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(21) International Application Number: PCT/NL98/00542 (22) International Filing Date: 18 September 1998 (18.09.98) (30) Priority Data: 97202858.3 18 September 1997 (18.09.97) EP (71) Applicant (for all designated States except US): ERASMUS UNIVERSITEIT ROTTERDAM [NL/NL]; Dr. Molewater- plein 50, NL-3015 GE Rotterdam (NL). (72) Inventors; and (75) Inventors/Applicants (for US only): VAN DONGEN, Jacobus, Johannes, Maria [NL/NL]; Parallelweg Zuid 147, NL-2914 LE Nieuwerkerk aan den IJssel (NL). PONGERS-WILLEMSE, Martha, Jacoba [NL/NL]; C. Kouwenbergzoom 197, NL-3065 HG Rotterdam (NL). (74) Agent: SMULDERS, Th., A., H., J.; Vereenigde Octrooibu- reau, Nieuwe Parklaan 97, NL-2587 BN The Hague (NL).		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>Without international search report and to be republished upon receipt of that report.</i>
(54) Title: DETECTION OF MINIMAL RESIDUAL DISEASE IN LYMPHOID MALIGNANCIES (57) Abstract The invention relates to the field of cancer diagnosis, more specific to the monitoring of disease development during and after treatment. The invention comprises a method for determining minimal residual disease comprising amplifying nucleic acid molecules using at least one primer reactive with a common gene segment and further comprising identifying malignancy-specific nucleic acid sequences by hybridising with a fluorogenic probe specifically and selectively reactive with said malignancy-specific nucleic acid sequences.		

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Title: Detection of minimal residual disease in lymphoid malignancies.

This invention relates to the field of cancer diagnosis, more specifically to the monitoring of disease development during and after treatment.

Cytostatic or cytotoxic treatment induces remission
5 in the majority of patients with lymphoid malignancies. Nevertheless many of these patients relapse. Apparently the current cytostatic or cytotoxic treatment protocols are not capable of killing all malignant cells in these relapsing patients, although they reached so-called
10 complete remission according to cytomorphological criteria. Since the detection limit of cytomorphological techniques is not lower than 1-5% malignant cells, it is obvious that such techniques can only provide superficial information about the effectiveness of treatment.
15 Techniques with a higher sensitivity to detect "minimal residual disease" or minimal disease (MRD) are needed to obtain better insight in the reduction of tumor mass during induction treatment and further eradication of the malignant cells during maintenance treatment (Figure 1).
20 Techniques for detection of "minimal residual disease" (MRD) at levels of 10^{-3} to 10^{-5} (one malignant cell between 10^3 to 10^5 normal cells) during follow-up of children with acute lymphoblastic leukemia (ALL) can provide insight into the effectiveness of cytostatic treatment. However
25 it is not yet clear whether and how MRD information can be applied to the clinical decision process, e.g. for stratification of treatment protocols. We monitored 240 childhood ALL patients who were treated according to national protocols of the International BFM Study Group.
30 Sixty patients relapsed and the patients in continuous complete remission (CCR) had a median event free follow-

up of 48 months. Bone marrow (BM) samples were collected at up to 9 time points during and after treatment: at the end of induction treatment, before consolidation treatment, before re-induction treatment, before maintenance treatment, three samples during maintenance treatment, at the end of treatment, and one year after cessation of treatment. Classical polymerase chain reaction (PCR) analysis of patient-specific immunoglobulin and T cell receptor gene rearrangements and *TAL1* deletions were used as targets for semi-quantitative estimation of MRD levels: $\geq 10^{-2}$, 10^{-3} , and $\leq 10^{-4}$ together with radioactive patient-specific junctional region probes. MRD negativity at the various follow-up time points was associated with low relapse rates (3-16%), but five to fourteen-fold higher relapse rates (41-86%) were found in MRD positive patients. The distinct MRD levels appeared to have independent prognostic value (p (trend) < 0.001) at all time points. Especially at the first two time points three-fold higher relapse rates were found in patients with high tumour loads ($\geq 10^{-2}$) as compared to patients with low tumour loads ($\leq 10^{-4}$). At later time points (including the end of treatment) also low tumour loads were associated with a high relapse rate. Positivity in CCR patients after treatment was rarely ($< 1\%$) observed, even when multiple sensitive PCR analyses were performed. Finally, using the combined MRD information of the first two follow-up time points, it was possible to recognise a low risk group comprising 43% of the analysed patients with a relapse rate of only 2% and a high risk group of 15% of patients with a relapse rate of 84%. Our MRD study unequivocally demonstrated that monitoring of childhood ALL patients at multiple time points gives clinically relevant insight into the effectiveness of treatment. Combined MRD information of the first 3 to 4 months of treatment allows identification of good prognosis and poor prognosis groups of

substantial size, which might profit from treatment adaptation.

However, MRD techniques need to have at least a sensitivity of 10^{-4} to allow for earlier identification of patients belonging to high and especially to low risk groups, allowing a clinical decision process that would allow assigning the proper treatment to the proper patient. This would allow to reduce false-negative results and avoid being too late with therapy when a patient with high risk has not yet been detected as such, and would allow reducing false positive results and avoid cumbersome therapy in patients with low risk.

Furthermore, more sensitive MRD techniques would allow sampling patients via routine blood sampling, instead of by the painful or cumbersome bone marrow aspiration currently employed.

Replacement of BM sampling by blood sampling has been a topic of debate in MRD studies for over a decade. Initial immunophenotyping studies in T-ALL and acute myeloid leukemias indicate that MRD levels in blood are generally less than one $10^{\log}$ lower than in BM. Recent PCR studies show that also in precursor-B-ALL this difference is 1 to 1.5 $10^{\log}$. This would imply that MRD techniques need to be at least approximately one $10^{\log}$ more sensitive (i.e. $\leq 10^{-5}$), when blood samples are monitored. Alternatively, bigger samples (i.e. more DNA) should be analyzed but this is too laborious and time consuming, unless a semi-automated system is used.

If multiple BM samples are analysed during follow-up, steady decrease of MRD levels to negative PCR results is associated with a favourable prognosis, whereas persistence of MRD generally leads to clinical relapse. Low MRD levels after therapy might be associated with late relapse, but absence of MRD at the end of treatment is not sufficient to predict that the patient is cured. A single time point of MRD analysis is not sufficient for

recognition of patients with a good prognosis and patients with a poor prognosis. However, information about the kinetics of tumour reduction is needed for MRD-based risk group identification and thereby provides new openings for treatment stratification. The MRD-based low risk patients might profit from treatment reduction. On the other hand, the group of MRD-based high risk patients might benefit from intensive treatment protocols, including stem cell transplantation. Therefore, follow-up samples need to be collected during and after treatment for obtaining insight into the kinetics of tumour reduction and for determining the risk of relapse per patient.

During the last decade several methods for detection of MRD have been developed and evaluated, such as cytogenetics, cell culture systems, fluorescence in situ hybridization (FISH), Southern blotting immunological marker analysis, and polymerase chain reaction (PCR) techniques. The detection limit of most techniques is not lower than 1-5% malignant cells. However, three types of techniques can detect so-called "minimal residual disease" at more sensitive levels:

1. flow cytometric immunophenotyping, detecting aberrant or unusual protein expression by the malignant cells;
2. polymerase chain reaction (PCR-) based detection of breakpoint fusion regions of chromosome aberrations; and
3. detection of clone-specific immunoglobulin (Ig) and T cell receptor (TCR) gene rearrangements via PCR amplification.

The last technique has the broadest applicability in lymphoid malignancies and is most frequently used in MRD studies so far (Table 1).

Rearrangements in Ig and TCR genes result in unique combinations of the many available variable (V), diversity (D), and joining (J) gene segments; the junctions between these gene segments form the so-called "junctional regions", which can be regarded as

"fingerprint-like" sequences due to deletion and random insertion of nucleotides during the rearrangement process. PCR analysis of junctional regions generally reaches sensitivities of 10^{-4} .

5 PCR analysis of a specific chromosome aberration can use primers at opposite sites of the fusion regions of the breakpoints e.g. TAL1 deletion, t(14;18), t(11;14), t(1;14), and t(10;14). At the fusion regions of breakpoints of chromosome aberrations in lymphoid
10 malignancies a random deletion and insertion of nucleotides may have occurred similar to the junctional regions of Ig and TCR gene rearrangements.

 Several retrospective and some limited prospective studies indicate that MRD detection in lymphoid
15 malignancies has prognostic value. So far, the prognostic value of MRD detection has especially been studied in patients with non-Hodgkin lymphoma (e.g. before and after bone marrow transplantation) and in patients with acute lymphoblastic leukemia (ALL). Absence of MRD in ALL
20 patients after induction therapy is suggested to predict good outcome. However approximately half of the ALL patients are still positive at that time. Therefore the level of MRD positivity was evaluated and found to have predictive value. If multiple bone marrow samples are
25 repeatedly analysed during follow-up, steady decrease of MRD levels to negative PCR results is associated with favorable prognosis, whereas persistence of MRD generally leads to clinical relapse. MRD data of a large prognostic study of ALL patients show that high tumor loads ($>10^3$) at
30 two successive time points in the early phase of therapy result in a RFS (relapse free survival) of only 25%. In contrast, low levels of MRD ($<10^{-4}$) or MRD negativity on both these time points result in RFS of -95%. This indicates that the kinetics of disappearing cells
35 predicts relapse and that the tumour load of a particular patient should be identified accurately, i.e.

quantitative MRD information is essential, therefore a need exists for a rapid, sensitive and quantitative method to determine the presence of malignant cells in patient, i.e. the detection of minimal residual disease.

5

The present invention provides a method for the detection of minimal residual disease by determining the presence of malignant cells in a sample comprising amplification of nucleic acid molecules corresponding to common rearranged gene segments using for example in a PCR a forward and/or reverse primer reactive with said gene segments and further comprising the identification of malignancy-specific nucleic acid molecules found among those rearranged gene segments by hybridisation with a fluorogenic probe specifically and selectively reactive with said malignancy-specific nucleic acid molecules (note that malignancy-specific is in most cases also patient-specific, since a malignancy in general occurs in a patient-specific manner).

20 The current MRD techniques, including immunological marker analysis and current PCR techniques have several disadvantages which make a prognostic assessment inaccurate. Immunological marker analysis is only applicable to a restricted set of patients for which appropriate immunological tools exist. Furthermore, the main problem with the current PCR protocols is the fact that PCR, albeit very sensitive, is mainly qualitative and its results can therefore not easily be related to precise frequencies of malignant cells. Only when limited dilution techniques are used in PCR or competitive PCR is applied, mixing a standard nucleic acid with the sample nucleic acid, semi-quantitative detection by endpoint analysis of relative amounts of amplified target. However, this is not only time-consuming and very costly but also inaccurate and difficult to standardize. Furthermore, the current PCR techniques are sensitive to

contamination, require a long processing time and often use radio-actively labelled probes.

The invention provides a method for the detection of minimal residual disease by determining the presence
5 of malignant cells in a sample by detecting amplified nucleic acid molecules via real-time quantitative nucleic acid amplification (e.g. using the ABI PRISM Sequence Detection System and the Light Cycler system), which
10 avoid using radio-active isotopes and contamination and have a short processing time, and allow detection of specifically amplified nucleic acid sequences.

Samples in which a specific nucleic acid molecule needs to be detected are subjected to amplification (for example by real-time quantitative (RQ-)PCR) using a
15 primer pair specific for said molecule. Detection of the thus amplified molecule occurs fluorogenically in real time (i.e. during each amplification cycle) via a fluorogenic probe consisting of one of more oligonucleotides. Only detection in real-time allows
20 reliable and reproducible quantification of MRD. RQ-PCR makes use of data generated in the early productive PCR cycles where the fidelity of the PCR is still high. It enables detection with a high throughput of samples allowing automated testing. Samples are not restricted to
25 bone marrow samples but can also be blood samples or other cell samples. In a preferred embodiment of the invention, a patient- or malignancy-specific fluorogenic probe according to the invention is used.

One example exploits the exonuclease activity of
30 the Taq polymerase. The so-called TaqMan fluorogenic probe consists of an oligonucleotide to which a reporter dye and a quencher dye are attached. During amplification, the probe anneals to the template molecule somewhere between the location of the forward and reverse
35 primer sites. However, during the amplification process the annealed probe is cleaved by the 5' nuclease activity

of the polymerase. This separates the reporter dye from the quencher dye, generating an increase in the reporter dye's fluorescence, which finally allows real time detection of the amplified molecule, with an increase of
5 the fluorescent signal per PCR cycle.

Another example is based on the proximity of two oligonucleotides, together forming a fluorogenic probe for fluorescence emission. The first oligonucleotide is labelled with a donor fluorochrome that is excited by an
10 external light source and emits light that is absorbed by an acceptor fluorochrome present on a second oligonucleotide. When the first and second oligonucleotide are in close proximity they together form a fluorogenic probe according to the invention which
15 comprises an oligonucleotide specifically and selectively reacting with a junctional or fusion region which is only found with a specific patient and which is characteristic for his or her malignancy. During amplification of the target molecule the so-called fluorescence resonance
20 energy transfer (FRET) probe consisting of both nucleotides hybridises to the nucleic acid between the two primers to detect the amount of target, resulting in emission of light by the acceptor fluorochrome. During the annealing phase of each PCR cycle the amount of
25 fluorescence is a measure of the amount of PCR product formed so far. The fluorescent signal disappears when the probe dissociates during the extension phase of the PCR cycle. In this way the fluorogenic probe allows real-time quantitative detection of the PCR-target during the
30 annealing phases.

Samples that contain the wanted nucleic acid molecules that are reactive with said forward and reverse primer are thus identified by the presence of amplified product which is detected in real time by using said
35 fluorogenic probe.

Another embodiment of the invention is a method wherein the fluorescence of said probe is detected in real-time, during execution of the amplification, allowing the sensitive and quantitative detection of minimal residual disease to allow for earlier identification of patients belonging to high or low risk groups, allowing a clinical decision process that allows assigning the proper treatment to the proper patient. This allows to reduce false-negative results and avoids being to late with therapy when a patient with high risk has not yet been detected as such, and allows reducing false positive results since the probe is malignancy-specific and cross-contamination is reduced due to the absence of post-PCR processing and avoids cumbersome therapy in patients with low risk.

The invention also provides a fluorogenic probe which comprises an oligonucleotide specifically and selectively reacting with a junctional or fusion region which is only found with a specific patient and which is characteristic for his or her malignancy. One embodiment of the invention, exemplified in the experimental part, is a method wherein said fluorogenic probe is linked to a reporter dye and to a quencher dye, however in another embodiment, said probe comprises an oligonucleotide-linked donor fluorochrome and acceptor fluorochrome. Another embodiment of the invention is a method wherein said fluorogenic probe is reactive with a junctional region or fusion region of a malignancy-specific rearranged gene segments of Ig or TCR gene rearrangements or chromosome aberrations. Such a probe provided by the invention and/or a method provided by the invention is specifically used to detect and quantify the level of MRD, for example by using the ABI PRISM sequence detection system or the Light Cycler.

The invention also provides said probes in the context of a diagnostic assay, comprising necessary means

and methods to use a method provided by the invention. Said diagnostic assays or kit may also comprise primers, enzymes, buffers or other components that are necessary for the amplification of the rearranged gene segments.

5 The invention is further explained in the experimental part, which cannot be seen as limiting the invention.

EXPERIMENTAL PART

10

INTRODUCTION

Thanks to advances in modern chemotherapeutic treatment regimens during last decades complete clinical remission
15 can be induced in virtually all children and 75-80% of adults diagnosed with acute lymphoblastic leukemia (ALL). However, one third of pediatric and more than half of adult ALL patients is suffering from disease recurrence and further treatment intensification leads to increased
20 treatment-related toxicity and secondary malignancies. In order, to further increase the survival along with an improvement of the quality of life by preventing late toxicities, an individualization of the treatment may be needed. This can be realized by applying the results of
25 minimal residual disease (MRD) that allows the detection of leukemia invisible to normal morphologic examination, thereby providing more insight in the efficacy of cytostatic treatment. MRD analysis can predict outcome by determining the reduction of the leukemic burden during
30 the first months of therapy. Methods that allow sensitive MRD detection are (i) flow cytometric detection of leukemia-specific immunophenotypes, (ii) polymerase chain reaction (PCR) amplification of leukemia specific chromosomal aberrations, and (iii) PCR amplification of
35 clonogeneic rearrangements of immunoglobulin (Ig) and T-cell receptor (TCR) genes. The last method has the

broadest applicability in ALL. Using PCR techniques it is possible to detect one leukemic cell in a background of approximately 10^5 normal cells. This is about 100 to 10,000 times more sensitive than obtained with morphology.

Immature B and T lymphocytes rearrange the V, D, and J gene segments of their Ig and TCR genes in order to achieve antigen diversity. A molecular fingerprint is provided by the deletion and insertion of random nucleotides between the joined gene segments, the so-called junctional regions. In principal, all cells from a leukemia have the same junctional region, since they derive from one oncogenic progenitor. Thus, junctional regions of Ig/TCR gene rearrangements can be regarded as leukemia specific DNA fingerprints. Yet, oligoclonality of Ig/TCR gene rearrangements at diagnosis may occur, since these rearrangements are not linked to the oncogenic process. Furthermore, continuing rearrangements and secondary rearrangements during the disease course might result in the loss of the junctional regions initially identified at diagnosis. It therefore seems important to monitor ALL patients with two or more independent monoclonal Ig/TCR PCR targets to prevent false-negative results during follow-up.

A patient specific PCR primer or probe is usually designed to the sequence of the junctional region in order to detect the leukemia within the background of normal cells that may have similar gene rearrangements but different junctional regions. Especially, the usage of a patient-specific junctional region probe has shown to be highly effective in the detection of MRD. After PCR amplification of the Ig/TCR gene rearrangement, the PCR product is hybridized with the radioactively labeled patient specific probe. For that purpose, the PCR product is blotted after gel-electrophoresis or directly spotted on a nitrocellulose membrane, the so-called dot-blot

method. Alternatively, the PCR product is not fixed but hybridized as free DNA in liquid hybridization.

Although the high sensitivity of these MRD-PCR techniques, they provide merely semi-quantitative data owing to the analysis of end-point results. The PCR technique has the ability to amplify target DNA up to a plateau, but it consequently is impossible to define precisely the initial amount of target DNA. Strategies to overcome the limited quantitative potential of the PCR are the performance of competitive PCR or limiting dilution. Quantitation by competitive PCR is performed by comparing the PCR signal of the specific target DNA with that of known concentrations of an internal standard, the competitor. Quantitative estimations with the limiting dilution assay are established by using serial dilutions in multiple replicates of the target DNA. The dilution endpoint defines the amount of initial target DNA via Poisson's law. Both approaches are laborious, require multiple PCR analysis per sample and are difficult to perform routinely.

By several large prospective clinical MRD studies in childhood ALL it was shown that it is important to precisely determine the level of MRD to be able to discriminate between low and high risk patients. This was especially true if early remission time points were analyzed; at later time points also low levels of MRD have a risk of disease recurrence. By using the kinetics of tumor reduction during the first three months of therapy it was possible to recognize a low risk group (~43% of patients) with a relapse rate of only 2%, an intermediate risk group (~43%) with a relapse rate of 25%, and a high risk group (~15%) with a relapse rate of 75%. In the light of these results we were aiming at a fast method that would generate reliable quantitative MRD data to provide the means for stratification of ALL patients in the near future.

Recently, a novel technology has become available, the 'real-time' quantitative PCR (RQ-PCR). This assay exploits the 5' - 3' nuclease activity of the *Taq* polymerase to detect and quantify specific PCR products as the reaction proceeds. Upon amplification, an internal fluorogenic TaqMan probe specific for the target sequence is degraded resulting in emission of a fluorescent signal that accumulates during the reaction. Because of the real time detection, the method has a very large dynamic range, over five orders of magnitude, of initial target molecule determination. Thus, eliminating the need for performing serial dilutions of follow-up samples. Quantitative data can be accomplished in a short period of time, since post-PCR processing is not necessary.

MATERIALS AND METHODS

Patients and Cell samples

Four precursor-B-ALL (2145, 5160, 5199 and 5257) were randomly picked for this study, based on the presence of one or more Ig/TCR gene rearrangements as detected by Southern blot analysis. The probe and restriction enzyme combinations used for Southern blot analysis comprised: for the *IGH* locus the *IGHJH6* probe in *Bgl*III and *Bam*HI/*Hind*III digests; for the *TCRD* locus the *TCRDJ1* probe in *Eco*RI and *Bgl*III digests; for the *TCRG* locus the *Jy1.3* and *Jy2.1* probes in an *Eco*RI digest and the *J1.2* probe in a *Bgl*III digest; for the *IGK* locus the *IGKJ5*, *IGKC*, and the *IGKDE* probes in *Bgl*III and *Bam*HI/*Hind*III digest. Mononuclear cells (MNC) were obtained from peripheral blood (PB) or BM samples at diagnosis by ficoll density centrifugation. MNC samples were frozen and stored in liquid nitrogen. Good quality medium molecular weight DNA was isolated from MNC samples

using the QIAamp kit (Qiagen Inc, Chatsworth, CA)
(Verhagen et al., manuscript in preparation).

Patient specific probes

5 Sequences of the junctional regions were obtained by
direct sequence analysis of the Ig/TCR gene
rearrangements with the dye terminator ready reaction
cycle sequencing kit on an ABI PRISM 377 automated
sequencer of PE Biosystems. The template DNA used in the
10 sequence reaction was either the PCR product or a homo-
(or hetero-) duplex band excised and eluted from a
polyacrylamide gel in case of a bi-allelic gene
rearrangement. Based on the obtained sequence data
patient-specific oligonucleotides were developed
15 complementary to the sequence of the junctional region
(Table 1). Oligonucleotides that were likely to form of
secondary structures were avoided.

 OLIGO5.1 software (Dr. W. Rychlik; National
Biosciences, Plymouth, MN) was applied to design the
20 probes that were radioactively end-labeled with [α -
 32 P]dATP according to standard protocols. The T_m 's of the
radioactive probes ranged between 65-68°C.

 Fluorochrome labeled TaqMan probes were designed
with the Primer Express software (PE Biosystems). The
25 TaqMan probe did not start with a G and contained more
C's than G's according to the guidelines of PE
Biosystems. The melting temperature (T_m) was around 68°C,
8-9°C above the T_m of the matching primers, to ensure
proper hybridization to the target sequence. FAM was
30 chosen as reporter dye at the 5' end of the TaqMan probe
and TAMRA as the quencher dye at the 3' end.

RQ-PCR analysis

Primer design

Of two ALL patients primers and probes were designed for the detection of MRD using this new technique. From patient 1 an IgH rearrangement was applied as PCR target, from patient 2 an incomplete TCR-delta rearrangement. The fluorogenic probes or TaqMan® probes were positioned at the junctional region of mentioned rearrangement; for patient 1

5'-TGGTACTACTACAACCCATCCGCCCATCT-3' and patient 2: 5'-ATCCCCCAGTCCGGGCACAGTA-3'. Compatible primers at opposite of the junctional region were chosen using the Primer Express program (Applied Biosystems, Foster City, CA, USA). For amplification of the IgH rearrangement in patient 1 the forward primer: 5'-CACGGCTGTGTATTACTGTGCAA-3' and the reverse primer: 5'-GGTCGAACCAGTACCCAATAGC-3' were chosen giving a PCR product of 78 base pares. For amplification of the incomplete TCR-delta rearrangement the forward primer: 5'-GTAAGCTTAAGATACTTGACCATCAGAGA-3' and the reverse primer 5'-GAAGCTGCTTGCTGTGTTGTTC-3' were chosen and give a PCR product of 190 base pairs.

Primers matching to the designed TaqMan probes were developed as above using Primer-Express software (PE Biosystems) and had Tm's of 58-60°C. *IGH*-1 primers: forward, 5'-CACGGCTGTGTATTACTGTGCAA-3' and reverse, 5'-GGTCGAACCAGTACCCAATAGC-3'. *IGH*-2 primers: forward, 5'-GAGGACACGGCTGTGTATTACTGT-3' and reverse, 5'-ACCTGAAGAGACGGTGACCAT-3'. *IGH*-3 primers: forward, 5'-GAGGACACGGCTGTGTATTACTGT-3' and reverse, 5'-AGACGGTGACCAAGGGTTCC-3'. *IGK* primers: forward for *IGK*-1, 5'-AGCAGGGTGGAGGCTGA-3', for *IGK*-2 5'-GGTCAGGCACTGATTTCACT-3', and reverse for both *IGK*-1 and -2 5'-AAAAATGCAGCTGCAGACTCA-3'. *TCRG* primers (*TCRG*-1 and -2): forward, 5'-GCATGAGGAGGAGCTGGA-3' and reverse, 5'-GGAAATGTTGTATTCTTCCGATACTTAC-3'. *TCRD*-1 primers:

forward, 5'-GTACTTAAGATACTTGCACCATCAGAGA-3' and reverse, 5'-GAAGCTGCTTGCTGTGTTTGTC-3'. TCRD-2 primers: forward, 5'-GCAAAGAACCTGGCTGTACTTAAG-3' and reverse, 5'-GTTTTGTACAGGTCTCTGTAGGTTTTGTA-3'. The forward and
5 reverse primers to detect and quantify MRD in follow-up samples of patient 5257 by were : 5'-TGTCAGCAGTATGGTAGCTCACC-3' and 5'-AGTGGATATGGCAAAAATGCA-3', respectively.

10 PCR conditions

In a reaction mixture of 50 microliters containing 1x TaqMan buffer, 300 microM dATP, 300 microM dCTP, 300 microM dGTP, 600 microM dUTP, 1.25 U TaqGold (Perkin Elmer, Norwalk, CT, USA), 2.5 pmol probe, 300 nM forward
15 primer, and 300 nM reverse primer, the rearrangements were amplified with the following cycling protocol: 10 min. 95°C, followed by 15 sec. 95°C and 1 min. 60°C for 40 to 70 cycles.

20 For RQ-PCR analysis the TaqManTM PCR core reagent kit was used (PE Biosystems). Reaction mixtures of 50 µl contained the RQ-PCR buffer with the ROX dye as the passive reference, 5 mM MgCl₂, dNTP's: 0.3 mM dATP, 0.3 mM dCTP, 0.3 mM dGTP, and 0.6 mM dUTP, 50-900 mM primers,
25 1.25 U AmpliTaq GoldTM (PE Biosystems), 1 U uracil-N-glucosidase (UNG) and 50-1000 ng of DNA. The two-step amplification protocol consisted of a 2 minutes incubation step at 50°C (digestion of PCR product contaminants by UNG), 10 minutes at 95°C (inactivation of
30 UNG, denaturation of target DNA, and activation of AmpliTaq GoldTM), followed by target amplification via 40-70 cycles of 15 seconds at 95°C and 1 minute at 60°C.

Real time information was obtained using the ABI PRISM 7700 Sequence Detection System containing a 96 well
35 thermal cycler (PE Biosystems). During the PCR the TaqMan

probe first hybridizes to the DNA target, followed by primer annealing. With the TaqMan probe still intact, the emission of the reporter dye is quenched, but during the extension phase of the reaction the TaqMan probe is
5 cleaved by the exonuclease activity of the *Taq* polymerase. Subsequently, a fluorescent reporter signal is generated per cycle, which is proportional to PCR product accumulation. The fluorescence intensity is normalized using the passive reference ROX present in the
10 buffer solution. Normalization corrects for fluorescence fluctuations which are PCR independent. A real time amplification plot is generated using the normalized reporter signal (R_n). The PCR product yield or ΔR_n is defined as the R_n minus the baseline signal established
15 in the first few cycles of the PCR and is at least ten times the standard deviation of the noise. The cycle threshold (C_T) is the PCR cycle at which a statistically significant increase in ΔR_n is first detected.

To correct for the quantity and quality of DNA in remission follow-up samples, we used the gene encoding albumin. Sequences for primers and TaqMan probe, were kindly provided by Dr. E. Wensink.

All RQ-PCR experiments were performed at least in triplicate. Before determining the sensitivity of the PCR
25 target, the RQ-PCR was optimized. The primer concentration affect the T_m of the primer and can thus be used to optimize amplification efficiency on a fixed annealing temperature. Therefore, the amount of forward and reverse primer was determined that resulted in the
30 highest yield of specific PCR product. In this primer-matrix experiment, nine combinations of 50, 300, and 900 mM for each primer were tested in triplicate, i.e. 50/50, 50/300, 50/900, 300/50, 300/300, 300/900, 900/50, 900/300, and 900/900.

PCR target sensitivity

To determine the sensitivity of the PCR target, DNA from the sample at diagnosis was diluted in 10-fold steps into DNA from normal mononuclear cells (MNC), down to 10^{-7} . To avoid skewed gene rearrangement patterns and to obtain a bulk of the polyclonal control, the normal MNC DNA consisted of equivalent mixtures from ten different healthy donors. The dilution series was subjected to (RQ)PCR analysis together with appropriate positive and negative controls. The furthest dilution of diagnosis DNA that gave a radioactive or fluorescent signal, in the absence of a signal from the polyclonal control (MNC DNA), was defined as the sensitivity threshold of the PCR-target. The sensitivity threshold, based on the theoretical calculations, can be 10^{-4} (~8 copies of the target gene) or 10^{-5} (~0.8 copies of the target gene).

Conventional MRD detection techniques**PCR amplification**

The primers used for the PCR analysis of the Ig/TCR gene rearrangements were described previously. For the amplification of *IGK*, *TCRD*, and *TCRG* gene rearrangements, 1 µg DNA of diluted diagnosis material was used and 30 pmol of each primer in reaction mixtures of 100 µl containing 1 unit AmpliTaq GoldTM was used (PE Biosystems), 1.5 mM MgCl₂, 200 µM dNTP (Pharmacia, Uppsala, Sweden) in a final volume of 50 µl. The cycling protocol consisted of 3 minutes of initial denaturation at 92°C, followed by 40 cycles of 45 seconds at 92°C, 90 seconds at 60°C, 2 minutes at 72°C, and a final extension phase of 10 minutes at 72°C.

Rearrangements of the *IGH* gene locus were amplified in 50 µl reactions containing, 1 µg DNA, 30 pmol of each

primer, 2 units Taq polymerase, 2 mM MgCl₂, and 200 µM dNTP. The cycling protocol consisted of 7 minutes of initial denaturation at 95°C, followed by 30 cycles of 30 seconds at 95°C and 45 seconds at 55°C, and a final
5 extension phase of 7 minutes at 72°C.

PCR products were examined after gel electrophoresis in 1% agarose and or 6-10% polyacrylamide gels and ethidium bromide staining.

10 Dot-blot hybridization

After PCR amplification of the Ig/TCR gene rearrangements, 5 µl of the denaturated PCR product was spotted in duplicate onto a Nytran N13 nylon membrane of 0.45 µm (Schleicher & Schuell, Dassel) and cross-linked
15 by UV-exposure. The filter was hybridized at 50°C for two hours with 0.5-1.0 pmol of the radioactively labeled patient specific probe per ml hybridization buffer. Filters were subsequently washed for 20 minutes in 3x SSC, 0.1% SDS at 50°C. Radioactive signals were evaluated
20 by phosphor imaging (STORM-820, B&L Systems, Maarssen).

Liquid hybridization

Five µl of PCR product was hybridized with
25 approximately 1 ng of the radioactively labeled probe in 2x SSC buffer for 15 minutes at 60°C after denaturation for 10 minutes at 95°C. Subsequently, the mixtures were size separated by electrophoresis through a 10% polyacrylamide gel. Radioactive signals were evaluated by
30 autoradiography after drying of the gels.

RESULTS

Only the amplified rearrangements of the leukemic cells in patient 1 and 2 were detected by the ABI PRISM 7700,
35 and not similar rearrangements in the MNC that were co-amplified, since the TaqMan probe only hybridizes with

the leukemia specific junctional region. For both patients a linear correlation was found between the initial amount of leukemic derived DNA and the threshold cycle. The threshold cycle is that cycle where the
5 fluorescence emitted during the amplification of the target molecule rises above a certain threshold.

Despite consumption of primers and dNTP by the amplification of similar rearrangements in MNC, in the 10^{-4} dilution still leukemic specific rearrangements could be
10 detected, both in patient 1 and in patient 2. Thus, using this method it was possible to detect at least one leukemic cell in 10,000 normal cells, which is satisfactory to obtain reliable MRD information for a diagnostic setting. This sensitivity is comparable to
15 what is achieved with current techniques using a radioactively labeled junctional region probe that detects the leukemic cells after PCR amplification of the rearrangement (MRD review Tomek, Leukemia manuscript).

The advantage of the new method is that two steps
20 (amplification and identification) are taken together, this saves time so that MRD information is earlier available for the clinic. The absence of post-PCR processing of amplified DNA drastically reduces the risk of contamination which otherwise can be a major problem
25 for MRD detection where samples are analyzed together with high and low tumor loads. Another advantage of the method is the substitution of radioactive probes by fluorescent probes. Finally, the most important advantage of this method is the possibility of precise
30 quantification of MRD in blood or bone marrow samples.

Nine Ig/TCR gene targets (3 *IGH*, 2 *TCRD*, 2 *TCRG*, and 2 *IGK*) of three precursor-B-ALL were examined for their sensitivity. For all gene rearrangements it was possible to develop primer/probe pairs that resulted in successful
35 amplification and real time detection upon RQ-PCR analysis. For 4 out of 9 PCR targets it was necessary to

design the TaqMan probe complementary to the reverse strand of the junctional region, due to the high extent of G's (Table 2). The initial primer pair to amplify *TCRG*-1 and -2 PCR targets resulted upon RQ-PCR analysis in low sensitivities (10^{-2}), which was unexpected because both gene rearrangements used the rarely used Vy7 gene segment. A new primer pair was designed, given in the Material and Methods section, which were used in combination with the initially designed TaqMan probes; subsequent RQ-PCR analysis resulted in better amplification with higher sensitivities for both *TCRG* targets (Table 2). All the other primer/probe combinations initially designed using Primer Express resulted in efficient amplification according to the amplification plots and sensitivities obtained.

In primer-matrix experiments different ΔR_n values were found for the different primer combinations, while the C_T value were the same. The 300/300 nM primer combinations were used in all sensitivity experiments performed, although other combinations sometimes had equal ΔR_n values.

Standard curves were established from the results of the dilution experiments and displayed for each PCR target a linear correlation between the C_T and the logarithm of the initial DNA target concentration (Figure 2). The dynamic range spanned over up to five orders of magnitude. Triple or quadruple experiments had similar values, but at low target concentrations some variation in the C_T was observed (Figure 2).

Comparison of sensitivities of the three detection methods

For dot-blot and liquid hybridization, the same PCR product and radioactive probes were used, but different hybridization methods. The sensitivity results obtained

with these conventional methods were compared with those of the RQ-PCR analyses, which used specially designed primer/probe combinations, but the same DNA dilutions (Figure 3). Since RQ-PCR was performed in triplicate, the sensitivity of RQ-PCR was based on at least two out of three positive experiments.

The sensitivities of RQ-PCR analysis varied between 10^{-2} and 10^{-4} (Table 3). The sensitivities obtained by RQ-PCR analysis were most similar to that of the dot-blot method (Table 3, Figure 3). Using the *IGK-2* PCR target even a higher sensitivity was found than obtained with dot-blot analysis, but for the other PCR targets similar or 10-fold lower sensitivities were found. In contrast, liquid hybridization was always more sensitive, either 10-fold or even 100-fold.

The lowest sensitivity was obtained with the TCRD-2 target containing a junctional region without insertion (Table 2). For this reason, the specificity of the patient specific probes were limited. To decrease background signals, the liquid hybridization temperature was increased to 64°C and an extra washing step (0.3x SSC, 0.1% SDS at 55°C) was necessary to remove background signal from the dot-blot. To increase the stringency in RQ-PCR analysis we performed another experiment in which the annealing/extension temperature was increased to 63°C and the amount of TaqMan probe lowered to 100 nM. However, this still resulted in a ΔR_n for the polyclonal MNC control, thereby limiting the sensitivity to 10^{-2} .

MRD analysis of follow-up samples using RQ-PCR

A total of 12 BM follow-up samples from patient 5257, taken during and after treatment, were available for MRD analysis. RQ-PCR analysis using a *VkIII-Kde* gene rearrangement was performed in triplicate, and diagnosis dilutions and follow-up samples were analyzed in parallel. In addition, diagnosis and follow-up samples

were checked for the amount and integrity of DNA by performing a albumin RQ-PCR. Quantities were determined using a standard curve of MNC DNA diluted in milli-Q. By dividing the diagnosis quantity by the follow-up quantity
5 a ratio is established that can be used to correct the MRD level generated by the leukemia specific RQ-PCR.

With a sensitivity of 10^{-4} the first four time points appeared to be MRD positive, the level of MRD slowly decreased form 1.3×10^{-2} at week 5 down to 2.2×10^{-4} at
10 week 33 (Table 4). All further follow-up samples were MRD negative including at the end of treatment (week 158) until relapse that emerged four years after diagnosis (Table 4).

15 DISCUSSION

The aim of this study was to test the value of RQ-PCR analysis using the TaqMan™ technology for sensitive and quantitative detection of MRD in follow-up samples using rearranged Ig and TCR genes as PCR targets. In our
20 analysis, we have chosen for a patient specific junctional region TaqMan probe and matching primers. This technique was applicable for all PCR targets tested, i.e. complete *IGH*, *IGK-Kde*, *TCRG*, and incomplete *TCRD* gene rearrangements. The majority of PCR targets tested (7 out
25 of 10) were derived from patient 5199. The reason for analyzing most (7 out of 8) of the Ig/TCR gene rearrangements of this patient was to prevent selection of "sensitive" targets and to obtain insight into the possibilities for finding suitable primers and TaqMan
30 probe combinations for RQ-PCR analysis. All junctional region specific TaqMan probes developed were able to detect leukemia derived DNA and did not interfere with the PCR efficiency.

The high number of PCR targets in patient 5199 might
35 be due to oligoclonality at diagnosis. This was not evident from Southern blot analysis, except for the three

rearrangements of albeit equal intensity in the *TCRG* gene locus. This may be explained by an extra chromosome 7 or by two clonal populations of similar size. Two other findings point towards oligoclonality: (i) a large proportion of the Ig/TCR rearrangements appeared not to be stable at relapse (data not shown) and (ii) The sensitivities obtained by dot-blot analysis in this patient are not representative, only 29% (2/7) reached a 10^{-4} , while in a series of over 300 dot-blot analysis 87% of the PCR targets reached a sensitivity of 10^{-4} . We accordingly used the PCR targets of patient 5199 for relative comparison, but not for determining the sensitivity of the technique. Hence, all three techniques reached sensitivities of at least 10^{-4} and 10^{-5} ; *IGH*, *IGK*, and *TCRD* PCR targets in patients 2145, 5160, and 5257.

Upon comparison, the sensitivities obtained with the RQ-PCR technique were without further optimization similar to those obtained with the dot-blot method. The liquid hybridization with radioactively labeled probes appeared to be most sensitive. In principle, it should be possible to reach even higher sensitivities with RQ-PCR since hybridization with the TaqMan probe is also a liquid hybridization, unless the total detection system based on fluorescence, is less sensitive as compared to radiography.

In another RQ-PCR application, one point mutation appeared to be sufficient for allelic discrimination by the TaqMan probe. The T_m of the TaqMan probe was in this case 5 to 6°C above that of the corresponding primers. This adaptation may also be necessary to increase the specificity for RQ-PCR analysis of the *TCRD-2* in this study, which lacked randomly inserted nucleotides. This would mean that for this target a new TaqMan probe should be developed.

An alternative approach to RQ-PCR analysis of Ig/TCR gene rearrangements might be to use a TaqMan probe

positioned at germline sequences (V, D, or J gene segments) in combination with one or two patient specific junctional region primers. A similar strategy has also been used for conventional MRD detection using Ig/TCR gene rearrangements as PCR targets. This RQ-PCR approach will be more cost-effective, since it would be not necessary to design TaqMan probes per patient but per type of rearrangement. However, standardization will be more difficult, since adaptation of the annealing temperature may be required per case to prevent aspecific amplification. Nevertheless, comparative studies should demonstrate which strategy gives best sensitivity, which may also vary per target type.

Twelve BM follow-up samples of a precursor-B-ALL were analyzed for MRD by RQ-PCR, using a VkIII-Kde gene rearrangement. The standard curve established with the diagnosis dilution series, was used to define the initial amount of leukemic DNA in follow-up samples based on their Ct. Subsequently, the MRD level was corrected relative to the diagnosis sample based on albumin RQ-PCR analysis. A slow decrease of tumor load was observed during the first year of treatment. According to the literature, this is indicative of a poor outcome; the leukemia is relative resistant to the given treatment. Indeed, this patient relapsed almost two years after cessation of therapy and more than seven months after the last time point analyzed, which was still MRD negative.

Except for MRD analysis of follow-up samples, RQ-PCR may also be helpful in the identification of suitable PCR targets at diagnosis. By precise quantification it should be possible to discriminate between true-allelic Ig/TCR gene rearrangements and minor gene rearrangements derived from subclones. Subclonal rearrangements are frequently reported in ALL, but are inappropriate PCR targets for MRD detection. Perhaps RQ-PCR can replace Southern blot analysis in the future, which is still considered as the

golden standard for MRD target identification but is laborious, time-consuming and requires large amounts of good quality DNA.

Our data show that real time PCR analysis allows
5 accurate definition of the level of MRD in bone marrow follow-up samples, which is a major improvement to the end-point PCR analysis of conventional MRD detection methods. Similar CT values were accomplished for quadruple or triple experiments indicating the high
10 reproducibility of the technique. The RQ-PCR technique takes advantage of the first productive PCR cycles for quantitation, where the PCR fidelity is still high; exhaustion of reagents does not yet exists and potential inhibitory effects are minimal. Due to the semi-automated
15 system it is easy to analyze more DNA per follow-up sample. This does not only result in a more accurate definition of the tumor load in the follow-up sample, but simultaneously increases the sensitivity of the MRD detection technique. The sensitivity ultimately depends
20 on the maximal number of cells tested; by performing the experiment in quadruple two to four μg of DNA may be analyzed, corresponding to approximately 5×10^5 cells.

The here presented results demonstrate that RQ-PCR is applicable for MRD analysis via detection of clone-
25 specific Ig/TCR gene rearrangements. RQ-PCR offers many advantages over currently used techniques. The dot-blot and liquid hybridization are dependent of using radioactive isotopes and require individual optimization of the hybridization or extra washing steps. In contrast
30 to these methods RQ-PCR analysis is simple and fast; data can be acquired as soon as the PCR is completed without any post-PCR handling, i.e. within 3 hours, instead of 5 days generally required for conventional methods. The closed system minimizes the risk of PCR product
35 contamination, which is very important in this kind of studies where minimal amounts of target are amplified.

Most importantly, this novel technique allows accurate quantification of MRD, essential for the discrimination between patients with good and poor prognosis. Therefore, we consider this sensitive and reproducible RQ-PCR
5 technique as an important step forward in the clinical application of MRD studies.

TABLE 1. Detection of MRD in cell samples from patients with a lymphoid malignancy.

Technique	Detection limit	Applicability	Requirements, limitations, and pitfalls
Immunological marker analysis			
- Multiparameter flow cytometry (scatter pattern and double or triple labeling of membrane markers)	10^{-2} to 10^{-4}	- 40% to 50% of childhood precursor-B-ALL - 70% to 80% of adult precursor-B-ALL - 90% of T-ALL and lymphoblastic T-NHL	- Only applicable in cases with unusual or aberrant immunophenotype (including ectopic and tumor-specific antigen expression) - Normal cell populations (especially in BM) will influence the detection limit
- T-cell marker/TdT double IF staining (fluorescence microscopy)	10^{-4} to 10^{-5}	- > 95% of T-ALL	- Experience with fluorescence microscopy and TdT staining patterns is advised
- Single Ig light chain expression: Igκ/Igλ ratio 10^{-1} to 10^{-3} (double or triple IF staining in B-cell populations or specific B-cell subsets)		all Ig light chain ⁺ B-cell malignancies	- Weak SmIg light chain expression on leukemic cells (e.g. in B-CLL) may hamper their detectability - Occurrence of normal SmIg light chain⁺ B-cells will influence the detection limit
- Vβ, Vγ, and Vδ expression: excess of TCR-V gene expression (double or triple IF staining in T-cell populations or specific T-cell subsets)	10^{-1} to 10^{-3}	all TCR ⁺ T-cell malignancies	- Occurrence of normal T-cells will influence the detection limit - Oligoclonal T-cell subsets might occur in the elderly

Continued on next page

Table 1 continued

PCR techniques

Junctional regions of rearranged Ig and TcR genes (DNA level)

10^{-3} to 10^{-6}	- > 90% of precursor B-ALL - > 95% of T-ALL - > 95% of chronic lymphoid leukemias - 60%-80% of B-NHL and multiple myeloma - > 95% of T-NHL	- Rearrangements have to be detected and identified precisely by use of well-designed PCR primers - The occurrence of somatic hypermutation of Ig genes in B-NHL and multiple myeloma might prevent proper primer annealing and thereby inhibit detection and identification of Ig gene rearrangements at diagnosis and during follow-up - Junctional regions have to be sequenced in order to design junctional region-specific probes for each individual patient - Oligoclonality and clonal evolution at Ig or TcR gene level may cause false-negative results - Occurrence of normal cells with rearrangements of the same gene segments as the malignant cells influence the detection limit
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Chromosome aberrations with well-defined breakpoints at DNA level

10^{-4} to 10^{-6}	- 20% to 25% of T-ALL - 25% to 40% of B-NHL	- Availability of well-designed PCR primers - Fusion region oligonucleotide probes are useful for identification of PCR products from different patients
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Chromosome aberrations resulting in leukemia-specific fusion genes and fusion mRNA (PCR analysis after reverse transcription into cDNA-RT-PCR analysis)

10^{-4} to 10^{-6}	- 30%-35% of childhood precursor-B-ALL - 35%-40% of adult precursor-B-ALL - 15% to 20% of T-ALL - 20%-25% of childhood NHL - < 5% of adult NHL	- Availability of well-designed RT-PCR primers - Cross-contamination of RT-PCR products is the main pitfall (even cross-contamination between samples from different patients appears to occur) - Detection limit is dependent on the abundance of fusion gene transcription and the efficiency of the reverse transcriptase step
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TABLE 2. PCR targets and junctional region probes used for MRD detection.

Patient	PCR target	gene rearrangement	Junctional region	Radioactive probe ^a	TaqMan probe ^a
2145	IGH-1	VH3-13 JH5b	0 / GATGGCG / -6 / DH2-2 / -3 / TGGGT / -3	5'-TACCAGCTGCTATTGGGT-3'	5' AGATGGCGGATGGGTTGTAGTAGTACCA-3', ^d
5160	TCRD-1	V82 D83 -	-9 / CGG / 0	5'-ACTGTGCCCGGACTGGGG-3'	5'-TACTGTGCCCGGACTGGGGAT-3', ^d
5199	IGH-2	VH3-30.3 JH3b	-1 / GAGGGCACTGCTGA / 0	5'-GGCACTGCTGATGATGCTTT-3'	5'-GGCACCTGCTGATGATGCTTTTGTATCTG3', ^d
	IGH-3	VH6-1 - JH4b	0 / GATTCCGATAG / 0 / DH7-27 / -1 / CCAACTAGG / 8	5'-GCCAAACTAGGGACTACTG-3'	5'-TGGGGGCCAAACTAGGGACTACTGGG-3', ^d
	IGK-1	V λ I1 - Kde ^b	-8 / CGAACGAAAG / -6	5'-GGTACACACCCGAAACGAAAGCTAGT-3'	5'-CACACCGAACGAAAGCTAGTGGA-3'
	IGK-2	V λ I1 - Kde ^b	-4 / TCCCGGAGG / -3	5'-CACACTGGCTCCCGAGG-3'	5'-ACTGGCTCCCGAGGGCCC-3'
	TCRD-2	V82 - D83	-10 / 0 / -1 ^c	5'-TACTGTGCTGGGGGATACGC-3'	5'-AAGGGTCTTACTACTGTGCTGGGGGATA-3', ^d
	TCRG-1	V γ 7 - J γ 1.3	0 / CCTGAGG / -16	5'-TGAGGGCTCTTTGGCAGTGG-3'	5'-AGCCTGAGGGCTCTTTGGCA-3', ^d
	TCRG-2	V γ 7 - J γ 2.3	-12 / CCCAGGC / -11	5'-CCCAGGCAGAAACTCTTTGG-3'	5'-ATCTATTACTGTGCCCCCAGGCAGAAC-3'
5257	IGK-3	V λ III - Kde	0 / GGTTCCCA / -18	5'-CGGTTCCCAACAGGGCGAC-3'	5'-CGGTTCCCAACAGGGCGAC-3'

a. Underlined sequences represent randomly inserted nucleotides of the junctional region.

b. V λ member was A17.

c. No insertion of nucleotides; deletion can also be judged as -7 / 0 / -4 or even -8 / 0 / -3.

d. TaqMan probe was designed to the reverse strand.

TABLE 3: Comparison of sensitivities obtained by RQ-PCR with those obtained by conventional MRD detection techniques.

Patient code	PCR target	<u>Sensitivity</u>		
		RQ-PCR	Dot-blot hybridization	Liquid hybridization
2145	<i>IGH</i> -1	10^{-4}	NT	10^{-5}
5160	<i>TCRD</i> -1	10^{-4}	10^{-4}	NT
5199	<i>IGH</i> -2	10^{-3}	10^{-3}	10^{-4}
	<i>IGH</i> -3	10^{-3}	10^{-4}	10^{-4}
	<i>IGK</i> -1	10^{-3}	10^{-3}	10^{-5}
	<i>IGK</i> -2	10^{-4}	10^{-3}	10^{-5}
	<i>TCRD</i> -2	10^{-2}	10^{-3}	10^{-3}
	<i>TCRG</i> -1	10^{-3}	10^{-3}	10^{-5}
	<i>TCRG</i> -2	10^{-3}	10^{-4}	10^{-5}
5257	<i>IGK</i> -3	10^{-4}	10^{-5}	NT

TABLE 4. RQ-PCR analysis of twelve bone marrow samples during follow-up of patient 5257 using an V χ III-Kde gene rearrangement as PCR target, with a sensitivity of 10^{-4}

5					
	Weeks after diagnosis	Cycle* threshold	Mean MRD level	Corrected MRD level	Standard deviation
10	5	27.14	1.0×10^{-2}	1.3×10^{-2}	0.25×10^{-2}
		26.67			
		26.35			
15	13	30.29	2.0×10^{-3}	3.3×10^{-3}	0.45×10^{-3}
		30.27			
		30.11			
20	23	33.34	4.8×10^{-4}	7.0×10^{-4}	0.67×10^{-4}
		33.18			
		33.44			
25	33	37.54	6.2×10^{-5}	2.2×10^{-4}	2.13×10^{-4}
		36.24			
		50.00			
30	69/82/95/ 110/116/ 145/158	50.00 50.00 50.00	0 0 0	0	-
	191	21.49	1.2×10^{-1}	2.2×10^{-1}	0.13×10^{-1}
	(relapse)	21.35 21.30			

* Data in triplicate

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Legends to the figures

Figure 1 Diagram of the putative relative frequencies of leukemic cells in blood and bone marrow of acute leukemia patients during and after treatment and during development of relapse. The detection limit of cytomorphologic techniques as well as the detection limit of immunologic marker analysis and PCR techniques are indicated. I-Rx = induction treatment; M-Rx = maintenance treatment

Figure 2 An RQ-PCR sensitivity experiment of a precursor-B-ALL patient (2145) was performed using an *IGH* gene rearrangement (VH3-JH5b; OGH-1). (A) The real time amplification plots of the diagnosis dilutions for one series of experiments. (B) The standard curve shows the linear correlation between the cycle threshold (CT) and the initial amount of DNA (tumor load) of all four experiments. With this *IGH* gene rearrangement a sensitivity of 10^{-4} was reached in 4 out of 4 experiments.

Figure 3. The three MRD detection methods for an *IGK* gene rearrangement of precursor-B-ALL 5199 (JGK-2). (A) Schematic diagram of the VκII-Kde PCR target with a patient specific junctional region of a total of 7 nucleotides deleted and 9 nucleotides randomly inserted. Given are the sequences and relative positions of the primers used for RQ-PCR and conventional MRD methods, as well as the patient-specific junctional region probes. (B) Result of the dot-blot hybridization with the radioactively labeled junctional region probe after PCR amplification of the VκII-Kde gene rearrangements in a diagnosis dilution series. With this technique a sensitivity of 10^{-3} was obtained. (C) Liquid hybridization of the same PCR product and the same probe gave

a sensitivity of 10^{-5} . (D) RQ-PCR analysis of the V_kII-Kde PCR target with a different primer set and a fluorogenic TaqMan probe. The experiment was performed in triplicate on the serial diagnosis dilution. Real-time information of PCR product accumulation is given at the left. The standard curve at the right illustrates the linear correlation between the cycle threshold and the initial amount of DNA. With RQ-PCR analysis a sensitivity of 10^{-4} was reached in 1 out of 3 experiments.

CLAIMS

1. A method for determining minimal residual disease comprising amplifying nucleic acid molecules using at least one primer reactive with a common gene segment and further comprising identifying malignancy-specific nucleic acid
5 sequences by hybridising with a fluorogenic probe specifically and selectively reactive with said malignancy-specific nucleic acid molecules.
2. A method according to claim 1 wherein said fluorogenic probe is linked to a reporter dye and to a quencher dye.
- 10 3. A method according to claim 1 or 2 wherein said fluorogenic probe is reactive with a junctional or fusion region of a malignancy-specific rearranged gene segment.
4. A method according to claim 1, 2 or 3 wherein the fluorescence of said probe is detected during amplification.
- 15 5. A method according to any one of claims 1 to 4 allowing quantitative detection of minimal residual disease.
6. A fluorogenic probe comprising an oligonucleotide specifically and selectively reacting with a junctional or fusion region characteristic for a patient's malignancy.
- 20 7. A probe according to claim 6 wherein said oligonucleotide is linked to a reporter dye and to a quencher dye.
8. Use of a method according to any of claims 1 to 5 and/or a probe according to claim 6 or 7 in the determination of the
25 level of minimal residual disease.
9. A diagnostic kit or assay comprising the use according to claim 8.

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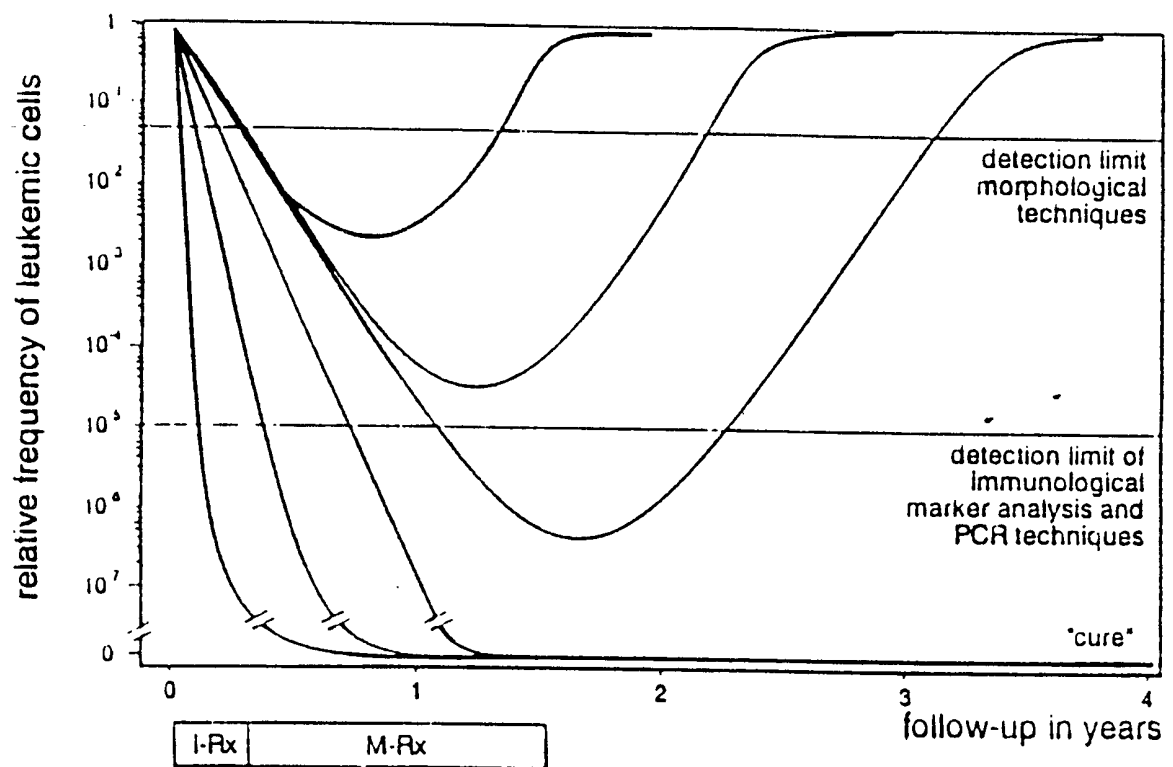
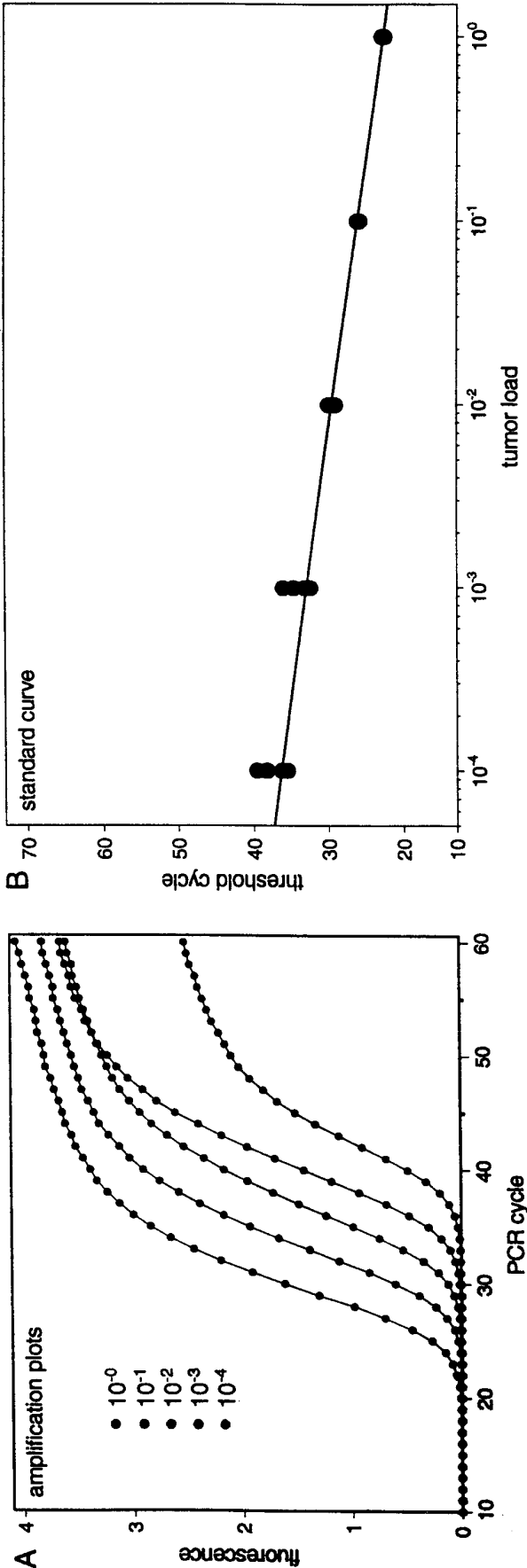


Figure 1

RQ-PCR of an *IGH* gene rearrangement



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Comparison of three MRD-PCR techniques

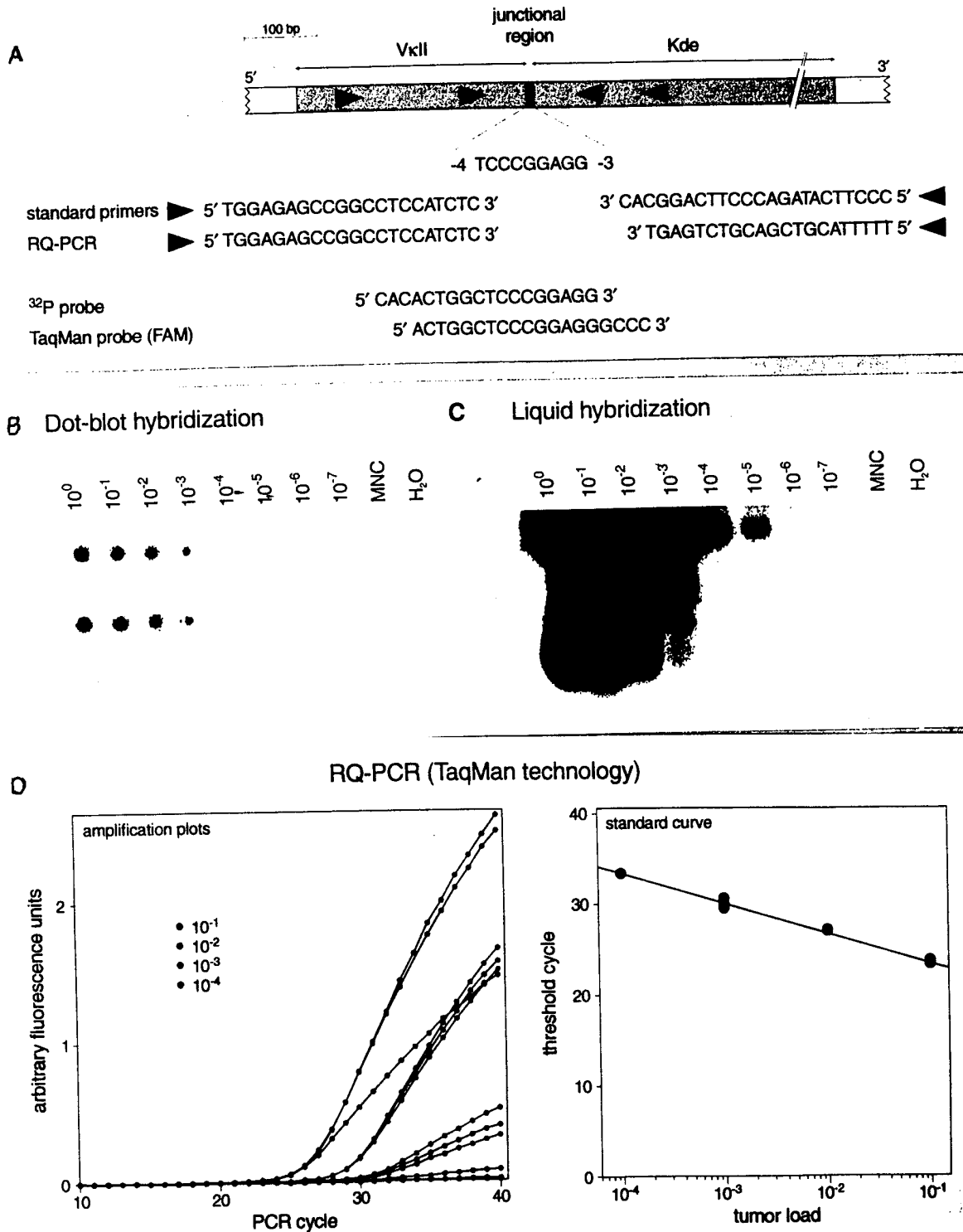


Figure 3