Quantitative Determination of Bone Marrow Transplant Engraftment Using Fluorescent Polymerase Chain Reaction Primers for Human Identity Markers


We have developed a quantitative, nonisotopic method using variable number tandem repeat (VNTR) and short tandem repeat (STR) markers for monitoring donor cell engraftment in marrow transplant recipients. Posttransplant DNA from the recipient is amplified with fluorescent polymerase chain reaction (PCR) primers for polymorphic markers that distinguish donor alleles from recipient alleles. The fluorescent PCR products are then separated on agarose or acrylamide gels on the Applied Biosystems 373A Sequencer (Foster City, CA). Using GeneScan® software (Applied Biosystems) to analyze the separated alleles, we can correlate allele peak areas to the percentage of donor or recipient DNA. We quantify engraftment in a mixed chimeric sample by mixing pretransplant recipient and donor DNAs in a range of percentages and amplifying the mixtures to produce a standard curve. By amplifying and analyzing the posttransplant sample DNA(s), we can determine the extent of engraftment by interpolating the percent peak area of the informative allele(s) from this standard curve. This approach provides a precision of measurement ranging, depending on the marker, from 3.5% to 8.0% (percent coefficient of variation) and an accuracy of engraftment determination ranging from 97% to 99%, with a sensitivity of detection of 1% donor or recipient DNA. We retrospectively analyzed a panel of 32 patients and found seven to be informative for some degree of mixed chimerism, indicative of either residual normal host cells or leukemic relapse. An analysis of different cell lineages obtained posttransplant showed different degrees of engraftment in myeloid and T-cell populations. In summary, this method can provide an accurate, quantitative assessment of mixed chimerism in patients posttransplant. Such information may be useful in the future in guiding early implementation of additional treatment designed to circumvent graft failure or suppress relapse.

TYPICALLY, bone marrow transplants (BMTs) are performed for patients with severe combined immunodeficiency disease (SCID), severe aplastic anemia, or leukemia. Physicians initially used cytogenetic markers or red cell phenotypes to assess chimerism and the extent of sustained engraftment. Recently, however, the discovery and characterization of polymorphic DNA loci that are capable of distinguishing individuals, such as the variable number tandem repeat (VNTR) and short tandem repeat (STR), makes available a wide range of genetic markers that can discriminate individuals independent of HLA or sex mismatching.

Several investigators have reported the use of the polymerase chain reaction (PCR) to monitor BMT engraftment by amplifying VNTR or STR loci. Typically, detection of the amplification products was accomplished by labeling with a radiolabeled deoxynucleotide during the extension step of the amplification reaction, or by Southern blot analysis of the PCR products with radioactive DNA probes. The gels or filters were exposed to autoradiographic film, and the images produced provided a qualitative assessment of mixed chimerism. To facilitate the monitoring of engraftment after transplantation, we sought to develop a rapid, accurate, nonisotopic, and quantitative method for determination of mixed chimerism after allogeneic BMT. This quantitative method can also provide clinicians with valuable information on which conditioning regimens are efficacious in eliminating host lymphohematopoietic cells and promoting complete engraftment, as well as monitoring for early evidence of residual malignant cells and relapse.

PATIENTS AND METHODS

The Applied Biosystems (AB; Foster City, CA) 373A DNA Sequencer provides sensitive detection of PCR DNA products amplified for human identity markers with fluorescent primers. The AB GeneScan 672™ software can also calculate the peak area of a fluorescent signal corresponding to a band on the gel. The peak area of a fluorescent dye molecule (fluorophore) is directly dependent on the emission energy of the fluorophore and the number of molecules present. Because each molecule of PCR product is conjugated to two molecules of dye (one from each primer), the peak area of the VNTR or STR allele band is directly dependent on the amount of PCR product DNA, and independent of the length. These data are exported to the AB Genotyper™ program, which provides the data reduction and tabulation of the GeneScan™ data of interest, eg, peak area of the allelic bands for a particular sample set. Determining the percent donor (or host) peak area for an informative allele provides a quantitative measurement of the degree of mixed chimerism. The instrument also provides the capability of simultaneous detection of four differently colored fluorescent dyes. This permits the analysis of more than one informative locus for a particular sample set or multiple samples sets (with each locus amplified with primers of different colors) on the same gel. Using the AB Genotyper™ software, one can selectively analyze the data from each locus independently of the other loci that may be separated on the same gel.

Our approach was as follows: (1) screen the pretransplant recipient and donor DNA samples with PCR-based VNTR or tetranucleotide STR markers; (2) quantify and match the concentrations of recipient and donor DNA before PCR with a spectrophotometer or QuantI-Blot™ (Perkin-Elmer Corp, Norwalk, CT); (3) check for amplifiability of pretransplant recipient and donor DNA by amplifying a PCR product of invariant length (eg, β-globin) for all samples; (4) prepare a standard curve (reconstruction) representative of a mixed

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chimeric sample by mixing varying percentages of pretransplant recipient and donor genomic DNA; (5) amplify the reconstruction, the posttransplant samples, and controls using fluorescently labeled PCR primers; (6) separate the PCR fragments on acrylamide gels to detect VNTR or STR alleles with the AB 373A Sequencer; (7) analyze the PCR fragments for peak area and export data to Geno-typer™; and (8) determine the percentage of donor or host DNA of the posttransplant sample from the standard curve.

The donor percentage peak area of the reconstruction samples was calculated and plotted versus percentage donor DNA amplified to determine the engraftment of the posttransplant sample(s). The donor percentage peak area is calculated as follows:

\[
\% \text{ Peak Area} = \frac{A_{Dn} - A_{2n}}{A_{Dn} + A_{2n} + A_{R1} + A_{R2}} \times 100, 
\]

where \( A \) is peak area; \( D \), donor allele(s); and \( R \), recipient allele(s). If an allele is shared, only the informative alleles (i.e., alleles that distinguish donor from recipient) are used in the calculation. The percent donor was interpolated from this standard curve, and the engraftment was expressed as either percent donor or percent host DNA. The percent host DNA was calculated as 100 minus the percent donor DNA.

**Patient samples.** Study specimens were derived from blood samples submitted to the Clinical Immunogenetics Laboratories of the Fred Hutchinson Cancer Research Center (FHCRC, Seattle, WA) for routine HLA typing before marrow transplantation and for chimerism testing by VNTR PCR analysis after marrow transplantation. A panel of 32 patients analyzed retrospectively with silver-stained gels, seven were determined to have some degree of mixed chimerism.

DNA samples tested by VNTR or STR analysis for the seven patients are shown in Table 3. Unique patient number (UPN) 6924 was a male who received two consecutive transplants for chronic myeloid leukemia/chronic phase (CML/CP) from his HLA-haploidentical mother, mismatched at DRB. At day 22 after the first transplant, a bone marrow aspirate showed 10% cellularity with trilineage engraftment, and studies with a Y chromosome-specific fluorescent probe by in situ hybridization (FISH) showed only 1% of the cells were of male origin. However, the peripheral white blood cell (WBC) counts (cells/µL) never rose above 300. At day 37 no hematopoietic elements were seen in a bone aspirate. FISH analysis of peripheral blood mononuclear cells (PBMC) on day 43 showed 98% male cells. The patient received a second transplant from the same donor on day 51. Although the peripheral WBC count (cells/µL) increased to 900 on day 58 (7 days post-second transplant) and reached a maximum of 2,300 on day 63 (12 days post-second transplant), the WBC counts began a persistent decline after day 73 (day 22 post-second transplant). A bone marrow aspirate on day 73 (day 22 post-second transplant) showed no evidence of engraftment or relapse, and FISH analysis of PBMC showed 30% male cells. The patient died on day 101 (50 days post-second transplant) of complications of graft failure. UPN 4752 was transplanted for acute myeloid leukemia (AML) from an HLA genotypically identical sibling (same sex). Engraftment was successful, and the patient remained disease-free until day 1,327 posttransplant, when blast cells were identified in peripheral blood and bone marrow. The patient achieved a remission with chemotherapy, and a second transplant from the same donor was performed on day 1,394 after the first transplant. Peripheral WBC counts indicated engraftment by day 16, and the patient remains disease-free through day 350 post-second transplant. UPN 6687 was transplanted from an HLA-identical sister for refractory anemia with excess blasts (RAEB-T) that had progressed to AML. Posttransplant, the WBC count indicated engraftment by day 11 posttransplant. On day 56 posttransplant, cytogenetic analysis of bone marrow showed 3 of 20 cells with the same clonal chromosomal abnormality detected before transplant. At day 71 posttransplant, 12 of 15 cells were abnormal, and by day 81, the marrow contained 50% blasts, although as of day 85, no blasts were detected in peripheral blood. The patient died of recurrent disease at day 97 posttransplant. UPN 6166 was transplanted for CML accelerated phase (AP) from an HLA haploidentical sibling, mismatched for HLA-A,B. At day 425 posttransplant, a bone marrow aspirate detected the presence the blast cells positive for the Philadelphia chromosome, indicating relapse. At day 495 after the first transplant, the patient, now in blast crisis, received a second transplant from the same donor. At day 45 after the second transplant, a bone marrow aspirate showed trilineage engraftment with no evidence of residual disease. At day 65 post-second transplant, the patient died of infectious complications of graft-versus-host disease (GVHD) but without evidence of leukemic relapse. UPN 6814 was transplanted for AML in second relapse from an HLA genotypically identical sibling (same sex). Analysis of peripheral WBC counts indicated engraftment by day 11 posttransplant, and the patient remained disease-free until relapse was detected at day 150 posttransplant by the presence of blast cells in the bone marrow. The patient died at day 157 posttransplant from relapsed leukemia. UPN 7246 was transplanted from an HLA-identical brother for CML/CP. On day 32 a bone marrow aspirate was hypocellular, with only 15% normal hematopoietic elements, but normal cytogenetics. The patient demonstrated slow engraftment, with a WBC count (cells/µL) less than 750 until day 35 posttransplant. Counts began to rise slowly until day 60, at which time the WBC count began fluctuating from 1,000 to 4,000, although the patient never became platelet-independent. A restriction fragment length polymorphism (RFLP) analysis for the BCR-ABL gene rearrangement characteristic of CML did not detect the translocation in peripheral blood on day 88 posttransplant. On day 103, the patient received a second marrow infusion from the same donor after conditioning with antithymocyte globulin (ATG). The WBC count indicated engraftment by day 122 (+20 days from second transplant). The patient is alive and well without evidence of disease as of approximately 10 months after the first transplant. UPN 6514 received two consecutive transplants for CML/CP. The first transplant was from an unrelated marrow donor matched for HLA-A,B and DRB, with the exception of a one-locus minor mismatch at DRB1. At day 21 transplant, a bone marrow aspirate showed no evidence of engraftment. The second transplant was performed with a related donor, mismatched at HLA-B and DRB1, at day 55 after the first transplant. At day 28 after the second transplant, the patient's peripheral WBC counts indicated engraftment, and 1 year later the patient was alive, fully engrafted, and without evidence of relapse.

**Sample preparation.** Unless otherwise indicated, DNA was prepared from the whole WBC fraction, according to the methods described by the 11th International Histocompatibility Workshop. Briefly, 20 to 50 mL of whole blood was subjected to three cycles of red cell lysis, the residual leukocytes were lysed with a Tris/sodium dodecyl sulfate (SDS)/proteinase K buffer, and DNA was precipitated by the salting-out method. White cell subsets were prepared in the case of UPN 6166 to identify the host or donor origin of T lymphocytes. In the day-447 posttransplant sample, whole blood was fractionated by Ficoll-Hypaque separation. The Ficoll interface cells were collected, and one aliquot was treated with LymphoQuik (One Lambda, Los Angeles, CA) to lyse the remaining myeloid cells. This sample set was identified as the lymphocyte-enriched fraction. The original Ficoll pellet was subjected to three cycles of red cell lysis and defined as the granulocyte-enriched fraction. The day-477 sample from UPN 6166 was fractionated by Ficoll-Hy-
paque, and T lymphocytes were isolated from the interface by positive selection via adherence to plastic plates coated with an anti-CD3 monoclonal antibody. The purity of T lymphocytes in this sample preparation was shown to be greater than 99% by flow cytometry analysis.

**DNA sample quantitation.** The concentration of the recipient and donor DNAs that are used to prepare the reconstruction standard curve was determined spectrophotometrically or with QuantiBlot™. The latter procedure is particularly useful if the amount of DNA available for quantitation is too limited for a spectrophotometric determination.

**Primer synthesis.** Fluorescently tagged oligonucleotide primers used for PCR were synthesized as described by Giusti and Adriano. PCR analysis. To ensure that equal amounts of sample DNAs were equally amplifiable, a β-globin control amplification was performed on all samples before tandem repeat analysis (data not shown). Samples were analyzed for peak area as described in Electrophoresis and GeneScan™ Analysis. Any DNA samples that am-
Table 1. Amplification Reaction and Cycling Conditions for VNTR and STR Markers

<table>
<thead>
<tr>
<th>Locus</th>
<th>D1S80</th>
<th>SE33</th>
<th>ApoB</th>
<th>D17S5</th>
<th>D21S11</th>
<th>Tho 1</th>
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<tbody>
<tr>
<td>Primers</td>
<td>CRX51</td>
<td>SE33A</td>
<td>RR17</td>
<td>SW26</td>
<td>T3</td>
<td>E2A</td>
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<tr>
<td></td>
<td>CRX66</td>
<td>SE33B</td>
<td>RR18</td>
<td>SW37</td>
<td>T4</td>
<td>E2B</td>
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<td>Primer concentration (µmol/L)</td>
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<td>0.5</td>
<td>0.25</td>
<td>0.5</td>
<td>0.5</td>
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<td>Dye</td>
<td>JOE</td>
<td>FAM</td>
<td>TAMRA</td>
<td>JOE</td>
<td>JOE</td>
<td></td>
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<td>Buffer, Tris, pH 8.3 (mM)</td>
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<td>10</td>
<td>10</td>
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<td></td>
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<tr>
<td>KCl (mM)</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>70</td>
<td>50</td>
<td>50</td>
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<tr>
<td>MgCl₂ (mM)</td>
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<td>2.0</td>
<td>0.85</td>
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<td>1.5</td>
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<td>dNTPs (µM)</td>
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<td>600</td>
<td>800</td>
<td>560</td>
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<td>200</td>
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<tr>
<td>Tag polymerase* (U)</td>
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<td>2.5</td>
<td>2.5</td>
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<td>DNA (ng)</td>
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<td>100</td>
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<td>94°, 1</td>
<td>94°, 1</td>
<td>94°, 0.5</td>
<td>94°, 0.5</td>
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<td>Annealing temperature (°C), time (min)</td>
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<td>65°, 1</td>
<td>62°, 1</td>
<td>68°, 4</td>
<td>60°, 0.5</td>
<td>60°, 0.5</td>
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<tr>
<td>Extension temperature (°C), time (min)</td>
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<td>72°, 1</td>
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<td>NA†</td>
<td>72°, 0.5</td>
<td>72°, 0.5</td>
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<tr>
<td>Cycle no.</td>
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<td>28</td>
<td>30</td>
<td>28</td>
<td>31</td>
<td>31</td>
</tr>
</tbody>
</table>

* Taq polymerase obtained from Perkin-Elmer Corp (Norwalk, CT).
† These markers were two temperature amplifications, with the anneal and extension steps consolidated into one temperature.

All reactions were 50 µL total volume, except for D21S11 and Tho 1, which were 25 µL each, and were overlaid with 50 µL of mineral oil to prevent evaporation. Samples were amplified with a Perkin Elmer 480 Thermal Cycler programmed with a step cycle thermal profile, followed by a 10-minute incubation at the extension temperature (except for ApoB, which was incubated for 20 minutes). Mineral oil was removed after amplification by extraction with an equal volume of chloroform.

A reconstruction consisting of mixtures of varying percentages of pretransplant recipient and donor will be representative of the degree of amplification of each allele in a mixed chimeric posttransplant sample. The percentages of recipient and donor DNA used for the reconstruction standard curve are described in the legend to Fig 1. For each experiment, the reconstruction samples, the transplantation sample set, and a negative control were amplified for the informative markers. All posttransplant DNA sample(s) were amplified in duplicate. In these reconstructions, while the percentages of donor and recipient DNA vary relative to each other, the total amount of DNA in the reconstruction remains constant. To ensure that there were no differences in mixed chimerism determinations that might be due to preferential amplification associated with high amounts of genomic DNA, each DNA sample in the transplantation sample set was also amplified with 60% of the amount of DNA used for the reconstruction and sample analysis described above. Each experiment was run twice, for a total of six measurements of the posttransplant samples, with the exception of UPN 6814. UPN 6814 was analyzed for the D1S80 and SE33 markers in three separate experiments, with the third analysis being a multiplex amplification for these two markers.

Amplification of human identity genetic markers. Table 1 lists the reaction constituents and amplification conditions for the VNTR and STR markers. D1S80 was amplified using primers described by Kasai et al.12 ACPBPT2 (hereafter referred to as SE33) was amplified using primers described by Polymeropoulos et al.13 When D1S80 and SE33 were amplified as a multiplex reaction, samples were amplified using 50 ng DNA and SE33 reaction cycling conditions. D17S5 was amplified using primers described by Walsh et al.14 For apolipoprotein B (ApoB), the primers sequences RR17 and RR18 are identical to those described by Boerwinkle et al.15 For D21S11 and Tho1, D21S11 primers were used described by Sharma and Litt.16 The Tho1 left-end primer was described by Edwards et al.17 The right-end primer sequence is 5'-GTTAATCCATTGG-CCTGTTCCCTC-3'.

Electrophoresis and GeneScan analysis. Unless indicated otherwise, 1 µL of PCR sample was electrophoresed using 7.5% Long Ranger gels (AT Biochem, Malvern, PA) with 1× Tris-borate EDTA (TBE) gel buffer and 0.6× TBE reservoir buffer7 at 40 W constant power for 2 hours on an Applied Biosystems 373A Sequencer with a 12-cm well-to-read distance. Gels were analyzed for peak areas with Applied Biosystems 672 GeneScan™ software.

RESULTS

Precision and accuracy. We examined four polymorphic tandem repeat loci, the VNTR loci D1S80, D17S5, and ApoB, and the STR locus SE33 to determine the precision and accuracy of this approach. To simulate a range of mixed chimerism that might be encountered in actual clinical posttransplant samples, we prepared mock transplant samples and a reconstruction standard curve by mixing pure genomic DNA from two unrelated individuals. The mock transplant sample was prepared to give a 50% mixed chimerism. The D17S5 locus was amplified from a different pair of unrelated individuals, as the original pair was not informative for this marker. The print of the gel analyzed for the ApoB locus in

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this experiment is shown in Fig 1. Figure 2 shows the Geno-
typer data for some of the ApoB reconstruction samples in
this experiment. The middle donor peak shows the relation-
ship of increasing donor allele peak area with increasing
percentage of donor DNA. Conversely, the upper recipient
allele peak area decreases as the percentage of recipient
DNA decreases. The percent peak area data for the informa-
tive alleles in the PBMC sample from day 29 after a second trans-
plant, indicative of a mixed chimerism. Interpolation of the
donor peak area in the standard curve for each marker (Fig 3)
was determined by interpolation of the percent peak area
from the reconstruction standard curve. Figure 3 also shows
that the donor peak area in the standard curve for each
marker is the peak area as calculated by the 672 Genescan™ Analy-
sis software. Data reduction (ie, peak area labeling) was performed
by Applied Biosystems Genotyper™ software.

Sensitivity. The use of fluorescently tagged PCR primers
for detecting amplified tandem repeat markers can provide
detection of as little as 100 pg of target DNA.
We typically applied 20-fold less amplified DNA when detecting fluo-
scently labeled PCR samples using the 373A sequencer than
when these samples were detected using ethidium bromide-
destained agarose gels (data not shown). The D1S80 and ApoB
markers typically provided the greatest sensitivity, possibly
due in part to the fact that these two markers amplify robustly
and exhibit low levels of preferential amplification. This low
level of preferential amplification permits detection of a 1%
minority genotype with amplification of 100 ng total geno-
ic DNA. The other polymorphic tandem repeat markers
that we have used typically provide detection limits of at
least 5% (data not shown). Figure 4 shows the sensitivity
obtained for the D1S80 VNTR marker.

Clinical samples. Clinical DNA samples from a panel
of 32 transplant patients were amplified for VNTR markers
and analyzed on silver-stained gels to select mixed chimeric
posttransplant samples (data not shown). Of the 32 patients
examined, seven patients showed some degree of mixed chi-
erism and were analyzed with quantitative reconstruction
analysis on the AB 373 Sequencer. The results for the seven
patients are shown in Table 3. Figure 6 shows one of these
patients as an example. Donor and patient pretransplant
samples from UPN 6924 were found to be informative with
D1S80. The donor and recipient sample contained the 18-repeat allele
and are distinguished by 21-repeat and 24-repeat alleles,
respectively. (The D1S80 samples were sized and genotyped
with the D1S80 allelic ladder from the Perkin Elmer D1S80
AmpilFLP kit [data not shown].) The posttransplant sample
alleles showed the presence of both donor and recipient al-
leles in the PBMC sample from day 29 after a second trans-
plant, indicative of a mixed chimerism. Interpolation of the
reconstruction plot (Fig 7) shows that the 40% donor peak
area of the posttransplant sample corresponds to 50% donor
DNA. UPN 4762, a patient with clinical relapse, had 12%
donor cells (88% recipient) on day +1,329. Analysis of a
sample from day 350 after a second transplant showed 100%
engraftment and demonstrated this patient to be in clinical
remission. Although UPN 6687 was known to be in relapse
from conventional marrow examination, examination of
PBMC showed 98.5% donor (1.5% recipient) cells by VNTR
analysis at day 81 posttransplant. For UPN 6166, a patient
with CML in clinical relapse, different subsets of blood leu-
kocytes were prepared and analyzed for donor chimerism. PBMC, granulocytes, and lymphocytes from day +447, showed 16%, 20%, and 39% donor cells present, respectively, and purified T cells at day +477 demonstrated 67% donor cells. A second transplant from the same donor was done on day +495, and 28 days later VNTR analysis documented 100% donor cell engraftment, with no evidence of relapse. The persistence of donor type cells in PBMC of UPN 6814 was documented with three markers: SE33, D1S80, and D21S11. SE33 consistently showed 25% donor, while D1S80 showed 48% engraftment, even when the samples were simultaneously amplified in multiplex reaction and analyzed in a third experiment. Analysis of this patient with D21S11 showed 45% donor cells (see Discussion). A series of posttransplant samples from UPN 7246 at days +24, +39, +64, and +74 showed the persistence of 8%, 1%, 1%, and 4% host cells, respectively. Clinically, the patient had poor graft function but no evidence of recurrent leukemia. A second marrow infusion was given, and subsequently, an analysis of both PBMC and marrow samples documented 100% donor engraftment. UPN 6514 showed no evidence of engraftment after a first transplant, and the D1S80 VNTR analysis showed 0% donor cells (100% recipient) at day 19 posttransplant. Analysis of a second transplant from a different donor showed 93.5% donor cells at day +76 (+21 after second transplant) and 100% donor cells at day +133 (+58 after second transplant).

**DISCUSSION**

The application of PCR to amplify informative tandem repeat markers as a means of detection or assessment of mixed chimerism in BMT patients is well documented.13,18 PCR is relatively simple and rapid and uses less sample DNA, yet provides better sensitivity than biochemical or RFLP methods. For example, PCR has been used for monitoring ex-vivo purging of T cells19 and determining levels of minimal residual disease.5 However, to date some issues associated with amplifying polymorphic length polymorphism markers in a mixed chimeric sample have not been addressed. These issues are important because they are related to measuring the degree of engraftment.

Preparing amplification reconstruction standard curves when using PCR is critical for determining engraftment, because of the potential for preferential amplification in the mixtures of alleles represented by a mixed chimeric sample. These preferential amplification effects can depend on a variety of factors: the inherent efficiency of the amplification system, the amount of DNA or enzyme used, the size ranges of the alleles, and the number and the relative size difference of the alleles present in the sample. We have shown, for example, that the reconstruction standard curves obtained for a specimen can vary between different markers. In addition, different donor or recipient samples can produce different standard curves for the same marker, presumably because of the size differences between the alleles. The accuracy of these standard curves is dependent on matching the amounts
of the individual DNAs that are used to prepare the mixtures. If the amount of DNA available is too limited for spectrophotometric analysis, simple and very sensitive methods, such as QuantiBlot, can be used to quantitate the DNA samples.

It is also important to account for potential differences in amplification efficiencies when amplifying equal quantities of sample DNA. For example, inhibitors of DNA polymerase that may be present in one of the samples could possibly decrease the product yield of the longer allele relative to the shorter allele, increasing the extent of preferential amplification. To control for this, we amplified each individual sample for a β-globin fragment of invariant length (so that there were no effects due to preferential amplification) before tandem repeat analysis.

While the sensitivity of our approach in detecting small percentages of host or donor DNA is comparable with silver-stained gels, in our experience, we have found it is often difficult to discriminate between different percentages (eg, between 5% and 20% donor DNA) of donor and recipient DNAs with silver-stained gels (data not shown). We find that GeneScan analysis of fluorescently labeled PCR fragments provides better accuracy and precision of measurement, perhaps because, as Ziegler et al report, "fluorescent signals are more linear over a much greater range of intensity than conventional autoradiography." While our fluorescent primer procedure does not approach the 0.1% level of sensitivity (obtained by labeled the PCR products during amplification with 32P-deoxycytidine phosphate) reported by Lawler et al, using fluorescently tagged deoxynucleotide analogs should increase the sensitivity of detection. A combination of this approach and/or reamplification of the posttransplant samples may provide sensitivity down to 0.1% host or donor DNA. The sensitivity could be further increased by first purifying, by positive selection, specific subsets of myeloid or lymphoid progenitor cells that may be present in very small numbers, eg, CD34+ precursor cells.

The quantitative capabilities of the Applied Biosystems 373A Sequencer and GeneScan and Genotype software provide very good precision in measuring the peak areas of donor and recipient alleles, and the data obtained for the mock transplant samples demonstrates that the standard curve provides an accurate determination of engraftment, regardless of the marker used.

In preparing the standard curves, one could plot the donor peak area versus the percent donor DNA added, but if any samples in the standard curve were underamplified relative
Table 3. Evaluation of Posttransplant Chimerism in Patients Receiving Marrow Allografts

<table>
<thead>
<tr>
<th>UPN</th>
<th>Diagnosis</th>
<th>Days Posttransplant (days post-second transplant)</th>
<th>Source</th>
<th>Marker</th>
<th>% Donor</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>6924</td>
<td>CML/CP</td>
<td>+80 (+29)</td>
<td>PBMC</td>
<td>D1S80</td>
<td>50 ± 5</td>
<td>Graft failure</td>
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<tr>
<td>4762</td>
<td>AML</td>
<td>+1,329</td>
<td>PBMC</td>
<td>D1S80</td>
<td>12 ± 3</td>
<td>Clinical relapse</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>D17S5</td>
<td>12 ± 3</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+1,744 (+350) Clinical remission</td>
</tr>
<tr>
<td>6887</td>
<td>AML</td>
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<td>PBMC</td>
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<td>+477 Clinical relapse</td>
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<td>T cells</td>
<td>67 ± 3</td>
<td>Clinical relapse</td>
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<td>+155</td>
<td>PBMC</td>
<td>SE33</td>
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<td>D1S80 Clinical relapse</td>
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<td>+24 Neutropenia and hypocellular marrow</td>
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<td>CML/CP</td>
<td>+39</td>
<td>PBMC</td>
<td>D1S80</td>
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<td>+64 Neutropenia and hypocellular marrow</td>
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<td>+74 Neutropenia and hypocellular marrow</td>
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<td>Lymph</td>
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<td>Marrow</td>
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<td>+123 (+21) Clinical remission</td>
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<td>+76 (+21) Clinical remission</td>
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<td>+113 (+58) Clinical remission</td>
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Abbreviations: Gran, granulocytes; Lymph, isolated from peripheral blood lymphocytes isolated from peripheral blood; T cells, T lymphocytes isolated from peripheral blood.

* Proven to be of host origin.

To the other (eg, due to well-to-well variation in the thermal cycler, primer-dimer formation, etc), the reduction in peak area from the underamplified samples would skew the reconstruction curve towards the underamplified sample. Because the percentage of donor and recipient DNA present in any chimeric clinical sample does not change, we control for any underamplified samples by plotting the percent donor peak area instead of an absolute value peak area.

Multiplex amplification of informative loci provides the ability to analyze more than one marker at a time, which increases confidence in the assessment of engraftment. Combined with four-color detection, multiple sets of samples or multiple loci for a single sample set can be analyzed on one gel. Additionally, by using loci that migrate in different parts of the gel, one can analyze either different markers and/or samples sets labeled with the same color.

Analysis of the panel of clinical samples provides some potentially illuminating results. Of the markers that we used for this set of patients, D1S80 was consistently the most informative, with all but one of the sample sets examined

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**Fig 6. Electropherograms for the clinical transplant DNA sample set.** The upper panel shows the peaks corresponding to the recipient alleles, the middle panel shows the peaks corresponding to the donor alleles, and the lower panel shows peaks from a posttransplant sample. The scale to the left of each electropherogram is the peak height; the scale along the top is the scan number.
having different size (informative) alleles. UPN 4762 showed the same degree of engraftment for both D1S80 and D17S5 markers (12%), and this finding is consistent with the results we obtained from the mock reconstructions. The difference in donor engraftment observed for UPN 6814 with the SE33 marker (25%) as opposed to the D1S80 marker (48%) was somewhat surprising. We consistently obtained this discrepancy, even when the specimens were simultaneously coamplified for the DIS80 and D17S5 markers (12%), and this finding is consistent with some 21, lead us to presume that the real degree of donor engraftment was approximately 45% to 48%. It is conceivable that the differences in apparent engraftment obtained for the SE33 marker may be due to cytogenetic abnormalities specific to this particular patient; for example, if the patient’s cells contained an extra copy of the chromosome 6 region containing the SE33 locus, a 50:50 mixture of donor and host cells would yield a 40% donor peak area for this genetic marker. Chromosomal abnormalities in leukemic cells are not uncommon and appear in 70% to 80% of AML patients, and the D1S80 and SE33 markers are located on different chromosomes (chromosomes 1 and 6, respectively12,13). UPN 6166, a patient with relapse of CML, also showed interesting results, with different levels of donor cell engraftment among myeloid and lymphoid cells. Detection of donor cells in this case was interpreted as showing that graft rejection had not occurred.

A prospective study performed early in the post-transplant period will be necessary to determine if this sensitive, quantitative method for detecting residual host cells can be useful in identifying patients at risk of early relapse. In patients transplanted for nonmalignant diseases, this method can provide information about the status of engraftment and may be useful for identifying patients at risk of graft failure. We hope that the methods and analysis described here will help in the future to answer these questions.

In conclusion, we believe that this method will have a range of applications not necessarily limited to the monitoring of engraftment of recipient allografts, but may prove useful for the quantitation of any marker or gene that may be present in a mixture or background of DNAs.

ACKNOWLEDGMENT

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Quantitative determination of bone marrow transplant engraftment using fluorescent polymerase chain reaction primers for human identity markers

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