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LEADING ARTICLE

Analysis of minimal residual disease by Ig/TCR gene rearrangements: guidelines for interpretation of real-time quantitative PCR data

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Most modern treatment protocols for acute lymphoblastic leukaemia (ALL) include the analysis of minimal residual disease (MRD). To ensure comparable MRD results between different MRD-polymerase chain reaction (PCR) laboratories, standardization and quality control are essential. The European Study Group on MRD detection in ALL (ESG-MRD-ALL), consisting of 30 MRD-PCR laboratories worldwide, has developed guidelines for the interpretation of real-time quantitative PCR-based MRD data. The application of these guidelines ensures identical interpretation of MRD data between different laboratories of the same MRD-based clinical protocol. Furthermore, the ESG-MRD-ALL guidelines will facilitate the comparison of MRD data obtained in different treatment protocols, including those with new drugs.
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Introduction

Several studies have shown that detection of minimal residual disease (MRD) in childhood and adult acute lymphoblastic leukaemia (ALL) is clinically relevant, both in *de novo* and relapsed ALL as well as in ALL patients undergoing stem cell transplantation.[1–12] Consequently, MRD diagnostics is currently incorporated in the stratification for several ALL treatment protocols.[13,14] In most MRD-based stratification studies, MRD is analyzed by real-time quantitative–polymerase chain reaction (RQ-PCR) analysis of 'leukemia-specific' junctional regions of rearranged immunoglobulin (Ig) genes and T-cell receptor (TCR) genes, which can be considered as 'DNA fingerprints' of the leukemic cells.[15,16]

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To ensure comparable MRD results between different MRD-PCR laboratories, standardization and quality control are essential. Based on the experience of the MRD Task Force of the International Berlin–Frankfurt–Münster (BFM) Study Group, performing the first pediatric ALL protocol including molecular MRD-based risk group stratification, the standardization is particularly needed for the interpretation of the RQ-PCR data.[17] In fact, guidelines for RQ-PCR data interpretation are not only essential to ensure comparable MRD results between different MRD-PCR laboratories within a single MRD-based treatment protocol, but also to provide a sound basis for the comparison of MRD results between different treatment protocols, including those with new drugs.

The European Study Group on MRD detection in ALL (ESG-MRD-ALL) was established in 2002 and now consists of 30 MRD-PCR laboratories spread across Europe, Israel, Singapore and Australia. The main aims of the ESG-MRD-ALL are the organization of a quality-control program twice per year, the collaborative development and evaluation of new MRD strategies and techniques, and the development of guidelines for the interpretation of RQ-PCR-based MRD data. The guidelines for data interpretation are based on theoretical considerations as well as practical applicability, and include guidelines for: (1) experimental set-up; (2) definition of quantitative range and sensitivity; (3) definition of MRD-positivity and MRD-negativity in follow-up samples; and (4) quantitation of MRD levels in follow-up samples.

Experimental set-up

A *standard curve* should be made by serially diluting the diagnostic DNA specimen in DNA obtained from mononuclear cells (MNC) from a pool of five to 10 healthy donors. For this purpose, MNC obtained from peripheral blood or bone marrow can be used. Owing to the much easier collection of peripheral blood MNC, most laboratories within the ESG-MRD-ALL use peripheral blood MNC. The serial dilutions should range from 10^{-1} to at least 10^{-5} (preferably with inclusion of a 5×10^{-4}

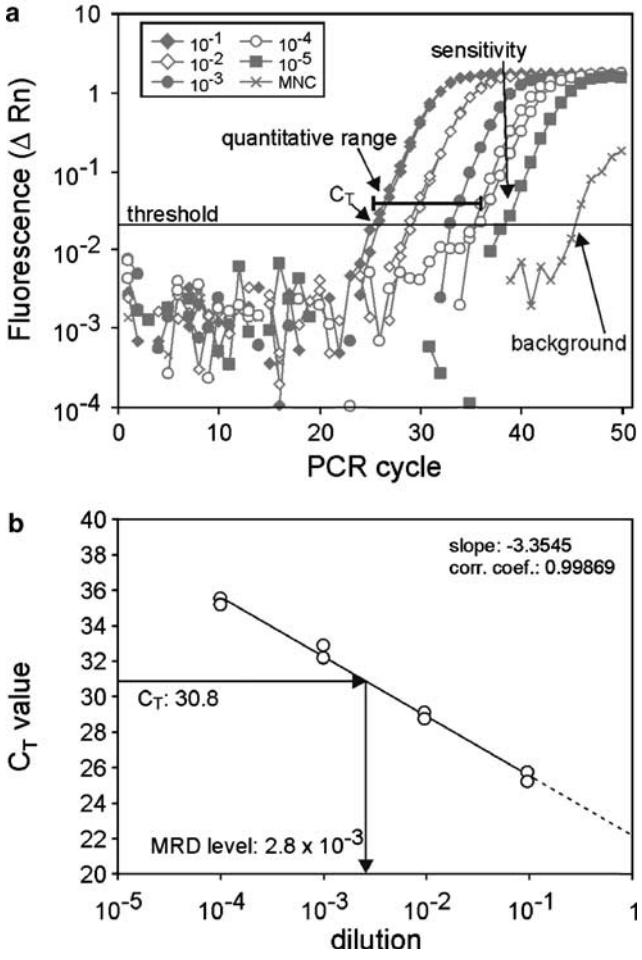

Figure 1 Technical definitions used for interpretation of RQ-PCR data. (**a**) Amplification plot of an RQ-PCR assay showing the position of the threshold and obtained C_T values, the 'quantitative range', 'sensitivity' and background (nonspecific amplification). (**b**) Standard curve constructed from the RQ-PCR assay. Based on the mean C_T value of the triplicate RQ-PCR analysis of a follow-up sample, the tumor load relative to the diagnostic sample can be determined.

step) and should be tested at least in duplicate. The first dilution step of the diagnostic sample should be based on the blast cell percentage determined in exactly the same sample (e.g. by flow cytometry), so that the 10^{-1} dilution really contains 10% blast cells. By plotting the logarithmic value of the dilution against the cycle threshold or crossing point (C_T), a standard curve can be obtained (Figure 1). If triplicates are used for the standard curve, all three values should be used, except for obvious technical reasons (e.g. broken capillary). As in most MRD-based protocols a sensitivity $\leqslant 10^{-4}$ is required, the ESG-MRD-ALL recommends the use of 500 ng DNA per reaction.

The *threshold* of the RQ-PCR assay should always be set in the region of exponential amplification across all amplification plots. This region is depicted in the log view of amplification plots as the portion of the curve that is linear. Often the threshold automatically determined by the instrument software can be used. However, if the threshold appears to be positioned outside the linear part of (some of) the amplification curves, adjustments may be made.

To determine the *background* of the RQ-PCR assay, that is, the nonspecific amplification of comparable Ig/TCR gene rearrangements present in normal cells, DNA obtained from MNC from a pool of five to 10 healthy donors should be used. As nonspecific amplification is generally only detected at a low level and outside the quantitative range of the RQ-PCR, nonspecific amplification controls should be run at least in $\geqslant$6-fold in each RQ-PCR analysis for each Ig or TCR marker. The lowest C_T value of these nonspecific amplification controls is specified as the (highest) background level. In addition, no template controls should always be included at least in duplicate in each RQ-PCR experiment.

Analysis of *follow-up samples* should be performed in triplicate and all three values should be taken into account.

Definition of the 'quantitative range' and the 'sensitivity'

The sensitivity of an RQ-PCR assay is dependent on several factors, including the type of rearrangement, the size of the junctional region and the amount of DNA in each reaction.[18–20] If a relatively high proportion of leukemic cells is present, the MRD level can be reliably quantified in the majority of cases. If the level of MRD is very low, the assay becomes less accurate. Thus, the variation in C_T values between replicates is generally <1.5, but this increases when the mean C_T value of the replicates is high (e.g. $C_T > 36$).[15] Within the ESG-MRD-ALL it was therefore, decided to define the *quantitative range* and the *sensitivity*. The 'quantitative range' reflects the part of the standard curve in which the MRD levels can be quantified reproducibly and accurately, whereas the 'sensitivity' reflects the lowest MRD level that still can be detected, although not reproducibly and accurately (Figure 1).

To determine the 'quantitative range' and the 'sensitivity' of the RQ-PCR assay, both the standard curve and the background need to be analyzed. As indicated above, nonspecific amplification is often only detected at a low level and outside the quantitative range of the RQ-PCR. Consequently, an accurate determination of the nonspecific amplification can be difficult. Furthermore, the nonspecific amplification observed in the normal MNC DNA may not be similar to nonspecific amplification present in the follow-up samples of patients as it is well known that follow-up samples can differ significantly in cellular composition, dependent on the follow-up time-point in the protocol. For example, high percentages of T cells can be found during induction therapy,[21] whereas high percentages of precursor B cells can be found post-induction and post-maintenance therapy.[22,23] Consequently, due to higher frequency of TCR or Ig gene rearrangements, respectively, the nonspecific amplification in follow-up samples may be higher than the generally used nonspecific amplification control (normal peripheral blood MNC DNA). It was decided, therefore, that within the ESG-MRD-ALL the 'quantitative range' should be sufficiently apart from the background, so that cellular composition-related variations in nonspecific amplification would not affect MRD analysis in the 'quantitative range'.

The standard curve of the RQ-PCR assay should theoretically have a slope of –3.3. However, in practice, several patient-specific RQ-PCR assays may perform somewhat less efficiently, resulting in a lower slope. In addition, due to experimental variation, the slope of the standard curve may sometimes be slightly higher than expected. Within the ESG-MRD-ALL, it was, therefore, decided to accept small deviations of the theoretical slope of –3.3.

The *quantitative range* is defined by the lowest dilution that meets all the following criteria:

- must give specific amplification, as determined by the shape of the amplification curve and (for the ABI Prism instruments) the multicomponent graph;

- must give reproducible amplification: ΔC_T of all replicates $\leqslant 1.5$;
- must have all C_T values $\geqslant 3.0$ lower than the *lowest* C_T value of the background (= amplification observed in normal MNC DNA); and
- must have a *mean* C_T value within a defined range from the *mean* C_T value of the previous dilution point: 2.6–4.0 C_T between 10-fold dilutions (e.g. 10^{-3}–10^{-4}); and 0.5–1.5 C_T between two-fold dilutions (e.g. 10^{-3}–5×10^{-4}).

The ranges between two dilution points were defined based on theoretical considerations (i.e. 1 C_T equals a twofold difference in amount of template) and practical applicability, as determined by analysis of over 1400 RQ-PCR assays.

The resulting *standard curve*, including the lowest dilution of the 'quantitative range' and *all* previous dilutions:

- must include at least three dilution points;
- must have a minimum range of two logs;
- must have a slope between −3.1 and −3.9; and
- must have a correlation coefficient $\geqslant 0.98$.

If an RQ-PCR does not fulfil the criteria for the 'quantitative range' and resulting standard curve, further analysis should not be performed because of the poor performance of the RQ-PCR.

The *sensitivity* is the lowest dilution that meets all the following criteria:

- must give specific amplification, as determined by the shape of the amplification curve and (for the ABI Prism instruments) the multicomponent graph;
- must have at least one positive replicate; ΔC_T of the replicates is not relevant;
- must have the *lowest* C_T value $\geqslant 1.0$ lower than the *lowest* C_T value of the background (= amplification observed in normal MNC DNA);
- must have the *lowest* C_T value <20 cycles from the undiluted sample or, if this undiluted sample is not included in the standard curve, from the intercept of the standard curve (representing the 10^0 dilution).

The 20 cycles in the last criteria reflect five log steps in case of a standard curve with a maximally accepted slope (−3.9).

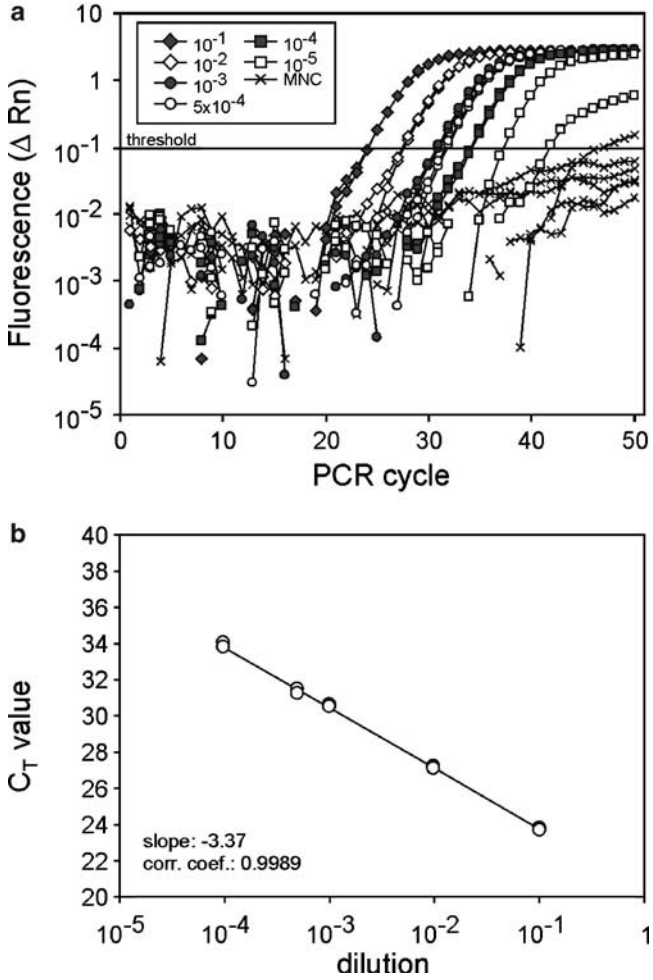

Figure 2 Definition of 'quantitative range' and 'sensitivity'. An *IGH* rearrangement was analyzed (in duplicate) using serial dilutions of the diagnostic specimen (10^{-1} to 10^{-5}). Nonspecific amplification (background) was determined by analyzing DNA obtained from MNC from a pool of five to 10 healthy donors (analyzed in sixfold). (**a**) Amplification plot of the RQ-PCR assay. (**b**) Standard curve constructed from the RQ-PCR assay. The 'quantitative range' of this assay was defined as 10^{-4}, as this is the lowest dilution $\geqslant 3$ C_T apart from the lowest C_T of the background ($C_T = 45.5$) and the C_T value of the replicates $\leqslant 1.5$. The mean C_T value (33.9) is within 2.6–4.0 from the mean C_T value of the 10^{-3} dilution (30.5). The resulting standard curve shows a slope of –3.37 and a correlation coefficient of 0.999, both being in the acceptable range. The 'sensitivity' of the assay was defined as 10^{-5}, as this is the lowest dilution with the lowest C_T value of the replicates $\geqslant 1$ apart from the lowest C_T of the background ($C_T = 45.5$). Furthermore, the lowest C_T value of the 10^{-5} dilution (37.1) was within 20 C_T from the intercept of the standard curve (20.4).

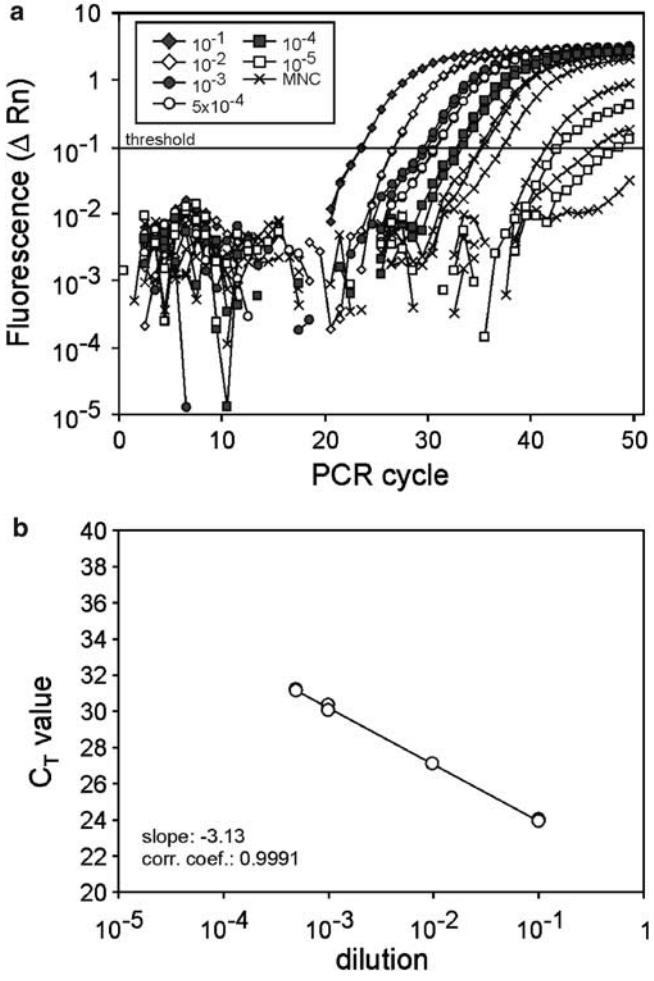

Figure 3 Definition of 'quantitative range' and 'sensitivity'. An *IGH* rearrangement was analyzed (in duplicate) using serial dilutions of the diagnostic specimen (10^{-1} to 10^{-5}). Nonspecific amplification (background) was determined by analyzing DNA obtained from MNC from a pool of five to 10 healthy donors (analyzed in sixfold). (**a**) Amplification plot of the RQ-PCR assay. (**b**) Standard curve constructed from the RQ-PCR assay. The 'quantitative range' of this assay was defined as 5×10^{-4}, as this is the lowest dilution $\geqslant 3$ C_T apart from the lowest C_T of the background ($C_T = 35.7$) and the C_T value of the replicates $\leqslant 1.5$. The mean C_T value (31.2) is within 0.5–1.5 from the mean C_T value of the 10^{-3} dilution (30.2). The resulting standard curve shows a slope of –3.13 and a correlation coefficient of 0.999, both being in the acceptable range. The 'sensitivity' of the assay was defined as 10^{-4}, as this is the lowest dilution with the lowest C_T value of the replicates $\geqslant 1$ apart from the lowest C_T of the background ($C_T = 35.7$). Furthermore, the lowest C_T value of the 10^{-4} dilution (33.2) was within 20 C_T from the intercept of the standard curve (20.9).

The five log steps were chosen because the theoretical sensitivity of an RQ-PCR assay using the recommended DNA input of 500 ng/reaction (corresponding to approximately 1×10^5 cells) is 10^{-5}.

Both the 'quantitative range' and the 'sensitivity' should be determined based on the results obtained in the RQ-PCR run, in which the follow-up samples are analyzed. Examples of two standard curves and their corresponding 'quantitative range' and 'sensitivity' are shown in Figures 2 and 3.

It should be noted that the 'quantitative range' of the RQ-PCR assay should be used to determine whether the RQ-PCR assay is sufficiently sensitive (generally $\leqslant 10^{-4}$) for reliable quantitative MRD analysis. However, when two Ig/TCR targets are required per patient, some protocols allow the second target to have a smaller 'quantitative range' (e.g. 5×10^{-4}), if the 'sensitivity' is $\leqslant 10^{-4}$. The required criteria for MRD-PCR targets need to be determined for each MRD-based clinical protocol as the cut-off MRD values for escalation or reduction of treatment can differ between clinical protocols.

Definition of MRD-positivity and MRD-negativity in follow-up samples

The interpretation of RQ-PCR results obtained in follow-up samples is most difficult if MRD levels are outside the 'quantitative range' of the assay. In these cases, it may not always be clear whether the signal observed is due to specific amplification from leukemic cell DNA or from nonspecific amplification of normal DNA. However, the decision to classify a follow-up sample to be 'MRD positive' or 'MRD negative' may have major clinical implications. Therefore, it was decided within the ESG-MRD-ALL to develop separate guidelines for (i) protocols that aim at therapy reduction and (ii) for protocols directed toward therapy intensification.

Clinical protocols that aim at therapy reduction

In studies aiming at therapy reduction, it is particularly important to prevent false-negative MRD results to ensure sufficiently

intensive treatment for all patients. Therefore, the guidelines are designed in such a way that a follow-up sample can be interpreted as 'MRD positive', even though the C_T value is so high that nonspecific amplification cannot be excluded completely.

A sample is considered to be MRD positive if

- the C_T value of at least one of the three replicates is $\geqslant$1.0 C_T lower than the *lowest* C_T of background and
- the C_T value of at least one of the three replicates is within 4.0 C_T from the *highest* C_T value of the 'sensitivity' (fulfilling all 'sensitivity' criteria).

Consequently, a follow-up sample is considered 'MRD negative', if no amplification is observed at all, if the lowest C_T value of the target is within 1 C_T from the lowest C_T of the background, or if all C_T values are more than four cycles separated from the highest C_T value of the 'sensitivity'.

Clinical protocols that aim at therapy intensification

In studies aimed at therapy intensification, prevention of false-positive results is a prerequisite to prevent over-treatment of patients. Therefore, a sample is considered to be 'MRD positive' only if it gives amplification at a level clearly apart from the background.

A sample is considered to be *MRD positive* if

- the C_T value of at least one of the three replicates is $\geqslant$3.0 C_T lower than the *lowest* C_T of background and
- the C_T value of at least one of the three replicates is within 4.0 C_T from the *highest* C_T value of the 'sensitivity' (fulfilling all 'sensitivity' criteria).

Logically, very low MRD levels (below the 'quantitative range') should always be judged with caution, especially if only one well of the three replicates is positive. In such case, re-analysis of the doubtful sample(s) may be performed, but one should be aware that by definition the results will often not be reproducible.

Examples of MRD data interpretation of follow-up samples are shown in Figures 4 and 5.

Quantitation of MRD levels in follow-up samples

Although RQ-PCR is in principle a quantitative technique, it does not mean that the data obtained can be quantified in each case. Within the ESG-MRD-ALL, it was decided that data can only be quantified if the MRD level is within the 'quantitative range'. Outside that range, data are no longer fully reproducible and therefore, cannot be quantified accurately.

Furthermore, in order to obtain accurate MRD data, the RQ-PCR data obtained for the Ig/TCR gene rearrangements should

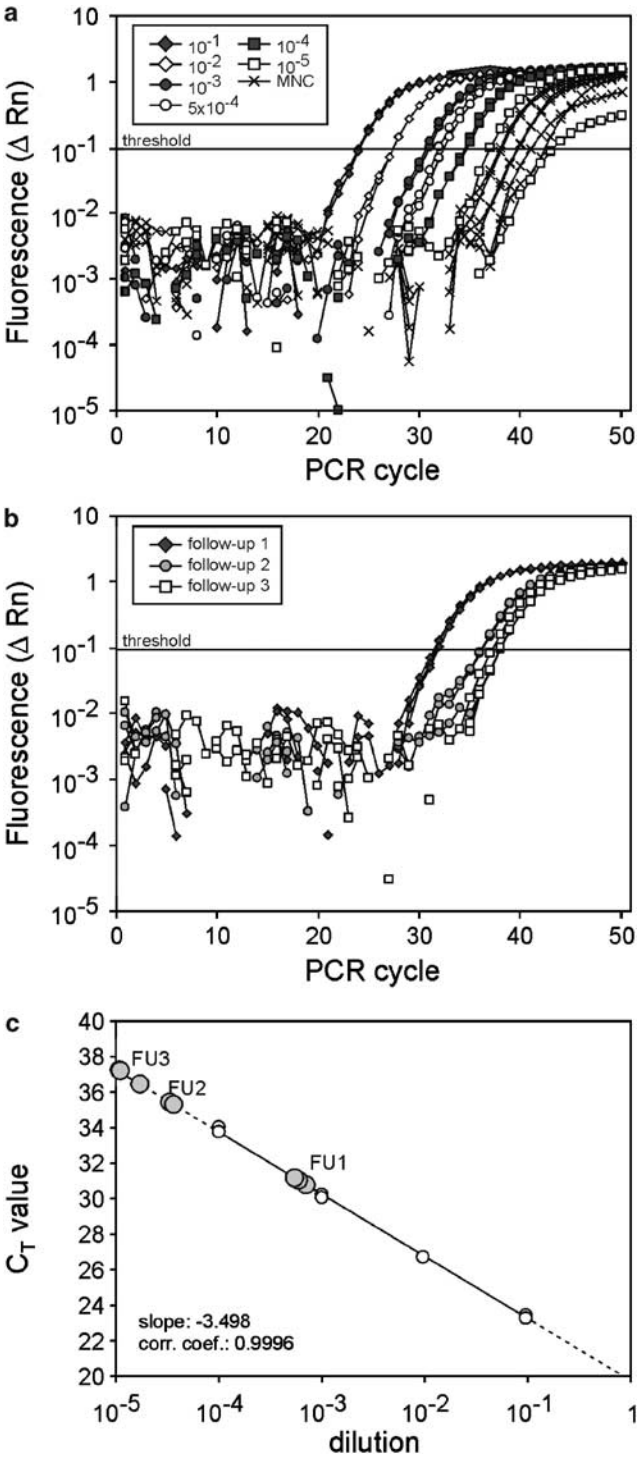

Figure 4 MRD data interpretation in follow-up samples. An *IGH* rearrangement was analyzed using serial dilutions of the diagnostic specimen (10^{-1}–10^{-5}), DNA obtained from MNC from a pool of five to 10 healthy donors (background), and three follow-up samples. (**a**) Amplification plot of the standard curve and the background. (**b**) Amplification plot of the follow-up samples. (**c**) Standard curve constructed from the RQ-PCR assay. The 'quantitative range' of this assay was defined as 10^{-4}; this resulted in a standard curve with a slope of –3.50, a correlation coefficient of 1.000 and an intercept of 19.8. The 'sensitivity' of the assay was also 10^{-4}, as both C_T values of the 10^{-5} dilution (42.2; 36.4) were within one C_T value from the lowest C_T value of the background (37.3). The highest C_T value of the 'sensitivity' was 34.0; the lowest C_T value of the background was 37.3. Follow-up sample 1 shows amplification in all three wells, the mean C_T value (31.1) being lower than the highest C_T value of the 'quantitative range' (34.0). This sample is considered MRD positive and the MRD level can accurately be determined using the standard curve of the assay, resulting in an MRD level of 6×10^{-4}. Follow-up sample 2 shows amplification below the 'quantitative range' in all three wells, two C_T values (35.5; 35.4) being $\geqslant$1 C_T from the lowest C_T value of the background. This sample therefore is considered MRD positive, but accurate quantitation is not possible. Consequently, this sample is considered 'MRD positive, $<10^{-4}$'. Follow-up sample 3 shows amplification in all three wells, but all C_T values are within 1 C_T from the lowest C_T value of the background, and therefore, this sample is considered MRD negative.

be corrected for the amount and 'amplifiability' of the DNA of the diagnosis sample and the follow-up samples. This should be done by analyzing a control gene in parallel to the Ig or TCR gene target. If the RQ-PCR of the control gene shows a lower amount of template than expected based on physical measurements (e.g. optical density measurement), special caution is needed as this lower value can be the result of inhibition, which can be found in a substantial number of bone marrow or peripheral blood samples (5–10%).[24] Addition of bovine serum albumin (BSA) prevents inhibition[24] and the ESG-MRD-ALL therefore recommends the inclusion of 0.04% BSA in all RQ-PCR reactions. Furthermore, as the addition of less amount of template will result in loss of sensitivity, the control-gene values of all samples need to be within predefined ranges (e.g. 250–1000 ng/reaction). This is especially relevant in the analysis of follow-up samples that seem to be MRD negative. If such samples have control-gene results outside the predefined range, they should be excluded from analysis.

MRD-positive samples can be quantified if

- the *mean* C_T value of the replicates is lesser than or equal to the *highest* C_T value of the 'quantitative range' and
- the ΔC_T of the replicates is $\leqslant 1.5$.

Quantitation is performed:

- using the standard curve, excluding dilutions outside the 'quantitative range', of the involved Ig/TCR target;
- using the *mean* C_T of the triplicates of the follow-up sample; and
- correcting the MRD level according to the DNA quality/quantity of the diagnostic sample and the follow-up sample as determined by RQ-PCR of the control gene.

For MRD-positive samples that *cannot* be quantified (i.e. ΔC_T of the replicates >1.5 and/or mean C_T value outside 'quantitative range'):

- data should be presented as 'positive, outside quantitative range' (e.g. positive, $<10^{-4}$).

For samples that cannot be quantified, the quality/quantity of the DNA should always be checked by RQ-PCR analysis of the control gene. These control-gene data should be within the predefined acceptable range, but are not used for correcting the MRD level.

All MRD data (either quantitative, positive but not quantifiable or negative) should always be reported together with the 'quantitative range' and the 'sensitivity' of the assay.

Examples of MRD data reporting of follow-up samples are shown in Figures 4 and 5.

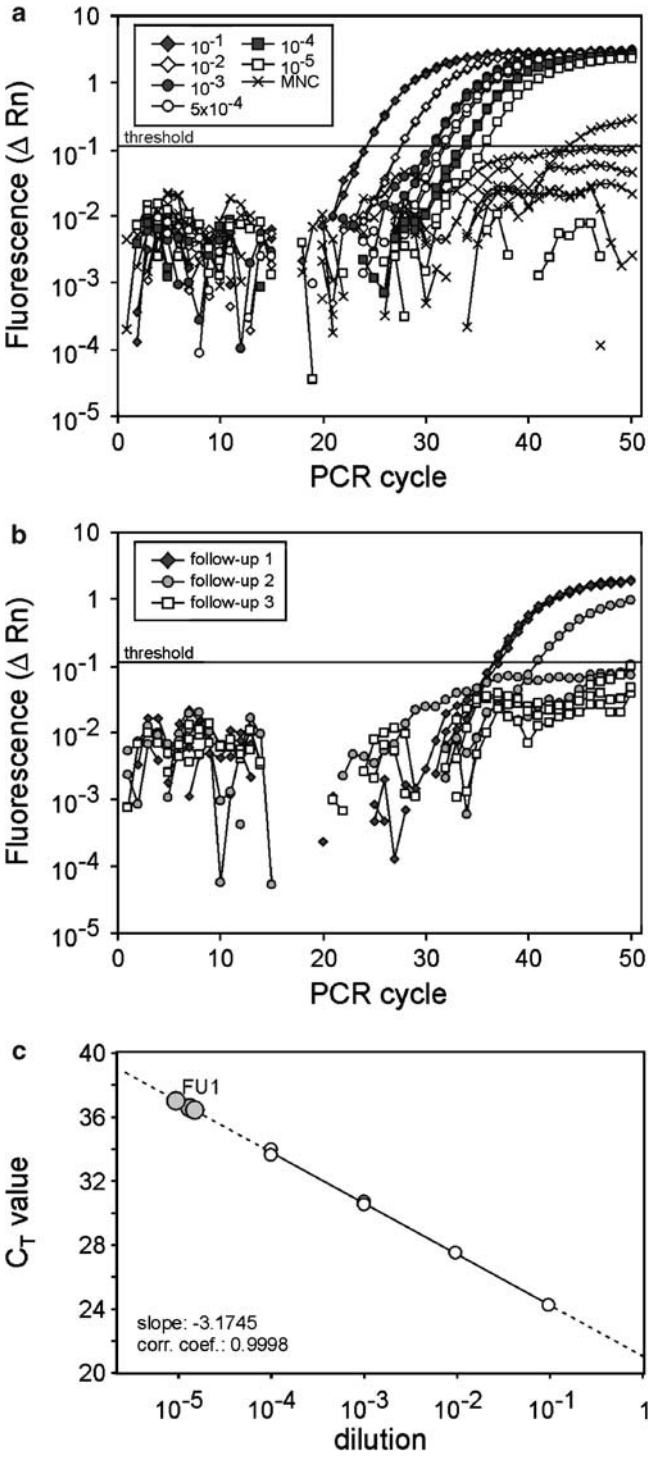

Figure 5 MRD data interpretation in follow-up samples. An *IGH* rearrangement was analyzed using serial dilutions of the diagnostic specimen (10^{-1}–10^{-5}), DNA obtained from MNC from a pool of five to 10 healthy donors (background), and three follow-up samples. (**a**) Amplification plot of the standard curve and the background. (**b**) Amplification plot of the follow-up samples. (**c**) Standard curve constructed from the RQ-PCR assay. The 'quantitative range' of this assay was defined as 10^{-4}; this resulted in a standard curve with a slope of –3.17, a correlation coefficient of 1.000 and an intercept of 21.1. The 'sensitivity' of the assay was 10^{-5}. The highest C_T value of the 'sensitivity' was 35.7; the lowest C_T value of the background was 43.9. Follow-up sample 1 shows amplification in all three wells, all C_T values (36.6, 37.1 and 36.5) being <4 apart from the highest C_T value of the 'sensitivity' (35.7) and more than 1 C_T apart from the lowest C_T value of the background (43.9). This sample is considered MRD positive. As the mean C_T value of this sample (36.7) is higher than the highest C_T value of the 'quantitative range' (34.0), the exact MRD level cannot be defined accurately; the sample is therefore, considered 'MRD positive, $<10^{-4}$'. Follow-up sample 2 shows amplification in one out of three wells only, with a C_T value of 40.9. This value is more than 4 C_T apart from the highest C_T value of the 'sensitivity' (35.7), and consequently this sample is considered MRD negative. Follow-up sample 3 shows no specific amplification at all, and is considered MRD negative.

Conclusions

The guidelines for RQ-PCR MRD data interpretation presented here can be used in all MRD studies that employ Ig/TCR gene rearrangements as MRD-PCR target. Their application is relatively simple and enables highly comparable interpretation of MRD data between different MRD laboratories, as determined in several quality control rounds performed within the ESG-MRD-ALL (manuscript in preparation). Logically, in some exceptional cases, interpretation of RQ-PCR data may not be appropriate using the ESG-MRD-ALL guidelines; in such cases one can deviate from these guidelines as long as this is clearly reported.

The application of the ESG-MRD-ALL guidelines will ensure uniform data interpretation in different MRD-PCR laboratories within the same MRD-based clinical protocol. Furthermore, the ESG-MRD-ALL guidelines will also facilitate the comparison of levels of residual disease achieved by different treatment protocols, including those with new drugs. Indeed, the ESG-MRD-ALL guidelines are already being utilized in several related MRD intervention clinical trials, which use the same induction regimen and identical MRD-based stratification, followed by treatment arms that differ per protocol.

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Appendix

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Representing the MRD laboratories of the following clinical ALL protocols:

AIEOP-BFM ALL 2000, ANZCHOG ALL Study-8, BFM-HR-ALL, DCOG-ALL10, Interfant 99, MRC-ALL 2003, MRC UKALL12, COALL 07-03, FRALLE 2000, EORTC-CLG 58951, NOPHO ALL–2000, MiniRisk (ALL-IC BFM), ALL-REZ BFM 2002, AIEOP REC 2003, MRC-UKALL R3, GMALL 07/03, GRALL 03/05 and MRC-UKALL12.