Quantitative polymerase chain reaction-based chimerism in bone marrow or peripheral blood to predict acute myeloid leukemia relapse in high-risk patients: results from the KIM-PB prospective study

Valentina Gambacorta,[1] Riccardo Parolini,[1] Elisabetta Xue,[1,2] Raffaella Greco,[2] Evelien E. Bouwmans,[3] Cristina Toffalori,[1] Fabio Giglio,[2] Andrea Assanelli,[2] Maria Teresa Lupo Stanghellini,[2] Alessandro Ambrosi,[4] Benedetta Mazzi,[5] Wietse Mulder,[3] Consuelo Corti,[2] Jacopo Peccatori,[2] Fabio Ciceri[2,4#] and Luca Vago[1,2#]

[1]Unit of Immunogenetics, Leukemia Genomics and Immunobiology, IRCCS San Raffaele Scientific Institute, Milano, Italy; [2]Hematology and Bone Marrow Transplantation Unit, IRCCS San Raffaele Scientific Institute, Milano, Italy; [3]GenDx, Utrecht, the Netherlands; [4]Vita-Salute San Raffaele University, Milano, Italy and [5]HLA and Chimerism Laboratory, IRCCS San Raffaele Scientific Institute, Milano, Italy

[#]FC and LV contributed equally as co-senior authors.

Correspondence: LUCA VAGO - vago.luca@hsr.it

doi:10.3324/haematol.2019.238543

SUPPLEMENTARY METHODS

Sample collection and preparation

Peripheral blood and bone marrow samples were collected at the pre-specified study time-points illustrated in **Figure 1A**, in concomitance to routine diagnostic procedures and upon specific written informed consent. Samples were centrifuged for seven minutes at 1500 revolutions per minute and the cell-enriched interphase was collected and stored at -80°C for subsequent use. Upon sample thawing, genomic DNA was extracted using the Qiamp Blood Minikit (QIAGEN, Venlo, The Netherlands) according to the manufacturer's instructions, checked for quality and concentration using a Nanodrop spectrophotometer (Thermo Scientific, Franklin, MA, USA), and stored at -20°C.

Hematopoietic chimerism analysis

Hematopoietic chimerism was analysed using a commercial qPCR-based system (KMRtype and KMRtrack assays, GenDx, Utrecht, The Netherlands) according to the manufacturer's instructions. Briefly, host-specific polymorphisms for subsequent chimerism monitoring were selected using the KMRtype genotyping assay, which probes for 29 insertion-deletion polymorphisms spread over 18 different chromosomes by 10 independent multiplexed TaqMan qPCR reactions. For each well, 10 ng of genomic DNA from the individual of interest were used in a final test volume of 20 µl.

For each donor-recipient pair, two markers positive in the patient and negative in the donor were selected as informative using the KMRengine software and employed in parallel for post-transplantation monitoring. Quantification of these informative patient-specific markers at each study time-point was performed using the KMRtrack qPCR assays. Each assay consists in a singleplex Taqman reaction probing an indel polymorphism.

Comparators for each reaction consisted in a pre-transplant sample from the patient and in a reference reaction, amplifying the invariant *RPPH1* gene. Percentage of host-specific chimerism was calculated according to the to the ∆∆CT formula: $2^{-\Delta\Delta CT}$x100%, in which ∆∆CT equals to: (CT (marker post-transplant sample) - CT (reference gene post-transplant sample)) - (CT (marker patient pre-transplant sample) - CT (reference gene patient pre-transplant sample)). All reactions were performed in duplicate, testing 50 ng of genomic DNA per well in a final test volume of 20 µl. All qPCR reactions were amplified and analysed using an Applied Biosystems 7500 Thermocycler, using the cycling conditions suggested by the assay manufacturers. For each time-point analyzed shown is the average between the two independent markers analyzed.

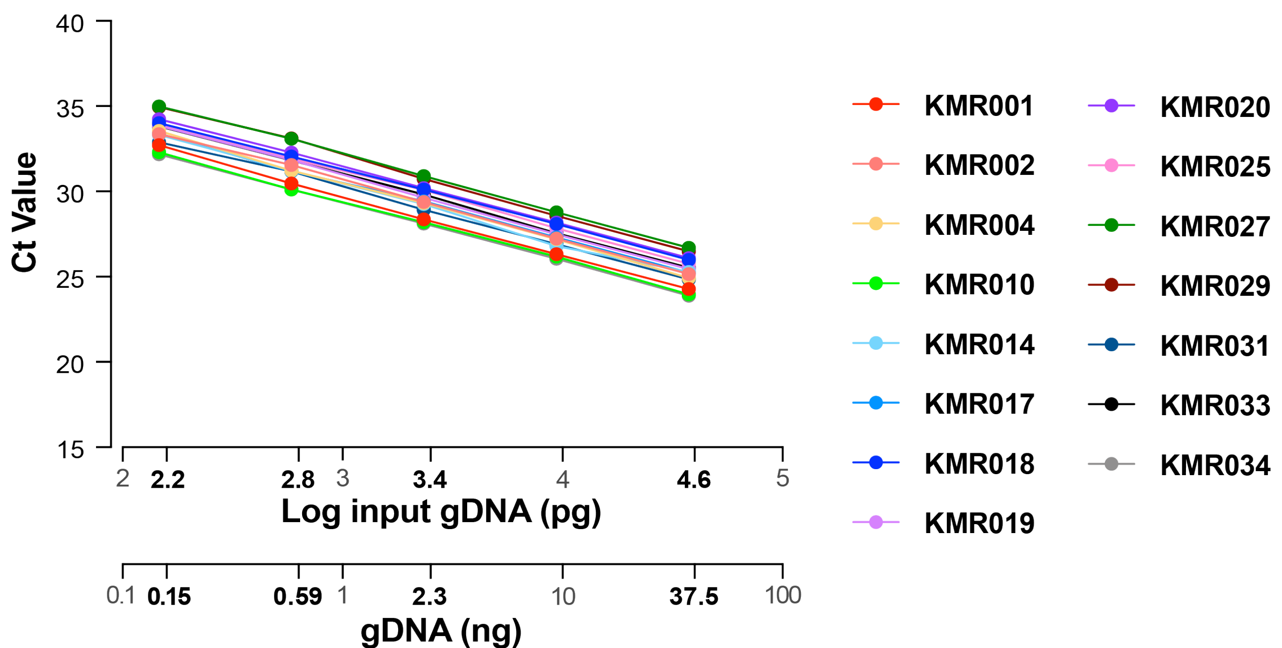
Characteristics of the assay

The KMRtype/KMRtrack chimerism monitoring system (GenDx, Utrecht, The Netherlands), is based on the determination and quantification of patient-specific polymorphisms by qPCR. Maximal reproducible sensitivity of the assay in the detection of a polymorphism varies as a function of the amount of the input DNA, as summarized in **Supplementary Table 1,** but for all available markers detection of DNA template positive for markers of interest remains reliable even at very low concentrations (**Supplementary Figure 1**).

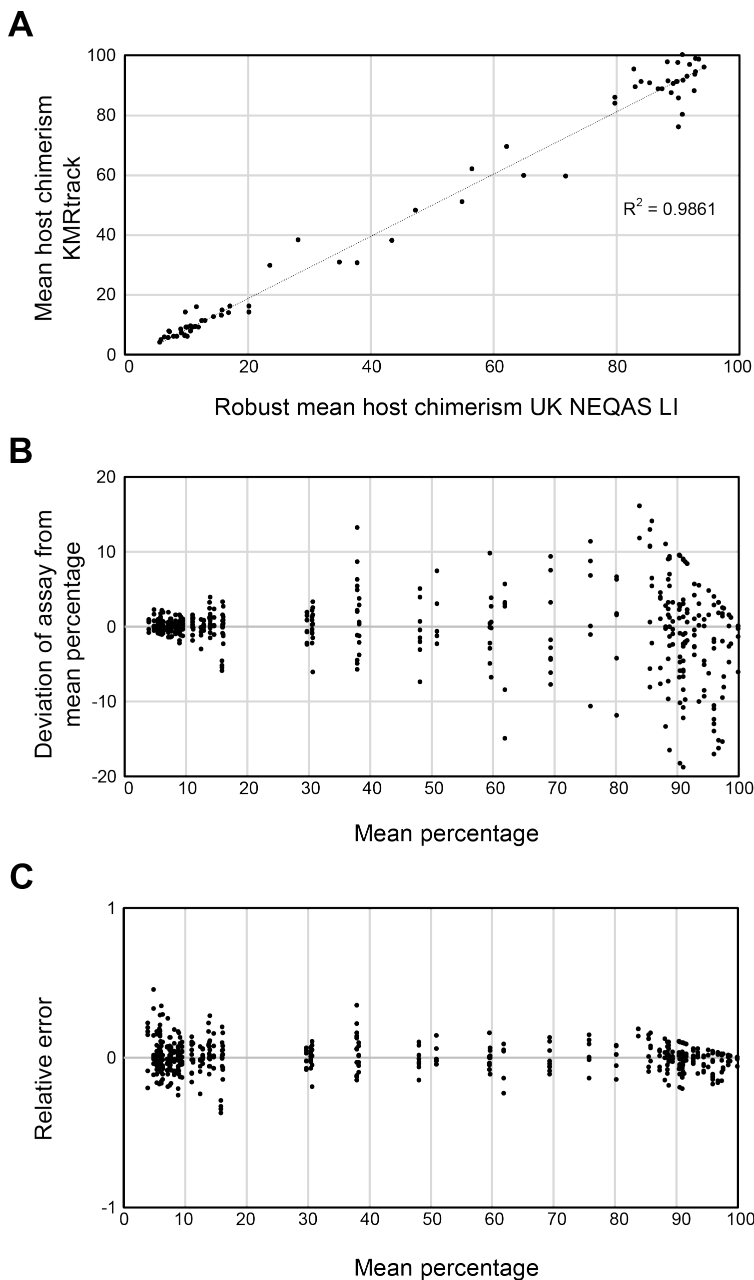
Experimental error intrinsic to this qPCR technique is directly proportional to the amount of host chimerism measured, with a constant relative error (defined as [experimentally determined chimerism – average of multiple determinations] / average of multiple determinations) quantified as 0.4±9.9% (**Supplementary Figure 2**). Based on this notion, the 95% confidence interval of variation can be calculated as 1.96*relative error stdev, and thus total to 19.5%.

Input DNA (ng)	#cells	Sensitivity (%)
150	22727	0.044
100	15152	0.066
50	7576	0.130
25	3788	0.260
10	1515	0.660
5	758	1.320

Supplementary Table 1. Relationship between input DNA, number of cells and sensitivity using the KMRtrack assay.



Supplementary Figure 1. Analysis of the performance of KMRtrack assays in relation to different concentrations of template DNA positive for the marker of interest. Note that linear proportion of detection values is maintained at all tested input DNA concentrations, including the lowest one.



Supplementary Figure 2. Analysis of KMRtrack performance and relative error on a panel of reference chimeric samples. KMRtrack was utilized to quantify hematopoietic chimerism in samples collected with permission from 19 studies from the UK NEQAS for Leucocyte Immunophenotyping (UK NEQAS LI) 'Post-Stem Cell Transplant Chimerism Monitoring' program, resulting in a total of 498 individual assay measurements. Overall, concordance with the robust mean result obtained from the UK NEQAS LI report was very high (R^2=0.9861, panel A). Absolute deviation between chimerism detected by an individual measurement and the mean percentage measured for each sample was then plotted against the mean percentage, demonstrating a higher absolute deviation at higher chimerism values (panel B). Next the percentage of deviation, or relative error, was plotted against the mean percentage measured for each sample, showing that this index is conversely constant throughout the whole range of detection (panel C).