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GENERATION OF PRECISE CALIBRATION CURVE FOR ABSOLUTE QUANTIFICATION OF DNA TARGET BY REAL-TIME PCR. A METHOD FOR ACCURATE MOLECULAR MONITORING OF T(14;18)-BEARING CELLS IN FOLLICULAR LYMPHOMA

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Abstract – *Objective:* *Determination of absolute DNA concentration by Real-Time PCR is mandatory for an accurate molecular diagnosis. The critical step is a precise and reliable calibration curve. Plasmid DNA containing cloned target sequences is widely used as standards in quantitative PCR. Usually, the initial molecular number of plasmid molecules is deduced by theoretical calculation and this can be severely limiting the accuracy of PCR assay.*

Materials and Methods: *We have developed a simple method to generate a precise standard curve made of plasmid DNA by competitive PCR approach. The procedure is the following: i) construction of competitor fragment; ii) generation of the stock standard by cloning the DNA target template in the plasmid vector; iii) determination of the number of molecules in the stock standard by competitive PCR; iv) analytical validation of standard curve, in terms of accuracy, sensitivity and specificity. We have used this procedure to quantify the genetic marker breakpoint, the t(14;18), associated to Follicular Lymphoma (FL) of Non-Hodgkin's Lymphomas (NHL).*

Results: *We found that the assessment of plasmid copy number, obtained by theoretical calculation, is overestimated compared to our competitive PCR approach (error of 22% +/-5). The standard plasmid DNA, used to generate the calibration curve, is stable and produces a high reproducible curve even after a long-term storage compared to genomic DNA curve.*

Conclusions: *The described procedure: i) generates robust standard curves for absolute quantification of any DNA target of interest; ii) is suitable with any molecular diagnostic platform; iii) contributes significantly to improve the accuracy of routine laboratory applications.*

KEYWORDS: *Molecular diagnostics, Standard curve, Competitive-PCR, t(14;18)BCL2/IgH Major Breakpoint Region.*

LIST OF ABBREVIATIONS: *qPCR, Quantitative Real-Time PCR; NHL, Non-Hodgkin's Lymphoma; FL, Follicular Lymphoma; Ct, threshold cycle number; LN, lymph nodes; BM, bone marrow; PB, peripheral blood; MBR, Major breakpoint region; dNTPs, deoxynucleotide triphosphate mix.*

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INTRODUCTION

Quantitative PCR (qPCR) is currently used routinely in a wide range of molecular diagnostics settings, including oncology, microbiology[1,2], haematology, forensic[3], botanic and food safety[4]. In onco-haematology, the importance of determining the absolute number of molecules of a cancer marker gene is essential in cases where the experimental variation may over- or under-estimate the nature and course of the disease[5].

Assay performed by qPCR can detect and quantify any DNA target and with increasing demand for the quantification of genes, simple, rapid, cheap and reproducible methods are required for use in clinical and routine laboratory applications. Sensitivity, reproducibility and accuracy represent quality measure of the assay; however, the application of quantitative methods in clinical laboratories requires parallel validation of the used approach and the development of techniques to enhance validity such as suitable reference standards and internal/external quality control in all steps[6]. Inter-assay reproducibility is often very low in different laboratories with the use of their own baseline control[7]. Determination of the absolute number of a DNA target molecule by qPCR is independent on the baseline control and can rely on a well proven and valuable approach with an increasing role in clinical diagnostic setting. To perform absolute qPCR, a homogeneous and pure template of well-known concentration, is used as standard for generation of a calibration curve. The choice and precise quantification of the template will determine the accuracy of the assay. Currently, a plasmid carrying DNA target sequence is the most widely used type of template in Real-Time PCR-based methods; determination of plasmid number of molecules is based on simple theoretical calculation[8]. Unfortunately, the accuracy is not always ensured for the following major reason: i) the spectrophotometric estimation yield may be higher for bacterial RNA and DNA contamination during plasmid preparation; ii) the performance of qPCR is strongly dependent on the specific primers design; iii) super-coiled plasmid structure (linearized and non-linearized plasmid), may be amplified with different efficiency from target genomic DNA[8]. However, there is no available information adequately addressing and solving the problem, alternative to theoretical calculation, to quantify the stock standards for generation of a precise calibration curve. However, MIQE guidelines (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) do lack a sufficient detail to build a calibration curve for absolute quantification[9,10].

We describe here a simple method to build an external standard curve by using recombinant plasmid DNA, precisely quantified by competitive PCR method. Competitive PCR is based on co-amplification of a target nucleic acid sample with an exogenous DNA competitor fragment that shares only the sequence primers sites with the DNA target. In this competitive PCR approach, the two templates (target and competitor) are subject to the same predictable and unpredictable variables that might affect PCR, and thus should amplify at the same rate. After amplification, the PCR products (target and competitor) can be visualized either by a difference in length on agarose gel electrophoresis[11] or they can be resolved by temperature gradient gel electrophoresis[12] or distinguished by real-time PCR[13]. Competitive PCR is a powerful tool for the absolute quantification of target molecules of DNA, but it suffers from a few drawbacks that somewhat limit its application. In particular, the technique is time-consuming for: 1. competitor construction and validation; 2. the necessity of assembling multiple competitive reactions for a single determination; 3. most notably, the need for a post-PCR electrophoresis-based detection and analysis step. This turns out to be particularly cumbersome for the analysis of a large number of diagnostic runs; in our methods, competitive PCR is performed only for generation of a precise quantification of the stock standard.

To validate qPCR for detecting tumor molecular markers, we have focused on the analysis of Follicular Lymphoma (FL) associated-t(14;18) rearrangement[14]. The neoplastic clone is characterized (70% of FL) by germinal center B cells carrying the t(14;18) involving BCL-2 gene (chromosome 18, q21). Specifically, the rearrangement generally occurs between the 3' untranslated portion of the third exon called "Major breakpoint region" (MBR) and J region of the Immunoglobulin heavy chain (IgH) gene (chromosome 18, q32). Monitoring the number of residual malignant cells carrying t(14;18) during the course of disease is critical since it may predict the early relapse[15], preceding the apparent clinical manifestation and may verify the efficacy of treatments[16].

It is worth noting that PCR-detected BCL-2/IgH rearrangements can be unrelated to the original FL clone at diagnosis since they can also be found occasionally in the blood of healthy individuals[17]. For this reason, it is crucial to determine whether the cells carrying the t(14;18) belong to the original malignant clone[18]. The development of reliable absolute qPCR strategy, allowing a clear separation between positive value (FL patients) *vs.* the negative (healthy subjects) cells, may overcome the potential pitfalls mentioned above.

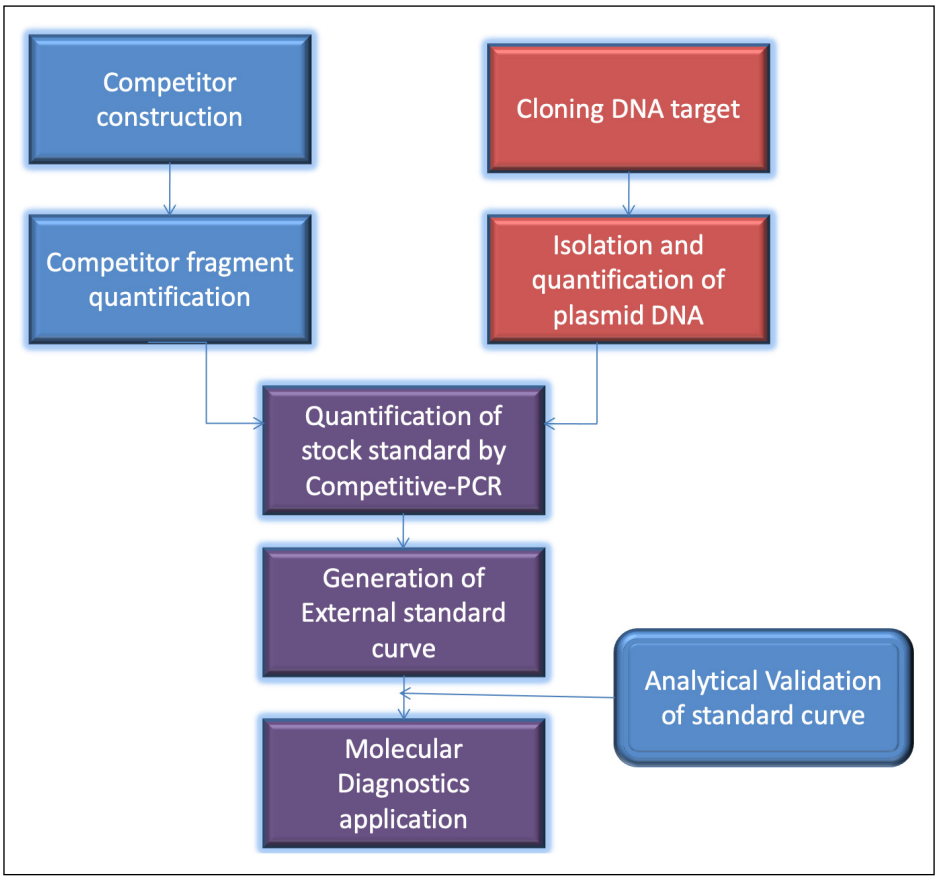

Fig. 1. Experimental design. The procedure covers all stage, from design of the composite primers for competitor construction and cloning of DNA target, to example of molecular diagnostics application.

We describe here the entire procedure, from design of the composite primers for competitor construction and cloning of DNA target, to example of molecular diagnostics application (Figure 1).

MATERIALS AND METHODS

DNA samples and DNA extraction

Surgical macro dissected lymph-node (LN) biopsies, bone marrow (BM) aspirates and peripheral blood (PB) samples were collected from patients with different clinical stages of NHL-FL. Lymphoma B-cell line DoHH2[19] carrying chromosomal translocation t(14;18)(q32;q21)MBR was used as a positive control for PCR studies. White Blood Cells (WBC) from a pool of healthy donors, known to be t(14;18)-negative is used as a negative control.

High molecular Weight genomic DNA (gDNA) was isolated from samples, by Wizard Genomic DNA purification Kit (Promega, Madison, WI, USA). Plasmid DNA (pDNA) was purified using the QIAprep midi-prep Kit (QIAgen, Hilden, Germany). Integrity of both DNAs was evaluated by visualizing them on Ethidium bromide-stained agarose gel. After spectophotemtrically determining concentration at 260/280 nm (SmartSpec 3000, BioRad, Hercules, CA, USA), gDNA and pDNA samples were shared and stored at -20°C for long term storage and +4°C after thawing.

Design and construction of competitor

Competitor fragment was made following by manufacturer's protocol of PCR-mimic construction Kit (Clontech, Palo Alto, CA). Briefly, a general consideration in order to make an internal heterologous competitor DNA-fragment, includes design of the composite primers for two steps PCR amplification (Figure 2A). In the First primary PCR amplification the entire sequence of gene specific primers is incorporate onto the ends of the competitor fragment, i.e., primers contain a 20-mer sequence that anneal on heterologous DNA fragment with linked 20-mer flanking sequence for gene specific primers. After 16 cycles the product of amplification was diluted 1/100 and used as template for secondary PCR (18 cycles) with gene-specific primers ***(Supplementary file)***. The molar quantity of the competitors' fragments (called MBRcomp) was determined by two ways: i) by densitometric comparison of the intensity electrophore-

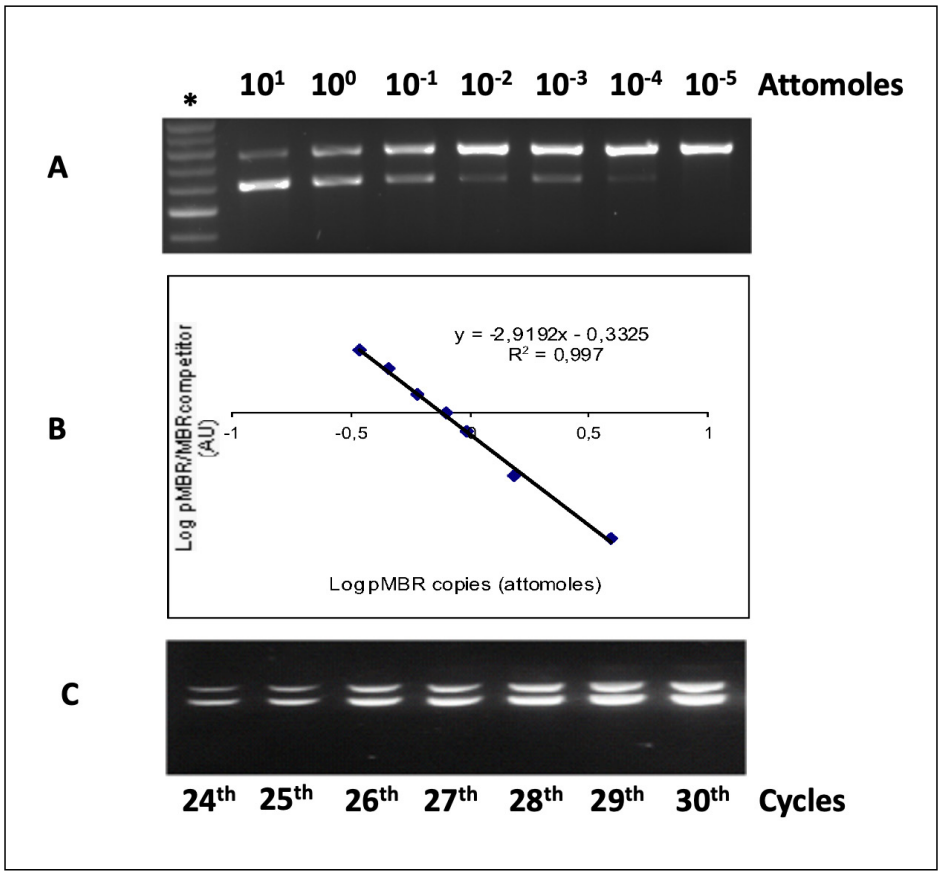

Fig. 2. Quantitative validation of plasmids standard by Competitive PCR. A) Competitive PCR: ten microliters of amplified product were run on a 2% ethidium bromide-stained gel: 10-fold serial dilution of Competitors (10 to 0.0001) was co-amplified with constant aliquots of pMBR (1 pg) and relative intensities of the bands corresponding to MBRcomp (size 595bp) and plasmids (size 798) were densitrometrically quantified by gel analyzer. The amount of specific amplified products was expressed in Arbitrary Units (AU).B) AU *vs.* competitors amount plot: The mean of the amount of specific amplified products for pMBR expressed in AU and the logarithm of their ratio were plotted as a function of the logarithm of respective quantity (attomoles). The equivalence point of pMBR and MBRcomp was inferred at 0.25 attomoles. It follows that 1 pg of pMBR corresponds to 150000 molecules or copies (see Avogadro's Number). C) Time-course competitive PCR: a kinetics validation of both Competitors and plasmids target was performed by co-amplifying in the same tubes 1 pg of pMBR and 0.25 attomoles of MBRcomp fragment. During amplification an aliquot of 5 □L of reaction mix was removed from 24th over to 30th reaction cycles. The relative intensity of the bands was quantified by gel analyzer and relative ratio value= 1 indicates a similar amplification efficiency for both templates.
*Marker 100bp DNA ladder (Invitrogen, Milan Italy)

sis bands of competitor, yielded 595bp, against those generated by known quantity of size DNA marker ϕX174/HaeIII digest; ii) by adding a^{32}P-dCTP into secondary PCR and measuring radioactivity of the competitor fragment amplicon. In both case, competitors' fragments were purified by spin column Microcon YM-50 (Millipore, Bedford, MA, USA) and diluted by TE (10mM Tris-HCl; 0,1 mM EDTA).

Furthermore, the competitor fragment yield, was 6.40 ng/uL. This result was confident with the theoretical estimation of manufacturer's protocol of PCR-mimic construction Kit made with radionuclide method (^{32}P-dCTP). Finally, competitor fragments were diluted (in 10 ug/ml ultrapure Glycogen solution) as a stock solution for 100 attomoles/uL and stored at –20°C.

Generation of the standard by cloning DNA target template in a plasmid TA-vector

Standard consists of plasmid DNA containing the sequence of interest (called pMBR), obtained from PCR amplicon DNA of DoHH2 cells lines carrying BCL2/IgH MBR rearrangement

Amplicon, yielded 798bp, was generated by the conventional PCR reaction mix containing: 10 X buffer (100 mM Tris-HCl pH 8.3, 0.5 mM KCl), 4 mM $MgCl_2$, 0.4 mM dNTPs, 200 nM of Primers *MBR forward* and *JH consensus reverse* (Invitrogen, Milan, Italy), 2.5 U Taq Gold (Applied Biosystem, Foster City, CA, USA) distilled water for volume 25 mL. PCR conditions were 10 minutes at 95°C followed by 35 cycles of 30 s at 94°C, 30

s at 58°C and 90 s at 72°C(extension) and a final extension of 5 minutes at 72°C.

Fresh PCR product was cloned into pCR 2.1 vector using the TOPO-TA cloning kit (Invitrogen, Milan, Italy). Purified pDNA samples were diluted with TE in a stock solution of 10 pg/mL and stored at –20°C (several aliquots). Rigorous precaution was taken to prevent cross contamination of sample and all experiments included negative control from all stage of the reaction.

Competitive-PCR

Competitive PCR was performed using primer set and thermal cycler conditions adopted for the conventional PCR: 10-fold serial dilutions of MBRcomp (10^{1} to 10^{-4} attomoles) were co-amplified with a constant aliquot of 1 pg of pMBR.

In addition, 1 pg of pMBR and 0.25 attomoles of MBRcomp were co-amplified in the same tubes, and 5 mL of the amplified products, from the 24^{th} over to 30^{th} reaction cycles, were run on agarose Ethidium bromide-stained gels. The relative intensities of the bands were densitometrically quantified by gel analyzer (VersaDoc, Bio-Rad, Hercules, CA, USA).

After correction in size difference between MBRcomp (595bp) and pMBR (798bp), the logarithms of their ratios were plotted as a function of the logarithms of the amount of MBRcomp added. This plot was used to determine the equivalence point (Figure 2A).

External Standard curve

Standard stocks were constructed by diluting 850000 copies of pMBR (5.67 pg) within 500 ng of DNA obtained from a pool of healthy donors. Standard stock was further serially diluted ($9x10^{5}$-$9x10^{0}$) in TE buffer, to build external standard curve. Same Standard stock providing to perform the standard curve of Albumin reference gene ($8.5x10^{4}$-$8.5x10^{0}$). However, a pDNA mixed in gDNA calibration curve model has the advantage that both target and reference gene templates undergone parallel real-time PCR steps.

The software SDS v3.2 provided by the PCR instrument AbiPrism 7900 HT (Applied Biosystems, Foster City, CA, USA) calculates the standard curve for each run based on the cycle threshold (Ct) for each magnitude of dilutions. Based on these values, a linear regression line is plotted, and the resulting equation is used to calculate the log starting quantity and copy number for the unknown clinical samples. Reproducibility of the calibration curves was examined by calculating mean and range cycle threshold (Ct) values for each dilution. The Ct values for the calibrators, obtained from each run, were assessed for outliers by use of calculation of the coefficient of variability (CV%). 44 different, daily prepared, standard curves have been analyzed over five years.

Real-Time PCR

The ABI/Prism 7900HT sequence detector (Applied Biosystem, Foster City, CA, USA) platform was used throughout this study. Quantitative PCR of t(14;18)MBR was performed on TaqMan Chemistry. DNA samples and calibrators were amplified in duplicate in the presence of 400 nmol/L primers MBR forward and MBR reverse, 300 nmol/L of dual labeled fluorogenic (FAM-TAMRA) MBR internal Probe, 250 mmol/L dNTPs, 1.25U AmpliTaq Gold DNA Polymerase and 4 mmol/L $MgCl_2$ in sample buffer A (Applied Biosystem) in a total volume of 25 uL. Sample was heated for 2 minutes at 50°C (for UNG digest) 10 minutes at 95°C and 50 cycles of 30 s at 95°C, 80 s at 60°C.

In parallel, Albumin reference reaction, was performed in duplicate in the presence of 300 nmol/L Albumin forward and Albumin reverse primers, 150 nmol/L dual labeled fluorogenic probe (FAM-TAMRA). Amplification condition and reagents were the same mentioned before. During a run, well-to-well variation can be normalized by use of a ROX (6-carboxy–X-Rodhamine) reference fluorescent dye, which remains constant through-out amplification.

Q-PCR amplification efficiencies and Normalization

The efficiency (E) of the reaction can be determined by slope values of pMBR standard curve. It is related to the logarithm of the slope by $E=10^{(-1/slope)}$.

To correct the differences in DNA load samples, Ct results from target t(14;18), were normalized to reference Albumin Ct results, by comparing the absolute amount of reference gene in each sample (see results). The amount of Albumin DNA copies present in tested samples is calculated referring to a Albumin-specific standard curve, considering that 500 ng of genomic DNA contains about 140000-180000 copies of such reference gene in duplicate allele, as described in literature[20,21]. The amplification efficiencies of the t(14;18) and reference Albumin samples must be the same to make similar slopes.

Statistical Analysis

To estimate reproducibility (inter-assay variability), quantification of calibrators and sample was performed in several times in different experiments (between-run variations). The Precision (intra-assay variability) was evaluated in duplicate quantification for calibrators and sample, of the same specimen during the same assay (in-run variations). The differences in order relative to Ct were valid if the lowest and highest value were 1.5. An Analysis of Variance (ANOVA) approach was used to identify sources of variability within the CV% data[22].

Assumptions and approximations

500 ng of human genomic DNA correspond to about 70000-90000 WBC as described above[21,23,24].
Each detected t(14;18)- rearranged gene is equivalent to one allele copy number.
DoHH2 cell line carrying 48 chromosomes, while we assumed than it has 46 chromosomes for uniformity to DNA from patient.

RESULTS

Theoretical calculation of plasmid copy numbers

The theoretical number of molecules of the pMBR (size 4729 bp) is calculated based on the following formula (*http://www.appliedbiosystems.com/support/tutorials/pdf/quant_pcr.pdf*):

$$n = (m \times NA)/M$$

in which n is the number of base pairs, m is the mass of the DNA target, NA is Avogadro's number (6.02×10^{-23} bp/mol) and M is the average molecular weight of a double-stranded DNA (660 g/mol).

Briefly we calculated that 10^6 copies correspond to 5.2 pg of pMBR:

m pMBR= 4729 bp x 1.096*10e-21g/bp = $5.2*10^{-18}$ g.

In conclusion, 1 pg of pMBR is equivalent to 192307 molecules of t(14;18).

Evaluation of the number of molecules of the stock standard by fine-tuned competitive PCR

Absolute quantification of the pMBR molecules was determined by fine-tuned competitive PCR. Ten-fold serial dilutions of MBRcomp (10^1 to 10^{-4} attomoles) were co-amplified with a constant aliquot of pMBR (1.0 pg). The relative intensities of the bands, emphasized by agarose ethidium bromide-stained gel, were densitometrically quantified by gel analyzer (Figure 2A), and the logarithms of their ratios were used to determine the equivalence point (ratio=1). The mean of equivalent point, derived from four experiments (0.24, 0.23, 0.28 and 0.26 attomoles) carried out (CV% 5.1) is 0.25 attomoles (Figure 2B). Regression analysis shows that y = – 2.19 and x – 0.332 (R^2=0.997). Clearly, by Avogadro's Number, since 1 attomole corresponds to 600,000 molecules of a given target, 0.25 attomoles is the equivalent of 150000 molecules of our target in 1 pg of pMBR. The number of 150000, referred to target copies present in 1 pg of pMBR, represents the starting point to generate the stock standard. To demonstrate that both templates display the same kinetics of amplification, a *time-course* competitive PCR was performed co-amplifying 0.25 attomoles of pMBR (1 pg) and 0.25 attomoles of MBRcomp. The amplification products, from 24th over to 30th of the PCR reaction cycles, were analyzed on agarose gel and relative intensities of the bands were densitometrically quantified. The relative ratio of densitometric value =1 in each amplification cycle indicates that both templates display comparable amplification efficiencies (Figure 2C).

Similarly, the number of attomoles of t(14;18) in 500 ng of DNA from DoHH2 cell line is 0.142 (+/- 0.05) attomoles (CV%= 5.6) corresponding to 85201 molecules of target (data not shown).

Analytical validation of External standard curve for absolute quantification of genomic DNA target

Standard curve performance was evaluated by using data collected from 44 pMBR daily standard curves during a 5-year period. The mean from 44 collected Ct for each dilution point is very similar, i.e., y = -3.27x+40.2 r^2 0.9982. As shown in Figure 3A, the standard curve is linear over five orders of magnitude, indicating a constant PCR efficiency over the concentration range studied. The reproducibility of measured pMBR was estimated by in-run variation (intra-assay CV), calculated on Ct value range which was 1.1-2.4 and between-run variations (inter-assay CV) which were 2.11–6.07% (Table 1). The lower quantification limits (reproducibility 90%, and CV%=6.07) of the method were 8.5 copies of pMBR. The lower detection limits (reproducibility 80%) of the method were 4.25 molecules (Data not shown).

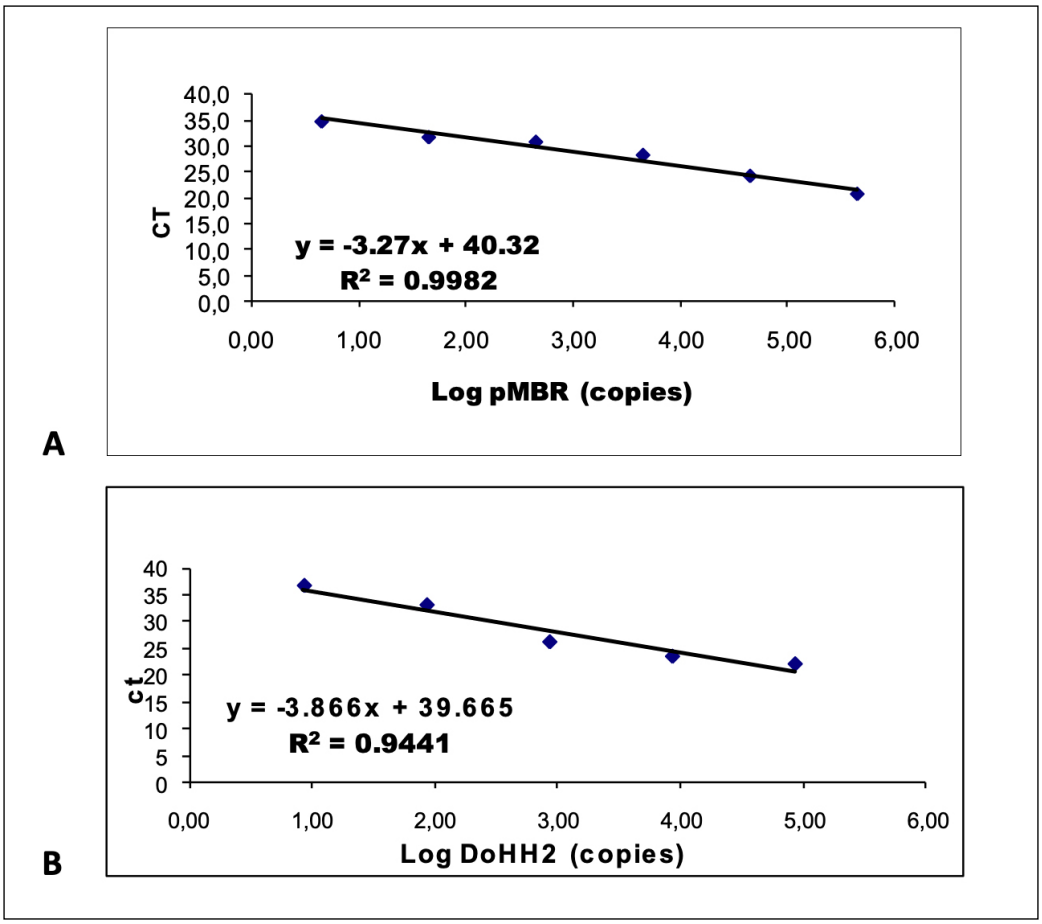

Fig. 3. Standard Curve performance. A) External Standard curve performance was evaluated with use of data collected from 44 pMBR daily standard curves over a period of 5 year about. pMBR (5.67 pg) was mixed into DNA from whole blood of healthy donor to a final concentration of 500ng/□L. The logarithm of ten-fold serial dilution of the stock standard (to 850000 molecules down 8.5), was plotted as function of Ct values. B) Sensitivity curve (data collected from 16 curves): the logarithm of ten-fold serial dilution of genomic DNA from DoHH2 (to 500 ng down 0.05 ng), was plotted as function of Ct values. 500 ng of DNA from DoHH2 correspond to 85000 t(14;18) molecules (see results), it follows that the lower sensitivity/quantification limit of the method is 8.5 cells on 500 ng of DNA.

Using pMBR standard curve, we quantified 500 ng of gDNA from DoHH2. We detected a mean Ct value 22.50 (CV = 4.84% n=16). This number corresponds to 85113 molecules of t(14;18) in 500 ng of DNA from DoHH2, normalized to Albumin gene Ct value. Since each cell harbor mono allelic t(14;18) molecules, this value can be translated in the number of the cells (approximated for convenience to 85000 cells). These results are in good agreement with empirical data, indicating that 70000-90000 WBC provide about 500 ng of human genomic DNA[21,23,24].

The PCR efficiencies were calculated by $E=10^{(-1/slope)}$ and the average was 2.13 (range, 2.07-2.19; n = 44) for the Albumin and 1.95 (range, 1.74–2.03; n = 44) for t(14;18). We found no significant differences between the PCR efficiencies of albumin and sample targets.

Comparison between standard curves of plasmid and gDNA

We compared the analytical performance of pMBR and gDNA from DoHH2-based standard curve. As shown in Table 1, a direct comparison of CVs for each magnitude of dilution (mean of Ct collected data) indicates that the pDNA quantification model display higher sensitivity, larger linear dynamic range, higher reproducibility, and higher stability compared to the gDNA model over time. The gDNA from DoHH2 calibration curve (n=16), is highly variable, y = -3.87x+39.66 r^2 0.994 (Figure 3B). It shows acceptable within-run variation (range CV% 1.4-3.1), but low reproducibility between-run (range 4.84–9.60%) in comparison to pMBR curve range 2.11-6.07%) (Table 2). In addition, comparing reproducibility between two standard curves, they show a CV

TABLE 1. Comparison between standard curves of plasmid and gDNA.

pMBR (n=44)		*DoHH2 (n=16)*	
Molecules	*CV %*	*Molecules*	*CV %*
85000	2.32	85000	4.84
8500	2.82	8500	8.97
850	2.11	850	9.60
85	4.06	85	6.01
8.5	6.07	8.5	4.99

1.5% (pMBR) and 7.7% (gDNA) calculated from the y-intercepts and slopes, 14.3% and 18.9% (given by the ABI/Prism 7900 Software after each new run), respectively. We conclude that the plasmid standard curve is more stable than genomic DNA to be used over time (p=0.0001).

Clinical diagnostics applications

Such approach was applied to quantify the genetic marker breakpoint t(14;18), associated to NHL-FL. The absolute amount of t(14;18) positive cells was comparatively assessed in 37 paired LN, BM and PB of FL at diagnosis. It is important to evaluate the qPCR efficiency with high precision in the particular biological samples studied, considering that a small error in the assumed PCR accuracy may cause large errors in the estimated number of molecules of a given gene target. To account for experimental error and variations in PCR efficiencies in the classification of samples, we estimated limits within which negative samples shall be found. The mean number of t(14;18) detected in 500 ng of DNA (85000 cells) from paired BM and PB at diagnosis, is very similar in our cohort of 48 patients (4591 and 1712 molecules respectively; p=0.09), while concurrent LN samples show a significantly higher tumor load value (59688 molecules p=0.018 compared to PB).

Of note, any PB sample of FL patients has been lower tumor load less than 10 neoplastic cells at diagnosis (Table 2). This value represents the threshold in a subject with clinical manifestations of disease (limit of quantification was 8.5 molecules). In subject without clinical manifestation of FL, these results could be confused with the unrelated values obtained from occasionally PB of healthy donors carrying t(14;18)[25]. We conclude that determination of the t(14;18) at diagnosis of FL patients, must be performed always by paired PB and BM; and when possible, supported also by LN biopsy.

DISCUSSION

With increasing demand for gene quantification, accurate and reproducible methods are required for use in clinical and routine laboratory applications. Since the gold standard method is depending by numerous variabilities, the rational selection of the better platform is needed[26]. To fulfill these criteria, we have developed a method for absolute quantification of genomic DNA based on external standard curve. The external calibration curve model has to be thoroughly validated since the absolute quantification in Real-Time PCR depends entirely on the accuracy of the standards.

TABLE 2. Absolute evaluation of t(14;18) copies in paired LN BM PB at diagnosis. Correlation between LN at High-Intermediate-low levels of t(14;18) copies on paired BM and PB.

	High (>850)	*Intermediate (>85-850)*	*Low (<8-85)*
p= 0.09	*LN High (30)*	*LN int. (6)*	*LN low (12)*
BM High (8)	8	0	0
BM int. (20)	15	5	0
Bm low (17)	4	1	12
PB High (4)	4	0	0
PB Int. (7)	7	0	0
PB low (23)	14	0	9

Standard design, production, determination of the exact standard concentration and stability over long time storage are not obvious and can be highly problematic. The most common methods used to quantify DNA standards are based on a simple theoretical calculation the number of DNA molecules[27,28] using the mass of the haploid human genome (about 3.5 pg). In fact, based on these assumptions, 1 ng of gDNA contains 286 copies of a single copy gene. The most agreed experimental protocol includes: 1. measurement of the bands' luminescence resolved by gel electrophoresis[11]; 2. hybridization methods[29]; 3. denaturing-HPLC [30]; 4. allele specific-PCR[31]; 5. labeled radionuclide-based assay[32]. All these described experimental methods were universally validated by competitive PCR, too[33]. We have chosen a competitive PCR to evaluate the exact number molecules present in the stock standard. However, theoretical calculation is a very simplistic model to assess plasmid DNA copy number and does not take into account all the chemical and physical events related to PCR that contribute to modify (including random factor) the efficiency of reaction and absolute quantification. It is well known that the accuracy of the standard curves is affected by several factors such as contamination of bacterial DNA and RNA residues (resulting in increased UV measurement), or different efficiency of amplification between plasmid standards and genomic DNA target. As results of these described events and probably other random factor, our data indicate that theoretical assessment of standard pDNA copy number (192307 copies in 1 pg of pMBR) grossly overestimates the number of DNA molecules compared to date obtained by competitive PCR (150000 copies in 1 pg of pMBR). Therefore, we estimate the error by using this approach to be about 22% +/-5.

Our calibration curve with the cloned recombinant plasmid DNA is very stable even after a long storage time, compared to genomic DNA (table 1). In addition, the circular plasmid is more resistant to degradation, but the efficiency of the few first cycles of PCR result decreased because of reduced performance of the primer annealing[34], compared to linear plasmid[35]. Repetitive testing of non-linearized pMBR, stored in small aliquot at -20°C, produces highly reproducible results compared to linearized plasmid obtained with restriction enzyme (unpublished data). This is the reason why, we decided to use un-restricted plasmid to avoid repetitive re-quantification procedure of the stock standard by tedious competitive PCR steps. In all cases, our method is suitable to build highly accurate external standard curve with both linear and circular plasmids.

However, pMBR mixed with gDNA calibration curve model has the advantage that both target and reference gene templates (Albumin gene) undergo parallel PCR steps. Simultaneous quantification of an internal reference standard can be used to further adjust measured target quantities, independently on the pre-PCR procedures. An effective combination of both external standard and internal reference standard provides the best procedure to accurately quantify gene markers[36].

Therefore, quantification with external standards requires careful optimization of its precision (replicates in the same PCR run, intra-assay variation) and reproducibility (replicates in separate PCR runs inter-assay variation) to assess the limitations within the specific application (e.g., Minimal Residual Disease in FL). As expected, high-copy samples are more reproducible than low-copy samples. One reason for the higher error is the higher number of cycles, which increases the variability of the amplification efficiency. For example, the TaqMan chemistry-based DNA quantification used in our experiments shows a coefficient of variation of 2.32% at 85000 copies/500 ng of DNA, 4.06% at 85 copies and 6.07% at 8.5 copies per tube (Table 1). At very low copy numbers, a different source of error becomes relevant; specifically, from 1 to 10 copies per tube, the random variation due to sampling error (also called Poisson error) becomes significant. In our hands, the analytical sensitivity was 8.5+/- 0.5 t(14;18) molecules/500 ng DNA.

We have validated our method by measuring the monoallelic t(14;18) in DoHH2 cell line DNA. We have detected 85113 molecules of t(14;18) in 500 ng of DNA by pMBR standard curve. This number of molecules is consistent with the empirical data indicating that one microgram of human gDNA derives from approximately 140000-180000 WBC. As consequence, by assuming that 1 cell carries one copy of t(14;18), we can translate BCL2/IgH allelic copies to the number of the cells bearing the translocation. Furthermore, by analyzing the appropriate standard curves of the endogenous Albumin reference gene and BCL2/IgH rearranged copies, we have calculated the ratio of specific BCL2/IgH gene copies to reference DNA (Albumin) in each sample. Since 500 ng of DNA correspond to 85000 cells, we can derive the number of BCL2/IgH positive cells over 85000 analyzed cells. The mean number of t(14;18) detected from concurrent BM and PB at diagnosis was very similar in our cohort of 48 NHL-FL patients (Table 2), while paired LN samples showed a significantly higher tumor load value. Similar values were obtained from volunteer blood donors who carr the traces of t(14;18), as described in lit-

erature, it is essential that determination of the t(14;18) in FL at diagnosis must be supported by paired BM and, where possible, LN biopsy, too[37]. Moreover, evaluation of neoplastic cell burden is NHL patients by molecular[38] and serum[39] markers is particularly essential for the neuronal metastasis prevention. In addition, these data were in agreements with EuroMRD worldwide standardization of the molecular methods to quantify t(14;18) by Real Time PCR[40].

We believe there are many advantages deriving from the use of pDNA mixed with gDNA from WBC as an external standard for absolute quantification of t(14;18), for example: 1. the structural similarity of High Molecular Weight gDNA from target samples; 2. the ability to use a wide linear dynamic range without excessive dilution, and; 3. the greater stability of the concentrated stock standards during long-term storage. The cost, time and efforts in the preparation of pDNA (cloning and precise quantification by competitive PCR) is worth if one considers the sensitivity and reproducibility of the standards curve[41,42]. In addition, pMBR is sensitive and specific enough to predict disease outcome, monitoring minimal residual disease and gaining relevant evaluation to readily distinguish healthy subject carrying t(14;18) from those affected by NHL-FL at diagnosis.

CONCLUSIONS

In summary, the described system can be used to build highly accurate external standard curve that can be used for any qPCR platform. It allows the absolute quantification of any DNA target of interest. Analytical validation of the standards displays a high intra-assay reproducibility, even after a long-time storage compared to genomic DNA curve.

Finally, we anticipate that this tool may contribute significantly to improve the accurate quantification of gene dosage, in clinical and routine laboratory applications.

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