External standard curve for absolute quantification of genomic DNA sequences by Real Time PCR

Raffaele Di Francia,[1] Ferdinando Frigeri,[1] Rosaria De Filippi,[1,2] Giancarla Iaccarino, [1] Gennaro Varriale,[3] Antonio Arbitrio[3] and Antonio Pinto.[1]
[1]Hematology-Oncology Unit, Istituto Nazionale Tumori, Fondazione 'Pascale', IRCCS, Naples, Italy; [2] Dip. di Biologia e Patologia Cellulare e Molecolare, University Federico II, Naples, Italy; [3]SENEBGENE, s.r.l., Naples, Italy

Background

Determination of the relative abundance of a DNA sequence is usually accepted as a reliable tool to measure differences among samples. However, absolute determination of the number of copies for a DNA target can result of a greater value for minimal residual disease (MRD) evaluation in hemopoietic neoplasms.
Absolute quantification of given DNA targets, originally present in live cells or tissues, remains technically difficult due to the lack of a methodology able to comprehensively account for all the variations that can occur during sample preparation. However, the quantity of target sequences in the samples can be assessed with a reasonable accuracy using proper external standards, i.e. plasmid DNA containing the target sequence, PCR-amplified target sequences and healthy DNA. Each of such standards has advantages and disadvantages in terms of complexity of the material, cost, accessibility, long-term stability and gene copy accuracy. In this study,we present a simple method to build an external standard curve by means of a recombinant plasmid DNA, exactly quantified by competitive PCR against a specific competitor, i.e. a non-human DNA fragment of known concentration. Such approach was developed to quantify two genetic marker breakpoints of non-Hodgkin's Lymphomas, i.e. the t(14;18) and t(11;14), respectively associated to Follicular (FCL) and Mantle Cell (MCL) lymphomas. Standard for the t(14;18) assay was represented by a vector (pFCL) containing theBCL2/JH MBR rearrangement, while standard for the t(11;14) was a vector (pMCL) containing the BCL1/JH rearrangement. Quantitative-PCR (Q-PCR) assay was performed by two different methods: i) TaqMan chemistry for t(14;18) and ii) SYBR Green for t(11;14).

Figure 1

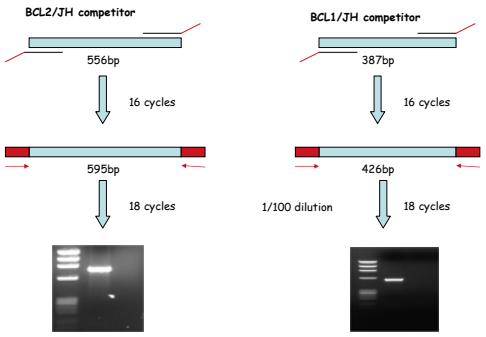
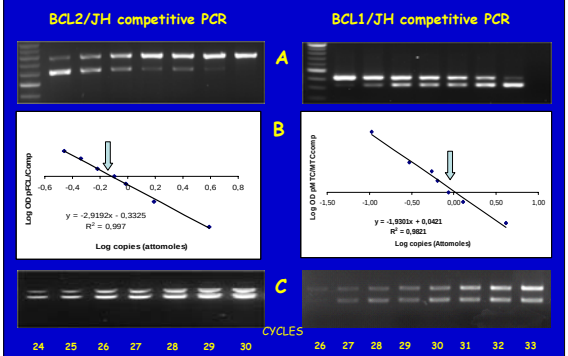


Fig.1 Contruction of competitor fragments and primers design
Competitor fragment was generated following by manufacturer's protocol of PCR-mimic construction Kit (Clontech, Palo Alto, CA). Briefly, the generation of the internal non-homologous competitor (BCL-1/JH and BCL-2/JH) was based on the design of composite primers for two successive PCR amplification. During the primary PCR amplification, our competitor fragment was amplified using the two composite primers, containing the up-down stream primers, for the target sequence linked to a 20-mer flanking sequence which anneals to the competitor fragment. After 16 cycles the amplification product was collected and diluted 1/100 to be used as template for the secondary PCR amplification (18 cycles). During this PCR amplification, only gene specific primers were used. The quantity of both PCR competitors was determined by running an aliquot on a gel and band intensity was assessed by densitometric analysis
We compared the intensity of the studied bands to a DNA marker of known size, fX174/HaeIII digest.

Figure 2: plasmids quantitation

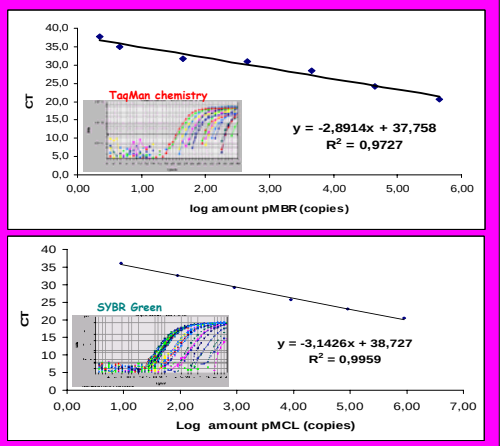


A.Competitive PCR: left panel: 10-fold serial dilution of BCL-2/JH competitor (10 to 10^{-4}) was co-amplified with constant aliquots of pFCL (1pg); right panel: 10-fold serial dilution of BCL-1/JH competitor (10 to 10-4) was co-amplified with constant aliquots of pMCL (1 pg). In both assays, ten microliters of amplified product were run on a 2% ethidium bromide-stained gel and relative intensities of the bands were densitrometrically quantified by a gel analyzer and expressed as Arbitrary Units (AU).

B.Validation curve of competitive PCR: after correction in size difference between BCL2/JH Competitor (size 595bp) and pFCL (size 798bp) left panel, and between the BCL1/JH Competitor (size426bp) and pMCL (350 bp), right panel , logarithms of OD ratios were plotted as a function of the logarithms of the amount of competitors added. Plots were used to determine the "equivalence point", which is, for pFCL, the point inferred to 0,75 attomoles (CV%=6.1) corresponding to 450000 copies (see Avogadro's Number) and for pMCL is the point inferred to 1.11 (CV%=7.4), corresponding to 666000 copies.

C.Time-course competitive PCR: a kinetic validation of both Competitors and plasmids was performed by Competitive PCR, co-amplifying, per each assay, in the same tube, 1 pg of pFCL and 0.75 attomoles of BCL2/JH competitor (left panel) and 1 pg of pMCL and 1,11 attomoles of BCL1/JH competitor (right panel), respectively. 5 mL of PCR reaction products, from 24 to 30 cycles, were removed and run on agarose Ethidium bromide-stained gels. The relative intensity of the bands was quantified by gel analyzer and a relative ratio value of 1 indicates a similar amplification efficiency for both templates.

Figure 3: External Standard curves



Quantitative-PCR (Q-PCR): assays were performed through the AbiPrism 7700 platform (Applied Biosystems) by two different methods: i) TaqMan chemistry for t(14;18) panel A and ii) SYBR Green for t(11;14) panel B.
Standard for the t(14;18) assay consists of a vector containing the sequence of interest, BCL2/JH MBR rearrangement, obtained from PCR amplicon DNA of DoHH2 cells. Standard for the t(11;14) assay was a vector containing the sequence of interest, BCL1/JH rearrangement, obtained from PCR amplicon DNA of JVM2 cells. Briefly, fresh PCR products, generated by conventional PCR, were cloned into a pCR 2,1 vector using the TOPO-TA cloning kit according to the manufacturer's instructions (Invitrogen, Milan, Italy). After purification and quantification, the plasmids (called pFCL and pMCL) were purified using the QIAprep midi-prep Kit (QIAgen, Hilden, Germany).
Standard stocks were constructed by diluting 9.0 x10^5 copies of translocation-specific plasmids and diluted with 500 ng of DNA from a pool of healthy donors. Standard stocks were then serially diluted (9.0 x10^5 - 9.0 x10^0) to build external standard curves for each of the targets, i.e. FCL curve (pFCL) and MCL curve (pMCL). Copy numbers of t(14;18) or t(11;14) were normalized by concurrently quantifying copies of the internal reference gene albumin for each dilution point.
As shown, the CT values were plotted as a function of the logarithms of the gene copies, producing a p FCL standard curve with a slope of -2.89 (R^2=0.97) and a pMCL standard curve with a slope of -3.14 (R^2=0.99). Mean and range values for each calibrator's Ct were calculated to show assay reproducibility. As shown, standard curves were linear over five orders of magnitude, indicating constant PCR efficiency over the concentration range studied. At very low DNA target concentrations, the variability will be greater because of the stochastic variation in target amplification. Both methodologies (i.e. TaqMan and SYBRGreen) were able to detect the translocation in sample serially diluted up to 10^{-5} -fold. However, as may be expected, sequences were inconsistently detected in 10^{-5} fold dilutions since this represents 4.5 target gene copies. We improved reproducibility by increasing the concentration of calibrators in the Q-PCR by using 2.0 pg of pFCL and 1.3 pg of pMCL. In our experiments, the maximal sensitivity of this Q-PCR strategy was of 9 rearranged gene copies/500 ng DNA (lowest limit), and we considered that value the benchmark of standard quantification.

Table 2: REAL-Time PCR analysis at diagnosis (n = 64)

Sample	N	x̄ Positive Cells (75000 cells analysed)
Lymph node	28	12044.0
Bone Marrow	24	189.1
Peripheral blood	12	65.4

Application of the described methodology for the absolute quantitation of t(14;18) positive cells in clinical material from patients with FCL.

The absolute number of t(14;18)+ cells was comparatively assessed in lymph nodes (LN), bone marrow (BM) and peripheral blood (PB) samples at diagnosis. The mean number of alleles detected in 500 ng of DNA (85000 cells) from BM and PB was very similar in our cohort of patients (189.1 and 65.4 respectively; p=0.09), while LN samples yielded a significantly higher mean value (12044.0; p=0.018) (Table 2).

Table 1: inter-assay variation

pFCL (n=24) a		DoHH2(n=6) b		pMCL (n=10) c		JVM2 (n=5) d	
Copies	CV%	Copies	CV%	Copies	CV%	Copies	CV%
90000*	3.78	85000§	4.84	90000#	3.98	85000§	6.84
9000	1.98	8500	8.97	9000	5.18	8500	10.97
900	3.10	850	9.60	900	3.55	850	11.60
90	1.58	85	6.01	90	4.01	85	8.01
9	5.29	8.5	4.99	9	6.19	8.5	7.90

Precision estimates were determined based on each PCR reaction mix combination at every copy number either of the plasmid or the positive cell lines, across all the performed experiments. Precision estimates were calculated in repeatability standard deviations of the Ct values from the amplification of each point dilution (5 per curve). Percentage coefficients of variance (%CV) have been used to represent precision estimates as a mean Ct value in all experiments. The mean values of these repeatability CVs are presented in Table 1 to provide a measure of the variance of our estimates. Plasmids and cell line DNAs, tested in this work, were compared and evaluated in terms of sensitivity, reproducibility and efficiency. Performance was evaluated either by collecting data from different daily-prepared curves (pFCL standard curves, n=24; DoHH2 curves, n=6; pMCL, n=10 and JVM2, n=5), over a period of 1 year. As shown, CV % values were calculated as mean CT from each run assay at each diluting point of PCR reactions.
a- Analysis of CV calculated on the slope values from the pFCL standard curves, displayed a high intra-assay (CV 1,7%) and inter-assay (CV 7,7%) reproducibility. The reproducibility of measured pFCL was estimated by a grand mean standard curve (y = -2,89x+37,76).
Within-run variation (intra-assay CV) was 1.1-2.4%, whereas the between-run (inter-assay CV) was 1.98-5.29%.
b- Analysis of CV calculated on the slope values from the pMCL standard curves, displayed a high intra-assay (CV 1,3%) and inter-assay (CV 11,8%) reproducibility. The reproducibility of measured pMCL was estimated by a grand mean standard curve (y = -3,14x+38,76)
Within-run variation (intra-assay CV) was 1.4-2.9%, whereas the between-run variation (inter-assay CV) was 3.98-6.19%.
The results indicate that standard curves contructed by our plasmids show a high stability even upon long-term storage (> 1 year).

Figure 4

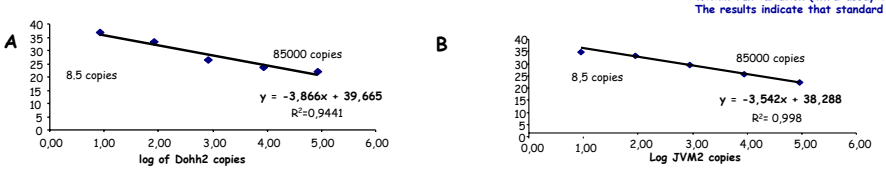


Figure 4. The sensitivity of the performed Q-PCR was assessed by evaluating the rearranged gene copy numbers on positive sample curves, i.e. DNA from positive cell-lines.
A- Starting DoHH2 t(14;18)-positive stocks were obtained by 500 ng of DoHH2 DNA serially diluted (500 ng to 0.005 ng) with 500 ng of DNA from a pool of healthy donors. Ct values were plotted as a function of the logarithms of the rearranged gene copies, producing a sensitivity curve with a slope of -3.8 (R^2=0.94). The Ct values of DoHH2, with use of our calibration curve, were converted in number of attomoles of t(14;18). The calculated mean resulted to be 0,14 (+/- 0.05, CV%= 5,6), and corresponded to the value of 85000 target gene copies.
B- Starting JVM2 t(11;14)-positive stocks were obtained by 500 ng of JVM2 DNA serially diluted (500 ng to 0.005 ng) with 500 ng of DNA from a pool of healthy donors. Ct values were plotted as a function of the logarithms of the rearranged gene copies, producing a sensitivity curve with a slope of -3.54 (R^2=0.99). The Ct values of JVM2, with use of our calibration curve, were converted in number of attomoles of t(11;14). The calculated mean resulted to be 0,13 (+/- 0.05, CV%= 4,9), and corresponded to the value of 83500 target gene copies (corrected to 85000).

CONCLUSION

The described system:
i) can be used to build robust standard curves for absolute quantification of any DNA target of interest;
ii) displays a high intra-/inter-assay sensitivity and reproducibility;
iii) is suitable for use with any Q-PCR strategy (TaqMan; SYBR Green).
With all these conditions, we anticipate that the use of pDNA as external standard will contribute significantly to the accurate quantification of gene marker copy number, in clinical and routine laboratory applications.

Ematologia Oncologica
Istituto Nazionale Tumori
Fondazione G. Pascale