

Highly sensitive quantification of human cells in chimeric NOD/SCID mice by real-time PCR

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We recently presented a real-time duplex PCR method to determine the amount of human cells in chimeric mice. Although this assay was suitable for the quantitative detection of 1% of human cells in the background of murine cells in tissue, blood and bone marrow of chimeric mice, there is legitimate interest in detecting even lower amounts of human cells in these animals reliably. Therefore, we developed a new assay that reliably detects as few as 5 human cells in 100,000 mouse cells (0.005%). The assay specifically amplifies a human and a murine endogenous retroviral sequence in two separate reactions. Based on the same primers, the assay can be performed either with 5' nuclease probes (TaqMan assay) or with a pair of hybridization probes (LightCycler assay), enabling the use of various real-time PCR instruments.

The use of immunodeficient mice in hematological studies has dramatically increased during the last years. One possible approach to quantify chimerism is based on specific detection of human DNA in chimeric tissue. Published PCR methods are either highly sensitive² or allow reliable real-time quantification¹; however, sensitivity is usually reduced at the expense of quantification. For the investigation of putative bone marrow engrafting cell types, kinetic studies of early engraftment events or the colonization of individual mouse organs with human cells, the combination of both, high sensitivity and reliable quantification, is required.³ Therefore, we established a new real-time PCR based assay, which combines i) all advantages of real-time PCR, including quantification, reduced contamination risk and specificity, with ii) an extremely high sensitivity. The assay is based on separate amplification of the human specific endogenous retroviral sequence ERV-3 and the mouse specific endogenous retroviral sequence ERV-L pol.⁴ For the detection of human ERV-3 a recently published TaqMan assay was modified and suitable hybridization probes were designed,⁵ whereas the mouse ERV-L assay was a completely new development. Both assays were optimized to be run under identical reaction conditions, permitting a simultaneous analysis of human and murine cells.

TaqMan PCR was performed in a Perkin Elmer 7700 Sequence Detection System in 96-well microtiter plates in a final volume of 25 μ L including 2.5 μ L 10x PCR reaction buffer, 4.5 mM MgCl₂, 1.0 mM dNTP, 1 U Platinum Taq DNA polymerase (all Invitrogen, Karlsruhe, Germany), 5 pmol of each primer, 3 pmol 5' nuclease probe and 1 μ M ROX (6-carboxy-X-rhodamine). 2 μ L template DNA were amplified starting with 3 min at 94°C, followed by 45 cycles of 94°C for 30 s and at 58°C for 30 s.

LightCycler PCR (Roche Molecular Diagnostics,

Mannheim) was performed in a final reaction volume of 20 μ L including 2 μ L LightCycler-Fast Start DNA Master Hybridisation Probes mix (Roche, Mannheim, Germany), 5 mM Mg²⁺, 6 pmol of each primer, 2 pmol of each hybridization probe and 2 μ L template DNA. Cycling conditions were 10 min at 95°C followed by 45 cycles of 8 s at 95°C, 8 s at 51°C and 8 s at 72°C. Table 1. shows primers and detection probes used for TaqMan and LightCycler PCR.

To prove the sensitivity of the assay, human MCF-7 cells were diluted stepwise in murine P388 cells from 100% human cells to 0.005% human cells. DNA preparation was performed as described previously.¹

Both assays were specific for each species. As shown

Table 1. Primers and detection probes used in this study

	Human endogenous retrovirus ERV-3*	M12140*	Tm†
HU-F	CATgggAgtCAAgggAACTATg	1801-1623	59,6°C
HU-R	CTCAgCaAgCAATACCAAAATCT	1735-1714	68,6°C
HU-TM	F-TCCTCCCTCgAACTgCACCATCAg p	1851-1675	67,3°C
HU-FL	CTCACATCAACgCCCTCCCTCgAACT X	1836-1664	70,7°C
HU-LC	TC-gCACCATCAAgTCagAgCATCTgTTCCTAAA p	1886-1695	68,0°C
	Mouse endogenous retrovirus ERV-L	AJ23384	
mu-F	gATgTggTgCCTTACTTgAgCA	64-88	65,2°C
mu-R	AAATCCCTCCAAAgTTCCTgCBA	260-237	65,9°C
mu-TM	AT-CTCTTgTggCTCTTgTAggCaggCAT p	160-133	68,1°C
mu-FL	gCTCTTgTggCTCTTgTAggCA X	161-139	61,1°C
mu-LC	TC-gCTCTTgTATATTTgTAggCTgTACCT p	137-108	61,7°C

*Abbreviations: T: 5-carboxytetramethylrhodamine (5-TAMRA) attached to 5-ethylamino-dThymidin, F: 6-carboxy-fluorescein attached to 5'-terminus (FAM), LC: LightCycler Red 640 attached to 5'-terminus, FL: 6,6-carboxy-fluorescein attached to 3'-O-ribose, p: phosphate group attached to 3'-terminus; X: fluorocytosine. †Nucleotide positions are based on sequence of the Human endogenous retrovirus 3 (accession number M12140) and the mouse endogenous retrovirus (accession number AJ23384). ‡Melting temperature was calculated by nearest neighbor method as described elsewhere⁶.

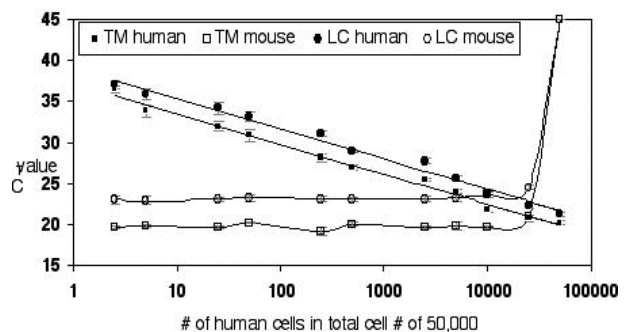


Figure 1. Correlation of human cell number and CT value. Serial dilutions of human cells in mouse cells were measured in triplicate with the TaqMan assay (human cells: filled squares, mouse cells: filled circles) and the LightCycler assay (human cells: open squares, mouse cells: open circles). Total cell number was adjusted to 50,000. Error bars indicate the intra assay variation. The linear correlation between CT value and human cell number for the TaqMan assay is described by $CT = -3.48 \log(\text{cell\#}) + 37.2$ ($r^2=0.99$) and for the LightCycler by $CT = -3.56 \times \log(\text{cell\#}) + 39.2$ ($r^2=0.99$).

in Figure 1., a linear correlation between C_T value and human cell number was obtained in a constant background of murine cells for the human TaqMan and LightCycler PCR. PCR efficiency was 94.0% for the 5' nuclease assay performed on the SDS7700 and 91% for the hybridization probe assay carried out on the LightCycler. C_T values for the background of murine cells were nearly constant. As few as 5 human cells diluted in 100,000 murine cells (0.005% human cells) were clearly detectable. Besides NOD/SCID mice, cells of several further mouse strains, such as NMRI-nu/nu, C57BL6, BDF-1 and DBA2, were also detected by the mouse specific assay. Although the LightCycler generally resulted in higher C_T values when measuring identical samples, the final sensitivity was not reduced. Variations of both assays were low, as indicated by the error bars in the calibration curves. The combination of the human and mouse specific assays to a duplex PCR was associated with a significant loss of sensitivity.

In summary, for quantitative detection of human cells in chimeric mice, the presented assays, based on amplification of species-specific endogenous retroviral sequences, display a detection limit of 0.005% of human cells in murine cells.

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