleotide probes (60 pmol) listed in Table 1 were end-labeled with 1 μ Ci of [γ -³²P]ATP using T4 polynucleotide kinase. The probes were denatured for 10 minutes at 95°C and then incubated with the filter for 4 hours at room temperature in 10 ml of fresh prehybridization buffer. After hybridization, the filter was washed four times for 15 minutes each with decreasing salt and increasing detergent until the final wash contained 1% SDS and 0.5X SSC at room temperature. Finally, the filter was placed in plastic wrap and exposed to a phosphorimager screen overnight. The screen was scanned and analyzed using a phosphorimager (Molecular Dynamics).

Results

The development of the ICS-PCR assay involved several steps. As an initial step, primers were screened and evaluated for specificity, sensitivity, and amplification of tumor-specific DNA in patient samples. This evaluation step is important because even though primer sequences are initially selected based on specific design criteria, the most effective primers can only be identified empirically. Primer pairs with the best performance characteristics were chosen for inclusion in the ICS, which was then assembled to satisfy PCR product size requirements. Once constructed, the ICS was used in a series of quantitative validation experiments to evaluate performance parameters, including accuracy, reproducibility, analytical range, and detection limits.

Primer Selection

The 90-kb deletion at the *tal-1* locus on chromosome 1 is associated with 25% to 30% of pediatric T-ALL patients¹³⁻¹⁶ (Figure 1A). There are two common 3' breakpoint regions: one used in *tal-1*^{d1}, which occurs in approximately 85% of rearrangements involving del(1), and

Table 1. Primer and Probe Sequences

Target and primer set	5' primer	3' primer	Probe
t(14;18) MBR			
A	TATGGTGGTTTGACCTTTAG ³⁵ (1)*	ACCTGAGGAGACGGTGAC (14)	GCCTGTTTCAACACAGACCCACCCAGAGCCCTCCT (IV)
B	CAGCCTTGAAACATTGATGG (8)	ACCTGAGGAGACGGTGAC (14)	
del(1) <i>tal-1</i> ^{d1}			
A	CGAGGTTTCTGACCAGTCAG (11)	AGAGCCTGTCGCCAAGAAGAC (21)	TCTGAGCTAAGGTATGTGAAAGAGGTTGCAGTCG (VI)
B	CTCCTACCTGCAAACAGAC (9)	AGAGCCTGTCGCCAAGAAGAC (21)	
C	CACACCAATACGTAACGGCG (6)	GAACGCACTCTCACAATCCC (20)	
t(14;18) mcr			
A	GATGGCTTTGCTGAGAGGTAT (2)	ACCTGAGGAGACGGTGAC (14)	GCAGCTATTGTTACTCTTCAGGGTCTTTAAGCAG (II)
B	TGCCTGGCATAAGCAAGCG (3)	ACCTGAGGAGACGGTGAC (14)	
del(1) <i>tal-1</i> ^{d2}			
A	CTCCTACCTGCAAACAGAC (9)	CATGGGAGTTCTGTTATCATC (19)	TCTGAGCTAAGGTATGTGAAAGAGGTTGCAGTCG (VI)
B	CGAGGTTTCTGACCAGTCAG (11)	GAGGGTCTCTGGGTTCAATC (22)	
t(11;14) MTC			
A	ATATTGCACTGTCTGGATGC (5)	ACCTGAGGAGACGGTGAC (14)	CAACCTTCACCTACTGAAGGACTTGTGGGTTGCT (III)
B	CATACGGTGTGTAGCCTTTTC (7)	ACCTGAGGAGACGGTGAC (14)	
Human β -globin	GAAGAGCCAAGGACAGGTAC ⁶⁴ (13)	GTCTCCTTAAACCTGTCTT ⁶⁴ (15)	GGGCATAAAAGTAGGGCAGGCCATCTATTGCTT (VII)
Human RNA polymerase II	GCATCAAATACCCAGAGACG (12)	CCACGTGAACACAGGCTTG (18)	TTGGAGCTGGGAGGAAAGCACTGGGTGTGTGCC (VIII)
Murine IL-10	TACTGCTAACCAGCTCCTTAATGC (4)	GGCGTCTCTAGATTTACC (17)	CTTGGGTTGCCAAGCCTTATCGGAAATGATCCAGT (I)
Murine C μ	GATCTGCCTTACCACAGAAG (10)	GACATGATCAGGAGACATTGTAC (16)	CTGAACCTGAGGGAGTCAGCCACAGTCACCTGCTT (IX)
ICS Probe			AGGCGACGTATCGATCCTGACGGTCTCGCAGATCAGTAA (V)

*Numbers after each sequence refer to designations presented in Figure 4 and Table 2.

the other used in *tal-1*^{d2}, which accounts for 10%.¹⁶ The 5' breakpoint region is the same for both the *tal-1*^{d1} and *tal-1*^{d2} rearrangements. These rearrangements can be amplified if primers are designed to flank the 90-kb deletion region. A common 5' primer can be used, but different 3' primers are needed for *tal-1*^{d1} and *tal-1*^{d2}, as the 3' breakpoints are separated by 2 kb.

A t(14;18) translocation exists in tumor cells in over 80% of patients with follicular lymphoma.^{19,20} On chromosome 14 at the immunoglobulin heavy chain locus (Figure 1B), a consensus sequence²⁷ at the 3' end of each of the six J_H regions is used for the 3' primer sequence. For the *bcl-2* chromosome, breaks usually occur in one of two tightly clustered regions, the major breakpoint region (MBR) involved in 60% of t(14;18) and the minor cluster region (mcr) involved in 25%.^{20,25,54} The MBR spans a 150-bp segment, and the mcr spans approximately 500 bp.^{20,25} Different 5' primers must be used to amplify breaks that occur in the MBR and mcr because they are 20 kb apart.²⁴

Primer sequences were initially chosen based on their sequence (see Materials and Methods) and location as described above. A panel of both newly designed and previously published primer pairs was evaluated for specificity and amplification efficiency. To evaluate these parameters, DNA mixtures were made with different concentrations of tumor-specific target diluted into nonspecific genomic DNA. Pairs were eliminated if amplification resulted in a weak signal, multiple nonspecific bands, or primer-dimer formation (data not shown).

Primer Evaluation

Once the best primer pairs were identified, the limits of detection were evaluated to determine whether they would be suitable for the detection of low tumor levels.

The specific target DNA was diluted into nonspecific genomic DNA at different ratios. For the *tal-1*^{d1} primer set (Figure 2A), the limit of detection was 50 targets in 500,000 cells, or a 10⁻⁴ ratio using an ethidium-bromide-

Figure 2. Limits of detection. **A:** Primer set A specific for the 90-kb *tal-1*^{d1} deletion found in T-ALL was evaluated to measure the limits of detection. PCRs were amplified with DNA isolated from mixtures of cell lines as the template. Each column represents a dilution of the tumor-specific DNA (CCRF-CEM) in *tal-1*^{d1}-negative genomic DNA (K562) as template, with PCRs containing either 500,000 (lanes 1, 3, 5, 7, 9, and 11) or 50,000 (lanes 2, 4, 6, 8, 10, and 12) total cell equivalents. The top panel shows an ethidium-bromide-stained gel of the samples; the bottom panel shows a Southern blot of the same samples using probe VI. **B:** A similar analysis was performed using MBR-specific primer set B, probe IV, and SUDHL-6 DNA as a specific target for the t(14;18) translocation.

Figure 3. Target detection in patient samples. **A:** The *tal-1^{d1}*-specific primer set A was evaluated for its ability to amplify DNA isolated from 24 T-ALL bone marrow samples demonstrated to carry *tal-1^{d1}* by Southern blot (lanes 2 to 25). Lane 1 contains a 123-bp DNA ladder size marker, and lane 26 contains a PCR sample run with no DNA template as a negative control. **B:** MBR-specific primer set B was evaluated for its ability to amplify DNA from follicular lymphoma patient samples suspected of carrying the t(14;18) MBR. Diagnostic bone marrow samples from 52 patients diagnosed histologically with follicular lymphoma were evaluated. Lanes 1 and 30 contain a 123-bp DNA ladder size marker, lane 55 contains a reaction with no DNA template, and lane 56 is a positive control sample with DNA from the SUDHL-6 cell line used as the template. Lanes 2 to 29 and 31 to 54 contain reaction products from the 52 patient samples.

stained gel (lane 7). By adding a hybridization step, or using fluorimaging with the more sensitive SybrGold intercalating dye (not shown), this was improved to 5 targets in 500,000 cells, or a 10^{-5} ratio (lane 9). Similar limits of detection were achieved for the amplification of other targets, including t(14;18) MBR (Figure 2B). In contrast to the amplification of *tal-1^{d1}*, amplification of t(14;18) resulted in multiple nonspecific bands in stained gels when the target DNA was a small fraction of the total DNA (eg, lanes 6 to 11). The fact that this was seen with different 5' primers (data not shown) suggests that this phenomenon is due to the use of a consensus *J_H* sequence as the 3' primer. For this reason, hybridization may be necessary in the setting of MRD for follicular lymphoma to achieve low limits of detection.

The primer pairs that exhibited the best specificity and lowest limits of detection were subsequently screened for their ability to detect translocation targets in patient samples believed to carry the given chromosomal rearrangements. For pediatric T-ALL, diagnostic patient samples were first screened for *tal-1^{d1}* by Southern blot (data not shown). Positive samples were then evaluated for detection by PCR using the selected primer pair. Of 24 patients found to be positive for *tal-1^{d1}* by Southern blot, 21 gave DNA bands of the predicted size after PCR amplification (Figure 3A). The identity of these bands was confirmed by Southern blot hybridization using probe VI (see Table 1), internal to the *tal-1^{d1}* primers. One patient (lane 20) gave a DNA product approximately 300 bp larger than predicted, but this also hybridized using the specific

probe, indicating a true positive with a breakpoint either downstream of the 5' cluster region, upstream of the 3' cluster region, or a combination of both. Of the two negative samples, one (lane 9) was found to have very little DNA as determined by A_{260} and using PCR reactions with primers specific for the RNA polymerase II large subunit control gene (data not shown). Thus, only one patient sample with an apparent *tal-1^{d1}* rearrangement determined by Southern blot was not detected by PCR. Additionally, 10 patient samples positive for *tal-1^{d2}* or t(1;14) by Southern blot were screened, and none were positive using the *tal-1^{d1}* primer pair (data not shown). Therefore, when comparing the PCR data with the standard molecular diagnosis of *tal-1^{d1}* in T-ALL, the Southern blot, the analytical sensitivity was 96% and the specificity 100%.

Similar screening procedures were performed for t(14;18) MBR primer pairs on bone marrow biopsy samples taken at the time of diagnosis. All patients had a diagnosis of follicular lymphoma (either follicular small cleaved, follicular mixed, or follicular large cell) by histological examination of biopsies derived from the primary tumor site or bone marrow. Using t(14;18) MBR primers, 27 of 52 gave a band in the predicted size range by fluorescence using the DNA intercalating dye (Figure 3B). As expected, the size of the tumor-specific band varied significantly from patient to patient, unlike those from the *tal-1^{d1}* analysis, reflecting the heterogeneity of the breakpoint sites found in follicular lymphoma as compared with T-ALL. Two additional samples were detected by in-

Table 2. PCR Product Sizes from LQ1 and Chromosomal Rearrangements

Target and primer set	3' primer*	5' primer*	LQ1 product size (bp)	Chromosomal rearrangement product size (bp)	Probe*
t(14;18) MBR					
A	1	14	586	640–780	IV
B	8	14	265	100–250	IV
del(1) <i>tal-1</i> ^{d1}					
A	11	21	469	590–610	VI
B	9	21	560	450–470	VI
C	6	20	734	590–610	VI
t(14;18) mcr					
A	2	14	558	400–900	II
B	3	14	523	150–650	II
del(1) <i>tal-1</i> ^{d2}					
A	9	19	501	440–460	VI
B	11	22	604	730–750	VI
t(11;14) MTC					
A	5	14	471	110–410	III
B	7	14	337	220–520	III
Human β -globin	13	15	275	324	VII
Human RNA Polymerase II	12	18	359	300	VIII
Murine IL-10	4	17	719	636	I
Murine C μ	10	16	410	345	IX
ICS Probe					V

*Primer and probe numbers correspond to sequence positions in the LQ1 standard (Figure 4).

creasing the assay sensitivity with hybridization using an MBR-specific probe (data not shown). Therefore, when comparing the PCR/hybridization data with the histology, the sensitivity was 56% for MBR detection, which is consistent with previously published data.^{20,54} These samples were also screened by an outside reference lab with the following results. Of the 52 patients, 58% (30/52) were determined positive. Two patients were determined positive in the outside lab but were negative in our lab (lanes 17 and 33), and one patient was positive in our lab (lane 6) but negative in the outside lab. The reasons for the minor differences between labs are unclear but may relate to the sensitivity of the individual assays, the position of the PCR primer pairs used, or the amount of patient DNA available. Overall, however, the results from the two independent labs correlated well.

ICS Construction (LQ1)

Primer pairs with the best performance characteristics were chosen for the design of the ICS. There were several factors to consider in the design of the ICS. The primer sequences were positioned in the ICS such that the length of product derived from the standard would differ in size from the length of the product derived from the chromosomal translocation by 10% to 30%. This would allow them to be easily separated by gel electrophoresis and yet similar enough in size that they should be amplified with the same efficiency. Because the precise break-point and thus the size of translocation product for the *bcl-2* translocation may vary from patient to patient by several hundred base pairs, the two primer pairs chosen targeted opposite ends of the cluster region; if the translocation product is similar in size to the ICS product using the first primer set, the second primer set can be used to give product sizes that differ. This circumvents the po-

tential problem of the ICS and translocation products being too similar in size or the products having greater than a 30% difference in size. Product sizes are listed in Table 2. In addition to the ICS product length considerations, the ICS standard was designed to contain probe sequences found near each chromosomal rearrangement so a hybridization step could be used to detect both the ICS and translocation products. The standard also contains a probe sequence unique to the standard to differentiate the ICS product from the translocation product. Once the primer and probe positions conformed to these criteria, the ICS standard sequence was then screened for potentially problematic self-complementary regions. A schematic of the Lymphoquant-1 (LQ1) ICS structure is shown in Figure 4A.

The LQ1 insert was synthesized by extension of overlapping primers (Figure 4B) as described previously.^{52,53} The final 1.1-kb product was cloned into a plasmid vector and sequenced, and mutations were corrected by site-directed mutagenesis (Clontech Laboratories, Palo Alto, CA). LQ1 contains primer sequences to both *tal-1*^{d1} and *tal-1*^{d2} for T-ALL, t(14;18) MBR and mcr for follicular lymphoma, t(11;14) MTC for mantle cell lymphoma, and controls: human β -globin, human RNA polymerase II large subunit, murine IL-10, and murine C μ .

ICS-PCR and Quantification

The basis for this approach to quantitative PCR is as follows: if the ICS and tumor-specific translocation targets are in the same tube and are amplified with the same primer sequences, factors affecting amplification efficiency are likely to affect both the ICS and the tumor targets equally. Indeed, for ICS-PCR to give accurate quantitative results, the ICS standard and translocation

Figure 4. Structure of the LQ1 ICS standard. **A:** Primer pairs were initially screened and selected for their ability to amplify various chromosomal rearrangements found in specific leukemias and lymphomas as described in the text. These primer sequences (vertical rectangles with Arabic numerals) were then positioned in the LQ1 standard so that they would generate products from the standard that differed in size from the products derived from the tumor-specific translocations. For example, amplification of LQ1 with primers 11 and 21 (*tal-1*^{d1} primer set A) will give an ICS-specific product of 469 bp, whereas amplification of genomic DNA from a T-ALL sample will give a *tal-1*^{d1}-specific product of 590 to 610 bp. Two or three primer pairs specific for each rearrangement were included in the design of LQ1; four examples are presented. Only one primer pair was included for each control gene. The product sizes for each primer pair are given in Table 2. LQ1 also contains a series of probe sequences (horizontal rectangles with Roman numerals) that will recognize PCR products derived from both the ICS and the specific chromosomal rearrangements. For example, probe VI will hybridize to both the ICS and translocation products generated using primers 11 and 21 described above. In addition, LQ1 contains one probe sequence (probe V) that is specific for the ICS. The sequence of each primer and probe is given in Table 1. Their locations within the *tal-1* and *bcl-2* loci are indicated in Figure 1. **B:** The synthetic insert used to produce LQ1 was constructed by oligonucleotide annealing and extension.^{52,53} Briefly, two 100-mer oligonucleotides that are complementary at their 3' ends by 20 nucleotides were annealed and extended by PCR amplification. The 180-bp product of this first step is shown in lane 2 after separation by agarose gel electrophoresis and ethidium bromide staining. A third 100-mer oligonucleotide complementary to this product by 20 nucleotides was then added to the mix. Amplification then extended the first product by an additional 80 nucleotides (lane 3). This process was continued for a total of 12 steps until the full-length 1.1-kb synthetic fragment was generated (lane 13). The entire sequence of this synthetic insert has been submitted to GenBank accession number AF116871.⁶³ Lane 1 contains a 123-bp ladder as a molecular weight marker.

targets must be amplified with the same efficiency. The relationship between the amount of product generated by PCR and the amplification efficiency of the reaction is given by the following exponential growth equations:

$$T_c = T_0(1 + f_T)^c \text{ and } S_c = S_0(1 + f_S)^c$$

or

$$\log T_c = \log T_0 + c[\log(1 + f_T)]$$

and

$$\log S_c = \log S_0 + c[\log(1 + f_S)],$$

where T_c and S_c are the amounts of product generated from the translocation and standard (ICS) targets after c

amplification cycles, respectively; f_T and f_S are the amplification efficiencies for the translocation and standard targets, respectively; and T_0 and S_0 are the initial copy numbers of the translocation and standard targets, respectively. Therefore, if $f_T = f_S$ and the initial number of ICS molecules, S_0 , is known, the number of translocation targets in the initial sample, T_0 , can be determined from the ratio the translocation and ICS product signals (T_c/S_c). (See Ref. 52 for an additional discussion of these relationships.)

To determine whether the amplification efficiencies are equal, the PCR cycle numbers are varied and the log of the product generated *versus* cycle number is evaluated. If the slopes of the lines generated for the translocation

Figure 5. ICS standard and translocation targets are amplified with the same efficiency. Primer set A, specific for *tal-1^{d1}* (A) or primer set B, specific for t(14;18) MBR (B) were used to amplify a mixture of the ICS and DNA isolated from normal human blood spiked with the T-ALL cell line CCRF-CEM (A) or the t(14;18)-carrying line SUDHL-6 (B). A total of 100 copies of ICS (determined by A_{260}) and DNA from the equivalent of 100 tumor cells mixed in 1 μ l of blood (determined by hemocytometer) were placed in each reaction tube. The reactions proceeded for the number of cycles indicated under standard conditions. Samples were separated by gel electrophoresis and analyzed by fluorescence imaging after staining with SybrGold (top). Bands corresponding to the translocation and ICS products are indicated. The amount of product generated from both ICS and translocation DNA was quantified and plotted versus cycle number (bottom left). The same data were also plotted as the ratio of translocation product to ICS product (T/S) versus cycle number (bottom right). For the *tal-1^{d1}* analysis, two separate experiments are plotted for T/S versus cycle number.

and ICS targets are equal, then the efficiencies are equal. An example of this type of analysis for the *tal-1^{d1}* target is shown in Figure 5A. The top panel shows the agarose gel in which increasing amounts of both ICS and translocation products were generated with increasing cycle numbers. In a plot of product amount (log scale) versus cycle number, lines with equal slopes were generated from each product. This indicates that the two targets were amplified with the same efficiencies. The validity of the quantification was further demonstrated by plotting T/S versus cycle number using the same data. By design in this experiment, $T_0 = S_0 = 100$, or $T/S = 1$; this relationship was maintained through the 34 cycles tested, again indicating that the two targets were amplified with the same efficiency. Similar results were seen for a t(14;18) MBR primer pair (Figure 5B).

ICS-PCR Validation

To evaluate reproducibility of ICS-PCR, precision tests were performed. Intra-assay precision was measured by preparing one master mix containing equal numbers of LQ1 and *tal-1^{d1}* targets (100 molecules each) derived from CCRF-CEM, dividing the mix into 20 tubes, and then placing the tubes in the same thermocycler. The inter-assay precision test included individual reactions run at different times using different thermocyclers. In experiments designed with a T/S ratio of 1, inter-assay and intra-assay quantitative analysis of the products resulted in an average $T/S = 0.92$ with a 16% coefficient of variation (data not shown).

To measure accuracy, ICS-PCR was compared to a gold standard. In this case, the gold standard used was hemocytometric determination of cell numbers. An example of this comparison for the *tal-1^{d1}* target is shown in Figure 6A. This experiment is performed by taking aliquots of reference human blood, spiking them with known numbers of tumor cells, isolating DNA, and using ICS-PCR to quantify the number of tumor cells in the starting sample. Here, the lower band is derived from 100 molecules of LQ1 (determined by A_{260}). The top band corresponds to the tumor-specific product derived from the T-ALL cells added to the blood. Each lane contains an ICS-specific band. The top band is related to the number of CEM cells present in the blood sample as well as the dilution factor, and therefore its intensity varied from lane to lane. For example, using undiluted DNA samples, when 10,000 CEM cell equivalents were present in the reaction, a strong upper band was observed (lane 1), whereas a weaker band was seen when 41 CEM cell equivalents were present (lane 21). As each DNA mix was diluted, the top band lost intensity until conditions occurred where the upper and lower bands were roughly equal in intensity, eg, the 1:10 dilution in the 3333-cell group (lane 6) or the undiluted sample for the 123-cell group (lane 17). For each initial cell number, dilutions giving roughly equal intensity bands were chosen, the bands were quantified, the dilution factors were calculated back in, and then the calculated values were plotted against the hemocytometer values. In the best-fit curve using data from two separate experiments for *tal-1^{d1}* (Figure 6A), the $R^2 = 0.97$, the slope = 0.9, and the y intercept = 0.1, indicating that there was minimal random error, proportional error, or constant error, respectively. Similar results were seen for the t(14;18) MBR analysis (Figure 6B).

ICS-PCR and Measurement of Tumor Burden in Patient Samples

Finally, to demonstrate the use of this technique on clinical samples, ICS-PCR was used to measure tumor burden in the diagnostic patient samples analyzed in Figure 3 (Figure 7). The median tumor load in the bone marrow of T-ALL patients at the time of diagnosis was 26.6% (range, 0.7% to 85.4%). Only one patient had a tumor burden in bone marrow of less than 10%. This unusual

Figure 6. Accuracy of tumor fraction quantification by ICS-PCR. Threefold serial dilutions of CCRF-CEM (A) or SUDHL-6 (B) were added to 200 μ l of whole blood, ranging from 10,000 tumor cells/ μ l to 41 cells/ μ l, to quantify *tal-r^{t1}* or t(14;18) targets, respectively. Tenfold serial dilutions of DNA isolated from these mixtures were then made, and 1 μ l was added to each reaction along with 100 molecules of LQ1. PCR was performed under standard conditions, and the samples were analyzed as described in Figure 5. Products generated from the translocation and ICS targets are indicated. The bands were quantified to determine the amount of tumor starting material and compared with the cell numbers determined by hemocytometric analysis. The equation of the best-fit line drawn through the points is indicated on the graphs.

patient was initially diagnosed with a primary lymphoma. Subsequent chest x-ray revealed a mediastinal mass that was found to carry the *tal-1*^{d1} rearrangement by Southern blot, suggesting that this patient had T-ALL rather than a primary lymphoma. However, the bone marrow biopsy was listed as negative for blasted cells by histology. The low tumor burden in the bone marrow of this patient by ICS-PCR is consistent with this clinical picture. The follicular lymphoma patients had a median tumor burden of 0.97% at diagnosis, with more variation and a wider range (0.01% to 68%) as might be expected for this disease that is likely to initiate as a solid tumor with variable bone marrow seeding.

Discussion

A complete therapeutic response in cancer is defined clinically by the absence of symptoms and detectable

Figure 7. Bone marrow tumor burden at diagnosis in T-ALL and follicular lymphoma patients as measured by ICS-PCR. Patient sample DNA was serially diluted and analyzed in reactions containing a constant amount of ICS (either 20 or 100 molecules). Quantification of tumor cell number was determined by comparing ICS and tumor target amounts in dilution samples with ratios closest to 1:1, as described in Figure 6; samples with higher tumor burden were quantified with higher dilutions, and samples with lower tumor burden were quantified with lower dilution (or even undiluted). For the follicular lymphoma samples, MBR primer set B was used except in cases where the translocation band was similar in size to the 265-bp ICS band (eg, Figure 3, lane 47). In those cases, MBR primer set A was used for quantification. Total cell numbers were calculated from the DNA concentrations determined by A_{260} , and confirmed by ICS-PCR using primers specific for RNA polymerase II large subunit. Only patient samples with detectable bands in Figure 3 were analyzed.

disease. Although many patients will have a complete response to therapy, some harbor residual tumor cells below the limit of detection of the evaluation methods used. These patients are likely to have an increased risk of relapse. Several assays can be used to measure disease burden in leukemia patients.⁷⁻⁹ They rely on the ability to distinguish between leukemic cells from normal cells based on morphology, phenotype, or genotype. Bone marrow biopsies and blood smears are evaluated histologically for the presence of abnormal blast cells in high numbers. However, when the level of leukemic cells is below 1%, this assay loses its reliability.³ In addition, histological evaluation is somewhat subjective and suffers from observer-to-observer variability. Flow cytometric identification of leukemic cells based on their unusual phenotypic characteristics provides a more objective measure of tumor burden and lowers the limits of detection by a factor of 10 to 100 depending on the sophistication of the analysis.^{3,11} The observation that neoplastic cells often carry characteristic chromosomal translocations is now being exploited in the analysis of tumor burden. The ability to use PCR to detect specific DNA alterations found in cancer cells promises to further improve the limits of detection and objectivity of MRD analysis.

The ability of PCR detection of MRD to predict relapse has been investigated for a number of translocations and diseases, including *bcr/abl* for CML, *pml/rar α* for APL, *aml1/eto* for AML, and *J ψ /bcl-2* for non-Hodgkin's lymphoma.^{7,9,29,55} Although many of these studies are still underway, some, but not all, show a correlation between PCR positivity and clinical outcome. For example, in T-ALL, MRD detection by PCR targeting the *tal-1* deletions after therapy was found to be predictive of relapse in two small series of four patients each.^{14,47} However, in CML, PCR positivity for *bcr/abl* early after bone marrow transplantation was found to have limited prognostic significance.^{56,57}

The analysis of *pml/rar α* in APL illustrates several points that are relevant to the use of PCR approaches for MRD detection in general. For APL patients in remission after treatment with chemotherapy and retinoic acid, all were PCR positive for the *pml/rar α* transcript when evaluated 1 to 3 months after therapy; at this time there was no correlation with clinical outcome. However, after 4 months, PCR positivity was highly predictive of relapse, and PCR negativity was usually associated with prolonged disease-free survival.²⁹⁻³¹ Thus, the time of evaluation is critical for useful prognostic information. In addition, elimination of cells carrying the translocation below a level of 1 in 10⁵ appeared to be required for stable remission,^{32,33} emphasizing the importance of quantification for this type of analysis. The prognostic value of quantitative detection of tumor markers has also been demonstrated using PCR detection of the t(8;21) fusion transcript in AML.⁴³ Indeed, a lack of quantification in many of the PCR studies published may be partly responsible for the discrepant prognostic results found in diseases such as follicular lymphoma.^{35,49,58,59} Thus, it appears that the predictive value of PCR detection of MRD will depend on the type of cancer involved, the structural details of the translocation, the time of analysis, accurate quantification, and limits of detection for the assay. Relevant prognostic parameters may include the magnitude of the initial therapeutic response or the tumor load at the time of diagnosis, after induction therapy, or at specific time points during maintenance therapy, all potentially monitored by quantitative PCR.

The great advantages of using PCR for MRD analysis are its extreme specificity, low limits of detection, and low cost. Low limits of detection are achieved due to the reiterative nature of the amplification process, giving rise to an exponential increase in target amounts. However, it is this reiterative aspect of the PCR reaction that complicates its use as a quantitative tool in the clinical laboratory. Thus, the effects of minor differences in amplification efficiency between samples are amplified to give large differences in product amounts at the end of the PCR amplification process. Without controlling for differences in reaction efficiencies between samples, comparative quantification using PCR is impossible. Unfortunately, many reaction components have been found to influence reaction efficiency, including primer structure, pH, Mg²⁺ concentration, carryover of organic solvents used during template preparation, and specific inhibitors present in patient samples. In some cases, inhibitors can reduce

amplification efficiency enough to give false negative results.

Here we have described a technique for quantifying DNA targets using an internal calibration standard (ICS) in each PCR reaction. This technique allows one to enjoy the advantages of nonquantitative PCR yet overcome the drawbacks described above. With the inclusion of an internal standard, ICS-PCR can rule out false negative results and accurately quantify initial target numbers in absolute terms (eg, targets/10⁶ cells) by controlling for differences in amplification efficiency. The ease of performing ICS-PCR is comparable to that of simple PCR, except that one needs to dilute the patient DNA in a manner similar to that shown for each blood sample in Figure 6. For a diagnostic sample where the tumor burden is high, three to four dilutions may be required to give similar product amounts for the tumor and ICS targets. However, in the setting of minimal residual disease where the tumor burden is very low, a single reaction carrying as much patient DNA as possible may be sufficient. As described, this technique demonstrates excellent performance characteristics for the quantification of tumor burden in terms of precision, accuracy, specificity, sensitivity, and low limits of detection. These characteristics should allow this approach to be used in any laboratory with comparable results.

We have shown that using ICS-PCR, we can identify patients carrying del(1) in T-ALL and t(14;18) in follicular lymphoma and quantify their tumor burden in diagnostic samples. The sensitivity when comparing the PCR data with Southern blot (T-ALL) or histology (follicular lymphoma) was 96% and 56%, respectively. These diagnostic patient samples have been quantified for tumor burden, and it will be interesting to determine how tumor burden relates to the survival of individual patients. For example, although one might expect that a high tumor burden at the time of diagnosis would be associated with a worse prognosis, it could be just the opposite; low tumor burden at diagnosis when clinical symptoms are apparent could be an indication of extremely aggressive disease.

Applications for this technology are vast. ICS-PCR can aid in diagnosis and determination of initial tumor load and can also be used to evaluate therapeutic responses, to monitor patients throughout maintenance therapy, and to follow the recurrence and growth rate of tumors during relapse. These are all potential prognostic factors that can be assessed by ICS-PCR to more clearly define each patient's risk for relapse and subsequently guide their therapy.

Acknowledgments

We thank Ian Magrath for providing us with the SUDHL-6 cell line, Jerry Radich and Brian Dawson for critical evaluation of the manuscript, Robert McKenna for helpful discussion, and all of the patients and physicians who agreed to participate in this study.

References

- George SL, Aur RJ, Mauer AM, Simone JV: A reappraisal of the results of stopping therapy in childhood leukemia. *N Engl J Med* 1979, 300:269-273
- Cheson BD, Cassileth PA, Head DR, Schiffer CA, Bennett JM, Bloomfield CD, Brunning R, Gale RP, Grever MR, Keating MJ, Sawitsky A, Stass S, Weinstein H, Woods WG: Report of the National Cancer Institute-sponsored workshop on definitions of diagnosis and response in acute myeloid leukemia. *J Clin Oncol* 1990, 8:813-819
- Wormann B: Implications of detection of minimal residual disease. *Curr Opin Oncol* 1993, 5:3-12
- Shuster JJ, Falletta JM, Pullen DJ, Crist WM, Humphrey GB, Dowell BL, Wharam MD, Borowitz M: Prognostic factors in childhood T-cell acute lymphoblastic leukemia: a Pediatric Oncology Group study. *Blood* 1990, 75:166-173
- Pui CH: Childhood leukemias. *N Engl J Med* 1995, 332:1618-1630
- Kersey JH: Fifty years of studies of the biology and therapy of childhood leukemia. *Blood* 1997, 90:4243-4251
- Campana D, Pui CH: Detection of minimal residual disease in acute leukemia: methodologic advances and clinical significance. *Blood* 1995, 85:1416-1434
- Carlo-Stella C, Mangoni L, Dotti GP, Rizzoli V: Techniques for detection of minimal residual disease. *Leuk Lymphoma* 1995, 18:75-80
- Drexler HG, Borkhardt A, Janssen JW: Detection of chromosomal translocations in leukemia-lymphoma cells by polymerase chain reaction. *Leuk Lymphoma* 1995, 19:359-380
- Sievers EL, Loken MR: Detection of minimal residual disease in acute myelogenous leukemia. *J Pediatr Hematol Oncol* 1995, 17:123-133
- van Dongen JJ, Breit TM, Adriaansen HJ, Beishuizen A, Hooijkaas H: Detection of minimal residual disease in acute leukemia by immunological marker analysis and polymerase chain reaction. *Leukemia* 1992, 6:47-59
- Rabbitts TH: Chromosomal translocations in human cancer. *Nature* 1994, 372:143-149
- Bash RO, Crist WM, Shuster JJ, Link MP, Amylon M, Pullen J, Carroll AJ, Buchanan GR, Smith RG, Baer R: Clinical features and outcome of T-cell acute lymphoblastic leukemia in childhood with respect to alterations at the TAL1 locus: a Pediatric Oncology Group study. *Blood* 1993, 81:2110-2117
- Jonsson OG, Kitchens RL, Baer RJ, Buchanan GR, Smith RG: Rearrangements of the *tal-1* locus as clonal markers for T cell acute lymphoblastic leukemia. *J Clin Invest* 1991, 87:2029-2035
- Breit TM, Beishuizen A, Ludwig WD, Mol EJ, Adriaansen HJ, van Wering ER, van Dongen JJ: *tal-1* deletions in T-cell acute lymphoblastic leukemia as PCR target for detection of minimal residual disease. *Leukemia* 1993, 7:2004-2011
- Brown L, Cheng JT, Chen Q, Siciliano MJ, Crist W, Buchanan G, Baer R: Site-specific recombination of the *tal-1* gene is a common occurrence in human T cell leukemia. *Embo J* 1990, 9:3343-3351
- Aplan PD, Lombardi DP, Ginsberg AM, Cossman J, Bertness VL, Kirsch IR: Disruption of the human SCL locus by "illegitimate" V-(D)-J recombinase activity. *Science* 1990, 250:1426-1429
- Bernard O, Lecoigne N, Jonveaux P, Souyri M, Mauchauffe M, Berger R, Larsen CJ, Mathieu-Mahul D: Two site-specific deletions and t(1;14) translocation restricted to human T-cell acute leukemias disrupt the 5' part of the *tal-1* gene. *Oncogene* 1991, 6:1477-1488
- Yunis JJ, Oken MM, Kaplan ME, Ensrud KM, Howe RR, Theologides A: Distinctive chromosomal abnormalities in histologic subtypes of non-Hodgkin's lymphoma. *N Engl J Med* 1982, 307:1231-1236
- Weiss LM, Warnke RA, Sklar J, Cleary ML: Molecular analysis of the t(14;18) chromosomal translocation in malignant lymphomas. *N Engl J Med* 1987, 317:1185-1189
- Bakhshi A, Jensen JP, Goldman P, Wright JJ, McBride OW, Epstein AL, Korsmeyer SJ: Cloning the chromosomal breakpoint of t(14;18) human lymphomas: clustering around JH on chromosome 14 and near a transcriptional unit on 18. *Cell* 1985, 41:899-906
- Tsujimoto Y, Cossman J, Jaffe E, Croce CM: Involvement of the *bcl-2* gene in human follicular lymphoma. *Science* 1985, 228:1440-1443
- Cleary ML, Smith SD, Sklar J: Cloning and structural analysis of cDNAs for *bcl-2* and a hybrid *bcl-2*/immunoglobulin transcript resulting from the t(14;18) translocation. *Cell* 1986, 47:19-28
- Cleary ML, Galili N, Sklar J: Detection of a second t(14;18) breakpoint cluster region in human follicular lymphomas. *J Exp Med* 1986, 164:315-320
- Ngan BY, Nourse J, Cleary ML: Detection of chromosomal translocation t(14;18) within the minor cluster region of *bcl-2* by polymerase chain reaction and direct genomic sequencing of the enzymatically amplified DNA in follicular lymphomas. *Blood* 1989, 73:1759-1762
- Lee MS, Chang KS, Cabanillas F, Freireich EJ, Trujillo JM, Stass SA: Detection of minimal residual cells carrying the t(14;18) by DNA sequence amplification. *Science* 1987, 237:175-178
- Crescenzi M, Seto M, Herzig GP, Weiss PD, Griffith RC, Korsmeyer SJ: Thermostable DNA polymerase chain amplification of t(14;18) chromosome breakpoints and detection of minimal residual disease. *Proc Natl Acad Sci USA* 1988, 85:4869-4873
- Baer MR: Assessment of minimal residual disease in patients with acute leukemia. *Curr Opin Oncol* 1998, 10:17-22
- Lo Coco F, Diverio D, Pandolfi PP, Biondi A, Rossi V, Avvisati G, Rambaldi A, Arcese W, Petti MC, Meloni G, Mandelli F, Grignani F, Masera G, Barbui T, Pelicci PG: Molecular evaluation of residual disease as a predictor of relapse in acute promyelocytic leukaemia. *Lancet* 1992, 340:1437-1438
- Huang W, Sun GL, Li XS, Cao Q, Lu Y, Jang GS, Zhang FQ, Chai JR, Wang ZY, Waxman S, Chen Z, Chen S-J: Acute promyelocytic leukemia: clinical relevance of two major PML-RAR α isoforms and detection of minimal residual disease by retrotranscriptase/polymerase chain reaction to predict relapse. *Blood* 1993, 82:1264-1269
- Miller WH Jr, Levine K, DeBlasio A, Frankel SR, Dmitrovsky E, Warrell RP Jr: Detection of minimal residual disease in acute promyelocytic leukemia by a reverse transcription polymerase chain reaction assay for the PML/RAR- α fusion mRNA. *Blood* 1993, 82:1689-1694
- Laczika K, Mitterbauer G, Korninger L, Knobl P, Schwarzingier I, Kapiotis S, Haas OA, Kyrle PA, Pont J, Oehler L, Purtscher B, Thalhammer F, Lechner K, Jaeger U: Rapid achievement of PML-RAR α polymerase chain reaction (PCR)-negativity by combined treatment with all-trans-retinoic acid and chemotherapy in acute promyelocytic leukemia: a pilot study. *Leukemia* 1994, 8:1-5
- Fukutani H, Naoe T, Ohno R, Yoshida H, Kiyoi H, Miyawaki S, Morishita H, Sano F, Kamibayashi H, Matsue K, Miyake T, Hasegawa S, Ueda Y, Kato Y, Kobayashi H, Shimazaki C, Kobayashi M, Kurane R, Sakota H, Masaki K, Wakayama T, Tohyama K, Nonaka Y, Natori H: Prognostic significance of the RT-PCR assay of PML-RARA transcripts in acute promyelocytic leukemia: The Leukemia Study Group of the Ministry of Health and Welfare (Kouseisho). *Leukemia* 1995, 9:588-593
- Dibenedetto SP, Lo Nigro L, Mayer SP, Rovera G, Schiliro G: Detectable molecular residual disease at the beginning of maintenance therapy indicates poor outcome in children with T-cell acute lymphoblastic leukemia. *Blood* 1997, 90:1226-1232
- Gribben JG, Neuberg D, Barber M, Moore J, Pesek KW, Freedman AS, Nadler LM: Detection of residual lymphoma cells by polymerase chain reaction in peripheral blood is significantly less predictive for relapse than detection in bone marrow. *Blood* 1994, 83:3800-3807
- Andersen NS, Donovan JW, Borus JS, Poor CM, Neuberg D, Aster JC, Nadler LM, Freedman AS, Gribben JG: Failure of immunologic purging in mantle cell lymphoma assessed by polymerase chain reaction detection of minimal residual disease. *Blood* 1997, 90:4212-4221
- Roberts WM, Estrov Z, Ouspenskaia MV, Johnston DA, McClain KL, Zipf TF: Measurement of residual leukemia during remission in childhood acute lymphoblastic leukemia. *N Engl J Med* 1997, 336:317-323
- Brisco MJ, Condon J, Hughes E, Neoh SH, Sykes PJ, Seshadri R, Toogood I, Waters K, Tauro G, Ekert H, Morley AA: Outcome prediction in childhood acute lymphoblastic leukaemia by molecular quantification of residual disease at the end of induction. *Lancet* 1994, 343:196-200
- Wasserman R, Galili N, Ito Y, Silber JH, Reichard BA, Shane S, Womer RB, Lange B, Rovera G: Residual disease at the end of induction therapy as a predictor of relapse during therapy in childhood B-lineage acute lymphoblastic leukemia. *J Clin Oncol* 1992, 10:1879-1888
- Foroni L, Coyle LA, Papaioannou M, Yaxley JC, Sinclair MF, Chim JS, Cannell P, Secker-Walker LM, Mehta AB, Prentice HG, Hoffbrand AV: Molecular detection of minimal residual disease in adult and child-

- hood acute lymphoblastic leukaemia reveals differences in treatment response. *Leukemia* 1997, 11:1732-1741
41. Gruhn B, Hongeng S, Yi H, Hancock ML, Rubnitz JE, Neale GA, Kitchingman GR: Minimal residual disease after intensive induction therapy in childhood acute lymphoblastic leukemia predicts outcome. *Leukemia* 1998, 12:675-681
42. Martin G, Mairs RJ: Synthesis of quantitation standards for nested competitive PCR for the determination of minimal residual disease in B-lineage acute lymphoblastic leukaemia. *Leuk Res* 1997, 21:807-809
43. Tobal K, Yin JA: Monitoring of minimal residual disease by quantitative reverse transcriptase-polymerase chain reaction for AML1-MTG8 transcripts in AML-M2 with t(8; 21). *Blood* 1996, 88:3704-3709
44. Nakao M, Yokota S, Horiike S, Taniwaki M, Kashima K, Sonoda Y, Koizumi S, Takaue Y, Matsushita T, Fujimoto T, Misawa S: Detection and quantification of TEL/AML1 fusion transcripts by polymerase chain reaction in childhood acute lymphoblastic leukemia. *Leukemia* 1996, 10:1463-1470
45. Cross NC, Feng L, Chase A, Bungey J, Hughes TP, Goldman JM: Competitive polymerase chain reaction to estimate the number of BCR-ABL transcripts in chronic myeloid leukemia patients after bone marrow transplantation. *Blood* 1993, 82:1929-1936
46. Lion T: Monitoring of residual disease in chronic myelogenous leukemia: methodological approaches and clinical aspects. *Leukemia* 1996, 10:896-906
47. Janssen JW, Ludwig WD, Sterry W, Bartram CR: SIL-TAL1 deletion in T-cell acute lymphoblastic leukemia. *Leukemia* 1993, 7:1204-1210
48. Meijerink JP, Goverde GJ, Smetsers TF, Raemaekers JM, Bogman MJ, de Witte T, Mensink EJ: Quantitation of follicular non-Hodgkin's lymphoma cells carrying t(14;18) in a patient before and after allogeneic bone marrow transplantation. *Ann Oncol* 1994, 5:43-45
49. Gribben JG, Freedman A, Woo SD, Blake K, Shu RS, Freeman G, Longtine JA, Pinkus GS, Nadler LM: All advanced stage non-Hodgkin's lymphomas with a polymerase chain reaction amplifiable breakpoint of *bcl-2* have residual cells containing the *bcl-2* rearrangement at evaluation and after treatment. *Blood* 1991, 78:3275-3280
50. Sykes PJ, Neoh SH, Brisco MJ, Hughes E, Condon J, Morley AA: Quantitation of targets for PCR by use of limiting dilution. *Biotechniques* 1992, 13:444-449
51. Koch DD, Peters T Jr: Selection and evaluation methods. *Textbook of Clinical Chemistry*. Edited by Burtis CA, Ashwood ER. Philadelphia, WB Saunders, 1994, pp 508-525
52. Scheuermann RH, Bauer SR: Polymerase chain reaction-based mRNA quantification using an internal standard: analysis of oncogene expression. *Methods Enzymol* 1993, 218:446-473
53. Bai X, Hosler GA, Rogers BB, Dawson DB, Scheuermann RH: Quantitative polymerase chain reaction for human herpesvirus diagnosis and measurement of Epstein-Barr virus burden in posttransplant lymphoproliferative disorder. *Clin Chem* 1997, 43:1843-1849
54. Bakhshi A, Wright JJ, Graninger W, Seto M, Owens J, Cossman J, Jensen JP, Goldman P, Korsmeyer SJ: Mechanism of the t(14;18) chromosomal translocation: structural analysis of both derivative 14 and 18 reciprocal partners. *Proc Natl Acad Sci USA* 1987, 84:2396-2400
55. Ito Y, Miyamura K: Clinical significance of minimal residual disease in leukemia detected by polymerase chain reaction. Is molecular remission a milestone for achieving a cure? *Leuk Lymphoma* 1994, 16: 57-64
56. Cross NC, Hughes TP, Feng L, O'Shea P, Bungey J, Marks DI, Ferrant A, Martiat P, Goldman JM: Minimal residual disease after allogeneic bone marrow transplantation for chronic myeloid leukaemia in first chronic phase: correlations with acute graft-versus-host disease and relapse. *Br J Haematol* 1993, 84:67-74
57. Roth MS, Antin JH, Ash R, Terry VH, Gotlieb M, Silver SM, Ginsburg D: Prognostic significance of Philadelphia chromosome-positive cells detected by the polymerase chain reaction after allogeneic bone marrow transplant for chronic myelogenous leukemia. *Blood* 1992, 79:276-282
58. Limpens J, Stad R, Vos C, de Vlaam C, de Jong D, van Ommen GJ, Schuurin E, Kluin PM: Lymphoma-associated translocation t(14;18) in blood B cells of normal individuals. *Blood* 1995, 85:2528-2536
59. Dolken G, Illerhaus G, Hirt C, Mertelsmann R: BCL-2/JH rearrangements in circulating B cells of healthy blood donors and patients with nonmalignant diseases. *J Clin Oncol* 1996, 14:1333-1344
60. Delabesse E, Bernard MC, Macintyre EA: Direct submission of *tal-1* 5' breakpoint region sequence. *European Molecular Biology Laboratory (EMBL) accession number Y07540*, 1997
61. Lecointe N, Ferrier R, Mathieu-Mahul D: Direct submission of *tal-1* d2 3' breakpoint region sequence. *European Molecular Biology Laboratory (EMBL) accession number X81027*, 1994
62. Mattila PS, Schugk J, Wu H, Makela O: Extensive allelic sequence variation in the J region of the human immunoglobulin heavy chain gene locus. *Eur J Immunol* 1995, 25:2578-2582
63. Hosler GA, Scheuermann RH: Direct submission of LQ1 internal calibration standard sequence. *GenBank accession number AF116871*, 1999
64. Liu J, Johnson RM, Traweck ST: Rearrangement of the BCL-2 gene in follicular lymphoma: detection by PCR in both fresh and fixed tissue samples. *Diagn Mol Pathol* 1993, 2:241-247