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# High resolution melting analysis of deletion/insertion polymorphisms: A new method for the detection and quantification of mixed chimerism in allogeneic stem cell transplantation

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This paper is dedicated to the memory of Prof. Claudio Orlando.

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ABSTRACT

Increasing mixed chimerism after allogeneic stem cell transplantation has been associated with a high risk of relapse and probable graft failure in patient with hematological malignancies as well as non-malignant conditions. We evaluated a new method for chimerism detection, based on the quantitative High Resolution Melting Analysis (HRMA) of deletion/insertion polymorphisms (DIPs).

The study consisted in the selection of a panel of DIPs, all generating genotype-specific melting curves, and in the use of samples containing opposite molecular species (homozygous INS/INS and DEL/DEL) mixed in different percentages to create a standard curve for each polymorphism.

The detection of mixed chimerism with the HRMA attained a sensitivity of <1%, as well as good accuracy and precision with Percent Errors and Coefficients of Variation not exceeding 30% in reconstruction experiments with DNA mixtures.

The present approach provides accurate and precise estimates of mixed chimerism and makes the method open to evaluation for its use in clinical practice.



## 1. Introduction

Allogeneic stem cell transplantation (SCT) is the treatment of choice for a wide range of hematopoietic disorders, with good chances of recovery [1–6]. Consequently, assessment of the chimeric status to predict relapse [7] has become a useful method in the follow-up of patients who underwent SCT. Furthermore, distinguishing between residual host hematopoiesis and donor hematopoiesis is a valuable tool to track the engraftment process in non-malignant conditions [8].

Thus, there is an increasing demand of reliable methods to monitor the success of engraftment in clinical routine laboratories. Methods for determining the persistence or the reappearance of a patient cell population have been reviewed [9,10] and include the analysis of restriction fragment length polymorphisms [11], variable number of tandem repeats [12], sex-specific mismatch [13] and microsatellites [14–16]. The sensitivities of these techniques vary greatly. Microsatellite analysis, which is the most used method, has a sensitivity estimated between 1% and 5% residual patient cells [17,18]. However, the accuracy of this approach is dependent on the choice of markers, since measurements may be complicated by the presence of stutter peaks arising from slippage of the DNA polymerase during PCR, or by spectral overlap of donor- and recipient-specific fluorescence signals. Other recently designed methods are based on the quantification of single nucleotide polymorphisms (SNPs) [19,20]. SNP differences between individuals have been used in microarray-based minisequencing [21], pyrosequencing [22] and real-time PCR [23–25] techniques. The real-time PCR method has a good accuracy and sensitivity estimated between 0.001% and 0.1%, but this approach is more expensive and complex than microsatellite analysis. Recently, a great number of diallelic polymorphisms due to the insertion or the deletion of short DNA sequences (DIPs) have been characterized in the human genome [26,27]. These polymorphisms were previously used for chimerism quantification using the technique of real-time PCR reaching an extremely high sensitivity around 0.001% [28,29].

High resolution melting analysis (HRMA) is an extension of previous DNA renaturation or melting analyses. It was recently introduced as a technique for genotyping SNPs [30–34] but lately

*Abbreviations:* SCT, stem cell transplantation; HRMA, high resolution melting analysis; DIPs, deletion/insertion polymorphisms; INS, insertion; DEL, deletion; SNPs, single nucleotide polymorphisms; dsDNA, double stranded DNA; Q-D-HRMA, quantitative differential HRMA; SD, standard deviation; CV, coefficient of variation.

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new horizons are developing for HRMA: detection and quantification of methylation degree in samples of tumor cells [35–38], determination and quantification of RNA editing [39], and the detection and quantification of mosaicism and heteroplasmy [40]. This closed-tube method is made possible by third-generation fluorescent double-strand DNA (dsDNA) dyes that do not inhibit PCR and can therefore be used at higher concentrations for greater saturation of the dsDNA sample that collects fluorescent signals with much greater optical and thermal precision [41].

We have been interested in assessing the potentiality of HRMA for the detection of mixed chimerism to follow-up patients who underwent allogeneic SCT. We propose here a new approach that exploits HRMA for the recognition to a defined sensitivity of a donor-recipient minor component. Informative DIPs, where the donor and the recipient presented opposite insertion or deletion alleles, give accurate and precise estimates of mixed chimerism in reconstruction experiments and make the method open to evaluation for its use in clinical practice.

## 2. Materials and methods

### 2.1. DNA samples

Institutional review board approval was obtained before starting the study. At the time of withdrawal, each subject gave informed consent for storage and future use of their own DNA for research purposes. Genomic DNA was extracted from fresh whole peripheral blood of 20 healthy subjects by QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's instructions. These samples were used to determine genotype-specific profiles of melting curves following HRMA, and to choose suitable DNA sources to create mixtures of known composition in order to construct standard curves for each DIP (see below). Blind DNA serial dilutions of a sham donor/recipient pair came from a recently held collaborative exercise [42] and were used to test the method.

### 2.2. DIPs

We selected 20 DIPs from the Marshfield Clinic database (www.marshfieldclinic.org/mgs/) on the basis of diallelic status, confirmation, high level of heterozygosity (close to 0.5) in most populations, variable size of DEL/INS (5–26 bp), uniform length of the amplicons (120 ± 60 bp), central position of the polymorphic site within the amplicon, and maximum CG content of the INS allele (Table S1). Among these DIPs we choose those generating genotype-specific melting curves to create a panel of 9 polymorphisms to be used in subsequent experiments for the quantification of mixed chimerism.

### 2.3. Quantitative differential HRMA

HRMA was carried out on a Rotor-Gene 6000 (Corbett Research). PCR was performed in a final volume of 15 μL containing 1.5 μL of AmpliTaq Gold Buffer (Applied Biosystems), 1 μL (10 pmol/μL) of each primer, 1 μL (200 mmol/μL) of dNTPs, 0.15 μL of AmpliTaq Gold (Applied Biosystems), 2 μL of DNA (50 ng/μL), 0.15 μL (50 pmol/μL) of SYTO 9 (Molecular Probes), and 8.2 μL of $ddH_2O$. The thermal profile was as follows: 10 min at 95 °C, then 40 cycles of 30 s at 95 °C, 30 s at 59 °C, 30 s at 72 °C, and a final step of 20 min at 72 °C. PCR products were subjected to thermal denaturation at 95 °C for 2 min and then rapidly cooled to 40 °C for 2 min, allowing the reassociation of homoduplex DNA in DEL/DEL and INS/INS homozygous samples as well as of varying proportions of homoduplex and heteroduplex DNA in DEL/INS heterozygotes and in both known and unknown mixed samples. Melting curve data were then acquired in a wide range of temperatures (75 °C–93 °C) at a ramping rate of 0.1 °C/s. Melting curves were normalized by setting temperature windows before and after major fluorescence changes, using the resident HRMA software. A differential profile was then evaluated for both known and unknown mixed samples by comparing fluorescence at the melting point against the value of the reference control (100% homozygous DEL/DEL).

### 2.4. Sequencing

Amplicons were purified using the QIAquick PCR Purification Kit (Qiagen). Direct DNA sequencing of purified PCR products was performed using dideoxynucleotide termination chemistry. Sequencing reactions were purified using the Dye Ex 2.0 Spin kit (Qiagen) and samples were run on the ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

### 2.5. Preparation of standard curves with HRMA

Sequence-confirmed homozygous samples for each DIP of our panel were used to prepare DNA mixtures of known composition in order to mimic donor/recipient mixed chimerism. We prepared serial dilutions of recipient DNA (INS/INS) in donor DNA (DEL/DEL) to create reconstituted samples at constant DNA concentration containing 50%, 25%, 12.50%, 6.25%, 3.13%, 1.56%, 0.78%, 0.39%, 0.20%, 0.10%, and 0.05% of recipient DNA in donor DNA. Full recipient DNA was added to the series for comparison, whereas full donor DNA sample served as reference. Quantitative Differential HRMA (Q-D-HRMA) let us to construct standard curves for each DIP by plotting the normalized differential fluorescence values against the serial dilutions. Each point in the standard curves is the mean of 5 independent Q-D-HRMA experiments. The associated source of variability served to measure inter-assay reproducibility.

### 2.6. Mixed chimerism estimate

Ten DNA mixtures of unknown composition were subjected to Q-D-HRMA of an informative DIP. For each sample, the differential fluorescence mean value of 3 replicates was used to estimate the proportion of recipient DNA by linear interpolation against the standard curve. The variability associated with the triplicates in a single Q-D-HRMA experiment served to evaluate intra-assay reproducibility.

### 2.7. Microsatellite analysis

Ten DNA mixtures of unknown composition (as above) were assayed with the gold standard microsatellite method. The AmpFlSTR Identifiler Amplification kit (Applied Biosystems) was used. PCR products were subjected to electrophoresis on the ABI PRISM 310 Genetic Analyzer (Applied Biosystems). The whole procedure was carried out according to the manufacturer's instructions. Size calling and quantification of peak areas were performed using the Genemapper software (Applied Biosystems). Donor and recipient DNA profiles were compared and peaks of different sizes were identified in the mock post-transplant samples. Recipient-specific peaks allegedly occurring at positions where stuttering and/or spectral overlap might bias the analysis were not considered. For each informative locus the areas of recipient and donor peaks were added and the percentage of the recipient peak areas over the total area was calculated. If recipient-specific signals at the expected positions were below 3 standard deviations (SD) of the average background noise, a zero value was computed. Donor chimerism was established by calculating the median value of all