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Evaluation and Automation of Hematopoietic Chimerism Analysis Based on Real-Time Quantitative Polymerase Chain Reaction

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ABSTRACT

Chimerism analysis is an essential tool in the follow-up of patients after allogeneic stem cell transplantation. High-resolution methods for chimerism analysis based on real-time quantitative polymerase chain reaction (RQ-PCR) with a detection limit of 0.1% marker-specific cells are especially valuable in the detection of patient-derived subpopulations for the monitoring of minimal residual disease. Using artificial chimeric mixtures of genotypically different cells, we optimized and evaluated the intrasample variation, accuracy, and detection limit of chimerism analysis based on RQ-PCR of short insertion and deletion polymorphisms. Furthermore, automated setup by robot was evaluated. The results were accurate, with acceptable intrasample variation at and above 0.1% marker-specific cells. The sensitivity was mainly limited by background values. Chimerism results based on RQ-PCR were similar to results based on PCR of short tandem repeats when samples from recipients of transplants with nonmyeloablative conditioning were analyzed. Furthermore, automated setup was feasible in a time-, labor-, and reagent-conserving manner.
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INTRODUCTION

Allogeneic stem cell transplantation with various conditioning regimens is a well-established treatment modality for many hematologic diseases [1-3]. Chimerism analysis distinguishing donor from recipient in hematopoietic cell subsets after allogeneic stem cell transplantation is routine in the follow-up of patients. Chimerism analysis early after transplantation reflects engraftment kinetics, whereas analysis after engraftment assists the interpretation of clinical events such as graft-versus-host disease, secondary graft rejection, minimal residual disease, and disease relapse. Information about chimerism status is especially important in the follow-up of patients undergoing allogeneic stem cell transplantation with nonmyeloablative conditioning, because immunomodulatory therapeutic intervention is an essential part of the management of these patients [4-11].

Chimerism analysis based on polymerase chain reaction (PCR) of polymorphisms such as variable number of tandem repeats or microsatellites/short tandem repeats (STR) with subsequent fluorescence detection of size-fractionated products is the standard method of examination. This method is well tested and has a detection limit of 1% to 5% [12-16]. New methods based on real-time quantitative PCR (RQ-PCR) of single nucleotide polymorphisms or short insertion/deletion polymorphisms have the advantage of a lower detection limit (approximately 0.1%) and are in some cases also time saving [17-23]. Validation and testing of the new methods are, however, necessary to confirm their applicability.

The purpose of this study was to optimize and

evaluate the intrasample variation, accuracy, and detection limit of chimerism analysis by RQ-PCR based on short insertion and deletion polymorphisms as previously described by Alizadeh et al [17]. In addition, automated setup by a robot was evaluated. The automated RQ-PCR assay was compared with chimerism analysis based on STR-PCR. Optimization and evaluation were performed on chimeric cell mixtures, whereas comparison of RQ-PCR–based with STR-PCR–based chimerism analysis was performed on blood samples obtained from patients who underwent peripheral blood stem cell transplantation after nonmyeloablative conditioning.

MATERIALS AND METHODS

Sample Preparation and DNA Extraction

Chimeric cell mixtures were made by serial dilutions of pregenotyped leucocytes from volunteers for testing of 17 of the 19 polymorphic genetic markers described by Alizadeh et al [17]. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density gradient centrifugation (Lymfoprep; Axis Shield PoC, Oslo, Norway) and counted twice in a hemocytometer after staining with methyl violet. A minimum of 300 cells were counted each time, and the mean count was used for subsequent dilution. For 15 of the evaluated allele-specific markers, PBMCs with the homozygous allele were diluted in PBMCs without the specific allele to obtain the following proportions of chimeric cell mixtures: 0%, 0.001%, 0.01%, 0.1%, 1%, 10%, 50%, 90%, and 100%. The markers S05B and S09B were tested by dilution of PBMCs heterozygous for the specific allele in the same cell mixtures. The 2 less informative biallelic markers (S05A and S09A) were not tested. The primers and probe for the marker S02 were substituted during the study, so that all analyses in the 384-well plates were performed with new primers and probe (new S02: forward primer, AACTTTTGCCGAAGCTCACAA; reverse primer, TTGGTTTTTCGCAGGCAGACT; probe, CAGGCGCCTGAGCCACCCTCA). DNA was extracted from each cell mixture by combined Triton-X/sodium dodecyl sulfate detergent and sucrose lysis, proteinase K digestion, 6 mol/L $NaCl_2$ extraction, isopropanol precipitation, and ethanol wash [24].

For the comparison of chimerism analysis based on STR-PCR versus RQ-PCR, peripheral blood samples were obtained from 11 randomly selected recipients of hematopoietic stem cell transplants after nonmyeloablative conditioning with fludarabine and 2 Gy of total body irradiation [25,26]. The leucocytes in the blood samples taken after transplantation were separated into specific lineages with immunomagnetic beads (Dynabeads; Dynal, Oslo, Norway) by using anti-CD15, anti-CD4, and anti-CD8 beads. DNA was

extracted as described previously. STR-PCR–based and RQ-PCR–based chimerism analysis was performed in parallel on 300 DNA samples. The study was approved by the local ethics committee.

Chimerism Analysis Based on RQ-PCR

To minimize the background signal while attaining sufficient DNA amplification, RQ-PCR conditions were optimized for each specific marker by evaluating triplet DNA standard amplification curves with a fixed probe concentration at 200 nmol/L and either a varying annealing temperature between 60°C and 62°C or a decreasing primer concentration (600, 100, 50, and 25 nmol/L).

Intrasample variation, accuracy, and detection limits of optimized RQ-PCR–based chimerism analysis were evaluated on the chimeric cell mixtures by use of the allele-specific marker only. To test the intrasample variation, triplicates of DNA extracted from the cell mixtures were analyzed twice for each allele-specific marker by using 96-well plates under the same conditions, and the mean results of the triplicates from the 2 analyses were compared. Accuracy was defined as the ability to obtain an RQ-PCR result corresponding to the cell mixture. Accuracy was evaluated both in 96-well and 384-well plates, because the final reaction volumes and, thus, the target DNA concentrations were different in the 2 types of plates (see PCR conditions, below). The detection limit was estimated as the minimal cell mixture percentage detectable in both 96-well and 384-well plates.

Optimized RQ-PCR analysis on patient DNA samples was finally compared with chimerism analysis based on STR-PCR. All patient samples were analyzed in the 384-well plates.

Optimization was performed on the ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA), and cell mixtures and patient samples were analyzed on the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). Parallel analyses on the 7900HT and 7700 apparatus showed no differences (data not shown). The baseline noise level was always set at 20 cycles, and the threshold at ΔR_n was 0.1, where ΔR_n was the normalized reporter signal minus the baseline signal.

PCR conditions were as follows: 100 ng of DNA mixed with 15 μL of Master Mix 2× Buffer (Applied Biosystems) (96-well system) or 10 μL of Master Mix 2× Buffer (384-well system), 100 nmol/L primer, and 200 nmol/L probe in a final volume of 30 μL (96-well system) or 20 μL (384-well system). PCR cycles were 2 minutes at 50°C followed by 10 minutes at 95°C and 50 amplification cycles (95°C for 15 seconds and 60°C-62°C for 60 seconds).

A standard amplification curve was made by serial dilutions of DNA with the allele-specific marker sus-

pended in DNA without the specific marker in each plate in the following dilutions: 1:0, 1:10, 1:100, 1:1000, 1:10 000, and 1:100 000. DNA was either heterozygous or homozygous for the allele-specific marker, depending on the composition of the cell mixture or patient sample being assessed. All samples were correlated with the standard amplification curve, and chimerism percentages were calculated by means of SDS 2.2 software (Applied Biosystems). The chimerism percentage was corrected for the number of DNA copies in each sample by using a genome-equivalent standard amplification curve made by serial dilution of DNA in the interval of 500 to 100 000 copies in an active reference system (the glyceraldehyde phosphate dehydrogenase gene or the albumin gene) [17,27].

The results of all analyses in the 384-well plates were furthermore calculated by the comparative method with the formula

$$QU/QC = (1 + E)^{-(\Delta CtU - \Delta CtC)},$$

where QU is the normalized quantity of DNA sequences in the unknown sample, QC is the normalized quantity of DNA sequences in the calibrator sample (ie, the patient or donor pretransplantation DNA sample), Ct is the threshold cycle, ΔCt is $Ct_{\text{allele-specific marker system}} - Ct_{\text{active reference system}}$, ΔCtU is ΔCt in the unknown sample, and ΔCtC is ΔCt in the calibrator sample. E describes the PCR efficiency, calculated by $E = 10^{(-1/\text{slope})}$. If the efficiency of the PCR was 100%, then the formula of the comparative method could be reduced to [17,28-31].

$$QU/QC = 2^{-(\Delta CtU - \Delta CtC)}$$

Results obtained by the 2 methods of calculation were identical (data not shown). Results obtained by the standard curve method were used subsequently, because it gives the opportunity to evaluate the efficiency and reproducibility of PCR cycling in each plate.

For comparison of RQ-PCR and STR-PCR, all patient samples were analyzed in triplicate with a patient and a donor allele–specific genetic marker on the same 384-well plate with the RQ-PCR method. The mean of triplicate values was used for comparison.

Background signals were defined as signals derived from DNA without the allele-specific marker analyzed in a given allele-specific marker system. Background DNA 100 and 300 ng without the allele-specific marker was analyzed on all plates.

When patient samples were analyzed, the sample curves were compared with the background curves to check for overlap. If the curves were overlapping, then the signal of the patient sample could not be considered as a signal of chimeric cells but was interpreted as possible nonspecific background noise and, thus, as a negative signal.

Automation of Chimerism Analysis Based on RQ-PCR

PCR analysis in 384-well plates was performed to evaluate an automated robot-assisted setup. Setup in 384-well plates was performed with a Biomek 2000 robot (Beckman Coulter, Fullerton, CA), which allowed direct transfer of premixed PCR reagents and DNA in known concentrations from patient and standard curve samples into the 384-well plate. This permitted simultaneous analysis of 39 samples from 1 patient in both the patient- and donor-specific genetic marker systems, including standard amplification curves, background controls, and active reference systems for all samples (the robot setup design and spreadsheet are available on request). Figure 1 illustrates the distribution of reagents and DNA on the 384-well plate.

Chimerism Analysis Based on STR-PCR

Patient samples were analyzed by fluorescence-based STR-PCR by using a combination of 7 microsatellite systems. The detection limit for the system as a whole was approximately 1% to 5% [14,32]. From each isolated patient cell linage, 100 ng of DNA was added to 50 µL of PCR reaction containing 2 mmol/L deoxyribonucleoside triphosphates, 200 mmol/L Tris-HCl, 500 mmol/L KCl, 50 mmol/l $MgCl_2$, 600 to 1200 nmol/L of the primers, and 1.25 U of *Taq* DNA polymerase (Platinum; Invitrogen, Carlsbad, CA), followed by amplification on the PTC-200 Peltier Thermal Cycler (MJ Research, Watertown, MA) with initial DNA denaturation at 94°C for 2 minutes. Cell cycling consisted of denaturation at 94°C for 60 seconds, primer annealing at 60°C for 60 seconds, and primer extension at 72°C for 60 seconds for 26 cycles. A final 10-minutes extension at 72°C followed the last cycle.

PCR amplification product (1-2 µL) was mixed with formamide and standard GeneScan 350 TAMRA (Applied Biosystems), denatured at 100°C for 4 minutes, and immediately chilled before analysis with capillary electrophoresis on an automated 310 Genetic Analyzer by using Performance Optimised Polymer 4 (Applied Biosystems). Data were analyzed by GeneScan software version 3.12 (Applied Biosystems).

Statistics

All chimerism results on patients, both STR and RQ-PCR based, are reported as donor percentages. The lowest chimerism value of the 2 marker systems was always used for determination of the chimerism percentage. That is, if the lowest chimerism value was the value calculated from the patient-specific marker system, then the reported chimerism percentage was calculated by subtracting this value from 100%. If the lowest chimerism value was the value calculated from
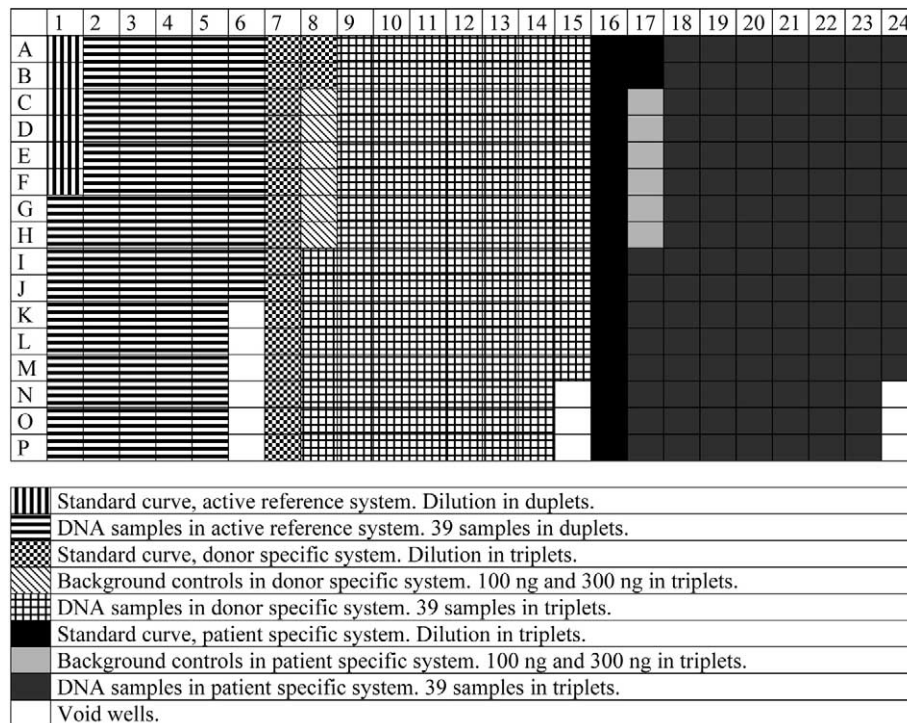
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 |

Figure 1. The 384-well plate setup.

Legend:

- Standard curve, active reference system. Dilution in duplets.
- DNA samples in active reference system. 39 samples in duplets.
- Standard curve, donor specific system. Dilution in triplets.
- Background controls in donor specific system. 100 ng and 300 ng in triplets.
- DNA samples in donor specific system. 39 samples in triplets.
- Standard curve, patient specific system. Dilution in triplets.
- Background controls in patient specific system. 100 ng and 300 ng in triplets.
- DNA samples in patient specific system. 39 samples in triplets.
- Void wells.

the donor-specific marker, then the value would be reported directly. This method of calculation circumvents the problem of the increased absolute variation of donor chimerism results seen with higher fractions of donor DNA in the PCR reaction. This problem can be illustrated as follows: a difference of 1 PCR cycle corresponds to 100% variation in estimated DNA in the sample. By using the comparative method and considering, for example, a 0.5 threshold cycle error, this will correspond to a variation in DNA of 50%. The result of a sample with a chimerism value of 1% will thus vary between 0.75% and 1.5%, a chimeric sample of 50% will vary between 37.5% and 75%, and a chimeric sample of 99% will vary between 75% and 150%.

Bland and Altman statistics were used for assessing the agreement between first and second analyses [33]. The differences and means of first and second analyses were plotted against each other to compare the results of the 2 analyses of RQ-PCR for information on intrasample variation. The limits of agreement were calculated as the mean difference between the 2 analyses ± 2 times the standard deviation. Because the variation of absolute difference increases as the chimeric value increases, all data were log-transformed. The same method was used to compare RQ-PCR–based with STR-PCR–based chimerism analysis.

The Wilcoxon matched pairs test was used to compare the results of the RQ-PCR–based chimerism analysis of the cell mixtures. Statistical analyses were performed with GraphPad Prism version 4 for Windows

(GraphPad Software Inc., San Diego, CA) and SAS version 8 for Windows (SAS Institute Inc., Cary, NC).

RESULTS

Optimization of RQ-PCR Conditions

Reduction of the primer concentration to less than 600 nmol/L resulted in fading background signals for most genetic markers. With a reduction to less than 100 nmol/L, a clear tendency toward declining amplification was seen in most systems independently of the annealing temperature. Consequently, a primer concentration of 100 nmol/L was chosen for all systems. Increasing the annealing temperature from 60°C to 62°C reduced the background proportions further for several markers without affecting the amplification. An annealing temperature of either 60°C (systems 01, 02, 04, 05, 06, 07, 09, 010, and 011) or 62°C (systems 03 and 08) was chosen.

Intrasample Variation, Accuracy, and Detection Limit of Chimerism Analysis Based on RQ-PCR

Intrasample variation was examined in the 96-well plates by 2 identical analyses on each cell mixture for each allele-specific genetic marker. Reproducible results were obtained from ≥0.1% marker-specific cells (Figure 2A). From 0.1% to 100% cell mixtures, the limits of agreement of the log-transformed difference between the 2 analyses were −0.22 to 0.25; ie, 95% of
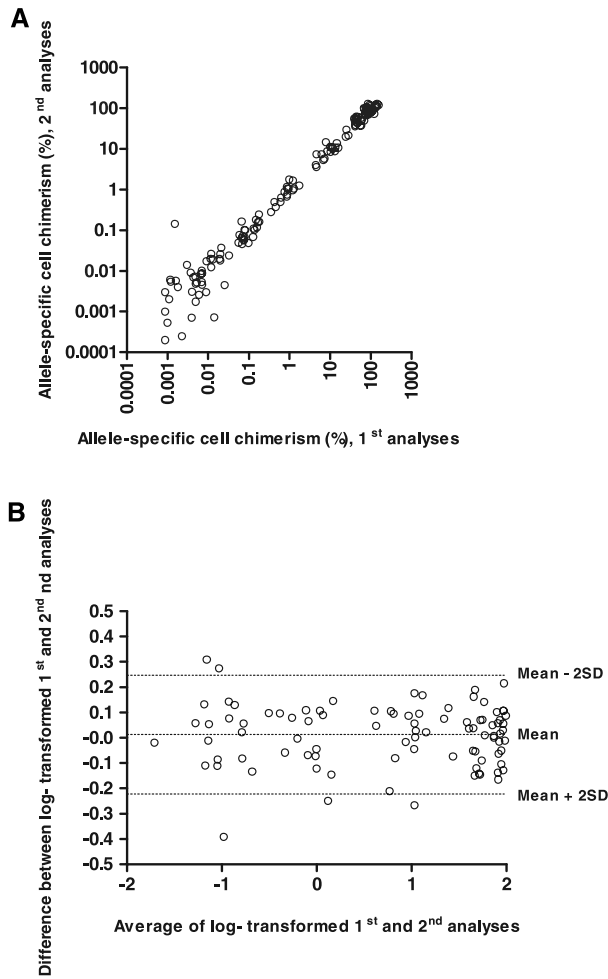
A

B

Figure 2. A, Intrasample variation of RQ-PCR–based chimerism analysis on cell mixtures from 0.001% to 100% allele-specific cells in each allele-specific marker in 2 different analyses in 96-well plates. B, Bland-Altman plot comparing log-transformed differences and means of first analyses and second analyses on cell mixtures from 0.1% to 100% allele-specific cells. On the x-axis, −2 corresponds to 0.01%, −1 to 0.1%, 0 to 1%, 1 to 10%, and 2 to 100% allele-specific cells.

cases of the second analyses will be between 0.60 and 1.77 times the first analyses of RQ-PCR (Figure 2B). At less than 0.1% marker-specific cells, the variation increased considerably.

Chimerism results on cell mixtures for evaluation of accuracy and detection limits are shown in Table 1. Accurate results were obtained with cell mixtures containing 0.1% to 10% marker-specific cells, except for 0.1% marker-specific cells in the 384-well plates, where a high coefficient of variation was found because of low sensitivity in a single genetic marker system (system S01B [17]). If system S01B was excluded from the 384-well plate, then the median value of RQ-PCR results at 0.1% marker-specific cells was 0.08% (range, 0.02%-0.21%), with a coefficient of variation of 56%.

At less than 0.1% marker-specific cells, the varia-

Table 1. *RQ-PCR–Based Chimerism Analysis of Artificial Cell Mixtures*

Variable	Artificial Cell Mixture (%)								
	0	0.001	0.01	0.1	1	10	50	90	100
96-well plate analyses									
Number of analyses	34 (2 × 17)	34 (2 × 17)	34 (2 × 17)	34 (2 × 17)	34 (2 × 17)	34 (2 × 17)	34 (2 × 17)	34 (2 × 17)	34 (2 × 17)
Median of RQ-PCR results (%)	0.0020	0.0038	0.012	0.08	0.9	9	44	86	94
Minimum of RQ-PCR results (%)	0.0000	0.0000	0.000	0.02	0.1	4	20	11	68
Maximum of RQ-PCR results (%)	0.1450	0.0760	0.068	0.24	1.8	16	69	136	158
Coefficient of variation (%)	340	189	93	51	48	36	28	34	22
384-well plate analyses									
Number of analyses	17	17	17	17	17	17	17	17	17
Median of RQ-PCR results (%)	0.0051	0.0025	0.013	0.08	0.9	10	52	97	112
Minimum of RQ-PCR results (%)	0.0000	0.0000	0.000	0.02	0.2	5	39	77	70
Maximum of RQ-PCR results (%)	0.9984	0.9643	0.998	1.15	2.1	19	200	149	177
Coefficient of variation (%)	338	330	283	164	53	36	60	20	24
Coefficient of variation (%) when excluding S01B	135	217	190	56	47	37	62	21	24

tion of RQ-PCR results increased considerably; ie, the accuracy failed. At ≥50% marker-specific cells, the coefficient of variation was constant or declining, but an increased absolute variation of chimerism results was observed, as expected.

In clinical transplantation settings, distinguishing mixed chimerism from complete donor chimerism is very important in relation to minimal residual disease. In the 96-well plates, 0.1% and 0.01% cell mixtures could be distinguished from 0% by RQ-PCR–based analysis ($P < .001$ and $P = .001$, respectively; Wilcoxon matched pairs test). No significant difference could be found between 0.001% cell dilution and 0% ($P = .804$). In the 384-well plates, virtually the same circumstances were demonstrated, as 0.1% and 0.01% cell mixtures could be distinguished from 0% ($P < .001$ and $P = .007$, respectively). No significant difference was found between 0.001% cell mixture and 0% ($P = .510$). This makes sense, because the PCR reactions contained only 100 ng of DNA in total (ie, approximately 16,000 DNA copies in total, leading to an average of only 0.16 DNA copies of the specific marker per well in the 0.001% mixture). In both the 96-well and 384-well plates, the 0.1% and 0.01% cell mixtures could be distinguished from each other ($P < .001$ and $P = .001$, respectively).

The median value of 100 ng of background DNA in the 96-well plates was 0.002% (range, 0%-0.145%). Three hundred nanograms of background DNA showed a slightly higher background value, with a median of 0.004% (range, 0%-0.007%). Results from the 384-well plates showed slightly higher values, with a median at 100 ng of background DNA of 0.005% (range, 0%-0.998%) and of 0.002% (range, 0%-0.445%) at 300 ng of background DNA. Again the one genetic marker system (S01B) with reduced sensitivity in the 384-well plates increased the background percentages. When the genetic marker S01B was excluded, background analyses showed a median value of 0.004% (range, 0%-0.084%) at 100 ng of background DNA and 0.002% (range, 0%-0.104%) at 300 ng of background DNA. Overall, especially taking the background values into consideration, we found that 0.1% marker-specific cells could be accurately and reproducibly detected.

Automation of Chimerism Analysis Based on RQ-PCR

The maximal number of chimerism samples that can be analyzed in a 384-well plate with the described setup is 39. Preparations for pipetting of 39 patient DNA samples, standard curve samples, and background DNA samples together with preparation of PCR reagents of both the patient- and donor-specific genetic marker system and the active reference system were performed within approximately 2 hours (exclusive DNA extraction); pipetting by the robot was per-
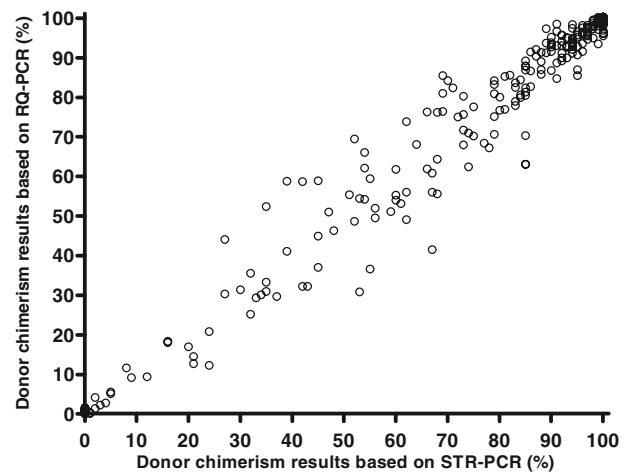

Figure 3. Comparison of donor chimerism results based on STR-PCR and RQ-PCR for 300 DNA extracts from recipients of hematopoietic stem cell transplants with nonmyeloablative conditioning.

formed within 1.5 hours. Analyses on the 7900HT apparatus were performed within 2 hours, and post-PCR data analyses were performed within 1 hour. In total, a duration of approximately 6.5 hours was thus used to analyze 39 patient DNA samples. In comparison, it would take ten 96-well plates to analyze 39 patient DNA samples manually. Preparation of samples and PCR reagents together with manual pipetting into one 96-well plate were performed within 1.5 hours, analyses on the 7900HT apparatus were performed within 2 hours, and post-PCR data analyses were performed within approximately 30 minutes. This sums up to approximately 40 hours for the same number of samples in the 96-well plate setting. The robot-assisted setup thus reduces the working time from 1 week to 1 day.

Apart from being time saving, analysis costs could be reduced by using 384-well plates with robot pipetting. Only 10 μL of Master Mix 2× Buffer (Applied Biosystems) was applied per well in the 384-well plates, compared with 15 μL of Master Mix 2× Buffer per well in the 96-well plates. Furthermore, fewer wells for standard curves and controls are used per sample in the 384-well plates compared with 96-well plates. Master Mix 2× Buffer is one of the major expenses of this analysis; nearly 4 mL of Master Mix 2× Buffer is required in a full 384-well plate, at a cost of approximately 360€, whereas the equivalent 96-well plate setting would require approximately 14 mL, corresponding to 1260€, or a factor of 3.5.

Comparison of STR-PCR and RQ-PCR for Chimerism Analysis

A high degree of correlation between chimerism results of patient samples obtained by STR-PCR and RQ-PCR was seen (Figure 3). Chimerism results were calculated on the basis of either the patient or the

donor allele–specific marker, as described in the "Materials and Methods" section, and always presented in donor chimerism. The distribution of corresponding chimerism analyses was skewed, with 34 corresponding analyses with less than 25% chimerism, 62 corresponding analyses with 25% to 75% chimerism, and 204 corresponding analyses with more than 75% chimerism because of the typical progression toward full donor chimerism after stem cell transplantation. Analyses resulting in 0% donor chimerism were excluded. Again, Bland and Altman statistics were used to calculate agreement between the 2 methods. Limits of agreement of the log-transformed difference between methods were −0.11 to 0.12; ie, 95% of cases of RQ-PCR–based analyses will be between 0.78 and 1.32 times the STR-PCR–based analyses (Figure 2b). Thus, the RQ-PCR–based analyses differed from the STR-PCR–based analyses from 22% below to 32% above, a difference corresponding to less than ±0.5 threshold cycles between the 2 methods. This means that, in a chimeric sample of 1%, 95% of the RQ-PCR–based results will lie between 0.8% and 1.3%, whereas in a chimeric sample of 50%, 95% of RQ-PCR–based results will lie between 39% and 66%. A chimeric sample of 99% has been calculated on the basis of 1% patient chimerism (100% − 1% = 99%); consequently, the difference also has to be calculated on this basis. That is, in a chimeric sample of 99%, 95% of RQ-PCR–based results will lie between 98.7% and 99.2%.

DISCUSSION

Determination of chimerism status is an essential and time-consuming analysis in the follow-up of patients after allogeneic stem cell transplantation [5-7,34,35]. It is therefore important to focus on the different methods available for analysis of this parameter. We evaluated a method based on RQ-PCR as an alternative or supplement to an analysis based on STR-PCR. The RQ-PCR–based method has shown promising results, especially in regard to the lower detection limit of this method compared with the more established STR-PCR–based method [6,17,18,21]. The difference in sensitivity between the 2 methods lies mainly in the smaller dynamic range of the STR-PCR method [36]. Furthermore, less nonspecific background might be expected in the RQ-PCR–based method because it is based on specific primers and probes that amplify patient and donor alleles in separate wells, whereas patient and donor alleles are amplified in the same tube with the same primers in the STR-PCR–based method [17]. The one decade lower detection limit in RQ-PCR–based methods compared with STR-PCR–based methods is especially attractive in the determination of low patient cell fractions as indicators of

minimal residual disease. The correlation between persistent patient-derived cell subpopulations and relapse of underlying disease after allogeneic stem cell transplantation has most clearly been demonstrated for the malignant myeloid diseases [14,37-39].

After conditions of PCR were optimized for each of the allele-specific markers, evaluation of intrasample variation showed reproducible results down to 0.1% marker-specific cells. Results in the 96-well and 384-well plates showed accurate values between 0.1% and 10% marker-specific cells. Below 0.1%, the system fails because of inaccuracy and overlapping background signals. Above 50% marker-specific cells, the system also fails because of inaccuracy, which can be explained by the higher amount of target DNA in the PCR reaction and, therefore, higher absolute variation. To circumvent this problem, we used the lowest chimerism value of the 2 specific marker systems in the analysis of the patient samples to calculate the chimerism percentage. Using this method of subtraction, the accuracy of chimerism results is satisfactory from 0.1% to 99.9%, with the highest absolute variation in the mid interval symmetrically around 50% chimerism, but with the same relative variation in the entire range. STR-PCR–based methods for chimerism analysis might be more accurate in the mid interval, because the PCR process is based on the comparative amplification of the same polymorphism between patient and donor in the same well, in contrast to the RQ-PCR–based method, which relies on different polymorphisms between patients and donors in different wells.

Background signals were one of the most important causes of restricting the detection limit, because overlapping background and chimerism values of approximately 0.01% render the detection of these very low chimerism values difficult. Furthermore, restriction of the study setup by inaccurate cell counting and dilution procedures in the making of chimeric cell mixtures has to be considered. In the light of this, the detection limit for the RQ-PCR–based chimerism analysis was estimated as 0.1% marker-specific cells, which is also demonstrated in other single nucleotide and short insertion and deletion polymorphism-based assays [6,17,18,21]. A better sensitivity can in some systems be accomplished by adding more DNA to the PCR reaction, but with the risk of higher background values and PCR inhibition, or by adding the procedure of cell sorting after transplantation, sorting the cells in different cell lineages, for example, examining the CD34$^+$ blasts.

The sensitivity in the 384-well plates seemed slightly inferior compared with the same chimeric cell mixtures analyzed in the 96-well plates. This was due to a single system (S01B) in the 384-well plates that did not function acceptably under the optimized conditions in the 384-well setting. If this genetic marker

is omitted, then the 96-well and 384-well plate systems function equally well. Automation of the setup in the 384-well plates allowed analysis of chimeric samples including various controls and standard curves in both the patient and donor allele–specific direction in a time-, labor-, and reagent-conserving manner compared with the setup of the same number of samples either by STR-PCR or in 96-well plates. Automated setup is most suitable in a research setting requiring analysis of many samples from a single patient, but automated setup in transplantation centers that perform many analyses a week could also be rational.

In conclusion, chimerism analysis based on RQ-PCR was accurate and had acceptable intrasample variation above the detection limit of 0.1% marker-specific cells. Results based on RQ-PCR were comparable to results based on STR-PCR from ≥1% chimeric cells. Furthermore, automated setup was feasible, accurate, and cost-effective.

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