Improved Quantitation of Minimal Residual Disease in Multiple Myeloma Using Real-Time Polymerase Chain Reaction and Plasmid-DNA Complementarity Determining Region III Standards

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ABSTRACT

The complementarity determining region III of the rearranged immunoglobulin heavy chain gene has been the target for tumor-specific PCR assays for the detection and follow-up of B-cell malignancies. Previously, these assays have relied on gel-based end point data collection methods (i.e., band densitometry) and, thus, have provided at best a semiquantitative assessment of tumor levels. We show the development of a novel, real-time TaqMan PCR assay to quantitate residual multiple myeloma cells in clinical samples after high-dose chemotherapy and autologous stem cell transplantation. We provide evidence that real-time PCR is reproducible, sensitive, and quantitative. In a 40-replicate PCR experiment targeting the β-actin gene, the coefficient of variation for threshold cycle data was 1.6%, whereas it increased to 13.6% and 31%, respectively, for end point fluorescence and gel densitometry. Moreover, in an experiment directly comparing standard curves obtained from band densitometry and threshold cycle data, the standard curve constructed from threshold cycle data had a multiple R2 value of 1.00 and demonstrated a dynamic range >4 logs, compared with the 2-log linear range of gel densitometry. Finally, we show that when a complementarity determining region III-specific PCR primer is used in conjunction with a consensus primer for the immunoglobulin heavy chain joining gene, plasmid DNA can be used as a readily available and effective substitute for clonal plasma-cell genomic DNA when preparing standards. By applying real-time PCR to the analysis of clinical samples, we are able to quantitate levels of tumor involvement with unparalleled reproducibility and statistical confidence. Real-time PCR technology may well provide the accuracy and reliability necessary for minimal residual disease detection to have real prognostic significance.

INTRODUCTION

Multiple myeloma is characterized by the clonal expansion of plasma cells in the BM, producing high amounts of monoclonal IgG or IgA. The Ig rearrangement that brings together the heavy chain VH, diversity (D), and J gene results in a region of hypervariability, the CDR3, and represents a unique DNA sequence for each myeloma clone (1). Naturally, the sequence of this DNA rearrangement has been the target for many variations of tumor-specific PCR assays for the detection and follow-up of minimal residual disease for B-cell malignancies (2).

A crude PCR technique for detecting the presence of a clonal population of B-cells is to use JH and VH consensus primers to look for evidence of a single predominant PCR product (3, 4); multiple bands or a "smear" as detected with agarose or PAGE indicate a polyclonal population of B-cells. Although simple, this assay is generally not able to detect a clonal population of cells unless it represents >1% of the total cells in the reaction (5).

More typically, JH and VH consensus primers are used to screen for myeloma clones in heavily infiltrated BM (5, 6) or flow-cytometry-sorted plasma cells (7). Once a unique clone is identified, the CDR3 region is sequenced and can be used for designing an allele-specific PCR primer (5, 8, 9). Generally, these CDR3 primers must be used in conjunction with a JH consensus primer to amplify the CDR3 sequence. The small size (<50 bp; Ref. 10) of this region precludes the use of both 5' and 3' allele-specific PCR primers. An alternative technique is to design a hybridization probe specific to the CDR3 sequence, which is then used in a Southern format (11) to detect the tumor-specific rearrangement in a VH/JH consensus PCR reaction (12–15).

Some attempts have been made to develop quantitative CDR3-specific PCR assays, which rely on end point DNA collection. For example, the amount of CDR3-specific product formation for a set of standards (as measured using band densitometry) can be correlated with known copy numbers of the target gene (5–7). From this data, standard curves can be constructed to estimate the number of target copies in an unknown sample using the process of regression discrimination (16). The limiting dilution PCR assay (17, 18) is an alternative and increasingly popular technique that does not rely on a standard curve. For this analysis, Poisson statistics are applied to PCR-positive serial dilutions of absolute cell numbers to predict the number of target copies in an unknown sample. However, both of these techniques are prone to wide variations in PCR product accumulation, which result in greatly expanded confidence intervals for the percentage of residual disease. Consequently, traditional end point measures of PCR product accumulation are of limited quantitative potential.

Recently, the 5' nucleic acid polymerase cleaves a double-stranded fluorogenic probe (20). These nonextendible probes (TaqMan probes; Roche Molecular Systems, Inc.), designed to hybridize internally to the PCR primers, are labeled at the 5' end with a reporter dye (i.e., FAM, 6-carboxyfluorescein) and at the 3' end with a quencher dye (TAMRA, 6-carboxytetramethyl-rhodamine). Fluorescence of the intact probe is quenched mainly by Förster-type energy transfer (21), and the nuclease degradation of the probe results in an increase in reporter dye fluorescence (22). The increase in fluorescence can be proportional to the concentration of template in the PCR.

The TaqMan method of PCR product detection has contributed to the development of extremely sensitive, accurate, and high-throughput assays for the detection of a number of DNA templates (23–25). With the introduction of analytical thermal cyclers that are capable of monitoring probe fluorescence real-time (i.e., during the course of the PCR), it is possible to develop truly homogenous and quantitative PCR assays (26, 27).

We provide evidence in this report of the significant advantage of real-time PCR. Specifically, we demonstrate that real-time PCR data...
has a high degree of reproducibility and provides a substantially increased dynamic range. Furthermore, we show that plasmid DNA is a readily available and an effective substitute for clonal B-cell genomic DNA in constructing patient-specific standard curves. Finally, we describe how these techniques are used clinically to provide accurate quantitation of minimal residual disease in a patient transplanted with autologous PB-HSC.

MATERIALS AND METHODS

Patient Samples

Multiple myeloma patients were participants in a Phase I/II clinical trial assessing the safety and efficacy of a tumor purging process in PB-HSC transplantation. The patients had received ±12 months chemotherapy at the time of study enrollment and provided informed consent.

Clinical PB samples for MRD analysis were obtained before and after cell processing. BM samples were collected at the time of enrollment and at 42, 100, 180, and 365 days after transplant. BM samples collected before mobilization were stained with CD38-FITC and CD45-PE antibodies (PharMingen, San Diego, CA) and enriched for CD38+CD45−cells using flow sorting (Becton Dickinson). Genomic DNA was isolated using the QiaAmp Blood Kit (Qiagen, Inc., Chatsworth, CA), and eluted in 50-200 μl of 10 mM Tris, pH 8.0 (BioWhittaker, Inc., Walkersville, MD). DNA concentration was determined using the picoGreen dsDNA Quantitation Kit (Molecular Probes, Eugene, OR) and a luminescence spectrometer (model LS-50B; Perkin-Elmer Corp., Norwalk, CT).

Ig Heavy Chain Consensus PCR

Consensus PCRs contained 0.1-0.5 μg of DNA in a total volume of 50 μl. Each reaction consisted of PCR Buffer #10 (10 mM Tris pH 9.2, 1.5 mM MgCl₂, 75 mM KCl: Stratagen, La Jolla, CA), 0.2 μM of each dNTP (dATP, dCTP, dGTP, or dTTP) in a premixed solution (Boehringer Mannheim Corporation), and 1.0 units of AmpliTaq DNA Polymerase (Perkin-Elmer Corp.). Primer sequence and concentrations are shown in Table 1. Standards and control reactions (described below) include a positive control of 0.1 μg of genomic DNA from the IM9 cell line (CCL-155; American Type Culture Collection, Manassas, VA), a negative control of 0.1 μg of genomic DNA from the RPMI 8226 cell line (CCL-155; American Type Culture Collection), and a no-DNA control. During reaction set-up, all reagents and DNA were kept on ice, and 0.2 ml of thin-walled PCR tubes (Applied Scientific, South San Francisco, CA) were stored in a metal TempBlock prechilled to -20°C (USA Scientific, Ocala, FL). Samples were then transferred to the thermal cycler (model 9600, PE/ABI) once the initial ramping temperature exceeded 80°C. Thermal cycler parameters included 2 min at 96°C, 40 cycles of (15 s at 95°C, 15 s at 57°C, 15 s at 72°C), 5 min at 72°C, and a 4°C soak.

Allele-specific CDR3 PCR

In allele-specific CDR3 PCRs (alternatively referred to as idiotype-specific amplification), all standards and unknowns were analyzed at least in duplicate and contained a total of 0.3 μg of DNA/50 μl of reaction (equivalent to 50,000 cells at 6 pg of DNA/cell). PCR reaction components were supplied by Perkin-Elmer Corporation and included 10× PCR Buffer (100 mM Tris pH 8.3, 500 mM KCl), 2.5-30 μM MgCl₂, 0.2 μM each of dATP, dCTP, dGTP, 0.4 μM dUTP, 0.5 units of AmpliTag Uracil N-Glycosylase, 1.5 units of TaqGold DNA Polymerase, and sterile water (Baxter Healthcare Corporation, Deerfield, IL). Primers and TaqMan probes were synthesized by the Oligo Factory (PE/ABI; Foster City, CA), and were used at the assay-specific concentrations shown in Table 1. TaqMan probes were labeled at the 5' end with the reporter dye molecule FAM (6-carboxy-fluorescein; emission λ 518 nm) and at the 3' end with the quencher dye molecule TAMRA (6-carboxytetramethyl-rhodamine; emission λ 582 nm). In addition, the 3' end of the probe was phosphorylated to prevent extension of the probe during the PCR.

Real-time PCR reactions were placed in 0.2 ml of MicroAmp optical tubes with caps (Perkin-Elmer Applied Biosystems Division, Foster City, CA), and data were collected using the ABI PRISM 7700 SDS analytical thermal cycler, as described below. Thermal cycler parameters included 2 min at 50°C, 10 min 95°C, and 40 cycles of [15 s at 95°C, 30 s at a patient-specific annealing temperature (60°C–62°C)].

Standards. PB1 DNA extracted from normal donors was used as a negative control. The DNA was diluted with 10 mM Tris (pH 8.0) to a final concentration of 30 ng/μl. Standard A (100% positive) was made by adding 4.5 μg of plasmid DNA (containing the patient-specific subcloned CDR3 insert) to 250 μl of negative control DNA. Then, Standard A was diluted 1:5 into negative control DNA to produce Standard B (20% positive). Standard B was serially diluted 1:10 into negative control DNA to yield Standards C-G (2%–0.0002%). Standard H consisted of 100% negative control DNA.

Real-Time versus End Point: Intra-assay Reproducibility

Forty replicates of a 3 ng of PBL DNA sample were analyzed in a real-time β-actin PCR assay. PCRs were performed as described above for the allele-specific CDR3 assays. Primer/probe concentrations and sequence data are found in Table 1. Thermal cycler parameters included 2 min at 50°C, 10 min at 95°C, and 40 cycles of (15 s at 95°C, 1 min at 60°C). Data collection protocols are described in the “gel densitometry” and “ΔΔRct” sections below.

CDR3-specific Standards: Plasmid DNA versus Genomic DNA

Two sets of standards were prepared. One consisted of genomic DNA from the IM9 multiple myeloma cell line. The other was made by subcloning the IM9 CDR3 consensus PCR product into the pCR2.1 sequencing vector, as described below. Both are in a background of genomic DNA from the RPMI 8226 cell line. The RPMI 8226 cell line lacks a heavy chain gene, and thereby, is an effective negative control for both the consensus PCR and the CDR3-specific PCR.

The genomic DNA standards A-G consisted of seven 5-fold serial dilutions of 0.1 μg/μl IM9 DNA into 0.1 μg/μl RPMI 8226 DNA to give final concentrations of 100%, 20%, 4%, 0.8%, 0.16%, 0.03%, and 0.006%, respectively. The final standard, H, consisted of only RPMI 8226 DNA. To construct

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*FAM, 6-carboxy-fluorescein; TAMRA, 6-carboxytetramethyl-rhodamine.*

**Concentration of primer = 0.25 μM in consensus PCR; 0.5 μM in 104 assay; 0.2 μM in 105 assay.**
the plasmid DNA and genomic DNA standards with equal target copy numbers (i.e., CDR3 rearrangements), it was assumed that a single copy of the 4 kb of pCR.1 vector containing the cloned IM9 CDR3 region was equivalent to one copy of the genomic CDR3 DNA sequence. Accordingly, it was determined that 0.5 pg of this plasmid DNA in a background of 1 pg of RPMI 8226 genomic DNA would have equal numbers of CDR3 target copies as 1 pg of IM9 genomic DNA (i.e., both would be “100%” positive). To complete the full set of standards based on copies of plasmid DNA, the 100%-positive sample was serially diluted into RPMI 8226 DNA as per the genomic DNA standards above.

The validity of replacing genomic DNA standards with plasmid DNA was assessed using either Vp/JH consensus primers (see Table 1) or CDR3-specific JH primers. Both assays included an IM9 CDR3-specific TaqMan probe and used the consensus PCR cycler protocol described above. After PCR, the samples were transferred to a white 96-well flat-bottomed plate (Perkin-Elmer Corp.), and fluorescence emission data were collected on the LS-50B luminescence spectrometer plate reader (Perkin-Elmer Corp.), as described previously (24). Standard curves were constructed by plotting fluorescence intensity (ARn) versus the log_{10} of cell equivalents of target DNA. Probability values evaluating differences in plasmid DNA versus genomic DNA standard curves were generated in SYSTAT (SPSS, Chicago, IL) using a multiple regression, dummy-coding technique (28).

**Data Collection Methods**

**Gel Densitometry.** After PCR, 8 µl of 6 × Tracking Dye (BioVentures Inc.) were pipetted into the 50-µl of PCR reaction. Each reaction (15 µl) was then loaded onto a 3% NuSieve/1% SeaKem GTG Agarose gel (FMC BioProducts, Rockland, ME) in 1 × TBE buffer (BioWhittaker). Electrophoresis was performed at 200 V for 30–45 min. Gel bands were visualized by staining with ethidium bromide (Life Technologies, Inc., Gaithersburg, MD), and the digital image was captured using the Eagle Eye II Still Video System (Stratagene). Band density was measured using One-Dscan software (Scanalytics, Billerica, MA), and regression analysis was performed with SYSTAT software.

**ΔRn Real-Time and End Point Data Collection.** Real-time data were collected using the ABI Prism 7700 SDS (Sequence Detection System, PE Applied Biosystems Division; Perkin-Elmer Corp., Foster City, CA). The SDS software calculated ΔRn using the equation ΔRn = (Rn^−) - (Rn^+) , where Rn^− equals the ratio of reporter and quencher dye at any given time during a reaction, and Rn^+ equals the ratio of reporter and quencher baseline emissions during cycles 3–15. A fluorescent threshold was manually set across all samples in the experiment such that it bisected the exponential phase of fluorescent signal increase (generally <0.1 arbitrary fluorescent units). The Ct was defined as the cycle number at which a sample’s ΔRn fluorescence crossed this threshold. As shown in Fig. 2, Ct is linearly related to the log of starting copy number. Consequently, assay-specific standard curves of Ct versus log_{10} of copy number were used to estimate the copy number of DNA targets in unknown clinical samples. End point ΔRn (Fig. 1) was simply the fluorescence measured at the final cycle of the PCR protocol.

**RESULTS**

**Real-Time versus End Point: Intra-assay Reproducibility.** Oligonucleotide primers targeting a 295-bp region of the human β-actin gene were used in conjunction with a doubly labeled fluorescent probe in a real-time PCR assay to investigate intra-assay variation between real-time and two end point PCR measures (ΔRn, band density). The Ct (Fig. 1A), end point fluorescence (ΔRn; Fig. 1A), and band density data (Fig. 1B) were collected for 40 replicates of a 3-ng PBL DNA standard (500 cell equivalents), as described above.

As shown in Fig. 1, the coefficient of variation, a direct measure of the 40-replicate intra-assay variability, was lower for real-time Ct data (1.6%) than for end point fluorescence data (13.6%) or band density data (31%). The potential outlier (§; Fig. 1, A and B) still crossed the threshold at about the same cycle number as the other replicates, although it did not exhibit the same fluorescent rate of increase. This particular PCR phenomenon, possibly due to late cycle inhibition, has been noted by others (26), but does not significantly affect the variability of real-time data.

**Real-Time versus End Point: Standard Curves.** Real-time PCR Ct data were compared directly to end point data (band density) in a CDR3 allele-specific assay for patient 104. Fig. 2A shows the gel and resulting standard curve obtained from end point band density data, whereas Fig. 2B depicts the threshold plots and the standard curve generated from real-time data. Both standard curves were fitted with 95% confidence intervals.

The greatly expanded confidence bands in Fig. 2A are partly the result of regressing only the 2-log linear portion of the data against the log of cell equivalents (©; range, between 1,000 and 10 cell equivalents). However, all six samples with a visible PCR band (A→F, E→F, F→F, G→H, H→H, and I→H) were measured. A comparison of the coefficient of variation of all three PCR measures demonstrates that real-time Ct data has increased sample reproducibility and reduced variation between replicates, *, a potential outlier among the 40 replicates.
Quantitative Real-Time PCR for MRD Standards

Fig. 2. A comparison between standard curves generated from real-time and endpoint data. Plasmid DNA containing the cloned CDR3 Ig heavy chain gene rearrangement for a multiple myeloma patient 104 was serially diluted into normal PBLs such that A→H represent 50,000; 10,000; 1,000; 100; 10; 1; 0.1; and 0 copies of plasmid DNA in a background of 50,000 PBLs. L, 100-bp DNA ladder. A, the post-PCR samples were loaded onto an agarose gel, and band densities were measured. Only the 2-log linear portion of the plot (range, between 1,000 and 10 cell equivalents) was fitted with a least-squares regression and 95% confidence intervals. B, the threshold plot depicts ΔRn versus cycle number for the same samples above. All six PCR-positive data points (A→F: 50,000 copies to 1 copy) were used to generate the log-linear regression plot of Ct versus cell equivalents. The 95% confidence intervals for this standard curve are extremely narrow and the multiple R² for the regression equals 1.00.

50,000 copies to 1 copy) were used to generate the log-linear regression plot of the data collected real-time (Fig. 2B). Therefore, this figure not only demonstrates the strong linear relationship between the Ct and the log of starting copy number (R² = 1.00), but reveals the >4-log dynamic range of the assay.

Plasmid DNA versus Genomic DNA Standards. The assay sensitivity and limit of detection were measured for both plasmid and genomic DNA standards in two assays, one with Vh/Jh consensus primers (Fig. 3A) and the other with CDR3-specific/Jh primers (Fig. 3B). Although both the variable and joining gene consensus primers (Cons-Vh and Cons-Jh in Table 1) showed 100% homology to the plasmid DNA subcloned from the IM9 consensus PCR product, they showed only <95% and 90% homology, respectively, to the IM9 genomic DNA sequence (29). The significant increase in PCR efficiency and limit of detection resulting from 100% matched versus mismatched primer/target DNAs can be seen in Fig. 3A (P = 0.001). However, when at least one primer was CDR3-specific and, therefore, 100% homologous to the target DNA (Fig. 3B), the sensitivity of both genomic and plasmid DNA standards was not significantly different (P = 0.2).

Clinical Application for Real-Time PCR. The 98-bp multiple-myeloma-specific CDR3 sequence of patient 105 (Fig. 4A) was amplified using consensus PCR primers. The locations of the consensus primers, CDR3 allele-specific primer, and fluorogenic TaqMan probe are shown in Fig. 4B.

The 100%-homologous CDR3 primer and probe were used with the Jh consensus primer in a series of quantitative real-time PCR assays to analyze MRD for patient 105. A representative standard curve for this patient-specific assay is shown in Fig. 5A. Each point of the standard curve was analyzed in duplicate, and the regression was fitted with 95% confidence intervals. A strong linear relationship was demonstrated for >4 logs (R² = 0.997; 50,000 to 10 cell equivalents).

Fig. 3. Plasmid DNA versus genomic DNA for allele-specific standard curves. Assay sensitivity was measured for both plasmid and genomic DNA standards in two assays, one with Vh/Jh consensus primers (A) and the other with CDR3-specific primers (B).
The limit of detection for a single replicate, 10 tumor cells/50,000 normal cells, was typical for this assay.

Fig. 5B compares the percentage of detectable CDR3-specific myeloma in sequential clinical samples for patient 105. The data represent the mean (± SE) of completely independent experiments (N, in the column at far right equals the total number of experiments performed for a given sample). Within each experiment, all samples were analyzed in at least duplicate (intra-assay coefficient of variation for estimated cell numbers ≥100 was typically <2%; data not shown). The PB-HSC samples (from days 1, 2, and 3 of apheresis collection) were negative for any detectable residual disease in all replicates available for analysis. Taking into consideration the limit of detection and the total number of cells analyzed for each of the apheresis collections, the assay sensitivity for days 1, 2, and 3 was estimated to be <0.0092%, 0.017%, and 0.0092%, respectively.

Fig. 5C is a notched-box-plot (30) of the data in Fig. 5B, except that only those data points derived from at least four experiments were included. This plot is analogous to a nonparametric t test for between-group differences. The vertical lines at the notch of each box represent the data median, and the spiked tips of the boxes represent 95% confidence intervals. It may be concluded with 95% confidence that two population medians are different if the tips of the boxes do not overlap. Fig. 5C shows that the 3.5% of residual disease detected in the BM samples after chemotherapy was significantly reduced by 2 logs, to a mean tumor load of 0.04% for the 3 days of apheresis. The percentage of residual disease detected in follow-up BM sampled at 42, 100, 180, and 365 days after transplant was 4.0%, 2.0%, 0.2%, and 0.7%, respectively.

DISCUSSION

Although high-dose chemotherapy and stem-cell transplantation together provide an effective short-term treatment for multiple myeloma (31–33), they rarely result in permanent remission. Patient relapse may be attributed to residual tumor cells present in the autograft (as a result of insufficient purity; Refs. 1, 18, 34, and 35) or chemotherapy-resistant myeloma cells remnant in the patient (36). Thus, quantifying the frequency of residual disease may be used as a prognostic factor and an indicator of a therapy's efficacy.

**Real-Time PCR.** This study presents a sensitive, specific, and quantitative means to detect MRD. Our approach, a modified, real-time PCR assay using TaqMan technology, has many advantages over traditional end point measures. A primary strength of the assay is its simplicity. For example, real-time PCR data are interpreted using simple regression analysis, where the degree of assay linearity is reflected in the multiple R² statistic. For most real-time PCR applications, R² ≥ 0.99 are typical, and R² ≤ 0.98 are usually indicative of a poorly optimized system and/or problems with operator technique (i.e., pipetting inconsistencies). We have developed more than a dozen real-time PCR assays (data not shown), and standard curves never generate R² < 0.99.

Furthermore, we have demonstrated that the dynamic range of real-time data can span ≥4 logs of target DNA concentration (Fig. 2B; Fig. 5A). Although it is possible to shift the 2-log dynamic range of a traditional PCR assay by manipulating target concentration and/or cycle number (5), this requires time-consuming sample-specific optimizations and increases the potential for variability inherent in the preparation of sample dilutions. In a study by Billadeau et al. (6), the percentage of MRD in the PB of multiple myeloma patients varied by as much as 6 logs (32–0.00088%). Certainly, for such studies an assay with a large dynamic range would be particularly convenient.

The single most important advantage of real-time PCR is its high degree of reproducibility. Whereas it has been reported that end point PCR measures based on limiting dilution analysis (18) or band densitometry (5) can vary by as much as 3–10-fold for the same DNA sample, we demonstrate herein that real-time PCR data, because it is collected during the phase of exponential product formation for each individual sample, has a high degree of both intra-assay (Fig. 1) and inter-assay reproducibility (Fig. 5). In addition, we and others (26) have observed that real-time Ct data does not seem to be affected by
Fig. 5. Quantitative analysis of residual disease in clinical samples (multiple myeloma patient 103). A. A representative standard curve with a regression fitted with 95% confidence intervals. Each point has been analyzed in duplicate ($R^2 = 0.997; n = 10$). %, data (log$_{10}$ of percentage of residual disease) represent the mean of completely independent experiments (error bars represent SE for interassay variation). Each data point (depicted as N, in the far right column) represents the mean of at least two replicates (intra-assay coefficient of variation for estimated cell numbers $\pm 100$ was typically $<2\%$). NEG., none detected. C, a notched-box-plot, analogous to a nonparametric $t$ test for between-group differences, for samples with data derived from at least four experiments. It may be concluded with 95% confidence that two population medians are different if the tips of the boxes do not overlap. *, significant between-group difference ($P \leq 0.05$).

presumably anomalous, stochastic PCR amplifications that result in reduced product formation (Fig. 1).

Although not immediately obvious, there are several benefits to real-time TaqMan PCR that are inherent in the technology. First, the assay is extremely rapid because post-PCR processing steps, such as Southern transfers and probe hybridization, are unnecessary. All relevant data are, indeed, collected real-time during the course of a typical 2-h PCR cycler program; data analysis can be completed in less than 10 min. Second, the majority of the assay, from reaction set-up to data collection and analysis, is a closed-tube system. Not only does this feature reduce the risk of false positives resulting from PCR product carry-over contamination, but it eliminates variation from additional pipetting steps. Moreover, this assay is amenable to enzymatic control for carry-over contamination, such as the dUTP/uracil-N-glycosylase system (37) that we routinely use for all of our quantitative PCR assays. Such a measure is of critical importance for any diagnostic assay that is optimized to detect single copy targets. Finally, the TaqMan PCR is highly specific for the gene target of interest. A positive fluorescent signal can only be generated from a PCR amplicon if both the primers and the fluorescent probe hybridize to the template DNA. Therefore, as we have previously demonstrated with other TaqMan assays (24), PCR artifacts such as nonspecific bands or primer-dimers, as detected on an ethidium-bromide-stained agarose gel, are not TaqMan positive.

**Plasmid DNA versus Genomic DNA Standards.** DNA isolated from clonal-plasma cells must be used not only to identify the CDR3...
sequence, but, ideally, to generate standards for subsequent CDR3 assays. Realistically, this may not be feasible. The frequency of tumor cells is generally quite low, especially in patients pretreated with high doses of chemotherapy and, thus, isolating a significant number of these cells could require a large-scale BM harvest. Moreover, we have found that using cell sorters to obtain pure plasma cells at best yields populations only enriched in myeloma cells (4–34%, based on TaqMan technology; data not shown). Consequently, the practical limitations of acquiring sufficient tumor cells has forced some investigators to report a limit of detection for patient-specific assays, which are based on standards made from cell-line DNA (38).

However, not only do thermal cycling protocols and target DNA concentrations affect PCR dynamics, but so, too, does the degree of primer/template sequence homology. One cannot make an absolute quantitative assessment of MRD when there is a disparity degree of primer/template sequence similarity between assay standards and unknowns. To maintain sequence homology between assay standards and patient samples, CDR3 sequences were cloned into a plasmid vector and used as standard templates. Although the consensus primers showed identical sequence homology to plasmid DNA but not genomic DNA, it was determined that standard curves obtained from genomic and plasmid DNA were not significantly different when at least one of the primers used was CDR3-specific (Fig. 3). Moreover, generating standard curves from cloned DNA, compared with standards made from heavily infiltrated BM (3), could yield more accurate data because the exact concentration of allele-specific DNA can be reliably quantified. Therefore, by relying on cloned CDR3 sequences to generate standard curves, the problems associated with using generic cell-line standards or isolating large numbers of pure, clonal, sample-specific tumor cells can be avoided.

**Clinical Application for Real-Time PCR.** Although detecting the presence of MRD is a critical part of many transplantation protocols, the correlation rather than detection of MRD may reveal relevant correlations with patient parameters and have important prognostic implications. Indeed, there have been a number of traditional, end point PCR assays developed specifically to generate quantitative MRD data. Some of these studies, whether for multiple myeloma or other B-cell malignancies, represent attempts to correlate MRD with clinical outcome (15, 18, 36, 38, 39), and others seek to evaluate the tumor purging efficacy of various treatment protocols (7, 40). Although some semiquantitative data have been generated for follow-up BM and PB samples (41), most studies have only reported the presence or absence of MRD (15, 38). For example, in one of the first studies to suggest that molecular detection of MRD can have prognostic significance, there was a clear association between disease-free survival and the lack of detectable MRD in BM after transplantation (36). In this study, it was found that 25 of 35 patients with consistently PCR-positive BM had relapsed, whereas 8 of 41 patients with MRD detected in only BM samples relapsed. Despite the heterogeneity of tumor involvement in the BM, these data suggest a possible correlation between the absolute level of residual tumor and frequency of relapse.

Real-time quantitative PCR can provide the necessary precision to not only correlate PCR negativity with clinical outcome but also to reveal the relationship between the patients’ measured residual tumor load over time, and their potential for relapse. For example, although the dynamic range of data for the follow-up BM samples in Fig. 5C is <1.5 logs and the confidence intervals for each time point are the result of interassay and not intra-assay variation, small changes in the percentage of MRD can be detected. Specifically, the precision of real-time PCR data revealed an increase in the percentage of detectable residual disease from 6 months (0.2%)-1 year (0.7%) after transplant. This increase in BM tumor load is currently being evaluated for clinical significance.

**Conclusion.** The use of real-time quantitative PCR is not limited to multiple myeloma. We have developed similar assays based on specific chromosomal translocations that are prevalent in other common B-cell malignancies, including the BCL-2 major breakpoint region and the BCL-1 major translocation cluster. These assays have an additional advantage over the current CDR3 approach in that they are disease-specific rather than patient-specific and can be universally applied (manuscript in preparation).

In this report, we show that the CDR3 real-time PCR assay clearly provides an advantage over routine diagnostic approaches such as nested PCR and limiting dilution analysis for detecting MRD. Once optimized, it is a rapid, closed-tube system that requires no post-PCR manipulation and fewer cells than other allele-specific assays. With its reduced risk of contamination, increased sensitivity, and expanded dynamic range, this assay is capable of generating reliable and quantitative data for assessing patient MRD and may well provide the level of accuracy necessary for such measures to have real prognostic significance.

**REFERENCES**


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