Leukemia (2003) 17, 1013–1034

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REVIEW

Detection of minimal residual disease in hematologic malignancies by real-time quantitative PCR: principles, approaches, and laboratory aspects

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Detection of minimal residual disease (MRD) has prognostic value in many hematologic malignancies, including acute lymphoblastic leukemia, acute myeloid leukemia, chronic myeloid leukemia, non-Hodgkin's lymphoma, and multiple myeloma. Quantitative MRD data can be obtained with real-time quantitative PCR (RQ-PCR) analysis of immunoglobulin and T-cell receptor gene rearrangements, breakpoint fusion regions of chromosome aberrations, fusion-gene transcripts, aberrant genes, or aberrantly expressed genes, their application being dependent on the type of disease. RQ-PCR analysis can be performed with SYBR Green I, hydrolysis (TaqMan) probes, or hybridization (LightCycler) probes, as detection system in several RQ-PCR instruments. Dependent on the type of MRD-PCR target, different types of oligonucleotides can be used for specific detection, such as an allele-specific oligonucleotide (ASO) probe, an ASO forward primer, an ASO reverse primer, or germline probe and primers. To assess the quantity and quality of the RNA/DNA, one or more control genes must be included. Finally, the interpretation of RQ-PCR MRD data needs standardized criteria and reporting of MRD data needs international uniformity. Several European networks have now been established and common guidelines for data analysis and for reporting of MRD data are being developed. These networks also include standardization of technology as well as regular quality control rounds, both being essential for the introduction of RQ-PCR-based MRD detection in multicenter clinical treatment protocols.

Leukemia (2003) **17,** 1013–1034. doi:10.1038/sj.fleu.2402922
Keywords: minimal residual disease (MRD); real-time quantitative PCR (RQ-PCR); leukemia; lymphoma; multiple myeloma; immunoglobulin; T-cell receptor; fusion genes

Introduction

During the last decade, a large number of studies have shown that detection of very low numbers of malignant cells, that is, detection of minimal residual disease (MRD), significantly correlates with clinical outcome in many hematologic malignancies. In certain categories of hematologic malignancies, MRD information is important for clinical decision-making (Table 1).[1–4] For example, detection of MRD during the initial phase of therapy in childhood acute lymphoblastic leukemia (ALL) allows significantly better stratification of patients into risk groups as compared with classical risk groups based on other relevant clinical and biological ALL characteristics.[5,6] In acute promyelocytic leukemia (APL) and chronic myeloid leukemia (CML), MRD information at specific time points enables effective early intervention treatment.[7,8] Based on these data, MRD detection is now becoming routinely implemented in several treatment protocols and is increasingly used for guiding therapy[9] or for evaluation of new treatment modalities, for example, the tyrosine kinase inhibitor Imatinib (STI571) for patient with Philadelphia chromosome-positive CML,[10,11] the CD20 antibody Rituximab for patients with B-cell non-Hodgkin's lymphoma (NHL),[12,13] or the CD33-calicheamicin conjugate Gemtuzumab Ozogamicin (Mylotarg) for patients with AML.[14]

Although qualitative MRD information can be highly significant (eg in APL),[15] it only gives limited information and does not allow precise analysis of tumor load kinetics. Therefore, several groups have developed (semi)quantitative methods that enable accurate assessment of the number of leukemic cells at consecutive follow-up time points. Quantitative MRD data indeed appeared to be crucial for appropriate evaluation of treatment response in ALL,[5,16] AML,[8,17–28] and CML.[7,27,30]

Quantitative MRD detection can be achieved by three main techniques: flow cytometric immunophenotyping using tumor-associated aberrant immunophenotypes,[31–34] PCR techniques using tumor-specific DNA (eg immunoglobulin gene rearrangements) targets, and reverse transcriptase (RT) PCR techniques using tumor-specific RNA targets (eg fusion-gene transcripts). In this review, we focus on PCR-based MRD techniques and the possibilities for quantitative MRD detection.

Quantitation of the MRD-PCR target can be performed by comparing the PCR signal (often after blotting and hybridization) with serial dilutions of a standard with known amounts of target DNA or RNA,[5,35] by limiting dilution experiments until negative PCR results are obtained,[36,37] and by competitive PCR.[28,29,38,39] However, 'real-time' quantitative PCR (RQ-PCR) methods have recently been developed and, together with recent GeneScan technology,[40–43] may replace the complex and time-consuming (semi)quantitative PCR analyses. Here, we will discuss the recent technical developments in RQ-PCR analysis, with emphasis on detection of MRD in hematologic malignancies.

RQ-PCR analysis: principles of the techniques

RQ-PCR permits accurate quantitation of PCR products during the exponential phase of the PCR amplification process, which is in full contrast to the classical PCR end point quantitation. Owing to the real-time detection of fluorescent signals during and/or after each subsequent PCR cycle, quantitative PCR data can be obtained in a short period of time and no post-PCR

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Received 12 November 2002; accepted 6 February 2003

Table 1 Clinical applicability of MRD detection in hematologic malignancies

	Type of MRD application			
	Early response to front-line treatment	*Continuous monitoring for therapy titration*	*MRD assessment before SCT*[a]	*MRD assessment after SCT*[a]
Lymphoid malignancies				
ALL[1,77,131,214–216]	++	+[b]	++	+
B-CLL[80]	—	+[c]	+	+
B-NHL[80]	—	+[c]	+	+
Multiple Myeloma[66]	—	+[c]	+	+
Myeloid malignancies				
APL[15]	++	++	+	+
AML[217–220]	++	—	+	+
CML[170]	+	+[d]	+	++

++Value of MRD detection proven by prospective studies.
+Potentially clinically relevant but not yet proven by large prospective studies.
−MRD detection has no additional value as compared with conventional cytomorphological techniques.
[a]SCT: stem cell transplantation.
[b]Late time points are potentially valuable for high-risk and intermediate-risk patients.
[c]Only relevant for patients treated with more aggressive protocols and/or including CD20 antibody.
[d]Monitoring of treatment protocols which include Imatinib.

processing is needed, thereby drastically reducing the risk of PCR product contamination. At present, three main types of RQ-PCR techniques are available.

RQ-PCR analysis using SYBR Green I Dye

The simplest RQ-PCR technique is based on detection of PCR products by the DNA-intercalating dye SYBR Green I (Figure 1a). This dye can bind to the minor groove of double-stranded DNA, which greatly enhances its fluorescence. During the consecutive PCR cycles, the amount of double-stranded PCR product will exponentially increase, and therefore more SYBR Green I dye can bind and emit its fluorescence (at 520 nm). The fluorescence signal will gradually increase during the extension phase, will be maximal at the end of each extension phase, and will be low or absent during the denaturation phase.

It should be noted that SYBR Green I-based detection of PCR products is not sequence specific and that consequently also nonspecifically amplified PCR products and primer dimers will be detected. To evaluate whether specific PCR products have been formed, a melting curve analysis can be performed (Figure 2).[44] In such analysis, the temperature is slowly increased from 40 to 95°C with continuous monitoring of the fluorescence. Fluorescence will be high at low temperatures when all DNA will be double stranded, but will drastically decrease around the melting temperature of the DNA products. PCR products of different length or sequence will melt at different temperatures and will be observed as distinct peaks when plotting the first negative derivative of the fluorescence *vs* temperature (Figure 2). If only the specific PCR product has been formed, only a single peak should be visible in the melting peak profile.

SYBR Green I is the most frequently used dye in nonspecific detection systems, but recently other dyes, such as Amplifluor,[45] have been developed.

RQ-PCR analysis using hydrolysis probes

RQ-PCR analysis with hydrolysis probes exploits the 5′→3′ exonuclease activity of the *Thermus aquaticus* (*Taq*) polymerase to detect and quantify specific PCR products as the reaction proceeds (Figure 1b).[46] The hydrolysis probe, also referred to as TaqMan probe or double-dye oligonucleotide probe, is conjugated with a reporter fluorochrome (eg FAM, VIC, or JOE) as well as a quencher fluorochrome (eg TAMRA) and should be positioned within the target sequence. As long as the two fluorochromes are in each other's close vicinity, that is, as long as the probe is intact, the fluorescence emitted by the reporter fluorochrome will be 'absorbed' by the quencher fluorochrome. However, upon amplification of the target sequence, the hydrolysis probe is initially displaced from the DNA strand by the *Taq* polymerase and subsequently hydrolyzed by the 5′→3′ exonuclease activity of the *Taq* polymerase. This results in the separation of the reporter and quencher fluorochrome and consequently the fluorescence of the reporter fluorochrome becomes detectable. During each consecutive PCR cycle, this fluorescence will further increase because of the progressive and exponential accumulation of free reporter fluorochromes.

Traditionally, the hydrolysis probes have been labeled with TAMRA as quencher. However, also several 'dark' fluorochromes have become available; these 'dark' fluorochromes absorb the energy that is emitted by the reporter fluorochrome and release the energy as heat rather than fluorescence. By using these dark quenchers, TAMRA can be used as an extra fluorochrome in multiplex approaches.[47]

RQ-PCR analysis using hybridization probes

RQ-PCR analysis with hybridization probe uses two juxtaposed sequence-specific probes, one labeled with a donor fluorochrome (eg FAM) at the 3′ end and the other labeled with an acceptor fluorochrome (eg LC Red640, LC red 705) at its 5′ end (Figure 1c).[48] Both probes should hybridize to closely juxtaposed target sequences on the amplified DNA fragment, thereby bringing the two fluorochromes into close proximity (ie within 1–5 nucleotides). Upon excitation of the donor fluorochrome, light with a longer wavelength will be emitted. When the two fluorochromes are in close proximity, the emitted light of the donor fluorochrome will excite the acceptor fluorochrome, a

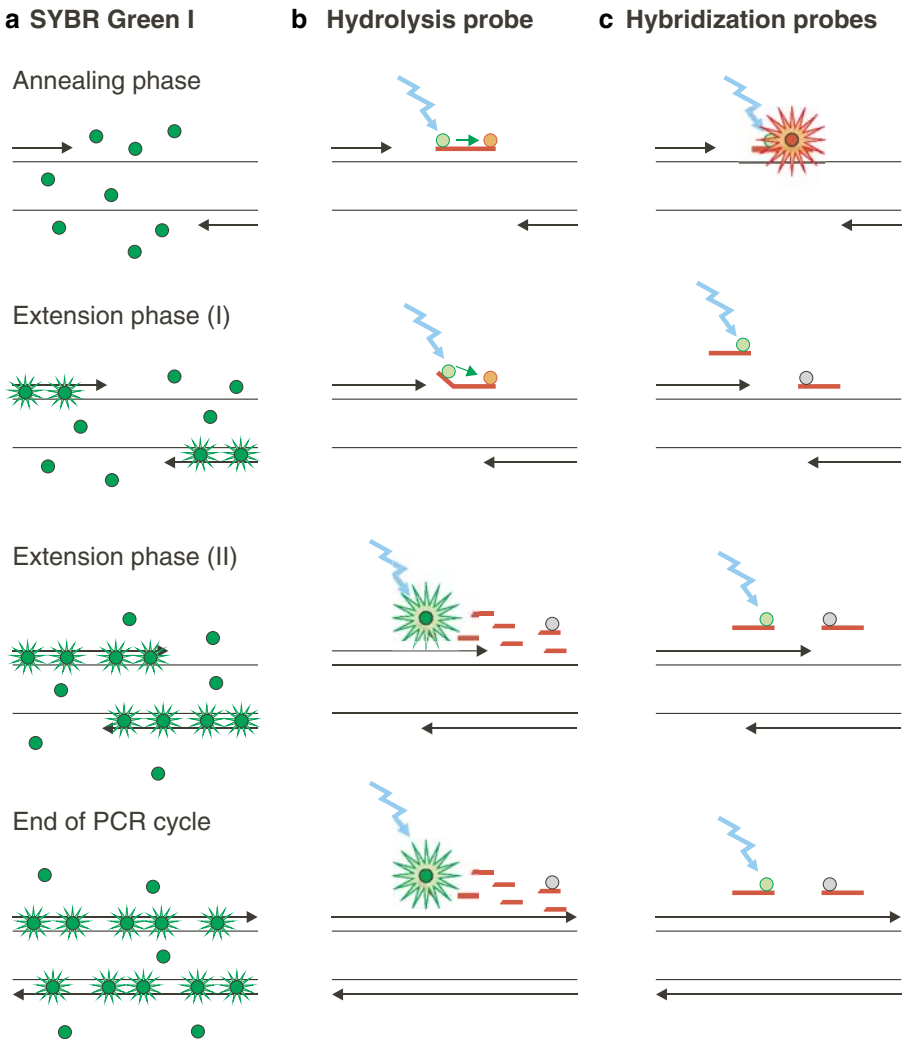

Figure 1 Principles of RQ-PCR techniques. (a) SYBR Green I technique. SYBR Green I fluorescence is enormously increased upon binding to double-stranded DNA. During the extension phase, more and more SYBR Green I will bind to the PCR product, resulting in an increased fluorescence. Consequently, during each subsequent PCR cycle more fluorescence signal will be detected. (b) Hydrolysis probe technique. The hydrolysis probe is conjugated with a quencher fluorochrome, which absorbs the fluorescence of the reporter fluorochrome as long as the probe is intact. However, upon amplification of the target sequence, the hydrolysis probe is displaced and subsequently hydrolyzed by the *Taq* polymerase. This results in the separation of the reporter and quencher fluorochrome and consequently the fluorescence of the reporter fluorochrome becomes detectable. During each consecutive PCR cycle this fluorescence will further increase because of the progressive and exponential accumulation of free reporter fluorochromes. (c) Hybridization probes technique. In this technique one probe is labeled with a donor fluorochrome at the 3′ end and a second probe is labeled with an acceptor fluorochrome. When the two fluorochromes are in close vicinity (ie within 1–5 nucleotides), the emitted light of the donor fluorochrome will excite the acceptor fluorochrome. This results in the emission of fluorescence, which subsequently can be detected during the annealing phase and first part of the extension phase of the PCR reaction. After each subsequent PCR cycle more hybridization probes can anneal, resulting in higher fluorescence signals.

process referred to as fluorescence resonance energy transfer. This results in the emission of fluorescence, which can be detected during the annealing phase and the first part of the extension phase of the PCR reaction.

RQ-PCR analysis using other probes

In addition to the three main approaches described above, other types of probes have recently been introduced. *Molecular beacons* are oligonucleotide probes that emit fluorescence when hybridized to a target sequence of (c)DNA.[49] The molecular beacon probes contain a stem-loop structure, which keeps a fluorochrome and a quencher together. Upon binding to its target sequence, the molecular beacon probe undergoes a conformational change. Consequently, the fluorochrome and the quencher are separated and fluorescence will be emitted.

Scorpions combine a PCR primer with a stem-loop tail containing a fluorochrome and a quencher.[50] During PCR, the primer element of the Scorpion is extended at its 3′ end and the Scorpion becomes a full PCR product. The recognition sequence of the Scorpion then hybridizes to its complementary target sequence within the same strand of the PCR product, resulting in a conformational change of the Scorpion and the consequent separation of the fluorochrome and the quencher, followed by emission of fluorescence.

Minor groove-binding (MGB) probes are probes (often hydrolysis probes) that are conjugated to a molecule that can strongly bind to the minor groove of the DNA.[51] By the addition of the minor groove binder, the overall binding of the probe is

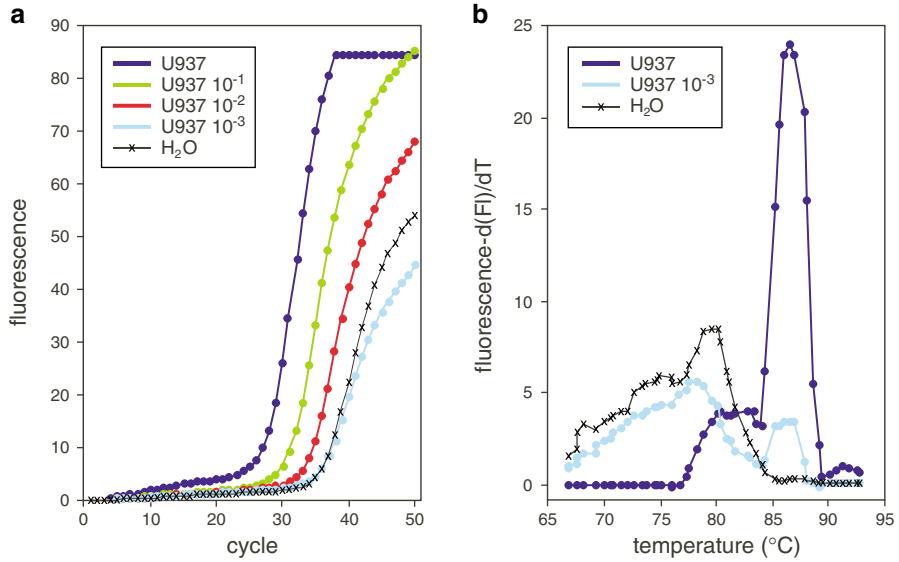

Figure 2 Melting curve analysis. (a) Amplification curves of several dilutions of the U937 cell line using SYBR Green I-based RQ-PCR analysis of the *ABL* gene. An increase in fluorescence is observed for all U937 dilutions, but also for the water control, suggesting nonspecific amplification. (b) Melting curve analysis of the same samples shows the presence of the specific PCR product (melting temperature approximately 86°C) in the U937 samples, but not in the water control, indicating that no specific PCR product has been formed. The increase in fluorescence apparently was because of nonspecific amplification or the formation of primer dimers.

largely enhanced and its melting temperature is increased. Consequently, MGB probes can be shorter and thereby potentially more specific than classical probes, especially within AT-rich regions.

Also ResonSense, Hy-Beacon, and Light-up probes[52,53] can be used, but these will not further be discussed because they are not routinely used yet. For more information, see http://www.eurogentec.be/upload/Q&Q_PCR_catalogue/q&q_pcr_cat_chap3_0302.pdf.

RQ-PCR terms and definitions

In all above-mentioned types of RQ-PCR analysis, the amount of fluorescent signal is exponentially increasing during the exponential phase of the PCR process. Based on the (background) fluorescence intensity, often determined during the first three to 15 PCR cycles, a cutoff level can be determined for specific fluorescence. This *threshold* (or *crossing line*) is used to calculate the *cycle threshold* (C_T) (or *crossing point*) of each sample, that is, the PCR cycle at which the fluorescence exceeds the threshold/crossing line for the first time (Figure 3a). The C_T value will be directly proportional to the amount of target sequence present in the sample.

Theoretically, the C_T values of a nondiluted and two-fold diluted sample should differ by one, whereas the difference between a nondiluted and 10-fold diluted sample should be 3.3. Consequently, the slope of a standard curve (using a ^{10}log scale for the dilutions) should approach −3.3 (Figure 3b). By plotting the C_T value of an unknown sample on the standard curve, the amount of target sequence in the sample can be calculated.

Like in all other PCR analyses, appropriate negative and positive controls should be used in RQ-PCR analysis.[54] Negative controls generally include *no-template controls* (eg water or RNA instead of cDNA sample), *no-amplification controls* (no *Taq* polymerase added), and negative controls for the PCR target (DNA or cDNA without the PCR target). Positive controls should provide information on the effectiveness of RNA extraction, cDNA synthesis, and (RT-)PCR analysis. Plasmid dilutions can be used to test the performance of the PCR process over time, whereas RNA from PCR target-positive cells or cell lines can be used to test the cDNA synthesis and PCR analysis. Alternatively, lysates of standardized cell line dilutions can be used to check the integrity of the whole system.

In several RQ-PCR analyses, an internal reference fluorochrome (eg ROX) is added to the PCR reaction in order to check for and control sample-related differences in fluorescence detection, for example, as a result of minor variations in volume, quality of the individual optical system, or plate characteristics. The fluorescence of the probe or dye is calculated relative to the fluorescence of the internal reference fluorochrome (*normalized fluorescence*; R_n). The increase in normalized fluorescence during the PCR is expressed as ΔR_n.

Currently available RQ-PCR equipment

At present, at least seven RQ-PCR instruments are commercially available. The most common ones are listed in Table 2. Roughly, the instruments can be divided in two categories: 'flexible instruments', with a relatively small number of sample positions but with high speed (LightCycler, SmartCycler), and 'high-throughput instruments', with a large number of sample positions but with lower speed (ABI Prisms, i-Cycler, MX-4000).

The choice of the instrument and the RQ-PCR approach is dependent on the requirements of the user. Parameters that may play an important role in the decision-making include the sample volume, speed, and number of samples to be analyzed simultaneously. Given the limited number of sample positions, the 'flexible instruments' are less suitable for MRD analysis, if more than six follow-up samples of one patient or if more than one patient should be analyzed simultaneously. However, they are convenient for fast analysis of a limited number of samples per patient ($\leq$6).

The costs of the different RQ-PCR instruments varies, but the cost of RQ-PCR analyses are not purely instrument dependent.

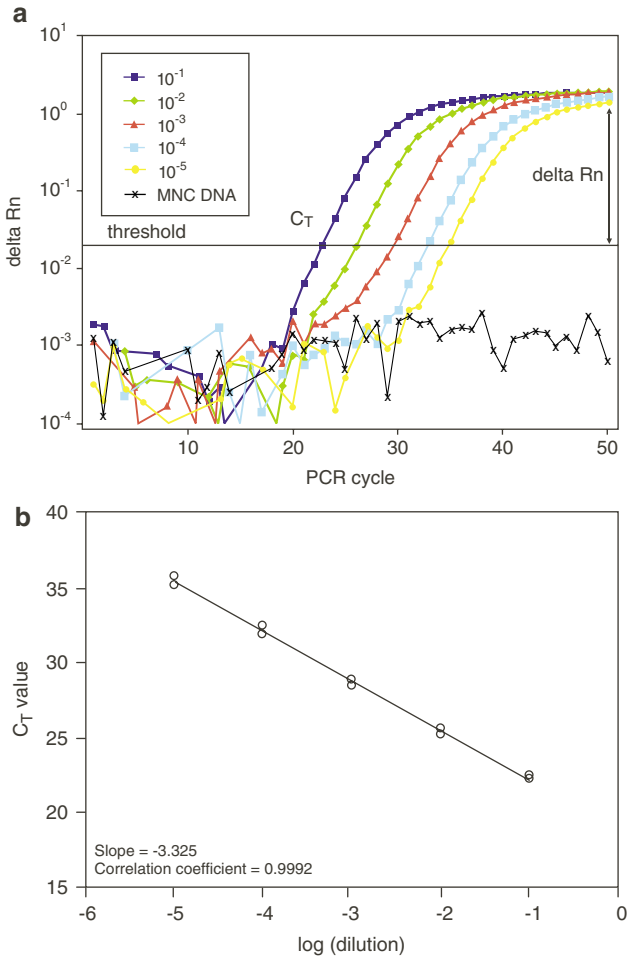

Figure 3 RQ-PCR plots. (a) An amplification plot of several 10-fold dilutions of a diagnostic leukemia sample is shown. The samples were diluted in normal mononuclear cell (MNC) DNA. Based on the (background) fluorescence intensity detected during the first three to 15 PCR cycles, a threshold is determined. The cycle threshold (C_T) is defined as the PCR cycle at which the fluorescence exceeds the threshold for the first time. The C_T value will be directly proportional to the amount of target sequence present in the sample. The increase in fluorescence, on the *y*-axis, is indicated as ΔR_n. (b) Standard curve prepared from the data in (a). The slope of the standard curve is close to the theoretical slope of −3.3. Unknown samples now can be plotted in the standard curve and based on their C_T value, the amount of template DNA can be calculated.

Costs are also dependent on the RQ-PCR approach (see below). Also the sensitivity that can be reached is more dependent on the choice of RQ-PCR approach than on the type of instrument.

It should be noted that the different RQ-PCR techniques in principle can be applied on all available RQ-PCR instruments.[55–57] However, which fluorochromes can be excited and what fluorescence can be detected is dependent on the light source and the filter combinations (Table 2). The routinely available filter combinations may not be sufficient to detect all fluorochromes, but filters may easily be adapted to the specific requirements of the user. Comparison of the TaqMan and the LightCycler instruments using hydrolysis probes for consensus regions of immunoglobulin (Ig) and T cell receptor (TCR) genes gave comparable results, both in dilution experiments and in MRD analysis, suggesting that at least some approaches can indeed be exchanged between different instruments (G Cazzaniga *et al*, *Hematol* J, 2001; **1** (Suppl. I): 233; abstract).[55]

MRD-PCR TARGETS

Several PCR targets are available for MRD detection in hematologic malignancies. The three main MRD-PCR target categories are: (1) rearrangements of Ig and/or TCR genes; (2) breakpoint fusion regions of chromosome aberrations and fusion-gene transcripts; and (3) aberrant genes or aberrantly expressed genes. Most of these MRD-PCR targets are highly specific and have no (or very low) background in normal cells, except for aberrantly expressed genes, which can also be expressed in subsets of normal cells. The applicability of these three MRD-PCR target categories varies per disease category (Table 3). For example, in CML and ALL a specific MRD-PCR target can be found in virtually all patients, whereas in AML a disease-specific MRD-PCR target can be found in only 30–50% of patients.

Ig/TCR gene rearrangements

During early B- and T-cell differentiation, the germline variable (V), diversity (D), and joining (J) gene segments of the Ig and TCR gene complexes rearrange and each lymphocyte thereby obtains a particular combination of V–(D–)J segments.[58–60]. Moreover, deletion of germline nucleotides by trimming the ends of the rearranging gene segments as well as random insertion of nucleotides between the joined gene segments creates an enormous junctional diversity. Therefore, the junctional regions of rearranged Ig and TCR genes are unique 'fingerprint-like' sequences that are assumed to be different in each lymphoid (precursor) cell. In principle, all cells of a lymphoid malignancy have a common clonal origin with identically rearranged Ig and/or TCR genes. Consequently, the junctional regions of these Ig/TCR gene rearrangements can be considered as 'DNA-fingerprints' of the malignant cells, which can be used as tumor-specific PCR targets for MRD detection.

To identify the various Ig and/or TCR gene rearrangements in each patient at initial diagnosis, clonal Ig and TCR gene rearrangements are amplified by PCR.[35] Discrimination between leukemia-derived and polyclonal PCR products is required and can be achieved by heteroduplex analysis or fluorescent gene scanning.[61,62] Monoclonal PCR products can be used for direct sequencing of the junctional regions of the Ig/TCR gene rearrangements. This sequence information allows the design of junctional region-specific oligonucleotides, so-called allele-specific oligonucleotides (ASO). Some clinical questions (eg recognition of high-risk ALL patients) can be answered by MRD techniques with limited sensitivity (10^{-2}–10^{-3}). Such detection levels might be achieved with high-resolution electrophoresis systems such as radioactive fingerprinting or fluorescent GeneScanning, without need for application of patient-specific oligonucleotides.[43,63] The latter approach is relatively rapid and cheap, because no sequence information of the junctional region is required.

Ig heavy chain (*IGH*), Ig kappa (*IGK*) light chain, TCR gamma (*TCRG*), and TCR delta (*TCRD*) gene rearrangements can be analyzed relatively easily with the PCR technique with a restricted number of oligonucleotide primers.[35,64,65] Also efficient PCR analysis of Ig lambda (*IGL*) and TCR beta (*TCRB*) gene rearrangements has become possible with the recent development of multiplex approaches for the many different V, (D), and J gene segments in *IGL* and *TCRB* gene complexes (by the BIOMED-2 Concerted Action BMH4-CT98-3936)(JJM van Dongen *et al*, *Blood* 2001; **98**: 543; abstract). If Ig genes are used for PCR analysis, one should be aware of somatic hypermutations

Table 2 RQ-PCR instruments and their main characteristics[a]

	Light source	*Detection channels*[b]	*Sample format*	*Number of samples*	*Sample volume (μl)*	*Speed (50 cycles) (h)*
Lightcycler (Roche)	LED (450–490 nm)	3 filters (530/640/710 nm)	Capillary	32	20	<1
Smartcycler (Cepheid)	LED (450–490 nm)	4 filters (520/550/585/710 nm)	Tubes	16–96	25–100	<1
Abi prism 7000 (Appl. Biosyst.)	Tungsten–halogen	4 filters (500–660 nm)	96 well	96	25–100	2–3
Abi prism 7700 (Appl. Biosyst.)	Argon laser (488 nm)	500–660 nm	96 well	96	25–100	2–3
Abi prism 7900 (Appl. Biosyst.)	Argon laser (488 nm)	500–660 nm	96 well/384 well	96/384	5–100	2–3
i-Cycler (Biorad)	Tungsten–halogen	5 filters (flexible)	96 well	96	25–100	2–3
MX-4000 (Stratagene)	Tungsten–halogen	4 (350–830 nm)	96 well	96	10–50	2–3

[a]Data obtained from instrument information booklets and from the web-sites of the different suppliers: http://home.appliedbiosystems.com/; http://www.biochem.roche.com; www.biorad.com/iCycler ; www.stratagene.com; www.eurogentec.com. Other RQ-PCR instruments not listed in the table include the Rotor-Gene (www.ozyme.fr) and the DNA engine cycler (www.biozymtc.com).
[b]Emission maxima FAM: 530 nm; SYBR Green I: 520 nm; VIC: 550 nm; JOE: 550 nm; TET: 550 nm; TAMRA: 585 nm; Cy3: 585 nm; LC Red 640: 640 nm; LC Red 705: 705 nm; ROX: 710 nm.

Table 3 Sensitivity and applicability of MRD-PCR targets in hematologic malignancies[a]

	Ig/TCR gene rearrangements	*Fusion genes (eg transcripts)*	*Other genes*		
			FLT3-ITD	*WT-1*	*HOX11L2*
Sensitivity	10^{-4} – 10^{-5}	10^{-4} – 10^{-6} (%)	10^{-4} – 10^{-5} (%)	10^{-4}	10^{-4}
B-lineage					
Prec.-B-ALL children	95%	40–50	< 2	ND[b]	<2%
Prec.-B-ALL adults	90%	35–45	< 2	70–90%	ND[b]
B-CLL	>95%	<5	< 2	<2%	ND[b]
B-cell lymphomas	>95%	25–30	< 2	<2%	ND[b]
Multiple myeloma	95%	10–15	< 2	<2%	ND[b]
T-lineage					
T-ALL children	>95%	10–25	< 2	ND[b]	20–35%
T-ALL adults	90%	5–10	< 2	75%	20–25%
T-cell lymphomas	95%	10–15	< 2	ND[b]	<2%
Myeloid lineage					
APL	ND[b]	>95	20–30	ND[b]	ND
AML children	5–10%	20–40	10–20	85–100%	<2%
AML adults	5–10%	10–20	15–30	85–100%	ND[b]
CML	ND[b]	>95	< 2	ND[b,c]	ND[b]

[a]Data based on references 66, 77, 80, 221 (Ig/TCR), 123, 222–225 (*WT-1*), 124, 125 (*HOX11L2*), 100, 103–106, 226–230 (*FLT3*), 1, 88, 93, 97, 231 (fusion gene/fusion-gene transcripts).
[b]ND: No data yet available or analyzed in limited patient series.
[c]*WT-1* expression in blast crisis seems to be higher than in chronic phase.

that can affect the binding sites of the primers used. The extent of these mutations seems high in myeloma (median of 8% nucleotides mutated), compared with 2% in chronic lymphocytic leukemia (CLL) and 4% in follicular lymphoma.[66]

Ig/TCR gene rearrangements in hematologic malignancies, in particular in ALL, might be prone to subclone formation. The problem of oligoclonality at diagnosis is the uncertainty which clone is going to emerge at relapse and should be monitored with MRD-PCR techniques.[67–70] Moreover, secondary Ig/TCR gene rearrangements (eg replacing pre-existing DH–JH, VH–JH, and Vκ–Jκ rearrangements) and ongoing Ig/TCR gene rearrangements (eg continuing VH to DH–JH joining and VH replacements) might occur in the time period between diagnosis and relapse, resulting in loss of leukemia-specific MRD targets.[71–75] Fortunately, during V to D–J rearrangements or V replacements the D–J junctional region remains unaffected, leading to the concept of designing the primers around the relatively stable D–J 'common stem' in order to prevent false-negative PCR results.[72–76] Furthermore, monoclonal MRD-PCR targets in childhood precursor-B-ALL are characterized by high stability, whereas oligoclonal MRD-PCR targets are often lost at relapse.[75,77] To reduce the number of false-negative MRD results, it is now generally accepted that in

clinical MRD studies preferably at least two Ig/TCR targets should be used per ALL patient.

Chromosome aberrations with fusion genes (DNA level)

Chromosome aberrations can be employed as tumor-specific MRD-PCR targets in which the PCR primers are chosen at opposite sides of the breakpoint fusion region.[78–80] Amplification of such hybrid sequences with 'standard-range' PCR is only feasible when the breakpoints of different patients cluster in relatively small breakpoint areas of preferably <2 kb. Despite the clustering of the breakpoints, the nucleotide sequences of the breakpoint fusion regions of chromosome aberrations differ per patient; such sequences therefore represent unique patient-specific MRD-PCR targets. One of the most widely studied chromosomal translocations is t(14;18), involving the *BCL2* and *IGH* genes, which occurs in 90% of patients with follicular lymphoma and is detectable by standard PCR procedures in 60–70% of cases.[81] In t(11;14), characteristic for most mantle cell lymphoma (MCL), the *BCL1* and *IGH* genes are involved and in 30–40% of patients the breakpoints are clustered within a restricted area (the MTC region), allowing easy identification at the DNA level by standard PCR analysis.[82] A third example concerns the submicroscopic 1p32 (*TAL1*) deletions, present in 5–15% of T-ALL patients, which also result in patient-specific breakpoints and can be used as MRD-PCR target.[35,79,83]

In many chromosome aberrations, the breakpoints of different patients are however scattered over large areas up to 200 kb.[84–88] This concerns both chromosome aberrations with aberrantly expressed genes (eg overexpression of *BCL1* and *MYC*) and chromosome aberrations leading to fusion genes with fusion-gene transcripts (eg *NPM–ALK, MLL–AF4, TEL–AML1*, and *E2A–PBX1*). New techniques for rapid and efficient screening of relatively large breakpoint regions, such as long-distance PCR and long-distance inverse PCR,[89–91] render breakpoint fusion sites into more feasible MRD-PCR targets. Particularly, if one of the two involved breakpoint regions is relatively small (eg <10 kb), it now becomes possible to identify the breakpoint fusion sites in many patients with a well-defined chromosome aberration, as was recently demonstrated by Wiemels *et al* for the t(1;19) with the *E2A–PBX1* fusion gene.[92]

Breakpoint fusion sites at the DNA level are highly attractive MRD-PCR targets for several reasons. Firstly, they are directly related to the oncogenic process and therefore stable throughout the disease course, which is in contrast to Ig/TCR gene rearrangements. Secondly, they concern PCR targets at the DNA level instead of at the RNA level, implying that these MRD-PCR targets are less sensitive to degradation. Thirdly, in contrast to fusion-gene transcripts but comparable to Ig/TCR gene rearrangements, only one PCR target is present per cell, which makes quantitation easier. Last but not least, the breakpoint fusion sites at the DNA level differ in each patient so that patient-specific RQ-PCR strategies can be applied. This implies that these MRD-PCR targets are less prone to false-positive results because of cross-contamination of PCR products between patient samples, a frequent but often underestimated problem in MRD-PCR strategies using fusion-gene transcripts.

Chromosome aberrations resulting in fusion-gene transcripts

Several malignancies with chromosome aberrations have characteristic tumor-specific fusion genes, which are transcribed into fusion-gene mRNA molecules that are similar between individual patients despite distinct breakpoints at the DNA level, which are generally located in introns. After reverse transcription into cDNA, these fusion-gene transcripts can therefore be used as appropriate targets for MRD-PCR analysis by choosing primers that are located in the exon sequences at opposite sites of the breakpoint fusion region.[88,93] Examples are *BCR–ABL* fusion-gene transcripts, that are especially observed in adult ALL cases with t(9;22) and CML,[94–96] and *NPM-ALK* fusion-gene transcripts in anaplastic large cell lymphoma with t(2;5).[97] Fusion-gene transcripts can be identified using a limited set of primers[88] and for RQ-PCR analysis a single hydrolysis probe or pair of hybridization probes can be used for the detection of several possible transcript variants (see below).

Aberrant genes and aberrantly expressed genes

In addition to the Ig/TCR gene rearrangements and the chromosome translocations, several other genetic aberrations in hematologic malignancies can be used as MRD-PCR target. A well-known example is the *FLT3* gene mutation, which concerns a variable internal tandem duplication (ITD) of the juxtamembrane domain-coding sequence of the *FLT3* gene.[98] In most patients, the *FLT3*-ITD involves exon 11, but in some cases intron 11 as well as exon 12 are involved. Of importance, not only an internal sequence is duplicated, but often additional nucleotides are randomly inserted, resulting in a truly patient-specific sequence.[99,100] The *FLT3*-ITD always consists of a multiplicity of three nucleotides, thereby retaining the reading frame. *FLT3*-ITD leads to ligand-independent autophosphorylation of the receptor, resulting in proliferation and inhibition of apoptosis. *FLT3*-ITD's have so far mainly been reported in myeloid malignancies, particularly in AML and APL (especially the M3 variant),[101] and appear to be associated with an increased risk of relapse.[100,102–106] Some recent studies suggest that *FLT3*-ITD may not be stable between diagnosis and relapse and consequently one should be cautious to use *FLT3*-ITD as MRD-PCR target.[107,108]

Wilms' tumor gene *WT-1* encodes a zinc-finger transcription factor that functions as a potent transcriptional repressor of several growth factors, including insulin-like growth factor-II and colony-stimulating factor-1.[109] Its expression is strongly regulated in a time- and tissue-specific manner. However, the *WT-1* gene is overexpressed in virtually all patients with AML and is thought to play a role in maintaining the viability of leukemic cells.[110–112] Overexpression of *WT-1* therefore can be regarded as a specific feature of the malignant cells and consequently can be used as an MRD-PCR target (Table 3).[113–118] However, it should be noted that *WT-1* expression in normal cells can cause a 'background' level.[119–123]

Recently, a new recurrent and specific cryptic translocation, t(5;14), has been identified in a subset of T-ALL.[124] As a result of the translocation the *HOX11L2* gene, encoding for a member of the homeobox-containing protein family, is transcriptionally activated. The *HOX11L2* gene is not expressed in adult spleen, adult peripheral blood, or bone marrow, but is expressed at very low levels in fetal thymus, fetal spleen, and adult thymus.[125] In 20–35% of T-ALL patients, but not in precursor-B-ALL or AML patients, high expression of *HOX11L2* was found.[125] Altogether, these data indicate that ectopic *HOX11L2* expression can be used as an MRD-PCR target (Table 3).

The chromosomal translocation t(11;14)(q13:q32), involving rearrangements of the *BCL1* locus, is closely associated with human lymphoid neoplasia affecting mantle cells (MCL, see

above). The putative *BCL1* proto-oncogene turned out to be the Cyclin D1 (*CCND1*) gene. Although the observed breakpoints in the *BCL1* locus are not tightly clustered, all of the known breakpoints leave the *CCND1* coding region structurally intact and result in increased *CCND1* transcripts and protein expression.[126] So far, quantitative analysis of *CCND1* transcript expression has only been used for the diagnosis of MCL and not for MRD detection. However, theoretically this might be possible.

Some recent studies have shown that the *PRAME* gene (preferentially expressed antigen of melanoma) is expressed at high levels in hematologic malignancies and might be used as MRD-PCR target.[127–130]

MRD-PCR targets: advantages and disadvantages

The different MRD-PCR targets have their specific advantages and disadvantages, as summarized in Table 4. It should be noted that some MRD-PCR targets can be analyzed at the DNA as well as at the RNA/cDNA level. This is the case for some fusion-genes and their corresponding fusion-gene transcripts (eg the *TAL1* deletion and *SIL–TAL1* fusion-gene transcripts) and for *FLT3*-ITD.

Ig/TCR gene rearrangements are frequently used MRD-PCR targets, as they are widely applicable in lymphoid malignancies.[66,77,80,131] The identification of Ig/TCR gene rearrangements at diagnosis and the design of patient-specific oligonucleotides for subsequent MRD detection during follow-up is, however, time-consuming and requires insight in the immunobiology of Ig/TCR gene rearrangements. As described above, loss of MRD-PCR targets may occur by secondary or ongoing rearrangements or by oligoclonality of the target. Although Ig/TCR gene rearrangements are patient-specific MRD-PCR targets, background amplification of comparable rearrangements in normal cells may hamper sensitive detection.[132] This background amplification can be dependent on the sample type used (bone marrow, BM *vs* peripheral blood, PB) and on the time point of sample collection. For example, BM samples obtained during induction therapy may contain high percentages of T cells,[133] whereas postinduction and postmaintenance BM samples

Table 4 Advantages and disadvantages of MRD-PCR targets

MRD-PCR target	Advantages	Disadvantages
Ig/TCR gene rearrangements (DNA level)	Patient specific Low-risk of contamination High stability of DNA DNA amount per cell is relatively constant	Labor intensive target identification Loss of target because of clonal evolution (oligoclonality and ongoing rearrangements) Background dependent on type of sample and follow-up time point Target not related to oncogenesis
Fusion genes (DNA level)	Patient specific Low-risk of contamination High stability of DNA DNA amount per cell is relatively constant Related to oncogenesis Stable targets No (or very low: $<10^{-6}$) background in normal cells Target not affected by type of sample and follow-up time point	Labor intensive identification of exact breakpoints, except for some translocations with small breakpoint regions, such as *SIL-TAL1*, 70% of *BCL2-IGH*, and 30% of *BCL1-IGH*
Fusion gene-transcripts (mRNA level)	Can be identified with limited set of primers Related to oncogenesis No (or very low: $<10^{-6}$) background in normal cells	Not patient specific Relatively high chance of contamination Expression level may be affected by therapy Instability of RNA
FLT3-ITD (DNA level)	Can be identified with limited set of primers Related to oncogenesis Patient specific High stability of DNA DNA amount per cell is relatively constant No background in normal cells	Target may not be stable
FLT3-ITD (mRNA level)	Can be identified with limited set of primers Related to oncogenesis Patient specific No background in normal cells	Target may not be stable Expression level may be affected by therapy Instability of RNA
WT-1 (mRNA level)	Easily identified	Expression level may be affected by therapy Expression level may differ per type of sample Instability of RNA Not tumor specific Background expression in normal cells
HOX11L2 (mRNA level)	Can be identified with limited set of primers Related to oncogenesis No background of normal cells	Expression level may be affected by therapy Instability of RNA

contain high percentages of regenerating precursor-B-cells.[134,135]

An advantage of using chromosome aberrations as tumor-specific MRD-PCR targets is their stability during the disease course. However, because of the high sensitivity of PCR techniques, *cross-contamination of RT-PCR products* between patient samples might severely hamper MRD detection, leading to false-positive results.[136] This cross-contamination is an underestimated problem, probably because cross-contamination is difficult to recognize, since leukemia-specific fusion-gene transcript PCR products are not patient specific. This is in contrast to PCR products obtained from breakpoint fusion regions at the DNA level, which can be identified by the use of patient-specific oligonucleotide probes. Consequently, strict precautions should be taken to avoid cross-contamination of RT-PCR products. Most importantly, sample preparation and PCR should be performed in separate rooms, dUTP should be used instead of dTTP, and heat-labile DNA glycosylase should be employed to hydrolyze all contaminating amplification products, but not the cDNA template, prior to the PCR. An additional disadvantage is that fusion-gene transcription might be affected by the cytotoxic treatment, potentially resulting in transcript levels that differ per treatment phase. Finally, one should be aware that RNA-based MRD results are often reported as gene expression levels and not as tumor load.

RQ-PCR approaches

Several approaches can be chosen for RQ-PCR-based detection of MRD in hematologic malignancies, such as nonspecific detection (using SYBR Green I) and sequence-specific detection systems (using probes). In practice, the choice will be dependent on the disease category, the available types of MRD-PCR targets, the required sensitivity, and the knowledge and experience of the involved MRD-PCR laboratory. The most frequently used approaches will be discussed with special emphasis on the position of the probes in sequence-specific detection systems.

Nonspecific detection using SYBR Green I

If RQ-PCR analysis with nonspecific detection is used (eg SYBR Green I), a melting curve analysis always should be made in order to discriminate between specific PCR products and nonspecific PCR products (see Figure 2).[44] Furthermore, a nested PCR approach may be needed for reaching sufficiently high sensitivity. In case of patient-specific MRD-PCR targets, such as Ig/TCR gene rearrangements, the first PCR can be performed using forward and reverse primers positioned in the germline parts of the involved gene segments. The second PCR then can be performed using one germline primer and one patient-specific (ASO) primer, in combination with SYBR Green I.[137] This procedure may result in a less precise quantitation, unless a limited number of PCR cycles are performed in the first PCR.[137]

By now, RQ-PCR analysis using SYBR Green I has been described for Ig/TCR,[55,137] *WT-1*,[116] t(11;14),[138] and t(14;18)[138] as MRD-PCR targets.

ASO probe approach

In the ASO probe approach (Figure 4a), the probe is positioned in the tumor-specific sequence, for example, the junctional region of Ig/TCR gene rearrangements[55,140,141] or the breakpoint area of fusion genes.[17,18,142] The probe is used in combination with a forward and reverse primer, which are positioned in germline sequences opposite of the tumor-specific sequence. If applied for Ig/TCR gene rearrangements, this approach can be compared with the classical dot-blot hybridization techniques[5] as both are based on specific detection of the MRD-PCR target within a background of normal PCR products. Primer competition between the MRD-PCR target and comparable Ig/TCR

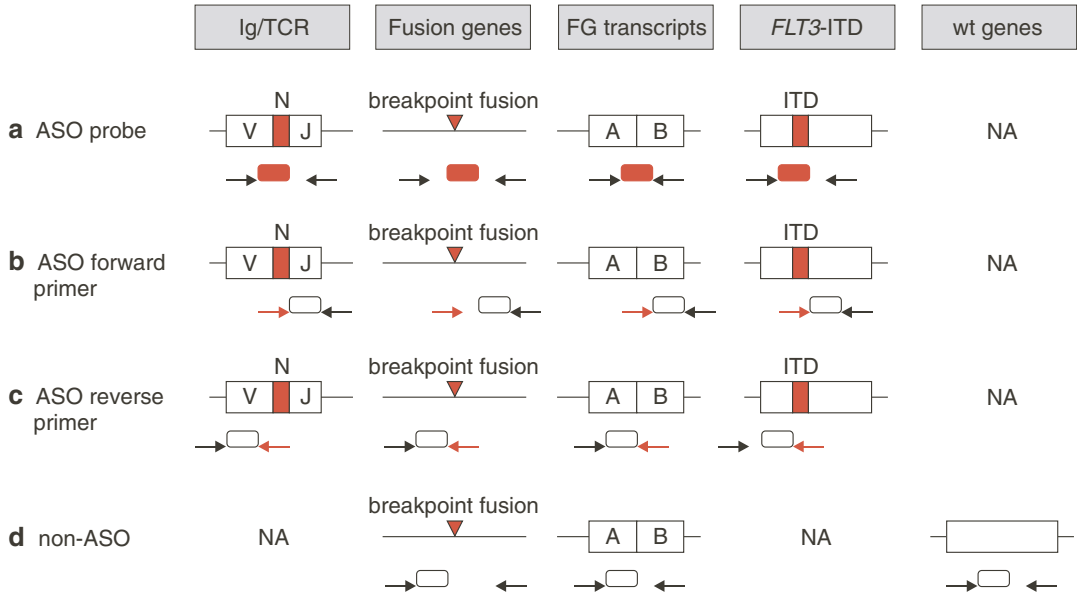

Figure 4 RQ-PCR approaches. (a) ASO probe approach. (b) ASO forward primer approach. (c) ASO reverse primer approach. (d) non-ASO approach. The four approaches are shown for the different types of MRD-PCR targets. Primers are indicated as arrows, whereas the probe (either one hydrolysis probe or two hybridization probes) is indicated by the oval symbol; the ASO probe or primer is indicated in red. NA: not applicable; FG: fusion gene; wt: wild type; ITD: internal tandem duplication.

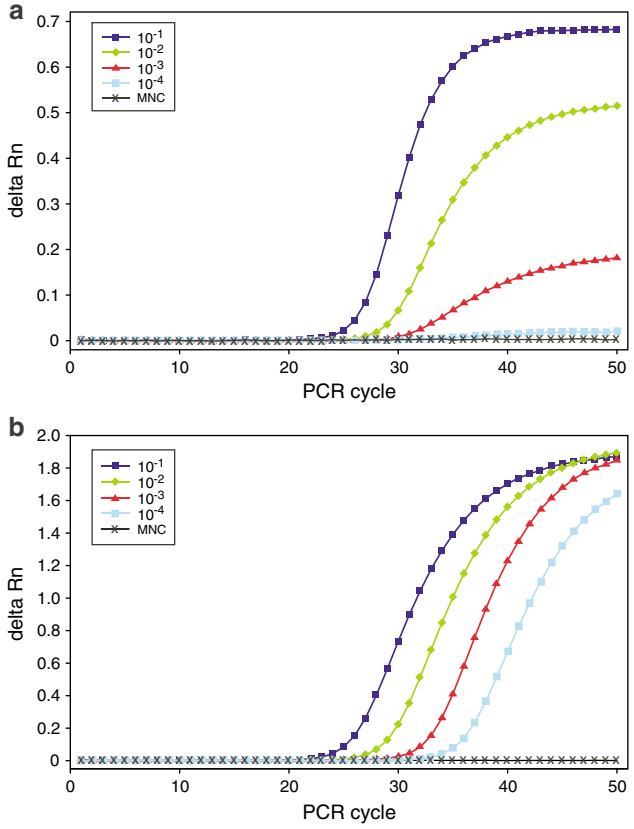

Figure 5 ASO probe and ASO primer approach. Representative example of a dilution experiment of a diagnostic sample from an ALL patient by RQ-PCR analysis of an Ig gene rearrangement using the ASO probe approach (a) and the ASO primer approach (b) Note the difference in the increase in fluorescence (ΔR_n) between the ASO probe approach (specific detection of leukemia-specific PCR product in the background of comparable PCR products derived from normal cells) and he ASO primer approach (specific amplification of leukemia-specific PCR products).

rearrangements in normal cells (involving the same V and J gene segments) may result in lower fluorescence intensities, if the percentage of malignant cells is low (Figure 5a).[143]

Although good sensitivities can be obtained by this approach, a major drawback is that for each individual tumor-specific MRD-PCR target, a fluorogenic probe has to be designed and ordered, which is particularly expensive in case of Ig/TCR gene rearrangements as RQ-PCR targets.

RQ-PCR analysis using the ASO probe approach has been described for Ig/TCR genes,[55,140,141] and the *AML1-ETO*[17,18] and *BCR-ABL*[142] fusion-gene transcripts.

ASO forward primer approach

The ASO forward primer approach (Figure 4b) employs a forward primer positioned in the tumor-specific sequence in combination with a germline reverse primer and a germline probe (eg in the J gene segment,[132,141,143–145] or in *FLT3* intron 11[146]) In contrast to the ASO probe approach, this approach is based on sequence-specific amplification of the MRD-PCR product and consequently the increase in fluorescence at the plateau phase of the PCR reaction is comparable for all percentages of malignant cells (Figure 5b).[143]

RQ-PCR analysis using the ASO forward primer approach has been described for *IGH*,[141,143–145] *IGK*-Kde,[147] *TCRG*,[132] *TCRD*,[148] t(14;18),[149] and *FLT3*-ITD.[146]

ASO reverse primer approach

The ASO reverse primer approach (Figure 4c) is comparable to the ASO forward primer approach, but with opposite location of the germline primer and probe relative to the tumor-specific sequence.[55,150–154]

If Ig/TCR gene rearrangements are chosen as MRD-PCR target, the ASO forward primer approach (using a probe generally located in the J gene segments) has several advantages over the ASO reverse primer approach (using a probe generally located in the V gene segments). Firstly, the number of J gene segments is lower than the number of V gene segments, and consequently a lower number of probes need to be made. Secondly, the ASO forward primer approach may be less susceptible to target loss, as a D_H–J_H stem can be retained in ongoing V_H to D_H–J_H rearrangements and V_H replacements.[72,76] Thirdly, the occurrence of somatic hypermutations in the V gene segment may result in less optimal primer and probe annealing.[155] On the other hand, one could argue that, because of the higher number of V gene segments as compared to J gene segments, the ASO reverse primer approach might sometimes be more sensitive. This is however not supported by our data or data in the literature.[132,143,144,151,154]

Allele-nonspecific (germline) primer and probe approach

For MRD-PCR targets that are not tumor-specific, such as *WT-1*, *HOX11L2*, and fusion-gene transcripts, it is not necessary or possible to apply ASO primers or ASO probes. If aberrant expression of a gene is analyzed, primers and probe are designed on the wild-type (germline) nucleotide sequence of the involved gene, for example, *WT-1*[116] or *CCND1*.[156–161] Germline primers and probes can also be applied for fusion-gene transcripts; in these cases, the forward primer is located in an exon from one fusion-gene partner, whereas the reverse primer is located in an exon sequence of the other fusion-gene partner (Figure 4d). If more than one type of fusion-gene transcript exists, additional primers may be designed.[88] The position of the probe is mainly dependent on the number and type of fusion-gene transcripts; in most cases one probe, capable of detecting all fusion-gene transcript types, can be designed in combination with several primers.[162,163]

RQ-PCR analysis using germline primers and probes is frequently used for fusion-gene transcripts (*E2A-PBX1*,[163,164] *BCR-ABL* p210,[27,56,57,163,165–170] *TEL-AML1*,[69,163,171–175] *BCR-ABL* p190,[27,163] *CBFB-MYH11*,[19–21,163] *PML-RARA*,[22,23,163] *AML1-ETO*,[24–26,163] *SIL-TAL1*,[163,176] and *MLL-AF4* [163]), fusion genes (t(14;18),[177–182] t(11;14),[182–184]) and for detection of wild-type transcripts such as *WT-1*,[117] *PRAME*,[128] and *CCND1*.[156–161]

Primer and probe design

For the design of primers and probes several software packages are available, such as Primer Express (Applied Biosystems), OLIGO (W. Rychlik, Molecular Biology Insights, Inc., Cascade, CO, USA), and LightCycler Probe Design (Roche). All oligonucleotides should anneal to target sequences free of direct

repeats, homopolymeric runs, and inverse repeats. Furthermore, all oligonucleotides should be checked for inter- and intramolecular dimer formation. For primers, special attention should be given to the 3′ sequence: this sequence should not form dimers or hairpins and the binding should not be too strong (relatively low ΔG of the 3′ end) in order to prevent nonspecific extension. Both hydrolysis and hybridization probes should have a melting temperature 5–10°C higher than the melting temperature of the primers to ensure strong binding of the probe during the annealing phase.[46,185] Probe binding, however, should not be too stable, because this may interfere with the amplification process by hindering the *Taq* polymerase and consequently may lower the sensitivity of the assay. Finally, attention should be given to frequent polymorphisms in order to avoid underestimation of PCR target.[186]

As indicated before, two probes are required for the hybridization probe approach. For RQ-PCR targets with a limited sequence area available for probe design, such as Ig/TCR gene rearrangements, the design of a single hydrolysis probe may be easier.[57]

SENSITIVITY OF RQ-PCR ANALYSIS

For MRD analysis it is not only important to obtain quantitative data, but the assay should also be sufficiently sensitive. The required sensitivity is dependent on the clinical application, but generally a sensitivity of at least 10^{-3}, but preferably 10^{-4}–10^{-5} should be reached. For example, sensitivities of at least 10^{-4} are required for recognition of MRD-based low-risk ALL patients,[5,16] but if one only aims at the recognition of high-risk ALL patients, a sensitivity of 10^{-2}–10^{-3} may be sufficient.[187]

Determining the sensitivity

To determine the sensitivity of the RQ-PCR assay, dilution experiments should be performed. This can be performed with diagnostic material of the patient, a reference standard ('calibrator', eg a cell line), or plasmids. For RT-PCR assays, cell line dilutions are frequently not appropriate since several cell lines express a higher level of fusion-gene transcripts than primary tumor cells.[163] The first two types of dilution curves are used for relative quantitation, whereas plasmids can be used for absolute quantitation. By plotting the logarithmic value of the dilution against the C_T, a standard curve with a slope of –3.3 should be obtained (see Figure 3b). For the analysis of the sensitivity, the following criteria should be taken into account:

- The amplification curve should reflect specific amplification as determined by the shape of the curve and (for the ABI Prism instruments) the multicomponent graph.
- For quantitative data, *the RQ-PCR analysis should be reproducible.* As shown in Figure 6, the variation in C_T values between replicates is generally less than 1.5 if the mean C_T value of the replicates is below 36, whereas the variation between replicates is (much) higher, if the mean C_T value of the replicates is higher. This implies that one could define two sensitivities: a reproducible sensitivity indicating the level up to which the data can be precisely quantified and a maximal sensitivity indicating the level that still can be detected, although not reproducibly.
- The specific amplification should be sufficiently separated from the nonspecific (background) amplification. Generally, nonspecific amplification is only detected at a low level and outside the reproducible range of the RQ-PCR (C_T value >36). Therefore, it is important to include nonspecific amplification controls (at least in triplicate) in each RQ-PCR analysis. The difference in C_T values between the specific and nonspecific amplification should probably at least be 1, but should preferably be ≥3 in order to limit the chance that a negative sample (with a C_T value slightly less than the C_T of the nonspecific amplification) is assumed to be positive. This is particularly important for follow-up samples, which might significantly differ in cellular composition, dependent on the time point in the protocol. For example, high percentages of T cells can be found during induction therapy,[133] whereas high percentages of precursor-B-cells can be found postinduction and postmaintenance therapy.[134,135] Consequently, because of high frequencies of TCR or Ig gene rearrangements, respectively, the nonspecific amplification may be increased as compared to the traditional nonspecific amplification control (normal PB MNC DNA).
- The standard curve obtained with the dilutions should have an acceptable slope and correlation coefficient (ie the position of the different dilutions relative to the standard curve). Theoretically, the slope of the standard curve should be –3.3 if 10-fold dilutions are used, but in practice a slope between –3.0 and –3.9 will probably be acceptable as long as the correlation coefficient is >0.95. For the determination of the reproducible sensitivity, one may apply stricter criteria for the difference in C_T values between two subsequent dilutions (eg ΔC_T between 3 and 4) than for the determination of the maximal sensitivity (eg ΔC_T between 2 and 5).

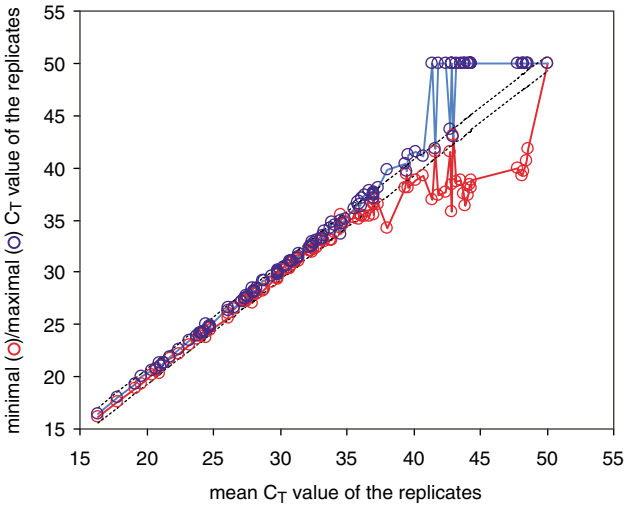

Figure 6 Variation in C_T values. From over 100 samples, the mean C_T value of the replicates (generally triplicates) was plotted against the minimum and maximum C_T value of the replicates. As can be seen, the variation in C_T values is very low (<1.5) for mean C_T values up to 35. However, at higher mean C_T values the variation increases significantly, because the RQ-PCR analyses reach their maximal sensitivity.

The above proposed criteria are based on the experience we obtained during the last 5 years in our own laboratories as well as during international meetings with other experienced laboratories: I-BFM-SG MRD Task Force (ER Panzer-Grümayer *et al*, T Flohr *et al*), DCLSG ALL9 (CE van der Schoot *et al*), European pre-BMT MRD Study Group (J Trka *et al*, H Madsen *et al*, N Goulden *et al*, P Bader *et al*). The described criteria should not be regarded as strict rules, but can serve as guidelines. Common international agreements on such

guidelines should be made in the future. This is one of the aims of our recently initiated European Study Group on MRD detection in ALL (ESG-MRD-ALL; coordinators: JJM van Dongen and VHJ van der Velden).

Several examples of dilution experiments, using either plasmids or diagnostic material, are shown in Figure 7. The obtained reproducible and maximal sensitivities are indicated. It should be stressed that the reproducible sensitivity should be used to determine whether the RQ-PCR assay reached the required sensitivity. However, in case of two or more Ig/TCR targets per patient, one could decide to apply less strict criteria (eg the maximum sensitivity) for the second MRD-PCR target.

Generally, the standard curve is constructed using the threshold suggested by the instrument. However, if the threshold appears to be positioned outside the linear part of the amplification curves, adjustments may be made in order to increase the reliability (and consequently the correlation coefficient). In addition, it may be necessary to standardize the way the standard curve is made, so that different experiments (either performed on different times in one laboratory or performed in different laboratories) can be

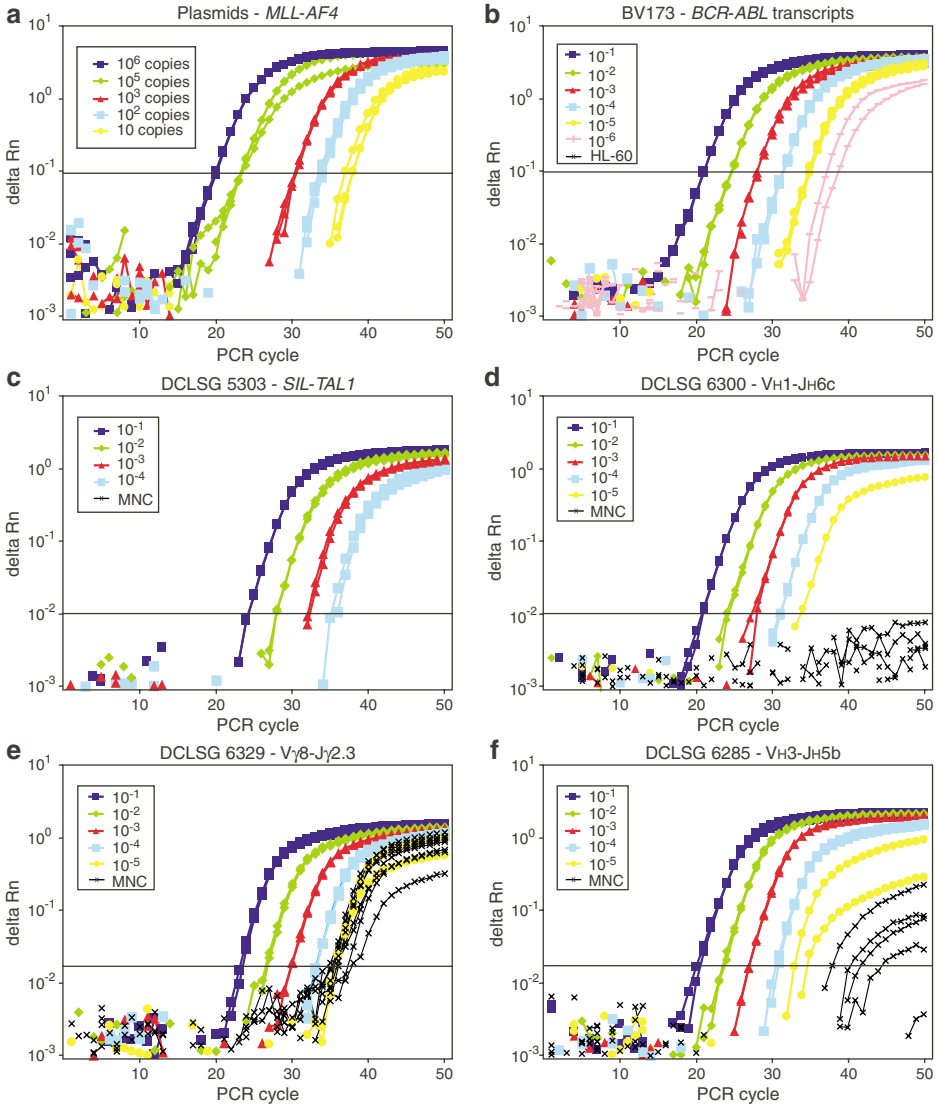

Figure 7 Sensitivity of RQ-PCR experiments. (a–f) show Δ examples of dilution experiments with different reproducible and maximal sensitivities. (a) *MLL-AF4* plasmids. The 10 copies dilution shows good amplification curves and the difference in C_T value between the replicates is <1.5. So, the reproducible sensitivity and the maximal sensitivity are 10 copies. (b) BV173 cell line with *BCR-ABL* transcripts. The 10^{-4} dilution shows good amplification with reproducible C_T values (difference between replicates <1.5), therefore the reproducible sensitivity is 10^{-4}. As also one of the 10^{-5} dilutions gives good amplification, the maximal sensitivity is 10^{-5}. (c) T-ALL patient DCLSG 5303 with the *SIL–TAL1* fusion gene (DNA level). The 10^{-4} dilution shows good amplification curves and the difference in C_T value between the replicates is <1.5. So, the reproducible sensitivity and the maximal sensitivity are 10^{-4}. (d) Precursor-B-ALL patient DCLSG 6300 with a V_H1–J_H6c rearrangement. The 10^{-4} dilution shows good amplification with reproducible C_T values (difference between replicates <1.5), therefore the reproducible sensitivity is 10^{-4}. As also one of the 10^{-5} dilutions gives good amplification, the maximal sensitivity is 10^{-5}. (e) T-ALL patient DCLSG 6329 with a Vγ8–Jγ2.3 rearrangement. Although the 10^{-4} dilution shows good amplification, it is to close (less than 3 C_T difference) to the nonspecific amplification observed in normal MNC DNA. Therefore, the reproducible sensitivity is 10^{-3}; the maximal sensitivity is 10^{-4}. (f) Precursor-B-ALL patient DCLSG 6285 with a V_H3–J_H5b rearrangement. Nonspecific amplification of normal MNC DNA is observed, but the lowest C_T is more than 3 C_T apart from the specific amplification of the 10^{-5} dilution. However, the difference in C_T value of the two replicates of the 10^{-5} dilution is >1.5 C_T. Therefore, the reproducible sensitivity is 10^{-4}; the maximal sensitivity is 10^{-5}.

Table 5 RQ-PCR for MRD detection via Ig/TCR targets using the ASO-primer approach and hydrolysis probes

Gene	*Germline probes*[a]	Reproducible sensitivity[b]	Frequency[c]	Background frequency[d]	Reference
IGH	3 Jh probes	10^{-4}–10^{-5}	85%	30–40%	Verhagen *et al*[143]
IGK	1 Kde probe	10^{-4}	85%	~30%	Van der Velden *et al*[147]
TCRG	2 Jγ probes	10^{-4}	30/60%[e]	~90%	Van der Velden *et al*[132]
TCRD	1 Dδ3 probe	10^{-4}	70%	~70%	Langerak *et al*[232]
	1 Jδ1 probe	10^{-4}	NA[f]	NA[f]	Szczepanski *et al*[148]

[a]Number of germline probes needed for RQ-PCR analysis of most rearrangements.
[b]Reproducible sensitivity: see text for criteria.
[c]Frequency of cases for which the indicated reproducibility can be obtained.
[d]Frequency of cases in which nonspecific amplification can be observed at an annealing temperature of 60°C.
[e]In precursor-B-ALL a reproducible sensitivity of 10^{-4} can be reached in approximately 30% of cases, whereas in T-ALL such sensitivity can be reached in approximately 60% of cases.
[f]NA, not yet available, experiments in progress.

compared more easily. Such standardization might particularly be important for MRD-PCR targets that are not patient specific, such as fusion-gene transcripts. If patient-specific MRD-PCR targets (such as Ig/TCR gene rearrangements or *FLT3*-ITD) are employed, standardization is less important as each individual target has its own standard curve. Standardization can be done by moving the threshold in such a way that the identical reference standards (eg a cell line) keep an identical C_T value. Alternatively, the threshold can be fixed at a particular value so that also C_T values can be compared. The latter method was used within the Europe Against Cancer Program (coordinator: J Gabert).[163]

Sensitivities of MRD-PCR targets

Using fusion-gene (transcripts) as MRD-PCR targets, detection limits of 10^{-4}–10^{-6} can easily be reached in most patients because nonspecific amplification can be avoided by selection of appropriate primers and probes and because fusion genes are generally not found in normal cells. In contrast, the sensitivity of MRD-PCR analysis of junctional regions generally ranges from 10^{-3} to 10^{-5} and is dependent on several parameters (see below). In Table 5, an overview is given of the reproducible sensitivities that were obtained for Ig/TCR gene rearrangements in our laboratory (Erasmus MC, Rotterdam) using hydrolysis probes. It can be seen that generally sensitivities of at least 10^{-4} can be reached. For other MRD-PCR targets, such as *FLT3*-ITD,[99,146] *WT-1*,[116,117] and probably also *HOX11L2* transcripts, generally sensitivities of at least 10^{-4} can be obtained.

Parameters that affect the sensitivity

The sensitivity that can be obtained in RQ-PCR analysis is dependent on several parameters. Parameters that are independent of the type of MRD-PCR target include the number of cells investigated, the total amount of DNA/RNA analyzed, the number of PCR cycles, and the use of a single or nested PCR approach.

The sensitivity of fusion genes as MRD-PCR target (DNA level) is mainly dependent on the potential presence of such fusion genes in normal cells. For example, the t(14;18) has been observed in healthy controls.[78,181,188] At the RNA level, the main parameters affecting the sensitivity are the expression level of the fusion-gene transcript and the potential presence of such fusion-gene transcripts in normal cells. Several studies now have shown that the expression level of a particular fusion-gene transcript can vary up to 100-fold between patients.[20,23,189] Furthermore, some fusion-gene transcripts have been found in healthy controls at very low levels[190–193] or in patients in long-term complete remission,[194,195] although these data are sometimes controversial.[196–198] Nevertheless, the presence of fusion-gene transcripts in nonmalignant cells may hamper detection of MRD, if the sensitivity of the RQ-PCR assay is extremely high ($<10^{-6}$).

When Ig/TCR gene rearrangements are used as MRD-PCR target, several parameters affect the sensitivity. Junctional regions of complete V–D–J rearrangements are extensive, whereas junctional regions of V–J rearrangements are generally three to four times smaller. Accordingly, the sensitivity of RQ-PCR-based MRD detection of complete *IGH* gene rearrangements is five to 10-fold more sensitive as compared to *IGK*–Kde targets (Table 5).[143,147] We also observed that the sensitivity of *TCRG* gene rearrangements in RQ-PCR studies is related to the number of inserted nucleotides in the junctional region.[132] The sensitivity of MRD-PCR analysis of Ig/TCR junctional regions is also influenced by the 'background' of normal lymphoid cells with comparable Ig or TCR gene rearrangements. For instance, Vδ1–Jδ1 rearrangements frequently occur in T-ALL, but also in a small fraction (0.1–2%) of normal peripheral blood T-cells.[199,200] VγI–Jγ1.3 and VγI–Jγ2.3 joinings comprise 50–60% of *TCRG* gene rearrangements in ALL, but are also found in a large fraction (70–90%) of normal T lymphocytes. Taking into account the abundance of normal T lymphocytes with polyclonal Vγ–Jγ joinings, particularly in postinduction follow-up samples,[133] it is not surprising that RQ-PCR analysis of short Vγ–Jγ junctional regions is generally less sensitive (10^{-2}–10^{-4}) than MRD-PCR analysis of long Vδ1–Jδ1 junctional regions (10^{-3}–10^{-5}). Similarly, *IGH* MRD-PCR targets involving the J_H4, J_H5, or J_H6 gene segments result more often in nonspecific amplification of normal cells,[143] as these JH gene segments are most frequently used in normal cells.[201–204] If nonspecific amplification of normal cells is observed, increasing the annealing temperature may increase the specificity and thus the sensitivity. Alternatively, a shorter ASO primer could be designed.

The sensitivity of *FLT3*-ITD and *HOX11L2* RQ-PCR analysis is mainly dependent on the selected primers and probe, as the sensitivity will not be affected by the presence of comparable sequences in normal cells. This is in contrast with RQ-PCR-based analysis of *WT-1* transcripts, which are also expressed in normal cells, albeit at low levels.[119–123]

CONTROL GENES

Selection of control genes

To obtain quantitative MRD-PCR data, it is crucial that control genes are included in the analysis to correct for the quantity and quality of the DNA or RNA/cDNA. When DNA sequences are used as MRD-PCR target, one should select a control gene that is located on a chromosome that is not frequently gained or lost in the studied type of malignancy. The albumin gene, located on chromosome 4, is often used,[132,140,147,181,205] but other genes (eg β-actin,[141,177,206] β-globin,[139,152] and *GAPDH* [153,154]) can be used as well.

Selection of an appropriate control gene for RNA/cDNA targets requires additional criteria. These include a similar expression level in different cell types, no relation with cell cycle or cell activation, a stability comparable to the MRD-PCR target, and an expression level comparable with the MRD-PCR target. Within the Europe Against Cancer Program, a detailed investigation has recently been performed for selection of control genes in leukemia studies.[207] After extensive analysis of *ABL*, *GUS*, and *B2M* in a large series of fresh samples, *ABL* appeared to be most appropriate since its expression was stable and comparable between BM and PB and between normal and leukemic samples (Beillard *et al*, submitted). Other control genes that have been described in various assays include *G6PD*,[27] *GAPDH*,[17,22,25] *TBP*,[175] *PBGD*,[23,173] *β-actin*,[18,117,118] *18S*,[20] and *HPRT*.[208] Since genomic DNA frequently contaminates RNA preparations, it is essential to ensure that the primers employed are specific to cDNA. Although some groups have used β-actin as a control gene, this is generally considered to be unsatisfactory in the context of MRD because of its high expression and the presence of multiple processed pseudogenes. Such processed pseudogenes are also known for *GAPDH*.[209,210]

Using control genes for quantitation

By analyzing a control gene, the MRD level as determined by the MRD-PCR target can be corrected for the amount and 'amplifiability' of DNA or RNA/cDNA in the sample. This can be done by two main methods: the standard curve method and the comparative C_T method.

In the standard curve method, dilution series from a calibrator are prepared for both the target and the control gene. For each patient sample, the amount of target and control gene is determined from the appropriate standard curve. The target amount is subsequently divided by the control gene amount to obtain a normalized target value. The calibrator can be a plasmid, the diagnostic sample from the same patient, or any other positive sample (eg a cell line).

The comparative C_T method uses absolute C_T values to calculate the control-gene-normalized amount of target relative to a calibrator. First, the difference in C_T (ΔC_T) between the target and the control gene is calculated for both the patient sample and the calibrator. Second, the difference in ΔC_T between the patient sample and calibrator is determined ($\Delta\Delta C_T = \Delta C_{Tsample} - \Delta C_{Tcalibrator}$). Finally, the control-gene-normalized amount of target relative to a calibrator is calculated by using the formula $2^{-\Delta\Delta C_T}$. For the $\Delta\Delta C_T$ calculation to be valid, the efficiency of the target RQ-PCR and the control gene RQ-PCR must be comparable. This can be determined by analyzing the ΔC_T for different dilutions of the template; if the efficiencies of the two RQ-PCRs are approximately equal, the plot of log input versus ΔC_T should have a slope less than 0.1.

If the control gene RQ-PCR shows a lower template amount than expected, special caution should be taken for several reasons. First, this lower value can be the result of inhibition, which can be found in a substantial number of BM or PB samples (5–10%).[211] The degree of inhibition of the control gene RQ-PCR is, however, not always identical to the degree of inhibition in the MRD target RQ-PCR, and consequently an overestimation or underestimation of the MRD level can be made. We have recently found that bovine serum albumin (BSA) prevents inhibition and therefore recommend the routine addition of 0.04% BSA to all RQ-PCR reactions.[211] Second, a lower input of template will result in loss of sensitivity. This especially will be relevant in follow-up samples that seem to be MRD negative. For such samples, it is important to estimate the maximal MRD level that can be detected. Such estimation can be made by calculating the difference in control gene C_T values of the sample and the calibrator (ΔC_T), followed by multiplying the sensitivity obtained with the calibrator by $2^{-\Delta C_T}$ (eg if $\Delta C_T = 2$, the sensitivity will be four times lower). This estimation cannot be made when plasmids are used as the calibrator. Alternatively, one could establish acceptable ranges for control gene values and use these ranges for exclusion of poor samples.[207]

EXPRESSION OF DATA

Possibilities for data expression

At present, at least three possibilities exist for the expression of MRD data. Firstly, MRD levels can be determined relative to the diagnostic sample. Secondly, MRD levels can be expressed relative to a calibrator, for example, a cell line. Thirdly, data can be expressed as copy numbers by using plasmid standard curves.

For tumor-specific MRD-PCR targets at the DNA level, such as Ig/TCR gene rearrangements[55,132,137,140,144,205] and FLT3-ITD,[146] expression of MRD data relative to the diagnostic sample is an often used and easy method, as no calibrators (either cell lines or plasmids) are routinely available. This method is also suitable for other MRD-PCR targets,[172] the advantage being that different MRD-PCR targets analyzed within the same patient can easily be compared. Another advantage is that data are relatively easy to understand, as they are presented as percentage of malignant cells relative to the diagnostic sample. Nevertheless, several studies have used cloned rearrangements for preparation of the standard curve,[55,151,152,154,155] which may especially be useful if diagnostic material is not or insufficiently available.

For MRD-PCR targets that are not patient specific, such as fusion-gene transcripts[17,25,56,142,166,173,175,176] or *WT-1* expression,[116,117] a calibrator can be used. Such a calibrator can be a cell line known to express the MRD-PCR target of interest. By making a standard curve of the calibrator, the MRD level of an unknown sample can be determined relative to the calibrator. Although this method may work well within one laboratory, it seems to be less appropriate for multicenter studies. Furthermore, the obtained data are more difficult to interpret and as they are relative to an arbitrary calibrator and consequently do not directly reflect the percentage of malignant cells.

Expression of MRD data as (normalized) copy numbers is most frequently used for fusion-gene transcripts,[18–20,22,23,26,27,57,162,165,168,169,171,174,212,213] but can also be used for other MRD-PCR targets.[117,180] Plasmids have the advantage that they are stable and robust, and thus can be used

for the analysis of intra- and interlaboratory RQ-PCR variations. On the other hand, using plasmids greatly increases the risk of contamination and thereby of false-positive results. Furthermore (normalized) copy numbers are more difficult to interpret, especially if it concerns RNA MRD-PCR targets. Firstly, expression levels of a particular fusion-gene transcript may vary between patients; secondly, expression levels of different fusion genes vary. Consequently, only experienced persons can interpret the obtained 'absolute' (copy number) data, because the result of RNA-based RQ-PCR MRD assays is dependent on both the number of residual tumor cells as well as the median expression level of the MRD-PCR target in the malignant cells. Nevertheless, the kinetics of response to therapy (ie MRD data obtained at multiple time points) are probably more important than absolute MRD data obtained at a single time point.

Quantitation of MRD data

Although RQ-PCR is a quantitative technique, it does not mean that the obtained data can be quantified in each case. In our opinion, data can only be quantified if the MRD level is within the reproducible range (ie higher or equal to the reproducible sensitivity). Outside that range, data are no longer fully reproducible and therefore cannot be quantified.

A sample can be considered as positive, if the C_T value of one or more of the replicates of that sample is clearly outside the C_T range of the negative controls (eg at least one cycle lower than the lowest C_T value of the nonspecific amplification) and within certain distance (eg four cycles) from the final dilution step used for the maximal sensitivity. If plasmids are used as calibrators, a sample can be considered as positive if the C_T value is lower than the C_T value of the intercept (defining the detection of one molecule) plus one.[163]

A sample can be considered as negative, if no amplification is observed at all, if the lowest C_T value of the target is within or close to the C_T range of the negative controls (eg within one C_T from the lowest C_T value of the nonspecific amplification), or if all C_T values are too far (eg more than four cycles) apart from the highest C_T of the maximal sensitivity.

A positive sample with a C_T value above the C_T value of the reproducible sensitivity should be reported as positive, with a maximal MRD level lower than the reproducible sensitivity (eg $+, <10^{-4}$). It should be noted that a correction for the sensitivity has to be made as described above, if the DNA/RNA quantity and quality as assessed by a control gene is not appropriate. A comparable approach has recently been described.[175] Logically, very low MRD levels (below the reproducible sensitivity) should always be judged with caution; especially, if only one well of the replicates is positive and if the employed MRD-PCR target is not tumor-specific. In such case reanalysis of the doubtful sample(s) may be required.

CONCLUSIONS AND FUTURE STEPS

Since their introduction in the late 1990s (1997/1998), RQ-PCR techniques have become rapidly implemented for MRD studies in patients treated for hematologic malignancies. Several RQ-PCR instruments are nowadays available and different principles and approaches can be used. The currently available MRD-PCR targets make it possible to detect MRD in most patients and for several diseases MRD monitoring is already used for therapy guidance in clinical protocols.

The introduction of RQ-PCR techniques for MRD detection in clinical treatment protocols needs the development of international guidelines and criteria for the data analysis and laboratory reports. Furthermore, quality control rounds are required to monitor the performance of the participating laboratories and to further improve and standardize RQ-PCR analyses. For these purposes, international collaboration is essential. In Europe, several networks have been established; the development of common guidelines and quality control rounds are essential parts of these networks. These networks include the Europe Against Cancer Program (RQ-PCR analysis of fusion gene transcripts; coordinator: J Gabert), the European Study Group on MRD detection in ALL (coordinators: JJM van Dongen and VHJ van der Velden), the MRD Task Force of the I-BFM-SG (coordinators: JJM van Dongen and M Schrappe), the International Study Group on Standardization of Residual Disease Detection in *BCR–ABL* positive leukemia (coordinator: A Hochhaus), and the International Collaborative Study for Characterization of a Reference Material for *BCR–ABL* (MBCR) RNA Nucleic Acid Quantification by Real-Time Amplification Assays (coordinators: J Saldanha and J Gabert). These collaborations should further facilitate the introduction of RQ-PCR-based MRD detection in multicenter clinical treatment protocols.

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