TITLE PAGE

A semi-nested real-time PCR method to detect low chimerism percentage in small quantity of HSC transplant DNA samples

Michelangelo Aloisio[a], Barbara Bortot[b], Ilaria Gandin[c] , Giovanni Maria Severini[b*], Emmanouil Athanasakis[b]

[a]Department of Life Sciences, University of Trieste, Trieste, Italy

[b]Medical Genetics, Institute for Maternal and Child Health, IRCCS "Burlo Garofolo", Trieste, Italy

[c]Department of Medical Sciences, University of Trieste, Trieste, Italy

[*]**Corresponding author**

E-mail: gianmaria.severini@burlo.trieste.it

Abstract

Chimerism status evaluation of post allogeneic hematopoietic stem cell transplantation samples is essential to predict post-transplant relapse. The most common technique able to detect small increments of chimerism is the quantitative real-time PCR. Although this method is already applied by several laboratories, the previously described protocols often lack of sensitivity and the amount of the DNA required for each chimerism analysis is too high. In the present study we compared a novel semi-nested allele-specific real-time PCR (sNAS-qPCR) protocol with our in-house standard allele-specific real-time PCR (gAS-qPCR) protocol. We selected two genetic markers and we analyzed the technical parameters (slope, *y*-intercept, R2, and standard deviation) useful to determine the performances of the two protocols. The sNAS-qPCR resulted having a better sensitivity and precision. Moreover, the sNAS-qPCR protocol needs, as input DNA, only 10 ng, about at least 10-fold less than the gAS-qPCR protocols described in the literature. Finally, the proposal sNAS-qPCR protocol could be very useful to perform chimerism analysis with a low amount of DNA samples as in the case of blood cell subsets.

Key words: allogeneic HSCT, chimerism, semi nested, real-time PCR, Sybr Green

Introduction

Allogeneic hematopoietic stem cell transplantation (HSCT) is one of curative treatment options for patients with hematologic malignancies. After HSCT, the determination of the genotypic origin of post-transplantation hematopoiesis can be made by chimerism analysis, that consists in determining the percentage of recipient or donor amount in a post transplant blood sample. Chimerism status evaluation, in particular in selected subpopulation blood cells, could provide information about early graft rejection risks, relapse and graft *versus* host disease in patients affected by malignant diseases (Bader et al. 2000, Matthes-Martin et al. 2003, Zeiser et al. 2005, Miura et al. 2006, Lion 2007, Ringden 2007, Breuer et al. 2008, Liesveld JL and Rothberg 2008, Lim et al. 2008).

Up to date, one of the most common sensitive techniques used to evaluate the chimerism is the quantitative real-time PCR (qPCR). For this purpose, from 2002, different in house qPCR methods (Alizadeh et al. 2002, Maas et al. 2003, Jiménez-Velasco et al. 2005, Bai et al. 2006, Gineikiene et al. 2009, Chen et al. 2011, Qin et al. 2011, Almeida et al. 2013) and a commercial kit, "AlleleSEQR chimerism assay" (Abbott Molecular, Santa Clara, CA, USA), based on single nucleotide polymorphisms (SNPs) and on insertion deletion polimorphisms (INDELs) amplification, were developed.

Since the chimerism evaluation represents a ratio between donor and recipient DNA, the qPCR analysis requires the increase of the amount of the input DNA in order to increase the accuracy and the precision of the test, especially during the quantification of small chimerism percentage.

The most common qPCR methods developed up to now, reach a small amount of recipient cells (0.001% to 0.1%) using a high amount of input genomic DNA. In most cases, 100 ng to 500 ng of DNA were used, determining the sensitivity in a Ct range (threshold cycle) between 35 and 40 cycles (Table 1).

However, high amounts of DNA are not always available, in particular when the evaluation of the chimerism on subpopulation blood cells is required. In these cases, the cell number available for chimerism analysis can be from a few hundreds to a few thousands. As a consequence, the amount of DNA can be as low as 1-10 ng (Lion et al. 2012).

The aim of this work was to set up a new reliable protocol useful to define low chimerism percentage when small amount of DNA input is available, especially in the case of rare blood cellular subsets. Our method is based on a semi-nested allele-specific qPCR (sNAS-qPCR) protocol and was compared with a standard genomic DNA based qPCR protocol (gAS-qPCR).

Material and Methods

The Ethics Committee of the Institute for Maternal and Child Health, IRCCS "Burlo Garofolo" approved the present study (approval number: Prot. 18/2015, Cl. M/11). Written informed consent was obtained from all the participants.

Considering that this paper is a proof-of-concept study, with the main aim to evaluate the proposed sNAS-qPCR protocol, and to perform a first comparison with the gAS-qPCR one, we selected two different type of genetic markers: the first one was a somatic tri-nucleotide INDEL polymorphism (rs3035969; -/ATC), usually used for chimerism quantification, and the second one was a SEX marker (AMGY) useful to discriminate male from female. The AMGY marker is composed by four nucleotides specific for Y-chromosome, located in the *AMELY* gene with GATA sequence, that differs at the same region from *AMELX* gene (AMGX marker: AAGG) (Roccazzello et al. 2004).

Thirty random DNA samples were sequenced to determine the genotype of the INDEL polymorphism, useful to build the 4 standard curves corresponding to the four informative allelic constellation, where at least one allele of recipient (R) was different from the donor (D): 1_R_INS/INS_D_DEL/DEL; 2_R_INS/DEL_D_DEL/DEL; 3_R_DEL/DEL_D INS/INS; 4 R_INS/DEL_D_ INS/INS).

The number of the sequenced samples and the probability to find at least 3 DNA samples for the genotype with the minor genotype frequency (INS/INS) was assessed considering the allele frequency (INS=0.338; DEL=0.662). Next, we chose 8 DNA samples: 2 heterozygous for the INDEL marker; 3 homozygous for the insertion (INS/INS); and 3 homozygous for the deletion (DEL/DEL). 3 out of 8 samples chosen were males and were used for the protocols with the SEX marker. We tested each ASD primer on 2 different allelic

constellations, choosing a different DNA every time, as reported in Table 2.

Finally, we applied the two protocols to 3 samples of a patient during his follow up (Table 2). All samples in all protocols were analyzed at least in triplicate.

The sNAS-qPCR protocol was composed by two PCR steps (called “first-PCR “ and “second-PCR”) while the gAS-qPCR only by one.

In the “first-PCR” step primers FB and REV were used , designed externally to each marker position in order to amplify without discrimination the different alleles sequences (INS and DEL; AMGY and AMGX) corresponding to recipient and donor, and to maintain the initial donor/recipient ratio when the PCR reaches the *plateaux* (Biffi et al. 2011).

In detail, for INDEL marker, the primer FB (5’-TGAGGTGGAGTAAGTTTCAGG-3’), and its primer REV (5’-AGATAAGGACATTGAGGCACAC-3’) generated an amplicon of 123 bp when the insertion polymorphism was present or an amplicon of 120 bp in the presence of the deletion polymorphism (Fig. 1). Instead, for the SEX marker, the primer FB-XY: (5’-CCTTTGAAGTGGTACCAGAGCA-3’) and its primer REV-XY (5’-TGACTCCAACCAGAGAAGCAG-3’) generated two different amplicons: 265 bp for the Y chromosome and 263 bp for the X chromosome.

This “first PCR” was performed with: 10 ng of gDNA sample; 7.5 µL of K2G Fast Hot Start Ready mix 2X (KAPA Biosystems, Wilmington, MA, USA) and 1.5 µL of each primer at 5 µM in a final volume of 15 µL. The amplification was performed on a Gene Amp PCR System 9700 (Life Technologies, Foster City, CA, USA) and was composed by 3 steps: the first was a DNA denaturation step performed at 96 °C for 3 min; the second was a touch-down step composed by 10 cycles with a denaturation temperature at 96 °C for 15 s, an initial annealing temperature at 63 °C for 10 s that decreased of -0.5 °C at each cycle and an extension temperature at 72 °C for 1 s; finally, the third amplification step was composed by 28 cycles with 96 °C for 15 s, 58 °C for 10 s and 72 °C for 1 s. The negative control using water instead of DNA template was included at each reaction plate. The products of the “first-PCR”, diluted at 1:8,000, were used as template for the “second-PCR” step.

The "second-PCR", useful to quantify the chimerism, was performed on a real-time PCR platform with the allele specific discrimination primers (ASD) and their respective reverse primers. The ASD primers allow us to discriminate and quantify a specific allele marker. In reference to the INDEL marker, the 3' end of the first one (primer ASD-INS: 5'-GGTGGAGTAAGTTTCAGGATC-3') binds at the 3 bases of the insertion polymorphism, while the 3' end of the second one (primer ASD-DEL: 5'-GGTGGAGTAAGTTTCAGGTAG-3') binds the 3 bases immediately downstream the deletion (Fig. 1). In the case of the AMGY-marker, the 3' end of the ASD primer (ASD-Y: 5'-GAAGTGGTACCAGAGCATGATA-3') binds 4 bases characteristics of *AMELY* gene.

The "second-PCR" reactions were performed with 5.5 µL of template diluted 1:8,000 derived from the "first-PCR"; 7.5 µL of SYBR Green PCR Master Mix 2X (Life Technologies, Foster City, CA, USA); and 0.75 µL of ASD primer and its corresponding reverse at 5 µM in a final volume of 15 µL. The amplification was performed on ABI 7900HT Fast Real Time PCR System (Life Technologies, Foster City, CA, USA) with the same protocol for both markers: a first DNA denaturation step performed at 95 °C for 10 min, followed by 30 cycles of amplification with 95 °C for 15 s and 65 °C for 1 min. The only one PCR step of the gAS-qPCR protocol was performed directly with the ASD primers and their reverse on ABI 7900HT Fast Real Time PCR System. In this step we used the lower quantity of genomic DNA (25 ng) reported in Table 1 (Kim et al. 2014); all other reagents of the reactions and the temperature protocol corresponded to those described in the "second-PCR" step of the sNAS-qPCR protocol, but with a raise of cycle numbers from 30 to 40. For both protocols, a standard ABI 7900HT Fast Real Time PCR final melting curve step was added to cycling protocol to certify the reaction's specificity.

All primers were designed using the Primer3web v4.0.0 on-line software (http://primer3.ut.ee/); the *in silico* specificity was tested by the web tool BLAT (http://genome-euro.ucsc.edu/index.html).

We built 4 standard curves for the INDEL marker and three of them were used for the SEX marker (Table 2). Each standard curve was composed by 6 points of a 4-fold serial dilution (100%, 25%, 6.25%, 1.56%,

0.39%, 0.0975%). All points were amplified with both protocols to obtain the slope, the y-intercept, the R2 and the standard deviation (SD) useful to evaluate the performance of the two methods.

The sensitivity of the assay was evaluated amplifying in 10 replicates the 3 lower dilutions (0.0975%, 0.39%, 1.56%) in order to analyze the distribution of the obtained values. The two protocols were tested for equality of variance using Levene's test. Significance level was set to 0.05.

In order to test the reliability of derived data, we examined the distribution of Ct values obtained for the 3 lower chimerism percentage dilutions and ensured that they remained within the linear region of the target concentration response.

The correlation between the two protocols was examined using the Spearman's correlation test. We compared the mean of each point in triplicate obtained from the six points amplified with both protocols *versus* the effective chimerism percentages loaded in the reaction.

Moreover, we evaluated 3 chimeric samples during the follow-up of a patient, in 3 different times (1, 2, 3 months) after allogeneic transplant. These samples resulted informative for both markers. We quantified the chimeric samples building a patient specific standard curve by diluting in 6 points of a 4-fold serial dilutions of the recipient pre-transplant DNA with the donor DNA, as described above. The standard curves and the post-transplant patient samples were amplified in triplicate with both markers and protocols.

Finally, since the nested PCR can result in an increased risk of contamination and false positives, for this study we applied the "Good Clinical Laboratory Practice (GCLP) for Molecular Based Tests Used in Diagnostic Laboratories" instructions (Raquel et al. 2011).

Results

To compare the two protocols and their performance to discriminate the INDEL polymorphism and the Y-specific region, all points of the standard curves were amplified with the two protocols. We used the ASD-INS (Fig. 2) and ASD-DEL primers (Fig. 3) for the INDEL genetic marker, and the ASD-Y primer for the AMGY marker (Fig. 4). All technical characteristics of each standard curve, for both markers, were reported

in Table 3. For both the qPCR protocols, in all cases the R2 resulted > 0.99, except for the SEX marker performed with the gAS-qPCR protocol in which it was 0.986. Regarding both genetic markers, with the sNAS-qPCR protocols the slopes were in the range of [-3.3 -6.6%] to [-3.3 +10%] (Fig. 2A and 2B, Fig. 3A and 3B), while with the gAS-qPCR protocol all ASD amplifications presented a slope in a range of [-3.3 +3%] to [-3.3 +21%] (Fig. 2C and 2D, Fig. 3C and 3D) (Table 3).

For the sNAS-qPCR the *y*-intercept was from 14.9 to 17.2 (range of Ct at 100% of recipient: 8-11; Ct at 0.0975% of recipient: 19-21) and it resulted minor than the one obtained for the gAS-qPCR, which range from 27.8 to 31.6 (Ct at 100% of recipient: 21-24; Ct at 0.0975% of recipient: 32-35).

The Ct SD of the triplicates of each point of the curves was ≤ 0.150 and ≤ 0.123 in the case of sNAS-qPCR protocol, for the ASD INDEL primers and AMGY primer, respectively. For the gAS-qPCR protocol, Ct SD results <0.245 and <0.187 in all the high chimerism percentage (from 100% until 1.56%) while results ≤ 0.840 and ≤ 0.945 at low percents of chimerism (0.39% and 0.0975%), for the ASD INDEL primers and AMGY primers, respectively.

For the sNAS-qPCR protocol, the Ct SD values of the 10 replicates for the three lower chimerism percentage points (1.56%, 0.39%, 0.0975%) were ≤ 0.220 and ≤ 0.129 for INDEL and sex marker respectively, while, for the gAS-qPCR protocol they were ≤ 0.697 and ≤ 0.825, respectively.

Moreover, the distribution of % of chimerism quantified was analyzed, in particular in terms of variability of the estimate. The test for homogeneity of variance (Levene's test) revealed that the variance in the gAS-qPCR protocol is significantly higher compared to the sNAS-qPCR method (average values of the 4 INDEL marker constellations: P 0.0975%=7.9e- 04, P 0.39%=6.3e-05, P 1.56%=5.5e-04; P 0.0975%=1.4e-04, P 0.39%=3.8e-02, P 1.56%=8.5e-04 for SEX marker).

The percentage of chimerism obtained with the semi-nested and genomic protocols was compared with the expected values. In both protocols we applied the Sperman's test and detected high correlation (Roh > 0.99, P < 0.005 regardless of the marker that was used).

Finally, we evaluated 3 chimeric samples during the follow up of a post-transplanted patient. The male

recipient results heterozygous for the INDEL marker and the donor homozygous (DEL/DEL). The results reported in Table 4 show that for low chimerism percentage, as in the case of the sample R3 (3 months), the sNAS-qPCR protocol has a Ct SD equal to 0.094 for the INDEL marker and 0.129 for the SEX marker. Instead for the gAS-qPCR protocol SD is 0.410 for the INDEL marker and 0.338 for the SEX marker.

Discussion

Since 2002, in different studies several panels of SNPs and/or INDELs were selected to evaluate chimerism post-HSCT with the qPCR method. The lowest chimerism percentage reached with those panels, ranges from 0.001% to 0.1% (Table 1) with an input DNA usually greater than 100 ng (Alizadeh et al. 2002, Maas et al. 2003, Jiménez-Velasco et al. 2005, Bai et al. 2006, Gineikiene et al. 2009, Chen et al. 2011, Qin et al. 2011, Almeida et al. 2013, Kim et al. 2014).

However, in some situations, it is necessary to isolate specific leukocyte subpopulations for chimerism analysis (Lion et al. 2012) and in these cases the number of isolated cells can be very low. Although a collection of larger patient samples may increase the number of leukocyte sub-populations (Almeida et al. 2013), but handling of large volumes is not always suitable, especially in pediatric patients. In all previous studies, where a standard qPCR method was used, there were two main critical issues: a) a high amount of input DNA was required; b) the smallest percentage of target DNA detected in a chimeric sample reported at high Ct (~ 35-40 cycles), reflecting to a high *y*-intercept and therefore to a low sensitive test (Table 1).

The advantage of the semi-nested qPCR is that the amplicons generated from the “first-PCR” step are used as template in the “second-PCR” step, in which the number of copies of amplicons is many fold greater than the number of copies of genomic DNA used in the “first-PCR” step; as a consequence, to accurately evaluate chimerism with the sNAS-qPCR protocol, only 10 ng of input DNA are required.

To compare the two methods, the same mixture of DNA with each ASD primer has been amplified with both protocols (sNAS-qPCR and gAS-qPCR) in order to obtain the following parameters: slope, *y*-intercept, R2 and SD. The slope of the regression line provides us with information about the efficiency and the accuracy

of the test. The optimal slope value is at -3.3, which corresponds to an efficiency of 100%. We have calculated the slope with six 4-fold dilutions in a linear dynamic range that goes from 100% to 0.975%, which include four orders of magnitude (100.0%, 10.0%, 1.0%, ~ 0.1%) (Bustin et al. 2009). The slope obtained with all ASD primers of both markers in both protocols, results similar. Indeed, the Sperman's test detects a high correlation (regardless of the marker that was used) between the effective chimerism percentage loaded in the reaction and the obtained values, with both protocols.

Moreover, two parameters provide information about the precision, the R2 and the replicates SD. R2 was calculated within the entire linear dynamic range (100% - 0.0975%) and was > 0.99 for both protocols and genetic markers; this means that there was a good correlation between the values of Ct and the logarithm of the chimerism percentage. Only when AMGY marker was amplified with gAS-qPCR protocol, the R2 resulted > 0.986 indicating a slight loss of precision in regard to this case.

The Ct SD estimated from the triplicate of all points of the sNAS-qPCR standards curve always results optimal (≤ 0.167). This means that more than 99.7% of the cases give a positive and specific amplification. Instead, for the gAS-qPCR protocol the Ct SD results in an acceptable range (≤ 0.250) for the high chimerism percentage (from 100% to 1.56%), but reaches high values, until 0.792, for lower chimerism percentage (0.39% and 0.0975%). This means that using the gAS-qPCR protocol, only in the higher percentage of chimerism more than 95% of the cases give a positive and specific amplification, instead for the lower dilution the gAS-qPCR protocol does not ensure the distinction between 2-fold dilutions with certainty.

To test the sensitivity, 10 replicates for the 3 lower dilutions (1.56%, 0.39%, 0.0975%) were performed with both protocols and markers. Using the sNAS-qPCR method, the Ct SD values are lower than the acceptable value. Instead, the gAS-qPCR presents a high Ct SD at 0.39% and 0.0975%. Moreover, the distribution of the percentage of chimerism, evaluated with the Levene's test, reveals that sNAS-qPCR protocol has a higher sensitivity.

As an additional sensitivity marker, we calculated the *y*-intercept: a low *y* indicates greater sensitivity at a

given cycle number (Saikaly et al. 2007). In regard the two protocols, the sNAS-qPCR presents a better *y*-intercept, close to 16, while the gAS-qPCR protocol close to 30 (Table 3).

In regard to the application of both protocols of a patient's samples, the low precision when low percentage chimerism is detected, as in sample R3, was confirmed.

From the comparison of all the above parameters we can conclude that the accuracy of the two protocols measured on a high number of replicates, appears to be comparable. However, we detected strong differences in terms of sensitivity, since the gAS-qPCR protocol showed significantly, higher variability when considering the amount of chimerism obtained for small percentage. This fact has important consequences in the clinical application of real-time for chimerism, where usually only 3 replicates are performed. Jacque et al. (2015), using a genomic real time approach with 250 ng of input DNA (Alizadeh et al. 2002) had demonstrated that an increase of chimerism of 0.1% could be useful to exclude all the cases of the relapse. However, considering the higher cost and time consuming of the proposed protocol, compared to the standard one, we suggest to use the sNAS-qPCR method only when low DNA input amount is available, as in the case of blood cell subset samples.

Finally, for clinical application of the semi-nested method, we suggest 3 or more informative genetic markers. Considering a heterozigosity of each marker close to 0.5, at least 8 markers must be screened to have a high probability to obtain 3 informative unrelated couples of transplant.

Acknowledgments

This work was supported by the Institute of Maternal and Child Health - IRCCS "Burlo Garofolo" grant (RC 16-2014). We would like to thank Dr. Elena Bortuzzo for English revision.

References

Alizadeh, M., Bernard, M., Danic, B., Dauriac, C., Birebent, B., Lapart, C., et al. 2002. Quantitative assessment of hematopoietic chimerism after bone marrow transplantation by real-time quantitative polymerase chain reaction. Blood, **99**(12): 4618-4625.

Almeida, C.A., Dreyfuss, J.L., Azevedo-Shimmoto, M.M., Figueiredo, M.S., De Oliveira, J.S. 2013. Evaluation of 16 SNPs allele-specific to quantify post hSCT chimerism by SYBR green-based qRT-PCR. J. Clin. Pathol. **66**(3): 238-242. doi:10.1136/jclinpath-2012-201224.

Bader, P., Stoll, K., Huber, S., Geiselhart, A., Handgretinger, R., Niemeyer, C., et al. 2000. Characterization of lineage-specific chimaerism in patients with acute leukaemia and myelodysplastic syndrome after allogeneic stem cell transplantation before and after relapse. Br. J. Haematol. **108**(4): 761-768.

Bai, L., Deng, Y.M., Dodds, A.J., Milliken, S., Moore, J., and Ma, D.D. 2006. A SYBR green-based real-time PCR method for detection of haemopoietic chimerism in allogeneic haemopoietic stem cell transplant recipients. Eur. J. Haematol. **77**(5): 425-431.

Biffi, S., Bortot, B., Carrozzi, M., and Severini, G.M. 2011. Quantification of heteroplasmic mitochondrial DNA mutations for DNA samples in the low picogram range by nested real-time ARMS-qPCR. Diagn. Mol. Pathol. **20**(2): 117-122. doi:10.1097/PDM.0b013e3181efe2c6.

Bortot, B., Athanasakis, E., Brun, F., Rizzotti, D., Mestroni, L., Sinagra, G., et al. 2011. High-throughput genotyping robot-assisted method for mutation detection in patients with hypertrophic cardiomyopathy. Diagn. Mol. Pathol. **20**(3): 175-179. doi:10.1097/PDM.0b013e31820b34fb.

Breuer, S., Preuner, S., Fritsch, G., Daxberger, H., Koenig, M., Poetschger, U., et al. 2012. Recipient chimerism in the T-cell lineage is indicative of impending graft rejection in pediatric patients undergoing allogeneic hematopoietic stem cell transplantation. Leukemia **26**(3): 509-519. doi:10.1038/leu.2011.244.

Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., et al. 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin. Chem. **55**(4): 611-622. doi:10.1373/clinchem.2008.112797.

Chen, D.P., Tseng, C.P., Wang, W.T., Wang, M.C., Tsai, S.H., and Sun, C.F. 2011. Real-time biallelic polymorphism-polymerase chain reaction for chimerism monitoring of hematopoietic stem cell transplantation relapsed patients. Clin. Chim. Acta, **412**(7-8): 625-630. doi:10.1016/j.cca.2010.12.018.

Gineikiene, E., Stoskus, M., and Griskevicius, L. 2009. Single nucleotide polymorphism-based system improves the applicability of quantitative PCR for chimerism monitoring. J. Mol. Diagn. **11**(1): 66-74. doi:10.2353/jmoldx.2009.080039.

Jacque, N., Nguyen, S., Golmard, J.L., Uzunov, M., Garnier, A., Leblond, V., et al. 2015. Chimerism analysis in peripheral blood using indel quantitative real-time PCR is a useful tool to predict post-transplant relapse in acute leukemia. Bone Marrow Transplant. **50**(2): 259-265.

Jiménez-Velasco, A., Barrios, M., Román-Gómez, J., Navarro, G., Buño, I., and Castillejo, J.A. 2005. Reliable quantification of hematopoietic chimerism after allogeneic transplantation for acute leukemia using amplification by real-time PCR of null alleles and insertion/deletion polymorphisms. Leukemia, **19**(3): 336-343.

Kim, S.Y., Jeong, M.H., Park, N., Ra, E., Park, H., Seo, S.H., et al. 2014. Chimerism monitoring after allogeneic hematopoietic stem cell transplantation using quantitative real-time PCR of biallelic insertion/deletion polymorphisms. J. Mol. Diagn. **16**(6): 679-688. doi:10.1016/j.jmoldx.2014.06.005.

Liesveld, J.L., and Rothberg, P.G. 2008. Mixed chimerism in SCT: conflict or peaceful coexistence? Bone Marrow Transplant. **42**(5): 297-310. doi:10.1038/bmt.2008.212.

Lim, Z.Y., Pearce, L., Ingram, W., Ho, A.Y., Mufti, G.J., and Pagliuca, A. 2008. Chimerism does not predict for outcome after alemtuzumab-based conditioning: lineage-specific analysis of chimerism of specific diseases may be more informative. Bone Marrow Transplant. **41**(6): 587-588.

Lion, T. 2007. Detection of impending graft rejection and relapse by lineage-specific chimerism analysis. Methods Mol. Med. **134**: 197-216.

Lion, T., Watzinger, F., Preuner, S., Kreyenberg, H., Tilanus, M., De Weger, R., et al. 2012. The EuroChimerism concept for a standardized approach to chimerism analysis after allogeneic stem cell

transplantation. Leukemia, **26**(8): 1821-1828. doi:10.1038/leu.2012.66.

Maas, F., Schaap, N., Kolen, S., Zoetbrood, A., Buño, I., Dolstra, H., et al. 2003. Quantification of donor and recipient hemopoietic cells by real-time PCR of single nucleotide polymorphisms. Leukemia, **17**(3): 621-629.

Matthes-Martin, S., Lion, T., Haas, O.A., Frommlet, F., Daxberger, H., König, M., et al. 2003. Lineage-specific chimaerism after stem cell transplantation in children following reduced intensity conditioning: potential predictive value of NK cell chimaerism for late graft rejection. Leukemia, **17**(10): 1934-1942.

Miura, Y., Tanaka, J., Toubai, T., Tsutsumi, Y., Kato, N., Hirate, D., et al. 2006. Analysis of donor-type chimerism in lineage-specific cell populations after allogeneic myeloablative and non-myelo- ablative stem cell transplantation. Bone Marrow Transplant. **37**(9): 837-843.

Qin, X.Y., Li, G.X., Qin, Y.Z., Wang, Y., Wang, F.R., Liu, D.H., et al. 2011. Quantitative assessment of hematopoietic chimerism by quantitative real-time polymerase chain reaction of sequence polymorphism systems after hematopoietic stem cell transplantation. Chin. Med. J. (Engl), **124**(15): 2301-2308.

Raquel, V.V., and Carole L. 2011. Wide Spectra of Quality Control, InTech, d.o.o., Rijeka, Croatia.

Ringden, O. 2007. Immunotherapy by allogeneic stem cell transplantation. Adv. Cancer Res. **97**: 25-60.

Roccazzello, A.M., Tringali, G., Barbaro, A., Cormaci, P., Barbaro, A., and Insirello, E. 2004. Simultaneous estimation of a Y-specific fragment, an X-specific fragment and sex determination of forensic studies in real-time PCR. Forensic. Sci. Int. **146**: Suppl S165-S166.

Saikaly, P.E., Barlaz, M.A., de Los Reyes, F.L. 3rd. 2007. Development of quantitative real-time PCR assays for detection and quantification of surrogate biological warfare agents in building debris and leachate. Appl. Environ. Microbiol. **73**(20):6557-6565.

Zeiser, R., Spyridonidis, A., Wasch, R., Ihorst, G., Grullich, C., Bertz, H., et al. 2005. Evaluation of immunomodulatory treatment based on conventional and lineage-specific chimerism analysis in patients with myeloid malignancies after myeloablative allogeneic hematopoietic cell transplantation. Leukemia **19**(5): 814-821.

Tables, Tables Titles and Legends

Table 1. Amount of input genomic DNA request for chimerism evaluation by qPCR.

Genetic Markers	Detection method	DNA input (ng)	Limit of detection (%)	Max Ct at detection limit	Reference
INDELs	TaqMan probes	250	0.1	~ 35	Alizadeh et al. 2002
INDELs	SYBR Green	100	0.1	nr[a]	Bai et al. 2006
SNPs	TaqMan probes	50 - 500	0.1 - 0.01	~ 35	Maas et al. 2003
INDELs	Hybridization probes	100	0.01	~ 36	Jiménez-Velasco et al. 2005
SNPs	TaqMan probes	100	0.1	~ 36	Gineikiene et al. 2009
SNPs and INDELs	TaqMan probes	250	0.003 - 0.006	~ 40	Chen et al. 2011
SNPs	SYBR Green	100	0.001	~ 35	Almeida et al. 2013
SNPs and INDELs	TaqMan probes	50 - 100	0.1 - 0.01	~ 35	Qin et al. 2011
INDELs	TaqMan probes	25 - 250	0.1 - 0.024	~ 37	Kim et al. 2014

[a]nr, not reported.

Table 2. DNA samples used for comparison of the two methods.

		Primer ASD-INS / Primer ASD-Y		Primer ASD-DEL / Primer ASD-Y	
DNA ID	**Genotype**	AC[a]: 1_R_INS/INS_ D_DEL/DEL	AC: 2_R_INS/DEL_ D_DEL/DEL	AC: 3_R_DEL/DEL_ D_INS/INS	AC:_R_INS/DEL_ D_INS/INS
DNA_01	INS/INS	Recipient (male)	-	-	-
DNA_02	INS/INS	-	-	Donor (female)	-
DNA_03	INS/INS	-	-	-	Donor (female)
DNA_04	INS/DEL	-	Recipient (male)	-	-
DNA_05	INS/DEL	-	-	-	Recipient (male)
DNA_06	DEL/DEL	Donor (female)	-	-	-
DNA_07	DEL/DEL	-	Donor (female)	-	-
DNA_08	DEL/DEL	-	-	Recipient (female)	-
[b]DNA_D	DEL/DEL	Donor (female)	-	-	-
[c]DNA_R	INS/DEL	Recipient (male)	-	-	-
[d]DNA_R1			-	-	-
DNA_R2			-	-	-
DNA_R3			-	-	-

[a]AC, allelic constellation; [b]DNA_D, DNA from the transplanted patient donor; [c]DNA_R, DNA from the pre-transplantation patient; [d]DNA_R1/R2/R3, DNA from the transplanted patient recipient.

Table 3. Technical characteristics of each standard curve, using both sNAS-qPCR and gAS-qPCR protocols.

		rs3035969								AMGY	
		Primer ASD-INS				Primer ASD-DEL				Primer ASD-Y	
		INS/INS vs DEL/DEL		INS/DEL vs DEL/DEL		DEL/DEL vs INS/INS		INS/DEL vs INS/INS		XY vs XX	
Protocol	Optimal / Acceptable values	sNAS-qPCR	gAS-qPCR	sNAS-qPCR	gAS-qPCR	sNAS-qPCR	gAS-qPCR	sNAS-qPCR	gAS-qPCR	sNAS-qPCR	gAS-qPCR
Y-Intercept	n.a.	15.6	27.8	16.6	28.7	14.9	29.9	17.2	31.6	17.0	31.3
Slope	-3.3 / -3.3 ±10%	-3.5	-3.6	-3.2	-3.4	-3.1	-3.7	-3.3	-4.0	-3.6	-3.7
R2	1.0 / >0.985	0.998	0.990	0.998	0.992	0.997	0.997	0.999	0.997	0.992	0.986

Table 4. Chimerism percentage results of patient samples during follow-up.

Marker	**INDEL**		**SEX**	
Genotype	R_INS/DEL_D_DEL/DEL		Recipient MALE; Donor FEMALE	
Protocol	sNAS-qPCR	gAS-qPCR	sNAS-qPCR	gAS-qPCR
Chimerism % of R1 (month 1)	6.1%	5.0%	7.1%	7.1%
Ct SD of R1	0.064	0.255	0.034	0.014
Chimerism % of R2 (month 2)	2.3%	2.7%	2.1%	3.6%
Ct SD of R2	0.071	0.100	0.076	0.226
Chimerism % of R3 (month 3)	0.6%	0.5%	0.6%	0.7%
Ct SD of R3	0.094	0.410	0.129	0.338

Figure Titles and Legends

Figure 1: Region of binding of the primers used in the semi-nested allele-specific real-time PCR and standard allele-specific real-time PCR protocols: primer FB and REV were used in the "first-PCR" step, amplifying the region containing the INDEL polymorphism. The allele specific discrimination primers (ASD-INS and ASD-DEL) were used to amplify and discriminate the INDEL polymorphism in the "second-PCR" step of the semi-nested allele-specific real-time PCR protocol and in the standard allele-specific real-time PCR protocol.

Figure 2: The standard curves of the ASD-INS primer, applied on two recipient genotypes, using the semi-nested allele-specific real-time PCR (sNAS-qPCR) and the standard allele-specific real-time PCR (gAS-qPCR protocols).

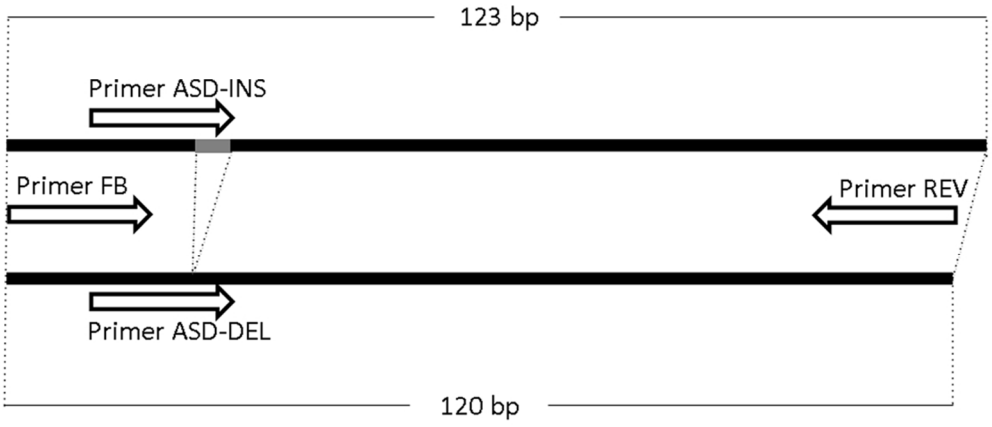
(A) Both recipient and donor are homozygous, (INS/INS) and (DEL/DEL) respectively; was used the sNAS-qPCR protocol. (B) The recipient is heterozygous (INS/DEL) and the donor is homozygous (DEL/DEL) and was used the sNAS-qPCR protocol. (C) Both recipient and donor are homozygous, (INS/INS) and (DEL/DEL) respectively; was used the gAS-qPCR protocol. (D) The recipient is heterozygous (INS/DEL) and the donor is homozygous (DEL/DEL) and was used the gAS-qPCR protocol.

Figure 3: The standard curves of the ASD-DEL primer, applied on two recipient genotypes, using the semi-nested allele-specific real-time PCR (sNAS-qPCR) and the standard allele-specific real-time PCR (gAS-qPCR protocols).

(A) Both recipient and donor are homozygous, (DEL/DEL) and (INS/INS) respectively; was used the sNAS-qPCR protocol. (B) The recipient is heterozygous (INS/DEL) and the donor is homozygous (INS/INS) and was used the sNAS-qPCR protocol. (C) Both recipient and donor are homozygous, (DEL/DEL) and

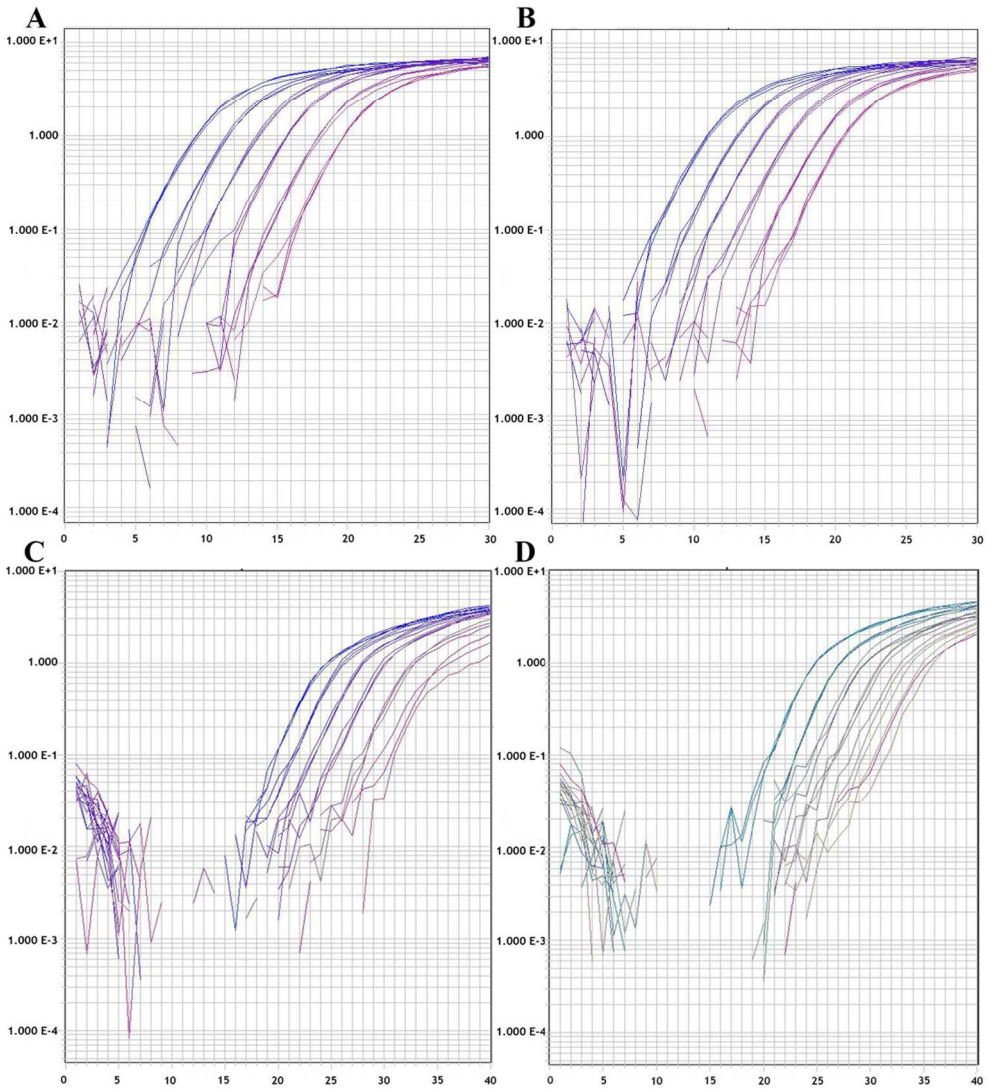
(INS/INS) respectively; was used the gAS-qPCR protocol. (D) The recipient is heterozygous (INS/DEL) and the donor is homozygous (INS/INS) and was used the gAS-qPCR protocol.

Figure 4: The standard curves of the semi-nested allele-specific real-time PCR (A) and standard allele-specific real-time PCR (B) protocols for the AMGY marker.



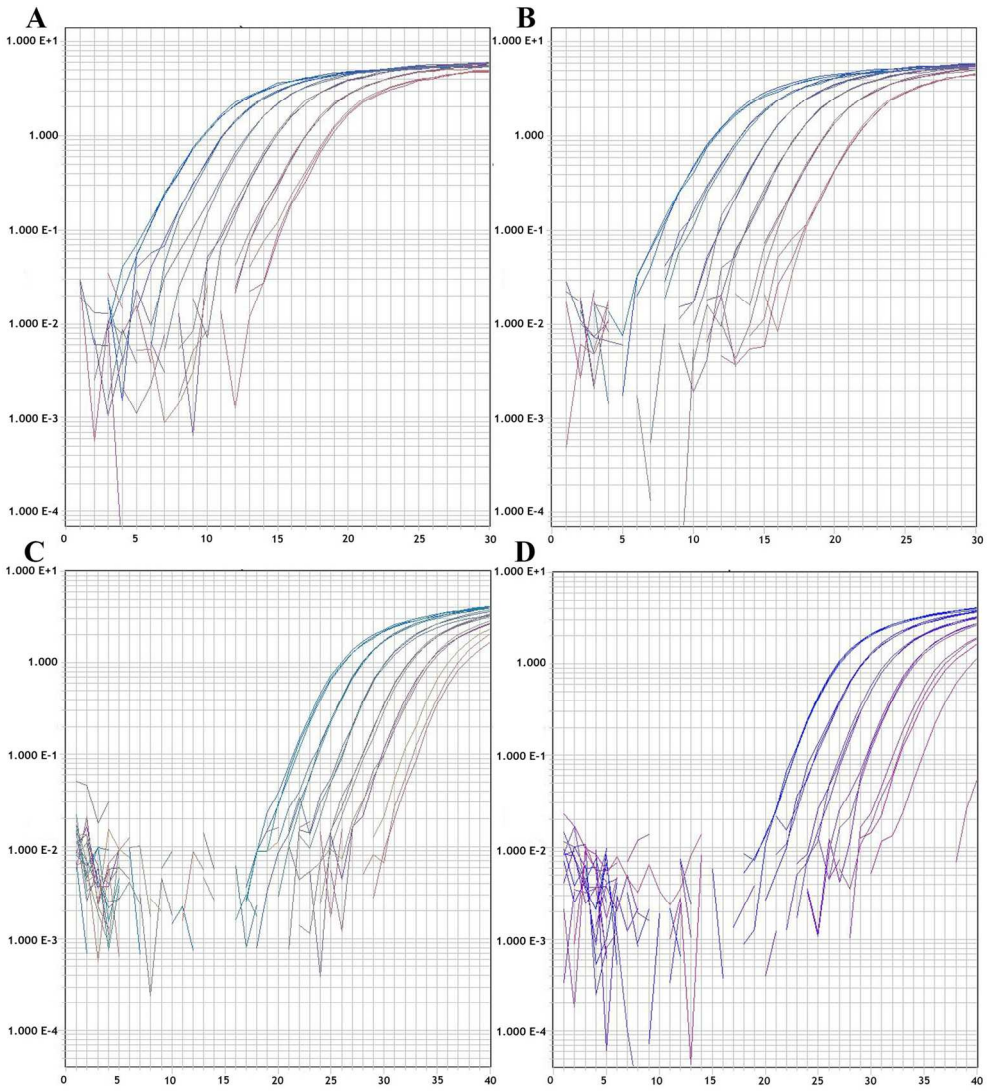
Region of binding of the primers used in the semi-nested allele-specific real-time PCR and standard allele-specific real-time PCR protocols: primer FB and REV were used in the "first-PCR" step, amplifying the region containing the INDEL polymorphism. The allele specific discrimination primers (ASD-INS and ASD-DEL) were used to amplify and discriminate the INDEL polymorphism in the "second-PCR" step of the semi-nested allele-specific real-time PCR protocol and in the standard allele-specific real-time PCR protocol.

99x44mm (300 x 300 DPI)



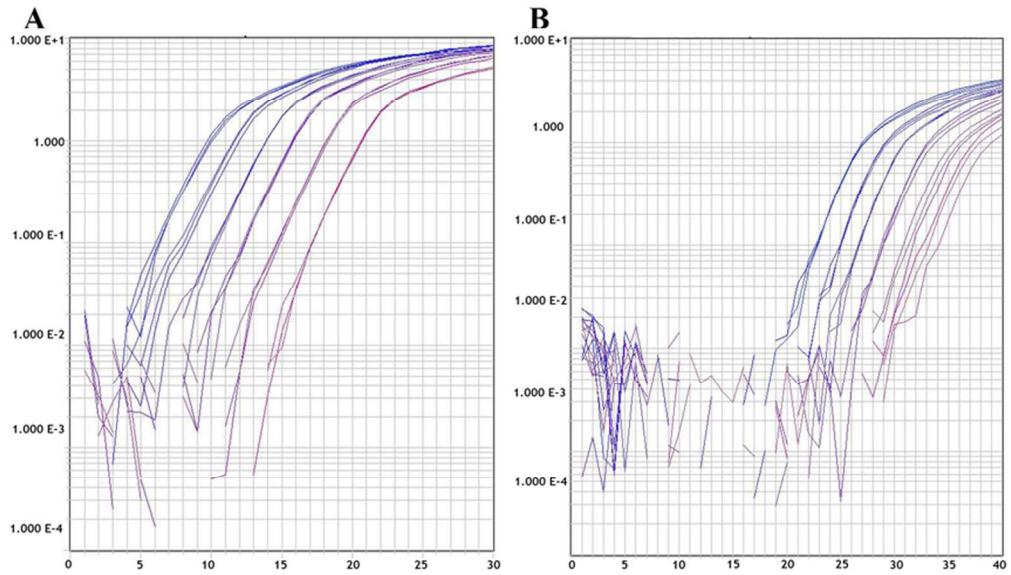
The standard curves of the ASD-INS primer, applied on two recipient genotypes, using the semi-nested allele-specific real-time PCR (sNAS-qPCR) and the standard allele-specific real-time PCR (gAS-qPCR protocols).
(A) Both recipient and donor are homozygous, (INS/INS) and (DEL/DEL) respectively; was used the sNAS-qPCR protocol. (B) The recipient is heterozygous (INS/DEL) and the donor is homozygous (DEL/DEL) and was used the sNAS-qPCR protocol. (C) Both recipient and donor are homozygous, (INS/INS) and (DEL/DEL) respectively; was used the gAS-qPCR protocol. (D) The recipient is heterozygous (INS/DEL) and the donor is homozygous (DEL/DEL) and was used the gAS-qPCR protocol.

150x164mm (300 x 300 DPI)



The standard curves of the ASD-DEL primer, applied on two recipient genotypes, using the semi-nested allele-specific real-time PCR (sNAS-qPCR) and the standard allele-specific real-time PCR (gAS-qPCR protocols).
(A) Both recipient and donor are homozygous, (DEL/DEL) and (INS/INS) respectively; was used the sNAS-qPCR protocol. (B) The recipient is heterozygous (INS/DEL) and the donor is homozygous (INS/INS) and was used the sNAS-qPCR protocol. (C) Both recipient and donor are homozygous, (DEL/DEL) and (INS/INS) respectively; was used the gAS-qPCR protocol. (D) The recipient is heterozygous (INS/DEL) and the donor is homozygous (INS/INS) and was used the gAS-qPCR protocol.

150x164mm (300 x 300 DPI)



The standard curves of the semi-nested allele-specific real-time PCR (A) and standard allele-specific real-time PCR (B) protocols for the AMGY marker.

150x85mm (300 x 300 DPI)